MARINE BACTERIA: WALL COMPOSITION AND OSMOTIC FRAGILITY

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INTRODUCTION

A major function of the bacterial cell wall is to maintain cellular integrity in adverse external environments. Thus, most bacteria can survive in media of low osmotic pressure, an ability dependent upon the presence of a cell wall rigid enough to prevent osmotic swelling and bursting of the bacterial protoplasm. The cell walls of Gram-positive organisms can withstand high internal osmotic pressures, e.g. 20 to 25 atmospheres in *Staphylococcus aureus* (Mitchell and Moyle, 1956). In contrast, this pressure in Gram-negative organisms is low, between 2 and 3 atmospheres as in *Escherichia coli* (Mitchell and Moyle, 1956), but it has been shown that these cells can be grown in salt-rich media and remain intact when placed in distilled water (Doudoroff, 1940).

This protective function of the cell wall is apparently absent in the marine bacteria which have been isolated and studied. Their cells lyse when put in distilled water, the cell wall rupturing allowing the cellular contents to escape (Johnson et al., 1943). The occurrence of lysis can be shown by a number of determinations such as decrease in turbidity and viability and release of protein and nucleic acids into the suspending medium. A marine bacterium has been estimated to possess an internal osmotic pressure of about 20 atmospheres (Johnson and Harvey, 1937; Johnson, Zworykin, and Warren, 1943) and the lysis
was the result of a critical difference in the osmotic pressure inside and outside the cells.

The seat of osmotic fragility has been shown (Boring, 1961) to be the weakness of the cell wall; whole cells and penicillin-induced spheroplasts of a marine bacterium were found to be nearly alike in osmotic fragility. It was suggested that the penicillin-sensitive component of the cell wall, while conferring a characteristic shape to the organism, was unable to protect it from osmotic lysis. In non-marine bacteria, it is this component which is believed to give the cell wall its rigidity and mechanical strength.

The cause of weakness in the cell walls of marine bacteria is not known but suggestions have been made that it is due to thinness, or to difference from ordinary cell walls in chemical composition or physical structure (Salton, 1956). A low hexosamine content in the cell wall of a marine bacterium has been implicated in osmotic fragility (Brown, 1960). This compound has been shown to be a component of the mucoprotein layer of the cell walls of *E. coli* (Weidel, Frank, and Martin, 1960), a layer responsible for the rigidity of the cell walls.

Some strains have been found to be less fragile osmotically than others (Tyler, Bieling, and Pratt, 1960; MacLeod and Matula, 1962) indicating the existence of a spectrum of osmotic fragility in these bacteria. If the weakness of the cell walls were due to a low hexosamine content, then it might be possible to correlate the wall hexosamine content with the degree of osmotic fragility exhibited by the marine organism.
This investigation was undertaken to establish and compare the cell wall compositions of three marine bacteria selected on the basis of their lytic behaviour in various test media, the extent of lysis being taken as an indication of their degree of osmotic fragility. Of the three organisms, M.B. 65 was the least and M.B. 98 the most susceptible to lysis while M.B. 29 was intermediate. The cell walls of a non-marine pseudomonad, Pseudomonas aeruginosa, were also isolated and analysed as earlier studies had indicated that the selected marine bacteria might be pseudomonads.

After considerable difficulty, a method was devised which gave clean cell walls of the marine bacteria. The fragile nature of the cell walls, especially those of M.B. 98 and M.B. 29, was evident from the electron micrographs. The analytical results indicated that the cell walls were predominantly lipoprotein in nature. They were completely soluble in phenol and were extensively disaggregated by sodium-dodecyl-sulfate. No sugars, except glucosamine, were detected; the hexosamine values were low as compared to those of P. aeruginosa and other Gram-negative bacteria.

The data suggested that the hexosamine content of the cell wall influenced the degree of osmotic fragility. From the electron micrographs, it was considered possible that thinness of the wall was partly responsible for the weak nature of the cell walls of the marine bacteria.
LITERATURE REVIEW

The literature, pertinent to the subject of this thesis, has been reviewed under two sections; (a) bacterial cell walls and (b) osmology of marine bacteria.

Bacterial Cell Walls

The external structures of bacterial cells, responsible for the rigidity and integrity of cells, are generally referred to as the "cell walls." In Gram-positive bacteria, the existence of a wall as a separate and distinct entity has been demonstrated in a variety of ways. This has not been possible in Gram-negative bacteria; whether they possess a wall distinct from the cytoplasmic membrane is uncertain.

Apart from their obvious mechanical function, very little is known about their biochemical activities. It is generally agreed that the isolated cell walls of Gram-positive bacteria are devoid of any enzymatic activity. The situation with the cell walls of Gram-negative bacteria is less certain; several reports on the enzymatic activities associated with the "envelope" preparations of such bacteria have appeared (Marr, 1960; Hunt, Rodgers, and Hughes, 1959; Salton, 1961a).
The removal of the cell wall, partially or completely by the action of agents such as lysozyme and inhibition of its synthesis by the action of penicillin on whole cells, results in the loss of the protective function and spherical bodies are produced. These are osmotically fragile in contrast to whole cells and have also lost the characteristic shape if they happened to be derived from rod-shaped cells. The bodies from Gram-positive bacteria, produced by the action of lytic enzymes, have been shown to be free from wall components (Freimer, Krause, and McCarty, 1959; Vennes and Gerhardt, 1959) and are called protoplasts. Gram-negative bacteria, on the other hand, give rise to bodies which retain some of the wall constituents (Shafa and Salton, 1958; Salton, 1958) and their membranes react positively with cell wall antibodies (Holme et al., 1960). The term spheroplast was suggested to describe such bodies.

Both protoplasts and spheroplasts are reasonably stable in media of appropriate osmotic pressure. They resemble whole cells in permeability properties and in their ability to carry out biosynthetic activities such as protein synthesis, indicating that the cell wall is not essential for such activities as long as protection from osmotic effects is provided. The presence of a cell wall, however, seems to be necessary for multiplication since these bodies have been found to be unable to reproduce. Cell walls may also act as reservoirs for certain metabolites; Gerhardt (1959) and Butler et al. (1958) obtained evidence suggesting that certain amino acids may be stored in the walls.
The rigidity and the mechanical strength of the cell wall is believed to be due to its mucopeptide component. Salton (1953) demonstrated this with the isolated cell walls of *Rhodospirillum rubrum*; treatment of the walls with lysozyme resulted in their becoming spherical in shape and the mucopeptide components were released. The formation of spherical bodies, by the action of lysozyme or penicillin on whole cells, is due to the disorganisation of the mucopeptide of the wall; lysozyme acts by degrading the mucopeptide enzymatically whereas penicillin interferes with its synthesis. In both the cases, the rigid part of the wall is weakened and the wall is unable to perform its mechanical function.

Attempts at isolating the cell walls were first made in 1887 when Vincenzi obtained what he believed to be the walls of *Bacillus subtilis* by extraction with 0.5 per cent NaOH. It is now known that treatment with alkali and acids leads to the degradation of part of the wall structure (Abrams, 1953; Baddiley *et al.*, 1958). Cell walls can be obtained by mechanical disintegration of cells followed by differential centrifugation (Salton and Horne, 1951a, b). The use of mechanical methods for disruption of bacterial cells is by no means of recent origin; as early as 1901, MacFadyen and Rowland had used agitation with fine sand for disrupting the typhoid bacillus. King and Alexander (1948) refined this method by using small glass beads. Dawson (1949) showed that the cells of *Staphylococcus aureus*, on vigorous shaking with glass beads, gave a preparation containing
cell walls. He used the Mickel tissue disintegrator (Mickel, 1946) for providing rapid and vigorous shaking of the cell suspension.

Salton (1956) and Cummins and Harris (1956a) have clearly shown that the residues from mechanically disrupted cells consist primarily of cell walls and this method is now widely used for obtaining bacterial cell walls. It is preferred since it avoids drastic chemical alteration of the walls. The method, as described by Salton and Horne (1951b), involves shaking of heavy cell suspensions with an equal volume of glass beads (0.15-0.20 mm in diameter) in a Mickel tissue disintegrator. The cell walls are separated from the suspension by differential centrifugation. They are then either washed repeatedly with appropriate solutions or treated with enzymes for the removal of cytoplasmic material from them.

Various solutions have been used for the cleaning of cell walls. Washing with 1 M NaCl or with phosphate buffer was found to be more effective than washing with water alone (Salton, 1953). Holdsworth (1952) used washing with sodium acetate solution followed by extraction with 90 per cent phenol for cleaning the cell walls of Corynebacterium diphtheriae. The cell walls of Gram-negative bacteria, however, have been found to be less amenable to cleaning by washing.

In their studies of bacterial cell walls, Cummins and Harris (1956b; 1958) employed enzymes for removing cytoplasmic material from cell walls. Since then, the use of proteolytic enzymes, ribonuclease, and deoxyribonuclease has become widespread. It is now known that
bacterial cell walls are not altered in physical appearance by these enzymes but it is possible that surface components are removed. Salton (1953) reported that trypsin removed the M protein from streptococcal cell walls and that the amino acid composition was simpler after trypsin treatment, sulfur-containing and aromatic amino acids being eliminated. The removal of surface protein antigen by the action of pepsin on Corynebacterium (Cummins, 1954) and solubilisation of about 40 per cent of isolated cell walls of Streptococcus by the action of trypsin (Barkulis and Jones, 1957) have been reported. Similarly Knox and Bandesen (1962) found that trypsin released a number of low molecular-weight peptides from isolated cell walls of Lactobacillus casei. Salton has recommended that the use of enzymes, especially crude enzymes which may contain wall degrading enzymes as well as other insoluble protein material, should be carefully controlled.

Other methods for obtaining bacterial cell walls are also available. Those involving disintegration of cells with sonic and ultrasonic vibrations, decompression rupture, and pressure cell disintegration suffer from the disadvantage of fragmentation and solubilisation of the wall (Slade and Vetter, 1956; Marr and Cota-Robles, 1957). Foster, Cowan, and Maag (1962) have recently described a device for rupturing of bacteria, under controlled conditions, by explosive decompression in a closed system. Autolysis and osmotic lysis of whole cells also yield cell walls; Weidel (1951) used toluene for autolysing the cells of E. coli and a lytic principle, associated with the cultures of Bacillus cereus, was found to digest cell contents of a number
of *Bacillus* spp. (Norris, 1957) giving clean cell walls. These methods have not been widely used because of the risk of degrading the wall enzymically.

The most widely used criterion for the purity of cell wall preparations is the absence of cytoplasmic material as determined with the electron microscope. Although it is not a very satisfactory criterion, wall preparations free from nucleic acids, electron-dense cytoplasmic material, and intracellular pigments can be obtained by careful control of the procedure employed. In certain cases, the purity of a wall preparation can be determined by using any special property of the cell walls under examination. Thus, with *Micrococcus lysodeikticus*, *Sarcina lutea*, or *Bacillus megaterium*, the purity can be determined by dissolving the walls with lysozyme and weighing the lysozyme-insoluble residue.

Isolated bacterial cell walls have been repeatedly examined by electron microscopy. They generally retain the shape and outline of the organism from which they had been derived. The walls of many Gram-positive bacteria, such as *Staphylococcus aureus* and *Streptococcus faecalis*, have a homogeneous appearance, although the walls of *Bacillus megaterium* give a vague impression of being fibrous (Salton and Williams, 1954). In the walls of some bacteria, such as *Rhodospirillum rubrum* and *Halobacterium halobium*, a spherical macromolecular type of fine structure has been observed (Salton and Williams, 1954; Houwink, 1956). In some bacteria, such as *E. coli*, the isolated walls appear
homogeneous by the usual method of examination in the electron microscope, but thin sections of cells have clearly established the multilayered nature of the wall. The walls of E. coli appear to have three layers visible in the electron microscope (Kellenberger and Ryter, 1958). The existence of these layers has been confirmed by their separation by chemical methods; Weidel et al. (1960) were able to separate an outer lipoprotein layer soluble in phenol, an inner insoluble and rigid layer containing the mucopolysaccharide constituents, and a middle lipopolysaccharide layer.

The thickness of bacterial walls has been estimated from measurements on thin sections of isolated walls or cells and on the shadows cast by the walls during electron microscopy. It varies from 100 to 200 Å, the walls of Gram-positive bacteria being thicker than those of Gram-negative organisms (Birch-Andersen and Maaloe, 1953; Kellenberger and Ryter, 1958). The cell wall accounts for about 20 per cent of the dry weight of the cell (Mitchell and Moyle, 1951; Cummins, 1956; Salton, 1956), but this value may vary depending upon the phase of growth or cultural conditions as in Streptococcus faecalis (Shockman et al., 1953; Toennies and Shockman, 1959).

Chromatography of cell wall digests has proved to be an invaluable tool in studies on the chemical composition of bacterial cell walls. Such studies have revealed a number of unusual components in the walls and the list is steadily growing. Most of the information available concerns the Gram-positive bacteria because of the relative
simplicity of their wall composition as well as structure and the ease with which they can be prepared. The cell walls of Gram-negative bacteria are more complex in nature, both chemically and structurally. Some excellent reviews on this subject have appeared within the last few years, the more recent being those of Work (1961) and Salton (1961a, b, 1962).

Bacterial cell walls have been found to contain a variety of chemical components: amino acids, lipids, carbohydrates, and phosphorus, but no nucleic acids, purines, and pyrimidines. A more complete picture of the chemistry of bacterial walls became available with the discovery of diaminopimelic acid (DAP) by Work (1951) and its detection in the cell walls of various bacteria, the isolation of muramic acid by Strange and Dark (1956) and its presence in the walls of all bacterial species so far examined (Salton, 1957; Cummins and Harris, 1956a; Work, 1957), the detection of D-amino acids (Salton, 1957; Snell, Radin, and Ikawa, 1955; Ikawa and Snell, 1956; Park, 1958), and the discovery of teichoic acids (Baddiley et al., 1958; Abrams, 1958) and teichuronic acids (Janczura et al., 1960, 1961).

The cell walls of Gram-positive bacteria differ noticeably from those of Gram-negative bacteria; the former contain a limited variety of amino acids, a small amount of lipid material, and a high amino sugar content, whereas the latter contain proteins with the usual variety of amino acids, a high amount of lipid, and a
small content of amino sugar. The cell walls of a large number of Gram-positive bacteria were examined by Cummins and Harris (1956a, b; 1958). The main components, invariably found, were glucosamine, muramic acid, glutamic acid, alanine, lysine, and diaminopimelic acid; in some cases there were also up to five sugars, one or two other amino acids, or galactosamine. A recurring type of "basal unit" was soon recognised (Worl, 1957) and the term "mucopeptide" was proposed by Mandelstam and Rogers (1959) to describe this unit. It is a complex of amino acids and amino sugars and is now recognised as the structural "backbone" common to the cell walls of Gram-positive bacteria.

In addition to these components, some other polymeric substances have been isolated from walls and partially or fully characterised. These include oligosaccharides, polysaccharides, teichoic acids, and teichuronic acid. These polymers are less widely distributed and teichuronic acid has been reported in walls of Bacillus subtilis (Janczura et al., 1960, 1961) only. The chemical structure of teichoic and teichuronic acids has been established. The name teichoic acid refers to the polymers of glycerophosphate and ribitol phosphate; the former type of polymer was first detected in the walls of Staphylococcus aureus (Mitchell and Moyle, 1951) and later on was found in the cell walls of other bacteria. Ribitol phosphate polymers were detected in the cell walls of Bacillus subtilis and Lactobacillus arabinosus (Baddiley et al., 1958). The teichoic acids
of *B. subtilis* (Armstrong *et al.*, 1961) and *S. aureus* H (Baddiley, Buchanan, Martin, and Rajbhandhary, 1962) contain ribitol units joined by phosphodiester linkages and most of the ribitol units carry ester linked D-alanine (Baddiley, Buchanan, Rajbhandhary, and Sanderson, 1961) and glucosyl residues. Teichuronic acid, isolated from the walls of *B. subtilis*, is composed entirely of N-acetyl-galactosamine and glucuronic acid (Janczura *et al.*, 1960, 1961).

Diaminopimelic acid (DAP) and muramic acid are two important constituents of bacterial cell walls which are generally absent in other types of organisms (Rhuland, 1960). DAP is present in most bacteria with the exception of some Gram-positive cocci and lactobacilli (Work, 1951; Hoare and Work, 1957). Muramic acid, first isolated from a product obtained from the exudates of germinating spores of *Bacillus megaterium* (Strange and Dark, 1956), is glucosamine carrying ether-linked lactic acid at the 3-position (Strange and Kent, 1959) and probably originates from glucosamine (Zillikin, 1959; Richmond and Perkins, 1960). The key role of this amino acid in the structure of the bacterial wall mucopeptides has been recognised and a broad outline of the mucopeptide structure has been established from the studies of products isolated from walls and mucopeptides after digestion with lysozyme and streptomycetes amidase (Salton, 1956, 1957; Chuysen and Salton, 1960; Chuysen, 1961; Primosigh *et al.*, 1961).
There is little doubt now that the mucopeptide forms the rigid backbone component composed of covalently bonded amino acids and amino sugars. The relation of other wall compounds to the mucopeptide component is less certain although it seems likely that they are attached to the mucopeptide by weak linkages. Evidence for this is provided by the extractibility of teichoic acids (Archibald et al., 1961) and teichuronic acid (Janczura et al., 1961) with trichloroacetic acid in cold and the removal of oligosaccharide and polysaccharide residues with both picric acid (Holdsworth, 1952) and formamide (Krause and McCarty, 1961). In all the cases the wall polymers have been obtained in solution leaving behind insoluble mucopeptide residues still possessing the structural rigidity and the appearance of the original cell walls as seen in the electron microscope (Archibald et al., 1961; Krause and McCarty, 1961).

The walls of Gram-negative bacteria show more complexity in chemical composition and structure than those of Gram-positive bacteria (Salton, 1961b). The protein, lipid, and polysaccharide complexes form part of the cell wall and, in addition, the specific mucopeptide constituents are also present. The existence of a rigid mucopeptide layer has been clearly demonstrated by the studies of Weidel et al. (1960) on the cell walls of *E. coli*, although the overall concentration of mucopeptide components is lower than in the walls of Gram-positive bacteria. Thus, there is now enough evidence that the walls of some Gram-negative bacteria possess a
mucopeptide of similar composition to that found in the cell walls of Gram-positive bacteria and it is the mucopeptide component which represents the "basal structure" of the walls of most bacteria.

The bacterial cell walls are disaggregated by the action of surface-active agents such as sodium-dodecyl-sulfate (SDS). The hemolytic action of SDS has long been known and the mechanism has been explained in terms of the "collapse" of oriented lipid and cholesterol layers in the red cell membrane (Schulman et al., 1955). The Gram-negative bacteria are generally resistant to the bactericidal action of certain surface-active, anionic compounds (Baker et al., 1941) but there have been reports of killing by high concentrations (0.2 per cent) of SDS (Lominsky and Lendrum, 1942). As the walls of these bacteria contain appreciable amounts of lipid, it is likely that the disaggregation of cell walls on exposure to SDS involves a physico-chemical change in the wall structure (Shafa and Salton, 1960). Complete disaggregation of isolated bacterial cell walls has been reported and it has been suggested that the mucopeptide complex of the walls forms a network extending across the multilayered wall rather than a continuous, separate layer (Shafa and Salton, 1960). However, Weidel et al. (1960) used SDS (0.4 per cent) during the isolation of the mucopeptide layer from the cell walls of E. coli, the detergent removing some of the protein and lipid from the outermost layers, a process which was completed by the action of phenol leaving behind the rigid mucopeptide layer.
The taxonomic importance of the cell wall composition has been stressed by some workers (Cummins and Harris, 1956a, b; Cummins, 1956, 1962). Each bacterial genus and even each species often has a particular pattern of amino acids, amino sugars, and sugars superimposed on the basal mucoprotein unit. Such patterns may prove useful in bacterial classification, although in some cases considerable variation in wall composition of a particular species has been reported (Slade and Slamp, 1962).

Osmolysis of Marine Bacteria

The internal osmotic pressure of one marine bacterium has been estimated to be about 20 atmospheres (Mitchell and Moyle, 1956) which approximately counterbalances that of the sea water. In media of low osmotic pressure, the cell walls of these bacteria are unable to withstand the internal pressure and the cells undergo lysis. In contrast, the terrestrial bacteria, though living in environments of low osmotic pressure, possess an internal pressure which may be as high as 20 to 25 atmospheres as in the case of Gram-positive bacteria (Mitchell and Moyle, 1956). In Gram-negative bacteria this pressure is low, between 2 and 3 atmospheres (Mitchell and Moyle, 1956). Their cells, after having been grown in media of high osmotic pressure, are protected against osmotic shock when transferred to distilled water (Doudoroff, 1940). While growing
in such media, the cells probably develop a high internal osmotic pressure (Christian and Ingram, 1959). Thus, the cell walls of terrestrial bacteria are strong enough to resist large differences in external and internal osmotic pressures.

Early studies on the osmolysis of marine bacteria were carried out with two luminous species, Photobacterium fisheri and Photobacterium harveyi. In 1915, Harvey made the original observation of the cytolysis of a marine bacterium while studying the phenomenon of biological luminescence. He demonstrated the dependence of luminescence on cellular integrity by showing that a dense cell suspension gave a bright light in oxygenated sea water, but no light in oxygenated tap water. He suggested that the cells were lysed in tap water, thus disrupting the system responsible for luminescence.

The osmolysis of P. fisheri was investigated by Hill (1929). He estimated lysis by measuring the disappearance of luminescence in diluted sea water and salt solutions. In distilled water containing 6 per cent sea water and in 0.0312 M NaCl, the luminescence disappeared within a few minutes but appropriate concentration of sucrose was found to protect it. He suggested that the disappearance of luminescence was an osmotic effect. The observation, that microscopic examination revealed little change in the appearance of cells which had ceased to give off light in diluted sea water, led him to suggest that the cells were surrounded by a rigid envelope
which did not swell but ruptured at a critical difference in osmotic pressure between the cells and the medium.

Korr (1935) pointed out that the disappearance of luminescence in hypotonic solutions might not indicate complete lysis and loss of viability. He found that some cells remained viable for several hours in distilled water. A majority of cells, however, undergo lysis in hypotonic media; Johnson and Harvey (1937) found that when dense cell suspensions were diluted with distilled water, the suspensions became clear and foamy, the motility and luminescence ceased, and the optical density and viability decreased considerably. They (1938) also made quantitative measurements of viability, respiration, and luminescence during cytolysis of P. harveyi. Their results, in general, showed that the above three functions showed a gradual decrease with progressive dilution of sea water with distilled water. Some salts and sucrose, at certain concentrations, showed protective effects.

Direct visible evidence of lysis of marine bacteria was furnished by Johnson et al. (1943). Electron micrographs of marine cells, which had earlier been placed in distilled water, showed evidence of lysis; the cell wall was found to be ruptured resulting in exudation of intracellular contents. That the cell walls are involved in osmotic fragility of marine bacteria is indicated by the recent work of Boring (1961). He compared the lytic patterns of whole cells and penicillin-induced spheroplasts of a marine
bacterium by estimating the degree of lysis in a series of graded concentrations of NaCl. The whole cells were protected from lysis in 0.06 to 0.08 M NaCl solutions, while the spheroplasts required 0.08 to 0.10 M solutions. These values indicated that as far as the osmotic fragility was concerned, whole cells and spheroplasts were almost equally fragile and the rigid, penicillin-sensitive component of the cell wall did not confer any added protection to the cells against osmotic lysis.

Brown (1960), in his studies on a marine bacterium, found that the hexosamine content of the isolated cell walls was lower than that reported for non-marine, Gram-negative bacteria. He suggested that the weakness of the walls of marine bacteria was due to the low content of amino sugar. In this connection, it is interesting to note that the cell walls of a non-marine bacterium, *Vibrio metchnikovii*, have been found to be low in amino sugar content (Shafa and Salton, 1953) and the cells of this organism are subject to osmotic lysis in distilled water. Amino sugars have been shown to be components of the mucoprotein layer of bacterial cell walls (Neidel et al., 1960) and a low amount of such components can result in a weakened mucoprotein layer.

In the present study, three marine bacteria were selected to represent a spectrum of susceptibility to osmotic lysis. Such differences in fragility in marine bacteria have been reported (Riley, 1955; MacLeod and Matula, 1962). It was hoped that a comparison
of the chemical compositions of the cell walls of selected marine bacteria might indicate some relationship between osmotic fragility and wall composition.
MATERIALS AND METHODS

The methods, used in this study, are presented in the following sections, each section corresponding with a particular phase of the investigation.

General Methods

Artificial sea water (ASW) was used in cultivating the three marine organisms used in this study. It was composed as follows: NaCl, 23.5 g; MgSO\(_4\cdot7\text{H}_2\text{O}\), 6.2 g; MgCl\(_2\cdot6\text{H}_2\text{O}\), 5.1 g; KCl, 0.75 g; distilled water, 1,000 ml. One per cent trypsinase (B.B.L.) in ASW was used as a source of nutrients for the organisms. Stock cultures were maintained on trypsinase-ASW slants containing 2 per cent agar. The same amount of agar was added to the basal medium whenever solid medium was used, unless otherwise stated. Nutrient broth was used for the cultivation of *Pseudomonas aeruginosa*. Two per cent agar was added to nutrient broth for preparing solid medium. All media were sterilized at 15 pounds pressure and 121 C for 15 min.

All glassware was initially cleaned in chromous acid solution; subsequent to any use, it was then cleaned with Haemo-sol (Meinecke and Company, Inc.) and rinsed several times with tap
water and three times with distilled water. For nitrogen estimation, the glassware was further rinsed with deionised water. All chemicals used were of reagent or chemically pure grade.

Organisms

The three marine organisms came from a collection of marine bacteria isolated by Bielling (1953) from coastal Atlantic waters off Florida. The isolation was based on the ability of the isolates to grow in media containing sea water or sea water salts, but not in media lacking in them. The organisms, designated as M.B. 29, M.B. 65, and M.B. 98, were selected mainly on the basis of their lytic properties.

A loopful from each of the three stock cultures, kept under oil, was inoculated into trypsinase-ASW broth. The growth was streaked on solid medium in Petri dishes and well-isolated colonies were picked and transferred to slants. The inability of these cultures to develop in trypsinase-distilled water medium was checked before starting work.

P. aeruginosa, used in the comparative study, was isolated by W. S. Silver in the department.

Growth Conditions and Harvesting

The marine bacteria were routinely cultivated in 1 per cent trypsinase-ASW broth. For obtaining small quantities of cells, Erlenmeyer flasks (250 ml) containing 50 ml of the liquid medium
were inoculated from starter broth cultures and shaken at 30 C; 4 to 6 hr old cultures were used.

For obtaining large crops of cells to be used for cell wall preparations, several Erlenmeyer flasks (1,000 ml) containing 200 ml of the trypticase-ASW medium were inoculated with starter cultures and the flasks were shaken at 30 C for 12 hr. The amount of inoculum was so adjusted that the culture was ready for harvesting when it had just reached the stationary stage.

An essentially similar procedure was used for the cultivation of P. aeruginosa with the exception that nutrient broth was used in place of trypticase-ASW medium and the temperature of incubation was 37 C instead of 30 C.

For centrifuging large volumes of cultures, a continuous flow centrifuge (Servall SS-1, type KSA-1) was used. For preparing the cell walls, 6 liters of a culture were handled at a time and the cells were used directly without washing.

Methods for Characterisation of M.B. 29, M.B. 65, and M.B. 98

Morphology.— The morphology of the marine organisms was studied under phase optics and wet mounts of young broth cultures were examined for motility under the light microscope. For determining the type of flagellation, the cells were fixed by adding
2 drops of a 1 per cent solution of osmic acid to 1 ml of a young broth culture. After standing for 5 min, the cells were centrifuged and resuspended in a few drops of distilled water. The suspension was sprayed on copper grids previously coated with a collodion film; the grids were allowed to dry and then examined with an electron microscope.

**Physiology.**--Certain physiological properties of the marine organisms were examined. The method of Hugh and Leifson (1953) was used for determining the type of carbohydrate metabolism. The medium was slightly modified to suit the organisms; trypsin, 0.2 per cent, was substituted for peptone and ASW for NaCl. Duplicate tubes of the medium were inoculated by stabbing; one tube was sealed with a layer of petrolatum and designated as the "closed tube." The tubes were incubated at 30 C and examined periodically over a period of one month.

The action of the following antibiotics on these bacteria was studied: chlortetracycline, chloramphenicol, erythromycin, penicillin, dihydrostreptomycin, triple sulfa, oxytetracycline, tetracycline, and the vibriostatic agent 0/129 (diamino-di-isopropylpterdine, supplied by Dr. J. M. Shewan). One drop of a young broth culture was spread over the surface of solid medium in a Petri dish and the inoculum was allowed to dry for a short time. Sensitivity discs were then placed on the surface of the agar and the plates were incubated at 30 C. For testing the vibriostatic
agent 0/129, a saturated solution was put in a small cavity in the agar surface on which the organism had already been spread. These plates were incubated in an upright condition. The various plates were examined after 24 hr and the presence of a zone of inhibition of growth around the disc or the cavity was taken as an indication of sensitivity.

The oxidation of tetramethyl-p-phenylene-diamine (oxidase test) was determined by Kovac's method (1956).

Some other physiological activities of these marine bacteria had previously been studied by Tyler et al. (1960) and some of the data have been included in this thesis to give a more complete picture of the physiology of these bacteria.

Lytic susceptibility. - The lytic susceptibilities of the marine organisms were examined according to the method of Tyler et al. (1960). The test solutions, in which the extent of lysis was determined, were distilled water, ASW, and 0.5 M and 0.05 M concentrations of KCl, NaCl, MgCl₂·6H₂O, and potassium phosphate buffer of pH 7.0. The test solutions were dispensed, in 10 ml amounts, in optically matched test tubes. Cells from a shaken broth culture were collected by centrifugation and were resuspended in sea water. The suspension was adjusted to give an O.D. of 0.80 at 500 μm when diluted 1:100; 0.1 ml was added to each tube. After mixing by inversion, the tubes were incubated at 37 C, and their optical densities were read at 500 μm, after 15 min, in
a "Spectronic 20" spectrophotometer (Bausch and Lomb). The per cent residual turbidities were calculated by taking the optical density of the control (ASW suspension) as 100 per cent residual turbidity.

Preparation of Cell Walls

The method finally adopted for preparing the cell walls of the marine bacteria was as follows. The cells were suspended in 0.5 M potassium phosphate buffer, pH 7.0, to give a heavy suspension. Equal volumes of the suspension (3 ml) and "Ballotini" glass beads, approximately 0.007 mm in diameter (C.A. Brinkman Co., New York), were shaken for 20 min at maximum oscillation in a Mickel tissue disintegrator. The disrupted and viscous cell suspension was transferred to a beaker; the cuvettes were washed with M/15 phosphate buffer, pH 7.0, and the washings were added to the beaker. The contents of the beaker were thoroughly mixed and then left standing, after addition of a drop of DNA-ase solution in buffer (0.05 mg per ml), for 20 min. The now watery suspension was then carefully decanted and the beads were washed 3 times with M/15 buffer, the washings being added to the supernate. The supernate was centrifuged at 1,100 x g for 10 min to remove unbroken cells and coarse debris. The crude cell wall fraction was removed and centrifuged at high speed and the cell wall residue was then washed 10 times with dilute buffer. After the final washing, the
cell wall suspension was again centrifuged at 1,100 x g for 10 min to remove any remaining cell debris. The supernate, which contained the cell walls, was examined by electron microscopy and the preparations, which were sufficiently free of cytoplasmic contamination, were pooled and centrifuged at high speed. The cell wall residues were resuspended in a small amount of suspending medium to give about 20 mg dry weight of cell walls per ml.

The cell walls of M.B. 29 were kept suspended in M/15 buffer. Electron microscopy showed evidence of disintegration of the walls when they were washed with distilled water. This was not observed in the case of the cell walls of M.B. 65 and M.B. 98; hence, after the final washing with dilute buffer, the cell walls were washed 4 times with distilled water and finally suspended in distilled water. The cell wall preparations were stored in tightly stoppered tubes at 0°C.

The cell walls of *P. aeruginosa* were prepared as follows. The cells, after harvesting, were suspended in distilled water to give a heavy suspension. Equal volumes of the suspension and "Ballotini" glass beads were shaken in a Mickel tissue disintegrator for 40 min. The contents of the cuvettes were transferred to a beaker, diluted with distilled water, and stirred. No increase in viscosity due to the release of intracellular DNA was observed. Presumably, the organism produces an extracellular DNA-ase; several strains of *P. aeruginosa* have been found to possess this property (Streitfeld et al., 1962).
The suspension was decanted from the glass beads and centrifuged at 1,100 x g for 10 min to remove the coarse debris. The supernate was poured off carefully and centrifuged at high speed; the residue was then washed 4 times with 1 M NaCl solution and 4 times with distilled water. The complete removal of the chloride ions was checked by adding a AgNO₃ solution to a small quantity of the suspension. The final suspension in distilled water was again centrifuged at 1,100 x g to remove any remaining coarse debris and the supernate was then centrifuged at high speed. The residue was resuspended in distilled water and examined with an electron microscope. It was stored at 0°C until used.

**Electron Microscopy of Cell Walls**

The cell wall preparations were mounted on copper grids previously covered by a collodion film. The preparations were shadowed with chromium at an angle of 25 degrees and then examined with a Phillips EM-100 electron microscope. The electron microscopy was performed by Mr. T. Carlisle and Mr. E. J. Jenkins, Physics Department, University of Florida.

**Disaggregation of Cell Walls With Detergent**

The disaggregation of the isolated cell walls in sodium-dodecyl-sulfate (SDS) was studied by adding 0.1 ml of a cell wall suspension to 10 ml of a 0.1 per cent solution of SDS. The contents
were mixed quickly by inverting the tubes and the optical densities
were read at 500 μm in a "Spectronic 20" spectrophotometer. The
tubes were again read after specified intervals up to a period of
30 min. The per cent residual turbidity was calculated for each
reading, taking the optical density in distilled water as 100 per
cent residual turbidity.

Action of Phenol on isolated Cell Walls

It was shown by Weidel et al. (1960) that phenol can solu-
bilise the lipoprotein and lipopolysaccharide components of the walls
of E. coli, thus exposing the rigid mucoprotein layer of the wall.
A similar reaction was tried with the cell walls of M.B. 29. About
200 mg cell walls, dry weight, were transferred to a 250 ml
Erlenmeyer flask and 10 ml of 95 per cent phenol were added and
the flask was shaken to suspend the walls homogeneously. An ad-
ditional 90 ml of the phenol solution were added and the flask was
put on a rotary shaker at 37 C. The cell walls were completely
solubilised giving a clear solution. Water was gradually added to
see if there was any precipitation. The mixture became milky and
two layers were found to separate after standing while a white
precipitate accumulated at the interface. The top layer was de-
canted and the remaining liquid was filtered. The residue was
scraped from the filter paper and washed several times with distilled
water. After dialysis against water for 24 hr, the material was dried first on filter paper pads and then in a previously weighed dish to a constant weight in an oven at 100°C.

The weight of the recovered material was determined and weighed amounts were used for estimating protein, lipid, hexosamine, and reducing substance using the methods described in the following section.

**Chemical Analysis of Cell Walls**

All quantitative analyses were done in duplicates with cell wall samples prepared at different times.

**Dry weights.** Aliquots of cell wall samples were dried, in weighing dishes, in an oven at 110°C. The dishes were weighed at intervals until they reached a constant weight.

**Total nitrogen.** Nitrogen was estimated colorimetrically by Nessler's reaction. One to 2 mg of cell walls (dry weight) were digested with 0.4 ml of 25 per cent (v/v) H₂SO₄ in Kjeldahl flasks. Digestion was continued until the mixture became brown. The flasks were then cooled, a drop of 30 per cent H₂O₂ was added and the heating was resumed until the solutions became colorless. The flasks were allowed to cool and the following solutions were then added to each flask in the order given: 1 ml of 6 per cent sodium citrate, 3.5 ml of 1 N NaOH, 20 ml of deionised water, and 1 ml of Nessler's reagent. After the last addition, the contents
were mixed quickly and the optical densities were read immediately at 505 μm in a "Spectronic 20" spectrophotometer.

The Messler's reagent was made from the commercial Folin and Wu reagent according to the directions given. A standard curve was prepared with known amounts of glycine and cell wall nitrogen was estimated by reference to this curve. Two samples, containing known amounts of glycine, were always run with the cell wall batches to serve as a check of the procedure.

**Total phosphorus.**- This was estimated by using a colorimetric method described by Fiske and Subbarow (1925). Between 2 and 3 mg cell walls (dry weight) were used for digestion with concentrated H₂SO₄. The amount of phosphorus was estimated by reference to a standard curve of K₂HPO₄ solutions.

**Total lipid.**- Lipid material was determined as described by Salton (1953) with a slight modification. The ethereal extract of the cell wall hydrolysates was washed 4 times with distilled water to remove the non-lipid material which was found to have been picked up during ether extraction of the hydrolysates. The lipid material was estimated by evaporating the solvent in a hood at room temperature and weighing.

**Reducing substance.**- The cell walls were hydrolysed as described by Salton (1953) and the hydrolysates were analysed colorimetrically for reducing substance using the anthrone reagent.
Glucose was used to determine a standard curve which served as a reference. The reducing substance was expressed in terms of glucose.

**Hexosamine.** This was estimated by using the Elson and Morgan reaction as described by Kabat and Mayer (1948). Known quantities of glucosamine hydrochloride were used to prepare a standard curve and the results were expressed in terms of glucosamine.

**Protein.** Protein was estimated by the method of Lowry *et al.* (1951) using the Folin-Ciocalteu reagent. About 4 mg of cell walls (dry weight) were suspended in 1 N NaOH and the suspension was incubated at 37°C overnight. A standard curve, using crystalline bovine albumin, was prepared and used as a reference.

**Paper chromatography.** Detection of amino acids and carbohydrates was done by paper chromatography. Whatman no 1 filter paper was used throughout. Glass museum jars were used for ascending and a chromatocab (Research Equipment Corp., Oakland, California) for descending chromatography.

**Amino acids and amino sugars.** Cell walls (20 mg dry weight) were hydrolysed in 6 ml of 6 N HCl in a sealed tube for 16 hr at 120°C. The hydrolysate was decolorised with activated charcoal, filtered, and then dried on a steam bath. The residue was dissolved in a small amount of distilled water and then redried in a vacuum desiccator over NaOH pellets and concentrated H₂SO₄. The final residue was dissolved in 0.5 ml of distilled water.
A platinum wire, with a small loop (2 mm diameter) at one end, was used for spotting on the chromatogram paper. The amino acids and amino sugars were separated by two dimensional chromatography. The first solvent used was n-butanol:acetic acid:water (60:15:25, v/v) and the second solvent was phenol:ammonia (1 ml ammonia added to 200 ml of phenol-water solution; 1 lb phenol + 113 ml water). After development, the solvents were removed by evaporation at room temperature in a chemical hood. For descending chromatography, the spotted paper was first equilibrated with the solvent for 10 to 15 hr before development.

The amino acids were detected by reaction with 0.25 per cent solution of ninhydin in acetone. After spraying the reagent, the paper was allowed to dry and then heated at 100 C for 10 min. The amino acids were identified by position or color and by comparison with chromatograms of known amino acids. For detecting the hexosamines, the Elson and Morgan reaction (Partridge and Westall, 1948) was used on the paper chromatograms.

Sugars.—Cell wall samples were hydrolysed in sealed tubes with 6 ml of 2 N H₂SO₄ for 2 hr at 100 C. The hydrolysates were neutralised with a solution of Ba(OH)₂ to pH 6.5; the precipitate of BaSO₄ was filtered off and the filtrate was evaporated in a vacuum jar containing NaOH pellets and concentrated H₂SO₄. The residue was dissolved in a few drops of distilled water.

The chromatogram papers were spotted using the platinum
wire loop and the solvent used was isopropanol:water (160:40). Multiple ascending development was used for obtaining a greater separation of sugars.

The reducing sugars were detected by spraying with aniline-hydrogen-phthalate reagent (Partridge, 1949). Other reagents such as naphthoresorcinol and phloroglucinol were used for the detection of ketopentoses (Smith, 1960). The colorimetric reaction of Dische (1953) was employed to detect any heptoses; the reaction was carried out with the extracts of cell walls prepared for the sugar chromatography.
EXPERIMENTAL RESULTS

This study involved the preparation and analysis of the cell walls of three selected marine bacteria. It was extended to include the isolation and analysis of the cell walls of a non-marine pseudomonad as well as the examination of some of the morphological and physiological properties of the marine organisms.

The marine bacteria, M.B. 29, M.B. 65, and M.B. 98, developed rapidly in shaken cultures at 30 C in trypticase-ASW broth and maximum growth could be obtained in 12 hr. The growth in stationary cultures, though less rapid, attained approximately the same level. A heavy pellicle was observed on the surface of the stationary cultures, especially in the case of M.B. 29 and M.B. 98. An abundant growth was obtained on trypticase-ASW slants in 24 hr. Twenty-four hr old colonies of M.B. 29 and M.B. 98 were about 2 mm in diameter while those of M.B. 65 were smaller. The colonies were smooth, entire, circular, and slightly raised; they were cream colored in the case of M.B. 29 and M.B. 98, and golden-yellow in the case of M.B. 65.

Shaken broth cultures, 4 to 6 hr old, were examined under phase optics (Plate 1). The marine organisms were pleomorphic in nature, from straight to slightly curved rods. The cells of M.B. 65
Plate 1  Phase contrast micrographs of M.B. 29, M.B. 65, and M.B. 93 (x 2,400).

Fig. 1  M.B. 29
Fig. 2  M.B. 65
Fig. 3  M.B. 93
were thinner and longer (1.5 to 3.0 microns long) than those of M.B. 29 and M.B. 93 (1.0 to 2.0 microns long). Some hemispherical and less phase-dense areas were observed in the case of M.B. 29; they appeared to be protruding from the cells. Their significance was not investigated.

The marine bacteria were found to be Gram-negative. Wet mounts of broth cultures of M.B. 29 and M.B. 93 showed the cells to be motile and the electron micrographs of their cells, fixed with osmic acid, showed the flagellation to be polar and monotrichous (Plate 2). Broth and slant cultures of M.B. 65 were examined at different stages of growth but no motility was observed.

The Hugh and Leifson technique (1953) was used for determining the type of carbohydrate metabolism (Table 1). M.B. 29 and M.B. 93 produced acid from some of the carbohydrates in the open tubes; no reaction was observed in any of the closed tubes. All the positive tests were visible within 24 hr and prolonged incubation revealed no adaptive response. M.B. 65 did not produce acid from any of the carbohydrates tested, in either the open or the closed tubes. The marine organisms gave a positive oxidase test, i.e. the oxidation of tetra-methyl-p-phenylene-diamine with the formation of a blue spot on the filter paper. The control tests with P. aeruginosa and E. coli were positive.
Plate 2  Electron micrographs of M.B. 29 and M.B. 98 showing flagellation.

Fig. 4  M.B. 29 (x 17,000).
Fig. 5  M.B. 98 (x 8,000).
### TABLE 1

CARBOHYDRATE METABOLISM OF THE MARINE BACTERIA

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Open tube</th>
<th>Closed tube</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.B. M.B. M.B.</td>
<td>M.B. M.B. M.B.</td>
</tr>
<tr>
<td>Xylose</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>-3</td>
<td>-</td>
</tr>
<tr>
<td>Glucose</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Mannose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galactose</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Mannitol</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Lactose</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Maltose</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Raffinose</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1 Incubated at 30 C for 72 hr.
2 - = alkaline or no reaction.
3 A = acid production.
and negative respectively. Some other physiological properties of these marine organisms had been previously examined by Tyler et al. (1960). Their data, along with the properties examined above, are given in Table 2.

The sensitivity of these organisms to antibiotics was studied and the data are given in Table 3. Chloramphenicol and erythromycin inhibited all three organisms, whereas triple sulfa inhibited M.B. 29 and M.B. 98 but not M.B. 65. The latter was inhibited by chlortetracycline and oxytetracycline. The vibrio-static compound 0/129, considered to be a specific inhibitor of vibrios, inhibited M.B. 65 but not M.B. 29 and M.B. 98.

**Lytic Properties of Cells**

The lytic properties of the marine organisms in various test solutions were examined by a procedure similar to that used by Tyler et al. (1960). The extent of lysis in test solutions, as measured by the per cent residual optical density, was taken as an indication of the degree of osmotic fragility. The results are presented in Table 4. They indicated that M.B. 65 was more resistant to lysis than M.B. 29 and M.B. 98. The solutions of 0.05 M K-phosphate buffer were most effective in lysing the cells, the per cent residual turbidities being 9 per cent and 8 per cent for M.B. 29 and M.B. 98 respectively as compared to 65 per cent for M.B. 65. In distilled water, M.B. 65 underwent very little
### TABLE 2

**PHYSIOLOGICAL CHARACTERISTICS OF THE MARINE BACTERIA**

<table>
<thead>
<tr>
<th>Property</th>
<th>M.B. 29</th>
<th>M.B. 65</th>
<th>M.B. 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigment</td>
<td>cream</td>
<td>cream</td>
<td>golden-yellow</td>
</tr>
<tr>
<td>Motility</td>
<td>+1</td>
<td>-2</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Starch hydrolysis*</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin liquifaction*</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrite from nitrate*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂S production*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indol production*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Data from Tyler et al. (1960).*

1+ = positive test.

2− = negative test.
## TABLE 3

SENSITIVITY OF MARINE BACTERIA TO ANTIBIOTICS

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone of Inhibition</th>
<th>Amount/disc</th>
<th>M.B. 29</th>
<th>M.B. 65</th>
<th>M.B. 98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlortetracycline</td>
<td></td>
<td>5ug</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>5ug</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td>2ug</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Penicillin</td>
<td></td>
<td>2 units</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td></td>
<td>2ug</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Triple sulphura</td>
<td></td>
<td>50ug</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td></td>
<td>5ug</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>5ug</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vibriostatic compound 0/129*</td>
<td></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*, **, *** = relative degree of inhibition.

- = uninhibited.

* a saturated solution was put in a small cavity in the agar.
## TABLE 4

**LYTIC PROPERTIES OF MARINE BACTERIA**

<table>
<thead>
<tr>
<th>Test Medium</th>
<th>Per cent residual turbidity$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.B. 29</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>62</td>
</tr>
<tr>
<td>0.05 M NaCl</td>
<td>73</td>
</tr>
<tr>
<td>0.5 M NaCl</td>
<td>93</td>
</tr>
<tr>
<td>0.05 M KCl</td>
<td>43</td>
</tr>
<tr>
<td>0.5 M KCl</td>
<td>83</td>
</tr>
<tr>
<td>0.05 M MgCl$_2$</td>
<td>112</td>
</tr>
<tr>
<td>0.5 M MgCl$_2$</td>
<td>115</td>
</tr>
<tr>
<td>0.05 M K-phosphate buffer$^2$</td>
<td>9</td>
</tr>
<tr>
<td>0.5 M K-phosphate buffer$^2$</td>
<td>91</td>
</tr>
</tbody>
</table>

$^1$Per cent residual turbidity
\[
\text{Per cent residual turbidity} = \frac{\text{O.D. in suspending medium}}{\text{O.D. in ASW}} \times 100
\]

$^2$pH 7.0
or no lysis as compared to 62 and 36 per cent residual turbidities of M.B. 29 and M.B. 98 respectively. In both concentrations of MgCl₂, there was no decrease in optical density; in fact, residual optical densities of more than 100 per cent were observed. The results of this experiment showed that M.B. 65 was the least susceptible to lysis with 90 per cent or more residual turbidities in all test media except 0.05 M K-phosphate buffer. M.B. 98 was found to be the most susceptible of the three organisms with M.B. 29 showing intermediate susceptibility.

Preparation of Cell Walls

Considerable difficulty was encountered in obtaining satisfactory preparations of the cell walls of the marine bacteria. Various methods for the disintegration of cells and for the removal of cytoplasmic material from cell walls were tried before a suitable method could be devised. The earlier attempts were confined to M.B. 29, and once the final method was adopted it was used without change for the preparation of the cell walls of the other two marine bacteria. No difficulty, however, was encountered in obtaining clean cell walls of P. aeruginosa.

The methods tried for the isolation of the cell walls generally consisted of the following steps: (a) the disruption of cells, (b) differential centrifugation to remove the coarse debris, and (c) the treatment of the residue, obtained from the supernate, by
different washing and enzymatic treatments. The cells of M.B. 29 were disrupted by lysis in cold or hot water, in 1 per cent aqueous n-butanol, in 0.1 per cent sodium-dodecyl-sulfate, and also by the treatment of cells with 5 per cent trichloroacetic acid (TCA). A large majority of the cells were disrupted as observed with the electron microscope. In the case of n-butanol, sodium-dodecyl-sulfate, and TCA, there was considerable fragmentation of the treated cells (Figure 6); hence these methods were discarded. In other cases, partly empty cell walls could be observed with considerable cytoplasmic material adhering to them.

Lysis of cells with water was carried out by a 20-fold dilution into distilled water of a heavy cell suspension in ASM. After removal of the coarse debris by centrifugation at 1,100 x g for 10 min, the supernate was examined by an electron microscope (Figure 7). Almost all the cells appeared to have lysed and in most cases the cell wall was clearly seen to have broken allowing the cellular contents to escape. The cell walls were, however, heavily contaminated with cytoplasmic material. These cell walls were then subjected to various treatments. Further washing with 1 M NaCl and/or distilled water did not have any effect; after washing 4 times with NaCl, there was some evidence of fragmentation into smaller, irregularly-shaped fragments which were flat in appearance and free of cytoplasmic material (Figure 8). Of particular interest was the presence of small circular discs which were, later on, observed in
Plate 3  Electron micrographs of M. B. 129 after lysing and washing with various procedures.

Fig. 6  An ASM suspension of cells was diluted 20-fold with 0.1 per cent SDS; the suspension was centrifuged at 1100 x g for 10 min. The supernate was centrifuged at high speed and residue was washed 4 times with water (x 9,000).

Fig. 7  An ASM suspension of cells was diluted 20-fold with distilled water and the coarse debris was removed as above; the supernate was examined (x 9,000).

Fig. 8  Cells were lysed and the coarse debris was removed as above. The residue from the supernate was washed 4 times with 1 M NaCl and 4 times with distilled water (x 8,000).
almost every preparation. These discs were of rather uniform size and flat in nature. Such discs have been observed in the cell wall preparations of Salmonella typhimurium (Herzberg, personal communication) obtained in this department. Their nature remained doubtful.

The adhering cytoplasmic material appeared to be protein in nature. Ultraviolet absorption spectra of TCA extracts did not show any appreciable absorption at the 260 mu wavelength at which nucleic acid shows absorption. In an effort to remove adherent matter, various enzymes (RNA-ase, trypsin, ficin, pepsin, bromelin), singly and in combination, were tried. All enzymatic treatments were carried out at room temperature with the material suspended in appropriate potassium phosphate buffer solutions of H/15 concentration. The time of incubation was usually 2 hr. When the material was to be treated with a second enzyme, the hydrogen-ion concentration of the suspension was adjusted and the second enzyme added. The suspensions were then centrifuged, washed 3 times with H/15 buffer, pH 7.0, and the final residues, still suspended in buffer, were examined by electron microscope.

The results did not show any detectable decrease in the amount of cytoplasmic material. With trypsin alone extensive fragmentation of the cell walls was observed (Figure 9). Most of the fragments were free from cytoplasmic contamination. With trypsin and RNA-ase together, the effect of trypsin was even less than when it was used alone; the cytoplasmic material was even more pronounced (Figure 10).
Plate 4  Electron micrographs of cells of M.B. 29 after lysis in water and treatment with various enzymes.

Fig. 9  Cells were lysed by dilution with water and coarse debris was removed by centrifugation at 1,100 x g for 10 min; the residue from supernate was suspended in M/15 buffer, pH 8.0, and trypsin (2 mg per ml of suspension) was added. Mixture was incubated for 2 hr at room temperature; suspension was centrifuged and residue washed 3 times with M/15 buffer, pH 7.0 (x 6,500).

Fig. 10  The procedure used was same as above, except that RNA-ase (0.05 mg per ml of suspension) was added with trypsin (x 13,500).

Fig. 11  The procedure used was same as above, except that only ficin (2 mg per ml of suspension) was used (x 9,000).
The same kind of results was obtained with ficin, alone or in combination with RNA-ase (Figure 11), and other enzymes.

In an attempt to improve the cleanliness of the wall preparations, the conventional method of breaking the cells by vigorous shaking with glass beads in a Mickle tissue disintegrator was employed. Here also a variety of conditions were tried. For suspending the cells during disintegration, 3 media were tried. These were ASW, 1 M NaCl, and 0.5 M K-phosphate buffer, pH 7.0. Washing procedures tried involved 1 M NaCl, M/15 K-phosphate buffer, pH 7.0, and distilled water. Along with these, various enzymes were tried under the conditions described earlier.

Equal volumes of a heavy cell suspension and "Ballotini" glass beads (0.007 mm diameter) were shaken in the Mickle tissue disintegrator for 20 min. The length of time used was arbitrary but later results showed it to be satisfactory. The increased viscosity was reduced by the addition of a trace of DNA-ase and, after removal of the beads, the suspension was centrifuged at 1,100 x g for 10 min. The supernate was then centrifuged at high speed and the residue was subjected to various treatments. Disintegration in sea water suspension followed by 4 washings with 1 M NaCl solution gave a heavily contaminated preparation (Figure 12). Similar results were obtained when 1 M NaCl or 0.5 M buffer was used as the suspending medium and 1 M NaCl solution was used for washing (Figure 13). However, better results were obtained.
when the suspending medium was 0.5 M buffer and the cell walls were washed with M/15 buffer (Figure 14). The cell walls were still contaminated but there was practically no fragmentation. The cell walls, obtained after this treatment, were used for further trials. Washing with water (Figure 15) and the use of various enzymes did not remove the cytoplasmic material. With RNA-ase and trypsin, there was considerable fragmentation of the cell walls as was observed earlier (Figure 16). Ficin, alone or in combination with RNA-ase, gave similar results (Figure 17), as did pepsin (Figure 18).

Since the results obtained by disintegration in the presence of 0.5 M buffer followed by washing with M/15 buffer were the best so far, further washing of the cell walls with the dilute buffer was tried. A gradual decrease in the amount of cytoplasmic contamination was observed and, after 10 washings, the suspension was found to contain cell walls which were practically free from cytoplasmic material (Figure 19). The preparation was considered to be satisfactory for chemical analysis, despite some fragmentation of the cell walls.

The same procedure was then used for preparing the cell walls of M.B. 65 and M.B. 98 and the appearance of the preparations obtained is shown in Figures 20 and 21 respectively. The cell walls of \textit{P. aeruginosa} were prepared as described in Materials and Methods. Clean cell walls were obtained in the first trial (Figure 22).
Plate 5  Electron micrographs of cell walls of M.B. 29 prepared by mechanical disintegration and washing with various procedures.

Fig. 12  Cells suspended in ASW and disrupted in Mickel tissue disintegrator; coarse debris was removed by centrifugation at 1,100 x g for 10 min. Cell walls were washed 4 times with 1 M NaCl and 3 times with distilled water (x 7,500).

Fig. 13  Procedure used was the same as used above, except that cells were suspended in 0.5 M buffer, pH 7.0, during disruption (x 7,500).

Fig. 14  Procedure used was same as for Fig. 13, except that M/15 buffer, pH 7.0, was used for washing in place of NaCl solution and distilled water (x 4,500).

Fig. 15  Procedure used was same as for Fig. 14, except that distilled water was used after washing with M/15 buffer (x 14,500).
Plate 6  Electron micrographs of cell walls of M.B. 29 obtained by mechanical disintegration and action of various enzymes.

Fig. 16  Cells were suspended in 0.5 M buffer, pH 7.0, and disrupted in Nickol tissue disintegrator. Coarse debris was removed by centrifugation at 1,100 x g for 10 min; residue from supernate was suspended in M/15 buffer, pH 7.0, and RNA-ase (0.05 mg per ml of suspension) and trypsin (2 mg per ml of suspension) were added; mixture was incubated for 2 hr at room temperature. Mixture was centrifuged and residue was washed 4 times with M/15 buffer, pH 7.0 (x 10,000).

Fig. 17  Procedure used was same as above, except that ficin (2 mg per ml of suspension) only was used (x 4,500).

Fig. 18  Procedure used was same as above, except that pepsin (2 mg per ml of suspension) was used and suspending buffer had a pH of 1.5 (x 5,500).
Plate 7  Electron micrographs of cell walls of M.B. 29, M.B. 65, M.B. 93, and *P. aeruginosa*.

Fig. 19  Cell walls of M.B. 29 (x 4,500).
Fig. 20  Cell walls of M.B. 65 (x 5,500).
Fig. 21  Cell walls of M.B. 93 (x 4,500).
Fig. 22  Cell walls of *P. aeruginosa* (x 11,500).
It might be emphasized here that the method finally adopted for preparing the cell walls of the marine bacteria and *P. aeruginosa* did not involve the use of any enzyme, except DNA-ase which was used to reduce the viscosity produced during the breakage of the cells of the marine bacteria.

From the electron micrographs, the walls of M.B. 29 and M.B. 98 appeared to be thin and fragile in nature as compared to those of M.B. 65 and *P. aeruginosa*. The cell walls of M.B. 65 appeared to be thicker than those of the other two marine bacteria but thinner than those of *P. aeruginosa*.

**Disaggregation of Isolated Cell Walls by Sodium-dodecyl-sulfate**

The extent of disaggregation of the isolated cell walls of the marine bacteria and *P. aeruginosa* was examined in 0.1 per cent solution of the anionic detergent. The marine cell walls were disaggregated to a greater extent than the cell walls of the non-marine pseudomonad. Thus, the per cent residual turbidity for M.B. 29 was 15 as compared to 55 per cent for *P. aeruginosa* (Figure 23). The values for M.B. 65 and M.B. 98 were 10 and 14 per cent respectively. The greater sensitivity of the cell walls of the marine bacteria to SDS was also evident during the attempts to prepare the cell walls of M.B. 29, when extensive fragmentation of the walls was observed by treating the cells with the detergent (Figure 6).
DISAGGREGATION OF CELL WALLS OF M.B. 29 AND \textit{P. aeruginosa}
BY SODIUM DODECYL SULFATE

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure23}
\caption{Figure 23}
\end{figure}
Action of Phenol on Isolated Cell Walls of M.B. 29

The isolated cell walls of M.B. 29 were shaken with phenol in an attempt to remove the lipoprotein component of the cell walls. It was hoped that an analysis of the insoluble material left behind might indicate the presence of the components detected in the rigid layer of the cell walls of E. coli. On shaking with phenol, the cell walls of M.B. 29 were completely dissolved by the solvent. The material, precipitated after the addition of water, was analysed. The data obtained were: protein, 75 per cent, lipid, 18.5 per cent, hexosamine (as glucosamine), 2.01 per cent, reducing value (as glucose), 0.97 per cent. This composition of the precipitate was found to be very similar to that of the cell walls of M.B. 29.

The cell walls of M.B. 65 and M.B. 93 were also dissolved when shaken with phenol. However, the material precipitated after the addition of water was not enough for quantitative studies.

Chemical Analysis of Cell Walls

Quantitative data. - The values for the quantitative analytical data have been expressed as per cent dry weight of the cell walls. The results are shown in Table 5.

The nitrogen contents of the cell walls of the marine bacteria ranged from 12.2 to 12.8 per cent as compared to 8.4 per cent...
### TABLE 5

**COMPOSITION OF CELL WALLS OF MARINE BACTERIA AND PSEUDOMONAS AERUGINOSA**

<table>
<thead>
<tr>
<th>Chemical constituents</th>
<th>Per cent dry weight cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M.B.</td>
</tr>
<tr>
<td></td>
<td>29</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>12.5</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.9</td>
</tr>
<tr>
<td>Protein</td>
<td>76.0</td>
</tr>
<tr>
<td>Lipid</td>
<td>19.4</td>
</tr>
<tr>
<td>Reducing substance*</td>
<td>1.8</td>
</tr>
<tr>
<td>Hexosamine**</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*expressed as glucose
**expressed as glucosamine
for the cell walls of *P. aeruginosa*. The protein contents of the marine cell walls were also higher, ranging from 74.8 to 76.2 per cent, in contrast to 65.3 per cent for the walls of *P. aeruginosa*. The lipid contents of the walls varied from 18.2 to 19.4 per cent which were about the same found in the walls of *P. aeruginosa*. However, in the marine bacteria the lipid and protein together comprised 93 to 96 per cent of the total dry weight of the cell walls. These values are considerably higher than 85 per cent for the walls of *P. aeruginosa*.

The amount of hexosamine in the walls of the marine bacteria ranged from 0.9 to 1.9 per cent as compared to 2.6 per cent for the walls of *P. aeruginosa*. The reducing values for the walls of the marine bacteria were close to the respective hexosamine values. Since no sugars were detected in their cell walls, the reducing values were a reflection of the hexosamine contents. The reducing value for the walls of *P. aeruginosa* was 10 per cent; the large difference between this and the hexosamine value was explained by the detection of glucose and rhamnose in the cell walls.

**Chromatographic analysis.** - The presence of amino acids, amino sugars, and sugars in the cell walls was detected by paper chromatography of the wall hydrolysates. The various substances identified are given in Table 6. They were identified by comparison with chromatograms of known compounds and from their *R*<sub>f</sub> values. No attempt was made to quantitate any of the constituents; however,
<table>
<thead>
<tr>
<th>Substance</th>
<th>M.B. 29</th>
<th>M.B. 65</th>
<th>M.B. 98</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugars</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Muramic acid*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glycine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Serine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proline</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Leucine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Threonine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Valine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cysteic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Methionine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Identification based on R_g value.*
the size of the spots and the depth of color with ninhydrin gave an approximate idea about the relative concentrations of some of these constituents.

On paper chromatograms, only hexosamine was detected in the walls of the marine bacteria. It gave a positive test with the Elson and Morgan spray reagent and was identified as glucosamine from its $R_g$* value (Smith, 1960). Glucose and rhamnose were found in the walls of P. aeruginosa. The spot of rhamnose was quite weak as compared to that of glucose. The presence of two hexosamines was also detected; their spots were close to each other and the one with the $R_g$ value of 64.2 was identified as glucosamine. The lower spot, with the $R_g$ value of 61, did not correspond with any of the known amino sugars that were used as references. From its $R_g$ value, it was believed to be muramic acid (Smith, 1960). This substance was not available for direct comparison.

The hydrolysis of the walls appeared to be complete since discrete spots were obtained by spraying with ninhydrin. However, poor separation of methionine and valine, and of leucine, isoleucine, and phenylalanine was obtained. The extent of migration of these two groups of amino acids is nearly the same in various

\[ R_g = \frac{\text{distance substance travels from origin}}{\text{distance glucose travels from origin}} \times 100 \]
solvents. The spots of these amino acids, obtained with the unknowns, were roughly comparable in size and shape to those found on the chromatograms of known amino acids.

Most of the amino acids, generally found in the hydrolysates of proteins, were detected in the cell walls of the organisms studied (Plate 8). Of the sulfur-containing amino acids, cysteic acid and methionine were found in *P. aeruginosa*; methionine was also detected in the walls of the marine bacteria. Proline and hydroxyproline were both present in M.B. 93; the former was absent in M.B. 29 and the latter in M.B. 65 and *P. aeruginosa*. Diaminopimelic acid was detected in the walls of all the organisms studied. Spots identified as due to glucosamine were detected in the case of M.B. 65, M.B. 93, and *P. aeruginosa* but not in M.B. 29, although it was found in the latter during sugar chromatography. From the size of the spots and the depth of color with ninhydrin, arginine, lysine, alanine, aspartic acid, and glutamic acid appeared to be present in greater concentrations than the rest of the amino acids; this was found to be true in the case of all the cell wall hydrolysates examined.
Plate 8  Chromatograms of ninhydrin positive substances in cell wall hydrolysates of marine bacteria and P. aeruginosa.

*diaminopimelic acid was identified by one dimensional chromatography in each solvent system.
PLATE 8

1 = alanine
2 = aspartic acid
3 = cysteic acid
4 = diaminopimelic acid
5 = glucosamine
6 = glutamic acid
7 = glycine
8 = serine
9 = hydroxyproline
10 = proline
11 = arginine
12 = lysine
13 = threonine
14 = tyrosine
15 = methionine
16 = valine
17 = phenylalanine
18 = leucine
19 = isoleucine

known amino acids

I BUTANOL : ACETIC ACID : WATER
DISCUSSION

The marine bacteria examined in this study can be tentatively placed in the family Pseudomonadaceae on the basis of the properties studied. These organisms occur both as straight and curved rods but, as noted by Hayes and Burkholder (Bergey's Manual, 1957, p. 90), the borderline between the straight rods found in Pseudomonas and curved rods found in Vibrio is not sharp. According to Shewan, Hodgkiss, and Liston (1954), a sharper differentiation between pseudomonads and vibrios can be made by the use of a vibriostatic agent reported to be a specific inhibitor of vibrios. Since M.B. 29 and M.B. 98 were insensitive to this agent as well as penicillin, were polarly flagellated, and metabolised carbohydrates oxidatively, they could be placed in the genus Pseudomonas.

If the salt requirements of the genus Halobacterium were less rigidly defined, M.B. 65 could be placed in this genus on the basis of production of a golden-yellow pigment (presumably carotenoid), its inability to produce acid from carbohydrates, and its pleomorphic nature. This genus includes species requiring at least 12 per cent salt for growth, a property not possessed by M.B. 65. The genus Pseudomonas has been placed in
the family Pseudomonadaceae together with bacteria which "attack glucose and other sugars either oxidatively or fermentatively." However, the Manual includes in this genus species (Pseudomonas relatica, Pseudomonas nigriaciens) which are without apparent action on sugars. With this precedent, and the fact that this genus includes some non-motile species, M.B. 65 could also be placed in this genus. These facts exemplify difficulties caused by delineating genera on the basis of a single physico-chemical property.

In some marine bacteria, the presence of a cell wall does not offer protection against osmotic lysis (Boring, 1961). If the osmotic fragility of these bacteria is due to the chemical composition of the walls (Brown, 1960), then it was considered possible that some relationship between the degree of osmotic fragility and wall composition might exist. The marine bacteria, selected for this study, differed in lytic susceptibility; such differences have been reported during studies on these bacteria (Tyler et al., 1960; MacLeod and Matula, 1962).

The difficulties encountered during the attempts to prepare clean cell walls of the marine bacteria were two-fold; the fragmentation of the cell walls which was observed during the various washing and enzymatic treatments, and the failure to remove the cytoplasmic material from the walls. The cell walls of Gram-negative bacteria are generally less amenable to various washing
methods which usually succeed in the case of Gram-positive bacteria. In the present study, methods which have been used for the preparation of cell walls of other Gram-negative bacteria were found to be unsuitable. Washing with NaCl and the use of various enzymes resulted in the fragmentation of the walls without the removal of the cytoplasmic material. The fragile nature of the cell walls was evident from these observations.

The proteolytic enzymes, such as trypsin, generally do not affect the integrity of isolated cell walls although they may remove some surface components such as the M protein in streptococcal cell walls. The fragmentation of the cell walls of M.B. 29 by the action of trypsin and other enzymes may be indicative of a nature different from that of other bacteria. It is possible that the predominantly lipoprotein nature of the cell walls of the marine bacteria, together with other features such as the absence of sugars, renders them susceptible to degradation by such enzymes.

The failure of enzymes to remove the adhering cytoplasmic material from the walls could not be explained unless it was assumed that either the material was somehow inaccessible to the enzymes, or it was of a nature not susceptible to such agents. From the electron micrographs, it was difficult to determine whether the material was trapped inside the walls or attached on the outside.

The method finally adopted was the simplest of the various methods tried. It did not involve the use of any enzyme except
DNA-ase which was used to reduce the viscosity produced during the breakage of cells. The method was found to be satisfactory for the other two marine bacteria, especially in the case of M.B. 65.

Some differences in the appearance of the cell walls of the organisms were noticed in the electron micrographs. The walls of M.B. 65 appeared to be thicker than those of M.B. 29 and M.B. 98 but thinner than those of _P. aeruginosa_. The cell walls of the latter two marine organisms were thin and fragile looking. Since M.B. 65 was found to be the least susceptible to osmotic lysis, it appeared possible that the thickness of the walls was partly responsible for resistance to lysis. From ultra-thin sections of cell walls and whole cells, it has been possible to measure the thickness of the walls of some bacteria. Similar studies on marine bacteria might give some useful information in this regard.

The analytical data revealed a close resemblance in the gross chemical compositions of the walls of the marine bacteria. Their nitrogen contents were considerably higher than those found in the cell walls of _P. aeruginosa_ and other Gram-negative bacteria. However, the cell walls of certain halophilic bacteria have a high nitrogen content (Gibbons et al., 1955), in which respect they resemble the marine bacteria. The lipid and phosphorus contents of the walls of the marine bacteria and _P. aeruginosa_ were comparable to those reported for other Gram-negative bacteria (Salton, 1953). Lipid and protein together comprised
about 94 to 96 per cent of the dry weights of the walls of the marine bacteria. Similar data on protein contents of walls of other Gram-negative bacteria are not available but, from their reported nitrogen contents, the amount of lipoprotein in their walls should be much like that of P. aeruginosa (85 per cent).

Some minor differences in the amino acid complement of the walls were observed but these may not be significant. Although no attempt was made to quantitate the amino acids, some of them appeared to be present in greater amounts than others; these were arginine, lysine, alanine, aspartic acid, and glutamic acid. Igramic acid, a characteristic component of the wall mucoprotein, was not detected in the walls of the marine bacteria though it was found in those of P. aeruginosa. It is possible that it was present in amounts too small to be detected by the methods used in this study.

The formation of spheroplasts from marine bacteria (Boring, 1961) by the action of penicillin on whole cells points towards the existence of a penicillin-sensitive component in the cell walls. The effect of penicillin, in the case of the cells of E. coli, is believed to involve an inhibition of the synthesis of the mucoprotein and its incorporation into the walls of the bacterium (Park and Strominger, 1957; Weidel et al., 1960). By this analogy, presence of a mucocomplex in the walls of the marine bacteria is indicated. Moreover, the detection of diaminopimelic acid (DAP)
in the marine cell walls indicated the presence of such a complex
since this amino acid can be considered as an indicator for the
presence or absence of the R-layer links (Weidel et al., 1960).

The cell walls of the marine bacteria were found to be solu-
ble in phenol and they were extensively disaggregated on treatment
with sodium-dodecyl-sulfate (SDS). Both these chemicals were used
by Weidel et al. (1960) for the isolation of the R-layer of the
cell walls of E. coli. In a paper published after the conclusion
of this study, Weidel, Frank, and Leutgeb (1963) have pointed out
that autolytic enzymes can damage the mucoprotein layer (R-layer)
if suitable precautions against their action are not taken during
the preparation of cell walls. They showed that the cell walls
of Salmonella gallinarum, prepared by using SDS, contained the
mucoprotein layer. However, if the walls were prepared by disruption
of cells in the Nickell tissue disintegrator followed by washing,
they were found to be deformed indicating damage to the R-layer.
They were also extensively disaggregated when treated with SDS.
The use of SDS during the preparation of the walls appeared to have
inactivated the autolytic enzymes. It is possible that such enzymes
could have damaged the walls of the marine bacteria during their
preparation resulting in their solubilisation in phenol and ex-
tensive disaggregation by SDS.

An unusual feature found in the walls of the marine bacteria
was the absence of sugars; only glucosamine was found to be present.
The absence of sugars was also evident from the low reducing values which were almost the same as the hexosamine values, the former values being a reflection of the hexosamine contents of the cell walls. A similar absence of sugars has been reported for the cell walls of three halophilic bacteria, *Vibrio costicolus*, *Micrococcus halodenitrificans* and *Pseudomonas salinaria* (Gibbons et al., 1955). However, in the cell walls of another marine bacterium, the only one previously analysed, glucose and a heptose have been reported (Brown, 1960). Evidently, the marine bacteria vary in this regard. The cell walls of *P. aeruginosa* were found to contain glucose and rhamnose; similar sugars have been found in the cell walls of other Gram-negative bacteria.

The cell walls of the marine bacteria were found to contain a lower amount of hexosamine than that found in the walls of *P. aeruginosa* and that reported for the walls of other Gram-negative bacteria. In this respect, the observations were similar to Brown's findings (1960). From the data, some relationship was indicated between the hexosamine contents of the walls and their osmotic fragility, although a perfect correlation was not obtained in the case of all the three marine bacteria. M.B. 98, the most osmotically fragile of the three, contained the lowest amount (0.9 per cent) of hexosamine in its walls; M.B. 65 and M.B. 29 contained 1.7 and 1.9 per cent respectively. Of these two, M.B. 65 was the most resistant to osmotic lysis.
However, if these data are considered from another angle, a more definite relationship is indicated. As mentioned earlier, the electron micrographs of the cell walls indicated that the cell walls of M.B. 65 appeared to be heavier looking, with more body to them, than those of M.B. 29 and M.B. 98 which appeared to be thin and fragile in nature. It is reasonable to assume that the total amount of hexosamine per cell wall would be greater in M.B. 65 than the other two bacteria and least in M.B. 98. On this basis, the hexosamine content of the cell walls would appear to influence the degree of osmotic fragility.

With our present limited state of knowledge regarding the finer details of the wall structure in bacteria, the exact role of hexosamine in determining the strength of the wall can only be speculated upon. There is little doubt, however, that hexosamine is an important component of the mucopeptide layer. Studies on this layer (R-layer) of *E. coli* cell walls (Weidel et al., 1960) have clearly shown that glucosamine, together with muramic acid, is a component of the links which join the spheres in the layer. A backbone of amino sugar, with chains of peptide linked through the -COOH group of muramic acid, has been visualised as the basic structure of the mucopeptide layer. The peptide chains link the adjacent spheres giving a comb-like layer. By the action of enzymes such as lysozyme and enzyme from bacteriophage T2, the links are broken resulting in the disengagement of the spheres from one another (Weidel et al., 1960). These facts point
towards the key role of hexosamine in the structure of the mucopeptide layer. A small amount of this sugar can affect the rigidity since polymers of such compounds are believed to be responsible for the mechanical strength of walls.

The results of this investigation suggested that the weak nature of the cell walls of the marine bacteria was due to a low sugar content. It was considered possible that the amount of sugar in the wall influenced the degree of osmotic fragility exhibited by these organisms. The cell wall appeared to be composed of a soft lipoprotein layer interspersed with a mucopeptide complex, the whole structure being able to confer a characteristic shape to the cells but not osmotic stability.
SUMMARY

Marine bacteria are osmotically fragile and their susceptibility to lysis varies from species to species. The cause of this fragility is believed to be the weakness of the cell wall, and the presence of a low amount of amino sugar in it has been suggested as the cause of this weakness. Three marine bacteria, differing in osmotic fragility, were selected and their cell wall compositions were compared. As these bacteria were suspected to be pseudomonads, the cell walls of Pseudomonas aeruginosa were also studied to provide comparison between marine and non-marine species.

The cell walls of the marine bacteria were prepared by shaking the cells, suspended in 0.5 M K-phosphate buffer, with glass beads in a Mickel tissue disintegrator followed by repeated washing with dilute buffer. The cell walls of P. aeruginosa were prepared by a similar method but the cell walls were washed with NaCl and distilled water. The electron micrographs showed differences in the appearances of the walls; the wall of the more osmotically fragile marine bacterium appeared to be thinner than those of less fragile forms.

The cell walls of the marine bacteria were predominantly composed of lipoprotein. No sugars, except glucosamine, were detected, the reducing values being low and comparable to the respective
hexosamine values. In the marine bacteria, the wall hexosamine content was lower than that of *P. aeruginosa* and of other Gram-negative, non-marine bacteria which have been reported. Some relationship between the hexosamine content of the walls and osmotic fragility was indicated, although a perfect correlation was not obtained.

The walls of the marine bacteria were probably composed of a soft lipoprotein coat; although muramic acid was not detected, the presence of mucopeptide was indicated by the presence of a penicillin-sensitive component and of diaminopimelic acid. The complete solubility of the walls in phenol suggested that the mucopeptide did not form a separate layer but was interspersed throughout the lipoprotein layer. The amount of hexosamine in the walls was related to the osmotic fragility exhibited by these organisms; it is possible that the thinness of the walls also played a role in this property.
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Inder J. Sud was born in Agra, India, on January 22, 1927. He graduated from St. John High School, Agra, in April, 1943. He attended the Agra University for six years, receiving the degree of Bachelor of Science in November, 1947, and the degree of Master of Science in November, 1949. He served as a teacher in Agra College until August, 1959, when he joined the University of Florida. He is now a candidate for the degree of Doctor of Philosophy.

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This dissertation was prepared under the direction of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

April 20, 1963

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