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ABSTRACT

The red marine psychrophile, formerly designated as NRC 1004, was identified as a Vibrio and given the name Vibrio psychroerythrus sp. n. Classification was mainly based on DNA guanine plus cytosine content, fermentation ability and sensitivity to vibriostat O/129. Thin section preparations of intact cells showed triple-layered outer and cytoplasmic membranes, each 6.0 to 7.5 nm wide; mesosomes were occasionally observed. Although a densely staining R layer could not be demonstrated in thin sections, the presence of muramic acid in mechanically-prepared envelopes suggests that the red psychrophile contains a glycosaminopeptide layer of the type found in other Gram negative bacteria. Rod-shaped organelles 10-15 nm wide and of varying length, tentatively identified as rhapidosomes, were frequently seen crossing the septum of dividing cells; inclusion bodies were also observed in the cytoplasmic matrix. Cell division proceeds with an initial constriction and centripetal growth of the cell membrane, and cell membrane septation proceeds to completion before the outer membrane begins to participate in the division process. Growth on enriched solid media and Ca^{+2}-supplemented liquid medium help preserve cellular fine structure; an increased number of membranes were observed in the cytoplasm of cells grown on enriched solid media.

V. psychroerythrus is stable in aqueous solutions of 0.5M NaCl plus 0.1M $MgCl_2$. Morphological and chemical studies

on the lytic susceptibility of the red psychrophile in solutions of low ionic strength showed that both Na^{+} and Mg^{+2} ions preserve the structural integrity of the outer membrane whereas the cell membrane is best stabilized by Mg^{+2}; Ca^{+2} but not K^{+} ions also appear to preserve cell membrane structure. Lysis at 37C induced considerable structural deterioration and heavy leakage; the effects of lysis in an "all salts" solution pH 5.0 were less pronounced. The nature of the particles released under different conditions of lysis was also studied. Fractionation of a 0.5M NaCl lysate yielded three homogeneous, red-pigmented bands on ficoll density gradients. Preliminary morphological and chemical characterization of the fractionated material did not permit us to identify the fractions as cell wall or cell membrane material.

Low beam intensity studies showed that the surface pattern of H. cutirubrum, seen as a regular hexagonal array of particles in shadowed preparations, occurs as a regular honeycomb network containing hexagonal holes 9.5 to 11.0 nm in diameter with a center-to-center repeat distance of 15.5 to 17.0 nm. These structures appeared to be negatively stained by NaCl or heavy cations absorbed from the growth medium. The network shares a number of characteristics with the perforate layer of Lampropedia hyalina.

Résumé

Des études physiologiques et biochimiques nous ont permis d'identifier le psychrophile marin NRC 1004 pigmenté rouge comme membre du genre Vibrio; nous proposons donc le nom Vibrio psychroerythrus sp. n. La position systématique du psychrophile a été basée en grande partie sur la teneur GC % de son ADN, sa capacité de fermenter le glucose et sa sensibilité au vibriostat 0/129. En coupe transversale, la membrane externe de la paroi cellulaire et la membrane cytoplasmique apparaissent comme des structures unitaires (deux feuillets sombres séparés par un feuillet clair) 6.0 à 7.5 nm d'épaisseur; quelques mésosomes ont été observés. Malgré l'absence en coupe transversale d'un feuillet dense aux électrons correspondant à la couche rigide (R) de la paroi cellulaire, la présence d'acide muramique dans l'enveloppe de Vibrio psychroerythrus suggère que le psychrophile possède une couche glucosaminopeptidique du type que l'on retrouve dans d'autres germes Gram négatifs. Des organelles cytoplasmiques en forme de batonnets 10-15 nm d'épaisseur et de longueur variable, provisoirement identifiés comme des rhapidosomes, ont été observés à plusieurs reprises traversant le septum de cellules-filles. Nous avons aussi noté la présence de nombreux corps d'inclusion dans le cytoplasme de cellules intactes. La première phase de la division cellulaire est marquée d'une constriction initiale suivie d'une invagination de la membrane cytoplasmique pour former un septum; la division cellulaire se poursuit avec une crois-

sance centripède de la membrane externe pour finalement libérer deux cellules-filles. La croissance de V. psychroerythrus sur milieu gélosé enrichi ou dans le milieu standard liquide additionné de 0.01M $CaCl_2$ favorise une meilleure préservation des détails morphologiques; la croissance sur milieu gélosé enrichi stimule aussi la formation de membranes dans le cytoplasme.

V. psychroerythrus est stable en solution saline contenant 0.5M NaCl et 0.1M $MgCl_2$. Des études morphologiques et biochimiques sur la lyse du psychrophile en solution saline à faible concentration ont démontré que le Na^+ et le Mg^{+2} maintiennent la structure unitaire de la membrane externe; par contre, le Mg^{+2} est plus efficace que le Na^+ dans le maintien de la membrane cytoplasmique comme structure unitaire. Le Ca^{+2} mais non le K^+ semble aussi stabiliser la membrane cytoplasmique. La lyse à 37C endommage considérablement l'ultrastructure des cellules et donne lieu à une perte importante de composants cytoplasmiques; les effets de la lyse en solution saline acide (pH 5.0) sont moins marqués. Les fragments cellulaires libérés à la suite de différents traitements lytiques ont aussi été soumis à une étude morphologique. Une suspension de cellules ayant subi la lyse en solution saline de 0.5M NaCl a été séparée en trois fractions homogènes sur des gradients de densité à base de ficoll. Des études préliminaires sur la morphologie et la composition chimique de ces fractions ne nous permettent pas d'établir leur origine c.a.d. matériel pro-

venant de la paroi cellulaire ou de la membrane cytoplasmique.

La surface ombragée de Halobacterium cutirubrum semble être formée d'un réseau hexagonal de sous-unités sphériques. Les études à rayons de faible intensité de cellules séchées à partir de solutions salines révèlent un réseau régulier en forme de nid d'abeilles; les trous hexagonaux, 9.5 à 11.0 nm de diamètre, se répètent centre à centre tous les 15.5 à 17.0 nm. Ces structures semblent être teintes négativement par NaCl ou par des cations de poids atomique élevé provenant du milieu de croissance. Ce réseau partage un nombre de caractéristiques communes avec la couche extérieure perforée de Lampropedia hyalina.

Preface

The aims of this research were to characterize, morphologically and chemically, the cell envelope of the red-pigmented marine psychrophile NRC 1004. This organism, originally isolated from flounder eggs by K. Eimhjellen of Trondheim, Norway, grows at 0-19 C but dies and rapidly lyses at 21C or higher. Possibly, elevated temperatures (25 and 38C) activate cell bound phosphatidases resulting in cell lysis and loss of most of the lipid phosphorus (Hagen, Kushner and Gibbons, 1964). The marine psychrophile is stable in cold seawater or in a solution containing 0.5 M NaCl and 0.1 M $MgCl_2$; cells are stabilized by some divalent cations but lyse in distilled water. Lysis in cold distilled water released 60-70% of the cell lipid-phosphorus and 80-85% hexosamine; most of these compounds could be recovered by high speed (100,000 xg) centrifugation (Korngold and Kushner, 1968). Acid, temperature and water-lysed cells showed an increased electrophoretic mobility. It was suggested that intracellular material released from cells lysed in water or at acidic pH is adsorbed on the surface thus increasing their electrophoretic mobility whereas the increased mobility of temperature-lysed cells is not due to adsorbed material but rather to an irreversible surface change (Madeley, Korngold, Kushner and Gibbons, 1967). Studies of the lipid composition have shown that the marine psychrophile contains a large proportion of monoenoic acids (mainly hexadecenoic acid), no cyclopropane acids and significant amounts of

phosphatidyl ethanolamine (Kates and Hagen, 1964). The organism also produces exocellular proteinases with relatively high temperature optima that hydrolyze casein, gelatin, B-lactoglobulin and haemoglobin (McDonald and Chambers, 1963). Purification of the proteolytic preparation yielded three enzymatically active fractions, two of which gave a similar electrophoretic band on polyacrylamide gel. From this and other evidence, Nunokawa and McDonald (1968) suggested that the red psychrophile produces two types of proteinase.

It was the purpose of this work to study the fine structure of the psychrophile and correlate the existing data on its lytic behavior at elevated temperatures, acidic pH and low ionic strength with morphological changes accompanying lysis. It was felt that such morphological studies could provide some information on the site of action of various cations, and possibly a means of isolating cell wall fractions through a non-degradative process (as obtained by Costerton, Forsberg, Matula, Buckmire and MacLeod 1967 with a marine pseudomonad). Although a good deal of work has been done on this organism, its classification was still uncertain and it was thought that a serious attempt should be made at assigning it generic standing.

The results of these studies will be presented in five separate chapters dealing with the taxonomy, general ultrastructure, structural changes occurring under various conditions of lysis and preliminary work on the isolation and chemical characterization of its cell wall. The last chapter

(Chapter 6) deals with some morphological aspects of the cell wall envelope of the red-pigmented, Gram negative, extreme halophile, Halobacterium cutirubrum, which resembles the psychrophile in that it requires Na^+ cations for its stability.

The Introduction (Chapter 1) will consist of a literature review of the structural and physiological roles of cations in marine and extremely halophilic bacteria, a major aspect of the present studies. Aspects of the morphology, chemistry and synthesis of the various layers of Gram negative cell envelopes will also be discussed.

Chapter 1

General Introduction

Chapter 1

I. Marine and Halophilic bacteria

1- Definition:

Over the past thirty years, salt-requiring bacteria have consistently attracted the interest of scientists. In 1946, 6.5% of the bacteria described in Bergey's Manual of Determinative Bacteriology were of marine origin (ZoBell, 1946) and this proportion increased to 20% by 1964 (Scholes and Schewan, 1964). It would not be surprising to see this figure increase within the next decade.

It was soon realized that the salt response of "halophilic" bacteria was not homogeneous. In an effort to confine the growing confusion, a number of classification schemes based on salt tolerance were proposed (Flannery 1956, Ingram 1957 and Larsen 1962). The following terminology, which will be used in this thesis, basically follows that of Baxter and Gibbons (1956) and Kushner (1968).

Non-halophiles - those organisms which grow without any added NaCl in the medium and do not grow in concentrations of NaCl above 0.5 M. They may well require trace amounts of Na^+ or other cations for growth, a requirement which could be easily satisfied by the contaminating ions of commercial media, as suggested by MacLeod (1965) and confirmed by Bovallius and Zacharias (1971).

Marine - those organisms which grow well in 0.2-0.5 M NaCl; below this level, the rate and extent of growth are roughly proportional to the amount of Na^+ present in the medium. Poor growth is obtained at about 1 M NaCl. (MacLeod 1965).

Moderately halophilic - those organisms which require 0.5 M NaCl for growth and survival; they grow well in 1-2 M NaCl and can grow in concentrations up to 3 or 4 M NaCl.

Extremely halophilic - those organisms which require 2.0-2.5 M NaCl for growth and survival; they grow well in 4.0-4.5 M NaCl and will grow in saturated or near saturated NaCl solutions (5.5-6.0 M).

Halotolerant - those organisms which have a low salt requirement for growth but which exhibit a salt tolerance range that can extend as high as 4 M NaCl.

It is then clear that a classification of organisms based on salt requirement and tolerance is only a working scheme. The great number of organisms included in this scheme and as great a number of salt responses cannot but introduce some overlapping in the terminology.

2- Distribution - Economic Importance:

The non-halophiles, usually isolated from soil and fresh water environments, are the most abundant and extensively studied group of organisms (Brown, 1964a). Their importance in industry and medicine has long been acknowledged.

Most of the organisms which fall in the marine category are of marine origin. In the past decade or so, they have captured the interest of an increasing number of investigators. Although the relationship between specific requirements for salts and the physiology of living matter is of great interest, the potential of marine bacteria in restoring the equilibrium of polluted offshore regions of the sea, which is rapidly becoming the universal septic tank, cannot be overlooked. Moderate halophiles such as Vibrio costicolus cause spoilage of salted products (Smith 1938, Larsen 1962). Some studies of their physiology have been carried out (Flannery and Kennedy 1962, Forsyth and Kushner 1970) though less interest has been shown in them than in extreme halophiles. Extreme halophiles have been isolated from salted meat products (Harrison and Kennedy 1922, Flannery 1956) and also occur in natural saline environments such as the Dead Sea (Volcani 1940) and the salt flats at the southern end of San Francisco Bay. The envelopes of many extreme halophiles contain bright carotenoid pigments which apparently protect the organisms from photochemical damage caused by direct sunlight (Dundas and Larsen 1963, Larsen 1962). Next to the non-halophiles, the halotolerant organisms are the most numerous group; this is not surprising because the inherent flexibility of "halotolerant" would permit some members of the marine and moderately halophilic groups to be classified as halotolerant. The salt tolerance of organisms isolated from various sources has been the subject of several reports (Shah and deSa 1964, Ingram 1957, and Forsyth, Shindler, Gochnauer and

Kushner 1971).

II. Cation requirements

1. Na^+ - Marine

Marine bacteria are generally more psychrophilic (ability to grow in the cold) in character than terrestrial species and prefer seawater or 3% NaCl to freshwater in the medium for growth (MacLeod 1965). Are these significant differences which make marine bacteria a unique group of organisms, distinctly set apart from their terrestrial counterparts? What physical and/or chemical characteristics of the marine environment are essential for the growth and proliferation of marine organisms?

The organic nutrition of marine bacteria in complex and synthetic media as studied by Ostroff and Henry 1939, MacLeod, Onofrey and Norris 1954 and Burkholder 1963 showed that although these bacteria appear to have a characteristic preference for amino acids as a carbon, nitrogen and energy source, there is nothing that could be considered unique about their organic nutritional requirement (MacLeod 1965).

If nutrients do not appear to be the distinguishing trait of marine bacteria, would it not be the salts or NaCl present in the marine environment? If such was the case, one would expect the salt requirement to be a stable character of marine bacteria. Korinek (1927) found that original differences between freshwater and marine bacteria were not eliminated after a one-year cultivation on laboratory media

and Stanier (1941) failed to train marine agar-digesting bacteria to grow at lowered seawater or salt concentrations. On the other hand, ZoBell and Michener (1938) and ZoBell (1946) presented evidence that most of their marine bacteria which required seawater in the medium on initial isolation developed a capacity to grow in freshwater media. Such confusing reports on the ability to train organisms of marine origin to grow on freshwater media did not support a definite physiological role of salts in the growth of marine bacteria. MacLeod (1965) later suggested that the nutrients used in these experiments were most probably contaminated with inorganic ions, the presence of which might conceivably have a bearing on the conflicting reports on the stability of the halophilic character of marine bacteria. MacLeod's suggestion proved to be correct following the work of Bovallius and Zacharias (1971) on the metal content of commercially prepared media. Richter (1928), Mudrak (1933) and Dianova and Voroshilova (1935) were among the first to observe a specific Na^+ requirement for the growth of marine bacteria; the most recent report was published by Baumann et al (1972).

Although it had been established that Na^+ was specifically required for the growth of marine bacteria, the specific role of Na^+ on their physiology and/or structure had yet to be elucidated. Since marine bacteria lysed when suspended in seawater diluted with distilled water, it was thought that Na^+ acted as an osmotic stabilizer. The limited ability of related monovalent cations and sucrose to replace

the Na^+ requirement for growth and stability of marine bacteria (MacLeod and Onofrey 1957, Payne 1958, Buckmire and MacLeod 1965, Korngold and Kushner 1968) suggests that the function is not only osmotic. However, an osmotic role for Na^+ has not been completely dismissed because a number of salts and sucrose reduce the Na^+ requirement for the growth of a marine Vibrio (Pratt and Austin 1963, Pratt 1963). From these considerations, it appears that the degree of specificity for Na^+ varies with the bacterial species, but that a minimal, irreplaceable requirement for Na^+ nevertheless remains.

Efforts to determine the function of Na^+ in the metabolism of marine pseudomonads revealed that Na^+ is specifically required for the oxidation of exogenous substrates (Tomlinson and MacLeod 1957, MacLeod, Claridge, Hori and Murray1958, Payne 1960 and MacLeod and Hori 1960) where the amounts of Na^+ required varied with the substrate to be oxidized (MacLeod, Claridge, Hori and Murray 1958); however, Na^+ was not specifically required by any of the enzymes tested in extracts derived from these cells (MacLeod, Claridge, Hori and Murray 1958, MacLeod and Hori 1960). Similarly, Pratt and Happold (1960) observed a Na^+ requirement for indole production from tryptophan by whole cells but not by cell extracts of a marine Vibrio. These findings suggested that Na^+ might be specifically required for the activation of a transport mechanism. Drapeau and MacLeod (1963) using the non-metabolizable substrates α-amino-

isobutyric acid and D-fucose to separate transport from metabolic processes, showed that the marine pseudomonad specifically required Na^+ for the uptake of these compounds; this system was further investigated by Drapeau, Matula and MacLeod (1966) and Wong, Thompson and MacLeod (1969). MacLeod (1965) suggested that previously observed differences in the quantitative requirements for Na^+ for the oxidation of various substrates by a marine bacterium (MacLeod, Claridge, Hori and Murray 1958) could be accounted for if one assumed the presence of different permeases in the cell membrane with quantitatively different Na^+ requirements for activation. In 1960, Payne reported a Na^+ - dependent glucuronate uptake mechanism in Pseudomonas natriegens; similar studies were later extended to other marine bacteria (Rhodes and Payne 1962). Recently, Webb and Payne(1971) confirmed earlier reports (Rhodes and Payne 1967 and 1968) of a Na^+ - dependent mannitol transport mechanism in Vibrio natriegens. It should be noted that in the course of Payne's studies on this organism, the generic standing of Pseudomonas natriegens was changed to Vibrio (Webb and Payne 1971). It would then appear that one of the functions of Na^+ in marine bacteria is to activate transport mechanisms. For more general information on transport mechanisms, the reader is referred to the excellent review of Oxender (1972). Pardee (1968) also reviewed some current ideas on the isolation and characterization of membrane transport proteins.

Structural roles of Na^+ in marine bacteria have been proposed. Studies on the stability of intact cells and envelope preparation of the marine Pseudomonas type IV when suspended in various solutions of inorganic salts and non-ionic solutes suggested that Na^+ stabilized the cell wall by shielding the negative charges of the outer layer (Costerton, Forsberg, Matula, Buckmire and MacLeod 1967) or of the mucopeptide layer (Buckmire and MacLeod 1965). Na^+ may also be required to maintain the cell membrane intact (Thompson, Costerton and MacLeod 1970, DeVoe, Thompson, Costerton and MacLeod 1970). Na^+ and Mg^{+2} ions contribute to the structural integrity of the cell wall of psychrophilic marine Vibrios (Kennedy, Colwell and Chapman 1970 and see Chapter 4).

Marine bacteria require Na^+ for their growth. The specific Na^+ requirement does not appear to be linked to an osmotic phenomenon but rather to an activation of a number of transport mechanisms and to the stability of the cell envelope of marine bacteria. Is the specific requirement for Na^+ a unique characteristic of the marine bacteria? This question will be answered in the following section.

Na^+ - Extreme halophiles

The Na^+ requirement is by no means unique to marine bacteria; there have been some well documented reports of non-halophilic species that require Na^+ for growth (Sistrom 1960, Bryant, Robinson and Chu 1959, Goldman, Diebel and Niven 1963). NaCl is essential for the growth and stability

of moderate and extreme halophiles (reviewed by Brown 1964, Larsen 1967 and Kushner 1968). Since extremely halophilic bacteria are of secondary importance to this thesis, I will not dwell at any length on this vast subject but only underline the salient features of extreme halophilism.

Extreme halophilism has already been defined (see "Definition") as a requirement of organisms for near-saturated or saturated NaCl solutions for growth. One of the first questions that comes to mind is what is the internal salt concentration of cells that live in an extremely halophilic environment? Do extreme halophiles possess an active pumping mechanism that would maintain a low ionic strength within the cell's cytoplasm? It appears that extremely halophilic bacteria do not exclude salt at all; in fact, the ionic strength within the cytoplasm is as high as the external environment (Brown 1964, Larsen 1967, Kushner 1968) though some ions, such as K^+, may be selectively transported into the cytoplasm at the expense of Na^+ (Christian and Waltho 1962).

Halophilic enzymes require high salt concentrations for activity and most of these enzymes are inactive in the absence of salts (Larsen 1967, Kushner 1968). Holmes and Halvorson (1965) have shown that this salt inactivation is reversible for malate and alanine dehydrogenases. The degree of enzyme activation by Na^+ and K^+ varies with each enzyme system. At equimolar concentrations of KCl or NaCl, higher activities were observed with the former salt in

citrate cycle enzymes of Halobacterium salinarium (Aitken and Brown 1969), in cytochrome oxidase, cysteine desulfhydrase and several dehydrogenases of Halobacterium salinarium (Baxter and Gibbons 1954, 1956, 1957) and in an aminoacyl t-RNA synthetase of Halobacterium cutirubrum (Griffiths and Bayley 1969). The aspartic transcarbamylase of Halobacterium cutirubrum was equally active in 3-4 M NaCl or KCl (Liebl, Kaplan and Kushner 1969) as was the $NADH_2$ oxidase of the halophilic strain AR-1 (Hochstein and Dalton 1968). Extracellular proteolytic enzymes of Halobacterium salinarium are more active in the presence of NaCl than KCl at equimolar concentrations (Norberg and Hofstein 1969); this is consistent with the high Na^+ and low K^+ ion content of the external environment of extreme halophiles. It would then appear that many enzymes of the extreme halophiles have a preference but not an absolute requirement for high concentrations of K^+ ions which is consistent with the observation that the intracellular concentration of K^+ is unusually high in these cells.

The 70S ribosomes of Halobacterium cutirubrum are composed of 60% RNA and 40% protein; in this respect, the ribosomes of Halobacterium cutirubrum are similar to those of Escherichia coli although the former differ in their high content of acidic proteins (Bayley and Kushner 1964). The halophilic ribosomes exist in their 70S state when suspended in 3-4 M KCl plus 0.1 M $MgCl_2$ and dissociate into subunits upon dilution of both salts. The KCl requirement for ribo-

somal integrity is quite specific and cannot be replaced with Na^+ or other monovalent cations. Bayley pointed out that the high concentration of cytoplasmic K^+ may neutralize mutually repulsive negative charges on the acidic proteins and RNA thereby enabling these components to aggregate and form stable and functionally active ribosome structures. It has been possible to reconstitute halophilic ribosomal particles (Bayley 1966) and elaborate a cell-free protein synthesizing system which required KCl, NaCl and NH_4Cl for maximum activity (Bayley and Griffiths 1968). Clearly, the halophilic nature of these ribosomes is a function of K^+ and Mg^{+2} ions rather than Na^+. Norberg et al (1973) recently suggested that low salt concentrations caused conformational changes in the aspartate transcarbamylase of H. cutirubrum.

Extreme halophiles require high concentrations of NaCl for their growth. This requirement does not appear to be directly linked to their enzymes and ribosomes which function better in KCl than in NaCl alone. As first suggested by Abram and Gibbons (1961), Na^+ ions may play a structural role by shielding negative surface charges of the halophilic envelope. The presence of more acidic than basic amino acids in the envelope proteins of halobacteria (Brown 1963, Kushner 1968), the excess of negative charges at the envelope surface of Halobacterium halobium as studied by titration techniques (Brown 1965) and the stability of protein extracted envelopes of Halobacterium cutirubrum in water (Kushner and Onishi 1966) strongly support the hypothesis of Abram and

Gibbons (1961). A more direct proof that Na^+ plays a structural role in stabilizing negatively charged envelopes was provided by Brown (1964b) who showed that the marine bacterium NCMB 845 whose envelope normally disintegrated at about 1-2% NaCl, required 5% NaCl to prevent lysis, following acylation with succinic anhydride. Thus, the addition of carboxyl groups to the surface made the envelope halophilic.

Using electron spin resonance (ESR) spin-labelling techniques, Hsia, Wong and MacLennan (1971) recently demonstrated that the envelope dissolution of Halobacterium salinarium in low NaCl concentration involves changes in protein conformation and lipid-protein interactions. This hypothesis is in keeping with previous ESR studies on synthetic lipid bilayers (Butler, Dugas, Smith and Schneider 1970).

High Na^+ concentrations may also activate transport mechanisms as suggested by the Na^+-dependent uptake of glutamate in Halobacterium salinarium (Stevenson 1966).

It would then appear that the need for high concentrations of NaCl for the growth and stability of halobacteria results from characteristics of their envelope rather than characteristics of their cytoplasm.

2. Mg^{+2} - Non halophiles

In non halophiles, the role of Mg^{+2} extends through various levels of cellular organization and function. Although such a topic is beyond the scope of this thesis, a few examples will be cited in order to provide some insight on the extent to which Mg^{+2} participates in non-halophilic

bacterial systems. Mg^{+2} (and Ca^{+2}) stabilized the envelope of Pseudomonas aeruginosa (Brown and Melling 1969 a and b) and affected the cell wall composition of Aerobacter aerogenes when the latter was grown in a Mg^{+2} - depleted medium (Ellwood and Tempest 1967). The degree and type of reaggregation of Mycoplasma membrane lipids is Mg^{+2} sensitive (Razin, Ne'eman and Ohad 1969). The absence of Mg^{+2} inhibits the respiratory activity of an Azotobacter sp. (Goucher, Sarachek and Kocholaty 1966) and adversely affects protein synthesis by dissociating ribosomes into subunits (McCarthy 1962).

Mg^{+2} - Marine

Marine bacteria, grown in a synthetic medium, usually require 4 to 8 mM Mg^{+2} for growth (MacLeod and Onofrey 1956) although some organisms, including the one described in this thesis, may require as much as 40 mM Mg^{+2} (Korngold and Kushner 1968). The Mg^{+2} requirement of marine bacteria is high compared to that of terrestrial species such as Escherichia coli (Young, Begg and Pentz 1944) and Bacillus subtilis (Feeney and Garibaldi 1948) which require 0.02 and 0.08 mM respectively. In 1956, MacLeod and Onofrey observed a marked interaction between Mg^{+2} and Ca^{+2} in marine bacteria; although both ions are required for growth, the requirement for one of the ions usually varied inversely with the level of the other in the medium. Lewis and Corpe (1964) also found that Ca^{+2} could partially replace Mg^{+2} for the growth of a red marine bacterium. Brown (1960 and

1962) observed an increased susceptibility of the marine bacteria NCMB 845 to proteolytic digestion when grown in a cation-depleted medium and concluded that protein conformational changes were responsible for the increased sensitivity. He further suggested that under such growth conditions, Mg^{+2} was not available to shield the carboxyl groups of the envelope proteins, thereby changing the conformation of the proteins. In his review, Brown (1964) states that divalent cations such as Mg^{+2} and Ca^{+2} can be expected to stiffen a lipoprotein membrane by forming salt bridges between neighboring carboxyl groups just as they were shown by Langmuir and Schaefer (1937) to stiffen fatty acid monolayers. Mg^{+2} cross-linkages of envelope components have been cited in order to explain the lytic behavior of some marine bacteria when suspended in solution of low salt concentrations (Costerton, Forsberg et al 1967 and DeVoe and Oginsky 1969).

Mg^{2+} - Extreme halophiles

Extreme halophiles require 100 to 500 mM Mg^{+2} for optimal growth (Larsen 1967, Kushner 1968). It would appear that Brown's hypothesis on the Mg^{+2} salt bridges in the envelopes of marine bacteria (Brown 1964) may also be valid for the extreme halophiles. As early as 1955, Brown and Gibbons suspected a structural role of Mg^{+2} in the halophilic envelope from the observation that the rod-shaped cells of Halobacterium salinarium changed to spheres when grown in a medium low in Mg^{+2} but normally high in Na^{+}.

Kushner and Onishi (1966) found that removal of lipids from the envelope of Halobacterium cutirubrum greatly increased the Mg^{+2} concentration required for stability; they interpreted these results to indicate that lipids were involved as Mg^{+2} binding sites. The work of McClare (1967) on the bonding between proteins and lipids in the envelopes of Halobacterium halobium lends strong support to Brown's hypothesis on the structural role of Mg^{+2}. McClare suggested that the lipids in the envelope of Halobacterium halobium are bound to at least two types of proteins by polar and non polar bonds and that the polar type of bonds may involve a Mg^{+2} chelate of protein and lipid head groups.

Extreme halophiles differ from marine bacteria in the amounts of Mg^{+2} required for optimal growth but are similar to marine bacteria in that Mg^{+2} is used to crosslink various components of their envelopes.

3. K^+ - Non halophiles

The physiology of K^+ in bacterial systems is poorly documented. In non-halophiles, K^+ is required for protein synthesis in intact and cell-free extracts of Escherichia coli (Ennis 1971) and for the incorporation of amino acids into polypeptides (Sachs 1957). There has also been a report that, in Escherichia coli, K^+ may not be free in solution but rather bound to proteins (Damadian 1969).

K^+ - Marine

Washing the marine pseudomonad B16 in 0.05 M $MgSO_4$ and suspending the organism in 0.2 M NaCl + 0.01 M KCl produced

an immediate increase followed by a slow decrease in optical density (i.e. 1st and 2nd phase of optical change). The 1st phase of optical change (increase in O.D.), resulting from a decrease in cell volume, was due to an interaction of NaCl with components of the cell envelope giving rise to a contraction of the envelope and shrinkage of the cell. The 2nd phase of optical change (slow decrease in O.D.), resulting from a gradual increase in cell volume, was shown to be a K^+ - dependent process in which the rate of the O.D. decrease was similar to the rate of uptake of isotopic K^+ by the cells. K^+ would appear to be osmotically active since its accumulation was accompanied by an increase in cell volume (Matula, Srivastava, Wong and MacLeod 1970). Thompson, Costerton and MacLeod (1970) came to similar conclusions in their morphological studies of the same system. That K^+ is osmotically active is not unique to marine bacteria for it has been observed in mitochondria (Agatha and Rasmussen 1966) and in cells of Bacillus coli communis (Orskov 1948).

K^+ - Extreme halophiles

Substantial amounts of K^+ are needed for the growth of extreme halophiles (Brown and Gibbons 1955, Gochnauer and Kushner 1969). The contribution of K^+ to the activity of halophilic enzymes and the stability of ribosome structure has already been discussed (see Na^+ requirement). Vitamins and carbohydrates are stimulatory to the growth of Halobacterium sp. if the low K^+ concentration of the normal growth medium (Onishi, McCance and Gibbons 1965) is increased

to 0.1% K^+ (Gochnauer and Kushner 1969), much of which becomes 'cell bound' (Gochnauer and Kushner 1971). One striking feature of Halobacterium sp. is their ability to maintain high intracellular K^+ concentrations for days in the absence of an energy source (Gochnauer and Kushner 1971) or oxygen uptake (Ginzburg, Sachs and Ginzburg 1970).

These preliminary reports suggest that K^+ is osmotically active (not bound) in marine bacteria and that the unusually high concentration of K^+ ions in Halobacterium sp., often exceeding that required to saturate their internal fluid, may occur in two physiological states: free in the cytoplasm and bound to proteins or other polyanions.

4. Concluding Remarks

Salt requirements (and possibly salt tolerance) are genetically stable characteristics of a bacterium which are expressed in the amino acid composition and sequence of proteins (Larsen 1962). Most marine bacteria are motile, gram negative rods and facultative psychrophiles (Hucker 1954); some of these organisms can grow at high hydrostatic pressures (Albright and Henigman 1971 and Palmer and Albright 1970). The question of the existence of specific marine bacteria may be linked to the presence of trace amounts of toxic heavy metals in the sea. Escherichia coli will survive and grow in natural or artificial seawater only if a chelating agent is present in the medium (Scarpino and Pramer 1962, Jones 1964). Marine bacteria require inorganic ions for their growth and metabolism

and for maintaining the integrity of their cell envelope. Na^+, which is specifically required for growth and whose intracellular concentration equals that in the growth medium (Matula et al 1970), activates Na^+ - dependent mechanisms for transporting substrates into the cells. Stability of the cell envelope of marine bacteria rests on the capacity of Na^+ to shield negative charges on the cell surface layers and of divalent cations to form cross linkages between various components of the cell envelope. A similar mechanism exists in the envelopes of extreme halophiles. The integrity of the ribosomes and the activity of many enzymes in the extreme halophiles is K^+ rather than Na^+ - dependent, a finding which is consistent with their unusually high internal K^+ concentration. The presence of an excess of acidic proteins in the envelopes and ribosomes of extreme halophiles may represent one if not the major adaptive response of these organisms to survival in an extremely halophilic environment.

III. Psychrophilism

1. Terminology and some physiological aspects

Growth temperature ranges have been used to divide microorganisms into thermophiles, mesophiles and psychrophiles. It is generally agreed that the former two are quite distinct from each other whereas there exists a considerable amount of confusion and lack of agreement on the dividing line between mesophilic and psychrophilic microorganisms.

The etymology of the word psychrophile stems from the Greek words psychros (cold) and philos (loving). The term psychrophile was coined by Schmidt-Nielsen (1902) to describe the ability of certain organisms to grow at 0C. Early investigators in the field objected to this name because it implied that these organisms preferred low temperatures whereas ample experimental evidence had already shown that psychrophiles grew better at 20C or higher (Ingraham and Stokes 1959). In fact, most psychrophiles are cold-tolerant rather than cold-loving. That freezing or near freezing temperatures were not the optimal growth temperatures of psychrophiles stimulated some workers to seek other descriptive epithets which would more accurately convey the temperature characteristics of this group of organisms. Among others, psychro-tolerant (Horowitz-Wlassowa and Grinberg 1933), eurythermic (Zobell 1934) and thermophobic (Edsall and Wetterlow 1944) had been proposed.

Most psychrophiles are Gram negative, asporogenous rods which belong to the genus Pseudomonas and, to a lesser extent, to the Vibrio, Achromobacter, Flavobacterium and a few other genera (Ingraham and Stokes 1959, Liston 1957, Witter 1961, Colwell 1962, Stokes 1963 and Larkin and Stokes 1966). Psychrophilic microorganisms are very widely distributed in nature having been isolated from air, water, soil, plants, animals and a great variety of foods (Stokes 1963). The greatly increased use of frozen and chilled foods in recent years and the increasingly longer periods of time between

their production and consumption have greatly enlarged the importance of psychrophilic bacteria in the food industries.

The question of the definition of psychrophiles is a difficult subject in which different conclusions can be reached depending on whether the minimal, optimal or maximal growth temperature is used as point of reference. In 1954, Hucker side-stepped this problem when he proposed two psychrophilic sub-groups not based on any of the three cardinal temperatures. According to his proposal, facultative psychrophiles were organisms that grew at 0C and 32C whereas organisms that grew at 0C but not at 32C made up the obligate psychrophilic group. Baxter and Gibbons (1962) also divided the psychrophiles into obligate and facultative sub-groups based on the capacity of the former to grow rapidly below 20C and the latter at or above 20C. That the unique property of psychrophilic organisms was the ability to grow well at 0C had been recognized from the very early studies of psychrophiles (Forster 1887, Schmidt-Nielsen 1902). Some definitions of psychrophilism specifically made use of this fact i.e. psychrophiles are organisms that produce visible growth following a two-week (Ingraham and Stokes 1959) or a one-week (Stokes 1963) incubation at 0C or organisms that exhibit a generation time of 48 hours or less at 0C (Ingraham 1958). From these considerations, the organism studied in this thesis has been designated as an obligate psychrophilic bacterium since it grows well in the temperature range 0-19C; at 21C, growth ceases and the

organism rapidly dies and lyses at higher temperatures (Hagen, Kushner and Gibbons 1964).

Physiological aspects of psychrophilism in microorganisms

Many so-called psychrophilic organisms have shown a surprisingly high maximum temperature for growth i.e. as high as 45C (Bedford 1933). Witter (1961) claimed that, since the maximum growth temperatures of organisms capable of low temperature growth are so variable, the inclusion of a limiting maximum growth temperature in the definition of a psychrophile would serve no useful purpose.

The minimum growth temperature of psychrophiles lies in the vicinity of -10C where growth is very slow (Bedford 1933, ZoBell 1934 and Smart 1935). Below this temperature, growth inhibition probably results from the progressive dehydration and the concomitant increase in salt concentration of the medium due to the progressive removal of water by freezing.

Psychrophiles have been defined most frequently on the basis of their optimum temperature for maximum growth rate; for most psychrophiles, this optimum temperature usually lies above 20C (Ingraham and Stokes 1959) and approaches that of mesophiles. Some authors (Hess 1934, Ingraham 1958) have found that a more representative optimum growth temperature can be obtained when it is defined in terms of the generation time of cells in the exponential phase of growth; it follows that the temperature at which the generation time is shortest is the optimum growth temperature for these cells.

Cell yield has also been proposed as a parameter in determining optimal growth temperature. However, this approach suffers from a serious drawback in that very long periods of incubation may be required to obtain maximal cell numbers (Hess 1934).

2. Psychrophily: a function of

A. Thermolabile enzymes:

Psychrophilism is an important aspect of marine life in view of the fact that, with the exception of surface waters which are subject to latitude and seasonal variations, the temperature of the sea is approximately 5C (Zobell 1946). Though psychrophily is not a characteristic unique for bacteria of marine origin, its physiological basis in marine bacteria and other microorganisms is of considerable interest. The ability of psychrophiles to grow at low temperatures may be due to some unique property of their enzymes and/or cell envelopes.

The van't Hoff-Arrhenius plot is commonly used in chemical kinetics to determine the activation energy of chemical reactions. If the natural logarithm of the reaction rate (k) is plotted as a function of the reciprocal of the absolute temperature (T), the slope of the straight line obtained is proportional to the energy of activation of the reaction (ΔE) i.e. $\ln K = -\frac{\Delta E}{RT} + C$ where C is an integration constant. Microbiologists have substituted bacterial growth rate for reaction rate in the van't Hoff-Arrhenius equation to obtain the temperature characteristic of growth (μ), a

quantity which is analogous to activation energy. It was hoped that this parameter, which indirectly measured the mean activation energy of the limiting enzyme systems of a cell population, would provide a novel approach to the definition of psychrophiles and mesophiles. Ingraham (1958) compared the growth of three psychrophilic strains of Pseudomonas with strains of mesophilic Escherichia coli and Pseudomonas aeruginosa and found the temperature coefficient (μ) for growth to be lower for the psychrophilic strains. The values of μ for the psychrophilic strains varied between 8,700 and 9,400 calories/mole while that of the mesophiles was 14,000 calories/mole. Ingraham suggested that this difference in temperature coefficient might be due to the different activation energies of psychrophilic and mesophilic enzymes or, stated differently, that the enzymatic activities of the psychrophiles were less affected by changes in temperature. However, Hanus and Morita (1968) obtained lower μ values for mesophilic than psychrophilic strains of Vibrio. From the discrepancies of their results with those of Ingraham (1958), Hanus and Morita (1968) concluded that no consistent differences existed in the μ values for mesophiles and psychrophiles and that attempts to try to correlate the μ of an organism's enzymes with its temperature range of growth would be futile. In fact, Brown (1957) and Ingraham and Bailey (1959) had previously found that the activation energies of corresponding enzyme systems in psychrophilic and mesophilic strains of Pseudomonas were

very similar for the enzyme examined.

Morita and Burton (1963) and Burton and Morita (1963) reported that the inactivation of malic dehydrogenase of Vibrio marinus, a facultative psychrophile with an optimum growth temperature of 24C and a maximum of 30C, was very rapid at 30C. The enzyme activity in cell-free extracts was 20 times greater than in intact cells although the cell-free enzyme lost almost twice as much activity with each 10C degree drop than did the intact cell enzyme. The authors suggested that an interplay between thermolabile enzymes and cell permeability may govern the maximal temperature at which this organism can grow. Morita and Robison (1964) came to similar conclusions from their work with an obligately psychrophilic strain of Vibrio marinus on the temperature inactivation of metabolic systems involved in oxygen uptake.

Grant, Sinclair and Nash (1968), following up earlier work by Sinclair and Stokes (1965) on the temperature-sensitive glucose fermentation in the psychrophilic yeast Candida gelida, confirmed that the heat-induced (35C) loss of fermentative activity was due to inactivation of a temperature-sensitive pyruvate decarboxylase, the only temperature-sensitive enzyme of the alcoholic fermentation pathway.

Malcolm (1969) ascribed the molecular basis of obligate psychrophily in Micrococcus cryophilus to two temperature-sensitive aminoacyl t-RNA synthetases which recognize their

cognate species of t-RNA only when the latter is in a low-temperature configuration. A few other reports have described thermolabile enzymes in psychrophiles (Hagen and Rose 1962, Langridge and Morita 1966, Purohit and Stokes 1967).

Though heat sensitivity of enzymes seems to be one, if not the principal, of the known factors limiting the maximum growth temperature of psychrophiles, it should not be taken for granted that all the enzymes isolated from psychrophilic organisms function best at low temperatures. The optimum temperature for the activity of some proteolytic enzymes of an obligate marine psychrophile ranges from 40-45C (McDonald and Chambers 1963) whereas a phosphatidase, in the same organism, functions at temperatures exceeding 20C (Hagen, Kushner and Gibbons 1964).

B. Cell permeability:

In addition to thermolabile enzymes, psychrophilism may also be linked to cell permeability. In the temperature range of 0-20C, little glucosamine uptake was measured in a mesophilic strain of the yeast Candida whereas the uptake was relatively unaffected in an obligate psychrophilic strain of the same organism (Baxter and Gibbons 1962). The authors commented that an increased permeability at low temperatures may be of prime importance for the survival of organisms that thrive in the cold.

Elevated temperatures adversely affect the structural integrity of psychrophilic cell envelopes. An obligate marine

psychrophile, which grows in the temperature range of 0-19C, rapidly lyses at temperatures above 21C releasing significant amounts of lipid-phosphorus and ultraviolet-absorbing material into the medium (Hagen, Kushner and Gibbons 1964, and see Chapter 4). Thermally-induced leakage has been reported in obligately psychrophilic strains of Vibrio marinus (Morita and Robison 1964, Haight and Morita 1966) and Candida nivalis (Nash and Sinclair 1968).

C. Lipid composition

Growth of microorganisms at low temperatures results in an increase in the proportions of unsaturated fatty acids (Kates 1964). The lipid composition of an obligate marine psychrophile (a marine Vibrio - see Chapter 2) differed from a mesophilic strain of Serratia marcescens in its lack of cyclopropane acids and a greater amount of unsaturated fatty acids when both organisms were grown at their optimal growth temperature (Kates and Hagen 1964). Over the temperature range of 43C to 10C, the proportion of monoenoic acids in E. coli cells grown in glucose-minimal and in yeast extract-supplemented media increased and the amount of cyclopropane acids decreased with decreasing temperature (Marr and Ingraham 1962). These findings suggest that the enzymatic transfer of a methylene group to the double bond of the monoenoic acids to form cyclopropane acids is either very slow or inhibited at low temperatures; other factors such as composition of the growth medium may also be involved in this saturation reaction (Kates 1964). The contribution of

lipid composition to psychrophilism is not clear at the present time.

A few isolated reports indicate that the salinity of the growth medium may affect the temperature tolerance of bacteria. When two psychrophilic strains of Vibrio marinus were grown in a medium containing 0.7% salts (lowest salinity permitting growth), the maximum growth temperature was 10C lower than in a medium containing 3.5% salts (Stanley and Morita 1968). Goldman, Deibel and Niven (1963) also reported that a number of strains of lactic acid bacteria isolated from meat-curing brines developed a requirement for NaCl at elevated temperatures.

IV. Morphology and Chemistry of the bacterial cell envelopes

The concentric layers that surround the cytoplasm make up the cell envelope; the number, morphology, localization and chemical composition of these layers may vary from one group of organisms to another. It is then not surprising that a number of texts (Gunsalus and Stanier 1960, Salton 1964, Rogers and Perkins 1968) and reviews (Glauert and Thornley 1969 on the "Topography of the Bacterial Cell Wall", Osborn 1969 on the "Structure and Biosynthesis of the Bacterial Cell Wall", Robertson 1966 on the "Design Principles of the Unit Membrane", Korn 1969 on "Cell Membranes: Structure and Synthesis" and Tomasz (1971) on "The Bacterial Cell Surface" have been devoted to the structural and chemical complexity of the bacterial cell envelope.

The major components of the bacterial envelope have been grouped under four headings: surface appendages, structured or amorphous outer layers, cell wall, and cell membrane.

1. Surface appendages

The flagella, thread-like structures responsible for cell motility, occur singly or in groups at the polar ends or the periphery of the bacteria. Bacterial flagella, attached to both the cell wall and cell membrane by a hook-basal body complex (DePamphilis and Adler 1971 a and b), consist almost solely of protein (Kerridge 1961, Iino 1969). The flagellins, a term coined by Astbury (1955) to designate the globular proteins of flagella, are of low molecular weight ranging from 20,000 to 40,000 in different species (Abram and Koffler 1964, Kerridge, Horne and Glauert 1962). Eucaryotic flagella and cilia exhibit a nine plus two organization of tubules (reviewed by Gibbons 1967, Hookes, Randall and Hopkins 1967) whereas no such arrangement has been observed in bacterial flagella. Electron microscope and X-ray diffraction studies suggest that bacterial flagella are constructed of three to five helices of flagellin units about 5 nm in diameter and wound around a common center (Kerridge, Horne and Glauert 1962, Lowry and Hanson 1965). Flagella grow by the addition of flagellins at their tips (Asakura, Eguchi and Iino 1968, reviewed by Kushner 1969 and Iino 1969).

2. Structured and amorphous outer layers

Capsules and slime layers lie external to the rigid cell wall of many microorganisms. Like the surface appendages, these extramural layers can usually be removed without affecting the viability or the morphological integrity of microorganisms (Salton 1964).

Production of capsules is subject to both phenotypic and genetic variations. The amount and chemical composition of capsular material is dependent on the organism and characteristics of its growth medium (Salton 1964). Microorganisms which produce capsules always form a layer of slime similar if not identical in composition to the capsular material (Rose 1965). It has been shown that the absence of capsules in a usually encapsulated organism may result from a mutation (Duguid and Wilkinson 1953 and 1954) or the production of capsule-degrading enzymes by the organism (Kass and Seastone 1944).

Polysaccharides are the principal component of capsules and slime layers although a D-glutamic polypeptide capsule has been found in Bacillus anthracis (Salton 1964); these components are usually quite distinct chemically from those of the cell wall (Salton 1960, Stacey and Barker 1960) although Salton (1964) does make reference to several instances of chemical overlap. Antigenic specificity has been associated with bacterial capsules; the texts by Boyd (1962) and Humphrey and White (1963) should be consulted for detailed information on this subject.

In addition to capsules and slime layers, the occurrence of intercellular cementing substances has been reported. Cellulose facilitates cellular cohesion in Sarcina ventriculi (Canale-Parola, Borasky and Wolfe 1961), whereas an unidentified polysaccharide acts as the intercalating material in Lampropedia hyalina (Chapman, Murray and Salton 1963). Cementing materials share some common features with capsules in that they are also polysaccharides and not essential for the survival of the organism.

Removal of non-structured layers, contiguous with the outer surface of the cell wall, has been described in a number of Gram negative bacteria. Treatment with sodium lauryl sulphate removed a lipoprotein layer in E. coli cells (Murray, Steed and Elson 1965) while lipopolysaccharide was liberated from Gram negative bacteria by washing in water or monovalent cations (Corpe and Salton 1966, Roberts, Gray and Wilkinson 1967). A loosely bound outer layer containing protein, lipid and carbohydrate, was removed from a marine pseudomonad by washing intact cells in 0.5 M NaCl (Forsberg, Costerton and MacLeod 1970). In none of the above cases did extraction of these external layers affect the viability of the cells.

A number of bacteria exhibit a structured outer layer. Negatively stained and shadowed preparations of intact cells and cell envelopes have revealed a regular hexagonal pattern in Halobacterium sp. (Houwink 1956, Kushner and Bayley 1963); this topic will be discussed in more detail in Chapter 6.

Other macromolecular layers have been observed in Spirillum serpens (Houwink 1953, Murray 1963), Micrococcus radiodurans (Work and Griffiths 1968), Lampropedia hyalina (Chapman, Murray and Salton 1963) and Bacillus polymyxa (Nermut and Murray 1967). For a more detailed account on this subject, the reader is referred to the review by Glauert and Thornley (1969). The subunits of the structured surface layer of Halobacterium halobium (Marshall, Wicken and Brown 1968) and of Spirillum serpens (Buckmire and Murray 1970) have been isolated and shown to consist mainly or entirely of proteins. At present, the structural and/or physiological significance of these layers is not clear.

3. Cell Walls

A. Introduction

The morphology and chemistry of the bacterial cell walls, the outermost layer of the cell envelope primarily responsible for cell rigidity, were extensively reviewed by Salton (1964), Rogers and Perkins (1968) and Ghuysen, Strominger and Tipper (1968); other reviews will be mentioned in the course of the discussion.

Purified cell wall preparations have been isolated following physical disruption of intact cells and treatment of the crude wall preparation with chemical agents such as trypsin, pepsin and nuclease (see Salton 1964 for a comprehensive review). The cell wall of Gram positive organisms, whose thickness ranges from 15 to 80 nm, accounts for 20 to 40% of the cell's dry weight while the Gram negative cell

wall, approximately 8.0 nm thick, contributes less than 15% of the cell dry weight (Salton 1964). The cell wall composition of these two groups of bacteria differs markedly; the Gram negative bacteria contain more lipids, less amino sugars and a full range of amino acids while only a few amino acid residues are present in the Gram positive species (Perkins 1963). Variations in the composition of the cell walls are of taxonomical significance; the cell wall lipid content permits Gram differentiation of bacterial species (Salton 1964), lipopolysaccharides often reflect the endotoxic and antigenic properties of certain bacteria (Simmons 1971) and the presence of phage receptor sites (Salton 1964).

The Gram negative cell wall will be discussed under three headings, corresponding to the three general areas of their wall: the outer membrane, the outermost layer of 'unit membrane' appearance, the periplasmic space, an electron transparent area between the outer membrane and the cytoplasmic membrane, and the glycosaminopeptide layer (R layer) which confers rigidity to the cell.

B. Outer Membrane

The spatial relationship of the outer membrane with other layers of the Gram negative cell wall is represented in the model profiles (based on the biochemical and ultrastructural studies of Martin (1963 - see figure 1) and DePetris (1967 - see figure 2). Models for the cell wall of Escherichia coli (Weidel, Frank and Martin 1960), Proteus vulgaris (Burge and Draper 1967) and other Gram negative

bacteria (Glauert and Thornley 1969) do not differ significantly with that presented in figure 2.

In thin section, the outer membrane appears as a 'unit membrane' (two electron dense layers separated by one of lesser density) 6.0 to 10.0nm thick and usually exhibiting a wavy, undulating outline which can be altered by different fixation methods (Murray, Steed and Elson 1965). Thin sections of phenol-extracted Gram negative cells no longer show the outer membrane (Weidel, Frank and Martin 1960) suggesting that lipopolysaccharides are associated with this layer. From his cytochemical and ultrastructural studies, DePetris (1967) proposed a model (figure 2) in which lipids form the basic layer in the outer membrane of E. coli with proteins and lipopolysaccharides in close association on both its inner and outer faces. This model is attractive because it explains the double-track staining pattern as a property of the polar heads of oriented sheets of lipids and phospholipid molecules similar to those postulated in the 'unit cytoplasmic membrane' which gives a similar staining pattern. Burge and Draper (1967) came to similar conclusions from their X-ray diffraction studies on the cell wall of Proteus vulgaris from which they suggest that the hydrocarbon chains of the oriented sheet of lipids in the outer membrane lie at right angles to the plane of the wall; subsequent work led them to conclude that the outer membrane is a lipopolysaccharide 'unit membrane' (Burge and Draper 1967a). The reader is referred to Osborn (1969) for a

Figure 1. Cell wall changes after treatment with lysozyme or penicillin.

a) Gram positive bacterium in which the cytoplasm (CP) within the cytoplasmic membrane (CM) is surrounded by a presumably pure glycosaminopeptide cell wall (MPW).

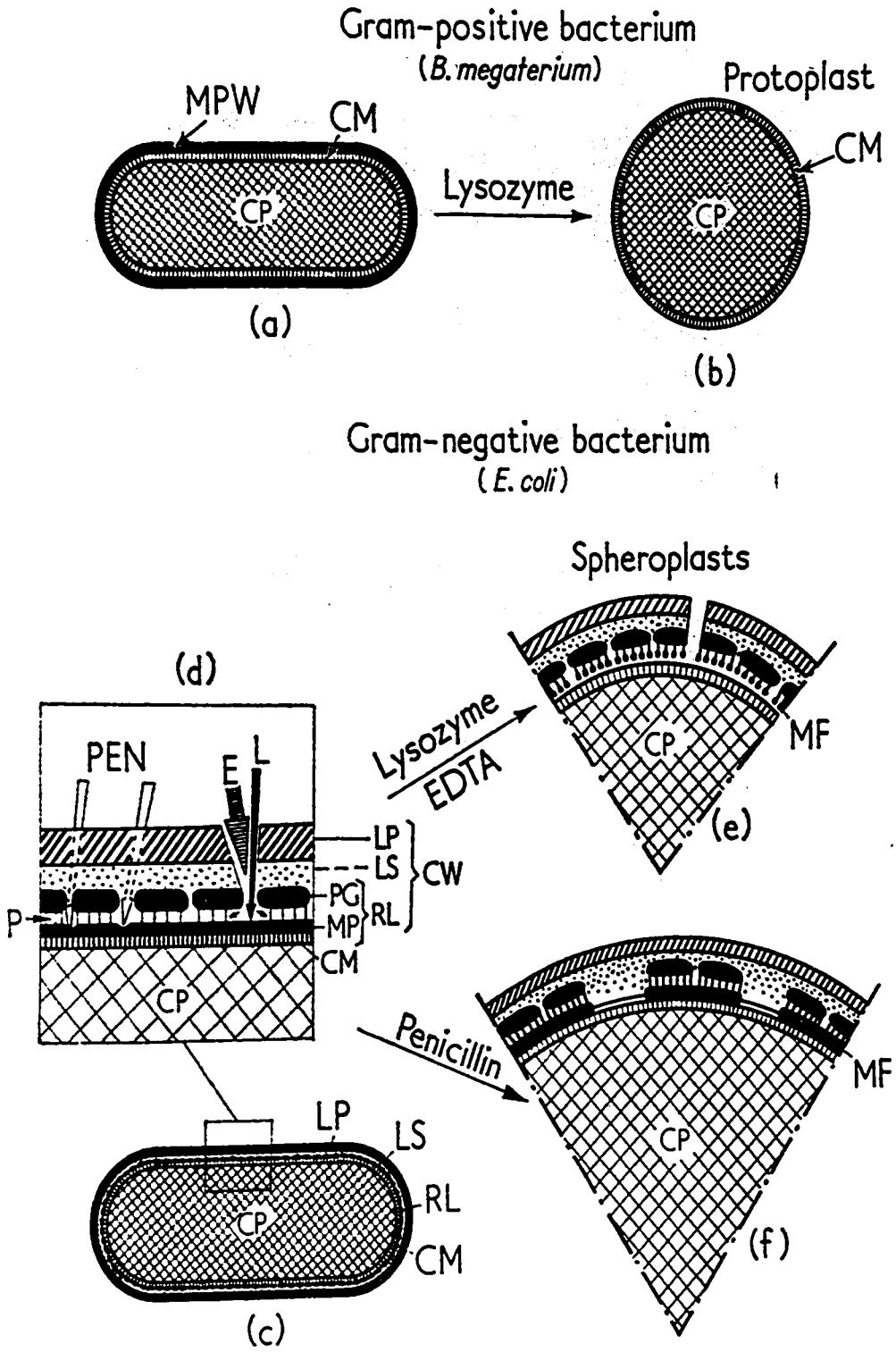
b) Lysozyme dissolves the cell wall and liberates the naked protoplast.

c) and d) Gram negative bacterium with complex triple-layered cell wall. Separation of the layers can be achieved by solvent extraction and by treatment with proteolytic (P) enzymes. d) Both EDTA(E) - lysozyme (L) and penicillin (PEN) treatments induce depolymerization of the glycosaminopeptide polymer (MP) to a different degree and by a different mechanism.

e) Small glycosaminopeptide fragments (MF), covalently linked to other cell wall components, remain in the wall after lysozyme treatment.

f) Penicillin spheroplasts may retain larger fragments of a non-rigid glycosaminopeptide (MF).

LP, lipoprotein; LS, lipopolysaccharide; RL, rigid layer; PG, protein granules. The LP and LS regions correspond to the molecular sieve layer and periplasmic space respectively. Illustrations are taken from H.H. Martin (1963).


Gram-positive bacterium
(B. megaterium)
MPW
CM
CP
Lysozyme
Protoplast
CM
CP
(a)
(b)
Gram-negative bacterium
(E. coli)
Spheroplasts
(d)
PEN
E
L
LP
LS
CW
PG
RL
MP
P
CM
CP
Lysozyme
EDTA
MF
CP
(e)
Penicillin
MF
CP
(f)
LP
LS
RL
CM
CP
(c)

comprehensive review on the structure and synthesis of lipopolysaccharides. The observations by Frank and Dekegel (1962) and DePetris (1967) that thin sections of lipopolysaccharides extracted with phenol from E. coli cell walls exhibit a 'unit membrane' appearance support the view of Burge and Draper (1967a). The outer membrane of a Gram negative marine bacterium, removed by simple manipulation of the cation concentration of the suspending medium, was found to be rich in proteins and phospholipids (Costerton, Forsberg, Matula, Buckmire and MacLeod 1967) and to contain hexosamines and non-amino sugars (Forsberg, Costerton and MacLeod 1970).

Present evidence suggests that the major components of the outer membrane of Gram negative bacteria are lipoproteins or lipopolysaccharides or both (Costerton 1970) arranged in some type of mosaic structure (Glauert and Thornley 1969). However, Costerton (1970) expressed his reservations on the complete identity of complexes obtained through the action of rigorous extraction procedures with those that exist in the intact cell. The observation that enzymatic disruption of one cell envelope component releases many other envelope components (Whiteside and Corpe 1969) supports Costerton's view.

The function of the outer membrane would seem to be that of a selectively permeable layer (Costerton 1970). Although the cell walls of Gram positive bacteria may retard the passage of certain molecules such as streptomycin and

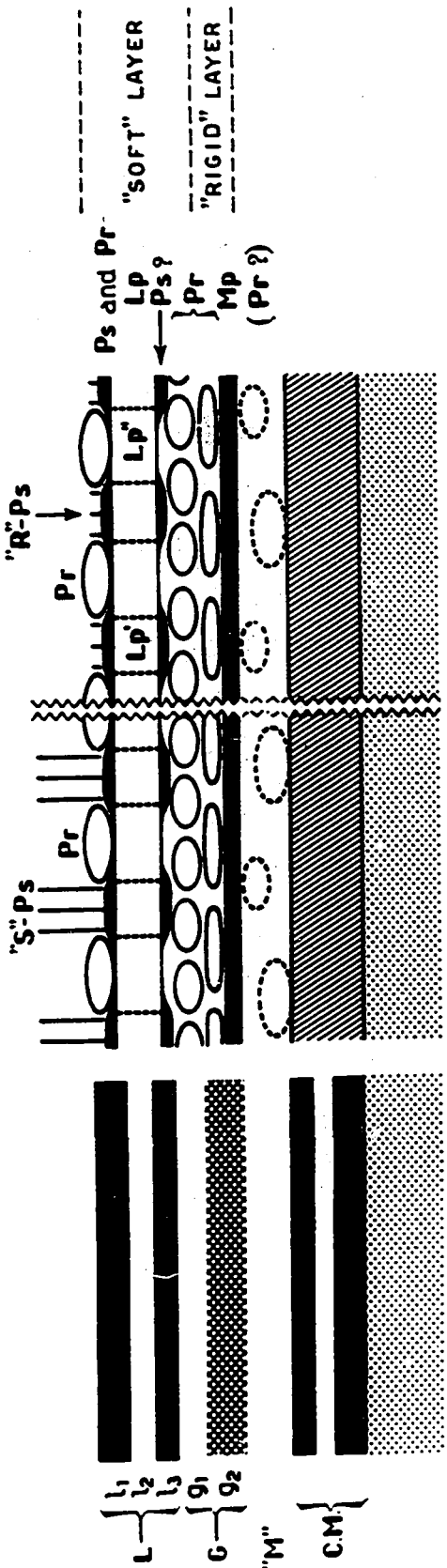
Figure 2. Model profile of E. coli cell wall.

The dimensions, shapes and arrangements of the actual single constituent elements of the cell wall layers are still unknown. The approximate correspondence of the various components with the structure visible in thin section is shown on the far left.

The left part of the diagram represents the wall of a 'smooth' cell in which the polysaccharides (S-Ps) are assumed to be composed of a basal structure and specific side chains; on the right, the wall of a 'rough' form in which the polysaccharides (R-Ps) lack the specific side chains.

Proteins (Pr) and polysaccharides (Ps) are considered to be distributed in a mosaic on the surface of the wall (l_1) and polysaccharides are possibly present at l_3. The bulk of the lipids (L_p) is situated at the level of the L membrane where Lp' and Lp" indicate schematically the existence of different classes of lipids associated either with polysaccharides or proteins. Globular proteins, covalently linked to the glycosaminopeptide layer (MP) are represented at the level of the G layer. The possible existence of other materials (proteins?) in the M layer between g_2 and the cytoplasmic membrane (CM) is also indicated.

Illustration taken from S. de Petris (1967).


"SOFT" LAYER
"RIGID" LAYER
Ps and Pr
Lp
Ps ?
Pr
Mp
(Pr ?)
"R"-Ps
Pr
Lp'
Lp"
"S"-Ps
Pr
L1
L2
L3
G
91
92
"M"
C.M.

neomycin (Kagan 1968), it is only in the Gram negative cell wall that we find a layer which acts as a "molecular sieve" (term coined by Mitchell 1961) in restricting the passage of certain large molecules (reviewed by Costerton 1970). The demonstration that spheroplasts, in which the outer membrane is demonstrably damaged, are able to carry on normal metabolic activities (Martin 1963) and that cells of a marine pseudomonad, from which the outer membrane is completely removed, are able to concentrate a non-metabolizable amino acid analogue at an undiminished rate (DeVoe, Thompson, Costerton and MacLeod 1970) suggests that the outer membrane does not play an important role in selective permeability or in active transport. Stated briefly, the Gram negative bacteria are not surrounded by two cytoplasmic membranes but by a cytoplasmic membrane within a molecular sieve layer.

The cell wall morphology of the Gram negative Halobacterium sp. is not typical of Gram negative bacteria in that it lacks a tripartite outer layer (see Chapter 6). Thin sections have revealed a fairly homogeneous layer approximately 7.5nm thick with a 'serrated edge' which corresponds to the regular surface pattern in sectional view (Cho, Doy and Mercer 1967, Steensland and Larsen 1969, and see Chapter 6). Chemical studies have indicated that the composition of the cell envelope is different from that of the cell walls of other Gram negative bacteria: in particular, the content of amino sugars (Stoeckenius and Kunau 1968) and simple sugars (Kushner, Bayley, Boring, Kates and Gibbons 1964) is

low. Envelopes of Halobacterium sp. also lack muramic acid and diaminopimelic acid (Brown and Shorey 1963, Kushner et al 1964) indicating that these bacteria do not have a rigid glycosaminopeptide layer of the type found in other Gram negative bacteria (see section on "Glycosaminopeptide"). Identification of the "rigidity factor" in extreme halophiles must await further experimentation.

C. Periplasmic space

The periplasmic space, a term coined by Mitchell (1961), describes the electron transparent area of the Gram negative cell wall between the cytoplasmic membrane and the molecular sieve layer (figure 1). The observation that proteolytic enzymes "unzip" the cell wall of intact E. coli at this level indicates that proteins are associated with this area (DePetris 1967). Periplasmic lipopolysaccharides have also been localized in E. coli and Salmonella typhimurium spheroplasts using ferritin-conjugated antibody (Shands 1966). Forsberg, Costerton and MacLeod (1970) reported the presence of proteins, lipids and carbohydrates in the 'underlying layer' of a marine pseudomonad following a non-degradative dissection of its cell wall.

A number of enzymes such as glucose-6-phosphatase (Mitchell 1961), DNA'ase and RNA'ase (Brockman and Heppel 1968) and a few others (cited by Costerton 1970) are associated with the periplasmic space. The most thoroughly documented case for the periplasmic localization of an enzyme is that of alkaline phosphatase based on the evidence

that the enzyme is not bound to either the membrane or wall fragments of E. coli cells (briefly reviewed by Brockman and Heppel 1968). The enzyme could not be detected immunologically at the outer surface of S. typhimurium cell walls (Schlesinger and Olsen 1968). The latter authors also found that when spheroplasts are formed, active alkaline phosphatase is released into the medium without a concomitant release of "intracellular" enzymes. The inactive subunits of this enzyme are synthesized in the cell cytoplasm and then transferred to the periplasmic space where they form active dimers (Torriani 1968).

Murray's comment (1968) that "things happen" in the gap between the cytoplasmic membrane and the innermost layer of the cell wall has been substantiated with the localization of a number of enzymes whose activities are important to cell function.

D. Glycosaminopeptide layer (R layer)

a. Morphology:

The constituent which is thought to be mainly responsible for the mechanical strength of the cell wall has alternatively been called murein, mucopeptide, mucocomplex, peptidoglycan and glycosaminopeptide (cited in Glauert and Thornley 1969). Salton (1964) strongly criticized the use of the terms mucopeptide and mucocomplex because they are chemically vague and physico-chemically misleading as the

wall fraction responsible for its rigidity is far from mucoid; the term glycosaminopeptide which more accurately describes the chemical nature of the rigid layer will be used in this thesis. Glycosaminopeptides have not been isolated from extreme halophiles and are absent in protoplasts and L-forms; otherwise, these compounds are present in all the bacteria so far studied (Glauert and Thornley 1969) and in other microbial groups such as blue-green algae and Rickettsiae (Salton 1964).

This layer accounts for 5-10% (w/w) of the Gram negative cell wall and as much as 80-90% of the Gram positive Micrococcus lysodeikticus (Rogers and Perkins 1968). The R layer has been visualized as a rigid 'bag-shaped' macromolecule surrounding the Gram negative (Weidel and Pelzer 1964) and Gram positive (Costerton 1970) bacteria. In thin section, the R layer of Gram negative bacteria is 3.0 to 8.0 nm thick and appears as a densely staining layer in the 'matrix' of the periplasmic space (figure 1 and 2). The R layer is sometimes closely allied to the inner surface of the outer membrane (Murray, Steed and Elson 1965). Another interesting morphological aspect of the R layer is the presence of granules on the surface of shadowed preparations of purified E. coli 'mucopolymer' (Martin 1963, and see figures 1 and 2). These granules can be dissolved by proteolytic enzymes liberating a cell-shaped, smooth, lysozyme-sensitive 'mucopolymer' layer (Frank and Martin 1960). The function of these protein granules has led to some speculation. DePetris

(1967) suggested that the R layer and protein granules could be the sites of 'surface-bound' enzymes; it is also possible that these granules have arisen from the aggregation of periplasmic enzymes.

b. Protoplasts, spheroplasts and mureinoplasts

The glycosaminopeptide layers of Gram negative and Gram positive bacteria are very similar in that they are disrupted by lysozyme digestion and their assembly is inhibited by penicillin (Martin 1963, Murray, Steed and Elson 1965).

As shown in figure 1, the Gram positive cell wall is solubilized by lysozyme digestion yielding an osmotically-sensitive protoplast; if the organism is originally rod-shaped, a concomitant transition from rod to sphere occurs (Martin 1963). In contrast, preparation of 'clean' Gram negative protoplasts has met with some difficulties (Salton 1964) although they have been obtained from sucrose-washed, EDTA-lysozyme treated cells of a marine pseudomonad (Costerton, Forsberg, Matula, Buckmire and MacLeod 1967). True naked protoplasts retain no residual cell wall demonstrable by chemical or immunological methods or by phage receptor activity.

Spheroplasts are lysozyme-treated cells that have been converted into spherical bodies, sensitive to a hypotonic medium, but which are still surrounded by a partially degraded, non-rigid cell wall (Martin 1963). Spheroplasting of Gram negative bacteria requires ethylenediaminetetraacetic acid (EDTA) which penetrates or partly dissolves the outer

membrane thus exposing the R layer to lysozyme attack (figure 1).

The term 'mureinoplast' has recently been proposed to describe Gram negative cells which have lost the outer layers of their cell wall and are surrounded only by the cytoplasmic membrane and the R layer (DeVoe, Thompson, Costerton and MacLeod 1970).

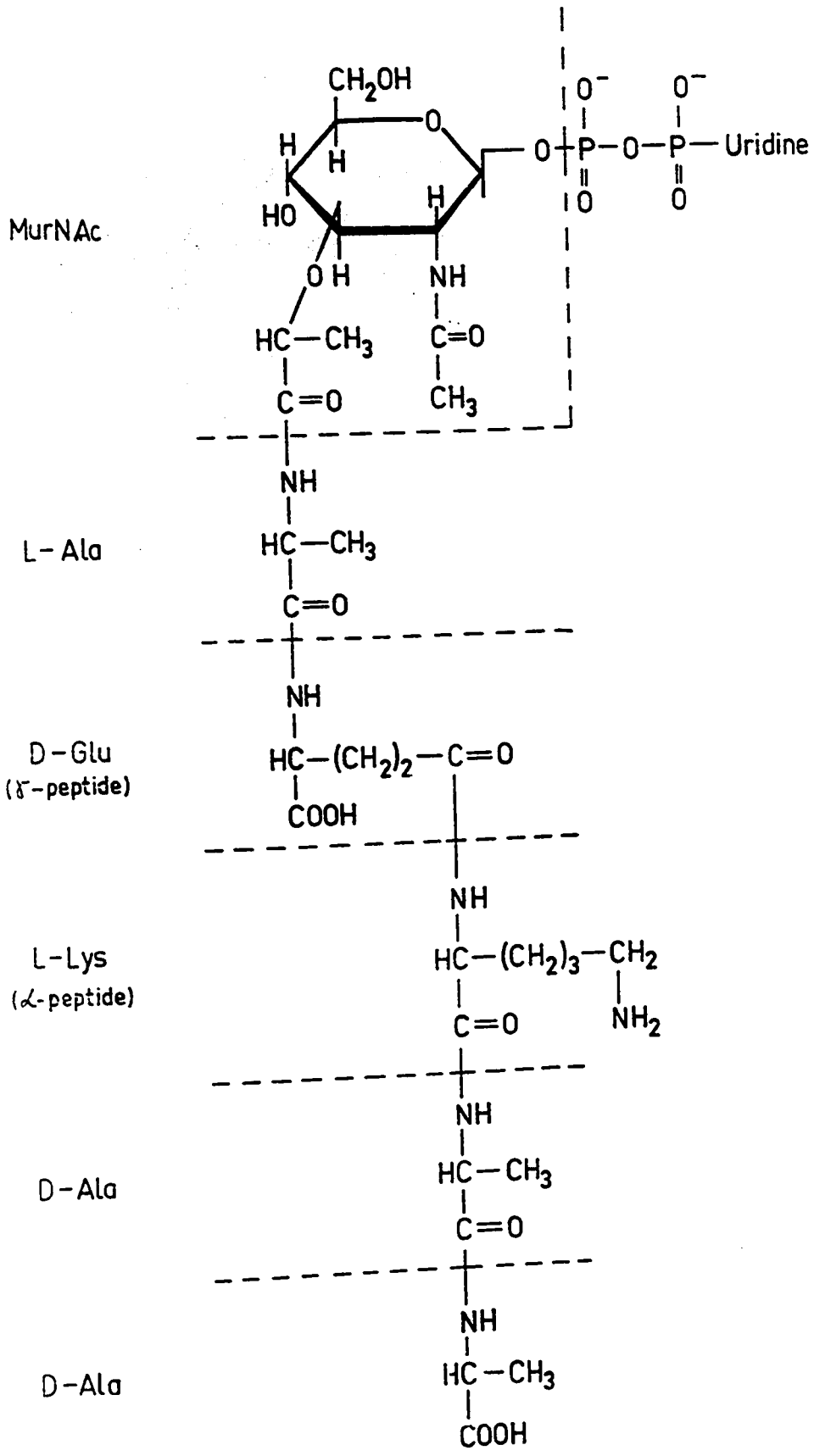
c. Composition in Gram negative bacteria

The chemical composition of partially purified R layers from Gram negative organisms (Weidel, Frank and Martin 1960, Mandelstam 1961 and 1962) although qualitatively similar to that of certain Gram positive bacteria (Costerton 1970) differs quantitatively from the Gram positive species. In the Gram negative species, the proportion of muramic acid is much lower and both lysine and DAP are present, whereas only one of these amino acids occurs in a given species of Gram positive bacteria (Mandelstam 1961). The glycosamino-peptide biosynthetic pathway appears to be analogous in both groups of organisms. From acetone-dried E. coli cells, Comb, Chin and Roseman (1961) extracted four uridine nucleotides of the "Park type" (see figure 3) containing uridine, phosphorus and N-acetylmuramic acid (Mur NA_c) linked to different molar amounts of alanine, glutamic and diamino-pimelic acids. Though the composition, the synthesis and possibly the structure of the R layer in Gram negative and Gram positive bacteria are very similar, much more information is available on the structure and biosynthesis of the Gram positive R layer.

Figure 3. Structure of UDP Mur NA_c pentapeptide

A "Park (1952) type" of nucleotide which serves as a precursor for glycosaminopeptide synthesis in Staphylococcus aureus.
Transpeptidation, the formation of cross-linkages between peptide units of neighbouring hexosamine chains, involves the release of the terminal D-ala (see text).

Illustration taken from H.H. Martin (1966).



d. Gram positive bacteria

i) Composition and structure

The glycosaminopeptide layer is composed of a glycan and a peptide portion. The glycan contains two alternating sugars, N-acetylglucosamine (Gl_cNA_c) and N-acetylmuramic acid (Mur NA_c), a 3-O-lactyl derivative of glucosamine first characterized by Strange and Park (1956). Early studies of the glycosaminopeptide in S. aureus Copenhagen (Ghuysen and Strominger 1963) and in M. lysodeikticus (Salton and Ghuysen 1960, Perkins 1960) led to a tentative identification of the disaccharide as a β-1,6 structure; comparison of the isolated material with authentic β-N-Gl_cNA_c-1,6-Mur NA_c (Flowers and Jeanloz 1963) showed that the original identification was incorrect and that the linkage was actually β-1,4 (Tipper, Strominger and Ensign 1967). It is currently assumed that the basic structure of the glycan chain is similar in all bacteria; the broad specificity of lysozyme and other muramidases is consistent with this assumption (Osborn 1969).

The amino acid composition of the peptide portion of the glycosaminopeptide is fairly constant in a wide variety of bacteria; D-ala, L-ala, D-glut and a dibasic amino acid, usually L-lys or meso-DAP, are present in approximately equal molar ratios (Salton 1964). These amino acid residues are usually linked in a tetrapeptide structure with a L-ala-γ-D-glut-L-lys (or DAP)-D-ala sequence (Ghuysen 1968 and see figure 3); the significance of the pentapeptide (figure

3) will be discussed under "glycosaminopeptide biosynthesis". Digestion of glycosaminopeptides with Mur NA_c-L-ala amidase has shown that the tetrapeptide units are joined to the glycan chain by amide linkages between the α-amino groups of L-ala and the lactyl carboxyl groups of muramic acid residues (Ghuysen 1968). The extent to which peptide subunits are cross-linked and the nature of the bridging element appear to be the major site of variability in glycosaminopeptide structure (reviewed by Osborn 1969).

Although the glycosaminopeptide has been shown to contain a number of covalent bridges and cross-links, what structural relationship does this layer maintain with other cell wall components? In Gram negative bacteria, protein granules (figure 1 and 2), covalently linked to the R layer, have already been mentioned; in Gram positive organisms, teichoic acids (figure 4), polymers of ribitol or glycerol phosphate (Armstrong, Baddiley, Buchanan, Carss and Greenberg 1958) are covalently linked to the glycosaminopeptide layer probably through phosphodiester bonds between the glycerol (or ribitol) of teichoic acids and muramic acid phosphorylated in the C6 position (Ghuysen 1968, Osborn 1969). Glycosaminopeptide-linked polysaccharides have also been reported in a few Gram positive organisms; the reader is referred to the review by Perkins (1963) and Ghuysen (1968) for a detailed discussion.

Figure 4. Teichoic acid

The teichoic acid of Bacillus subtilis (Armstrong), Baddiley and Buchanan 1960) consists of a polymer of D-ribitol units linked to phosphate residues through phosphodiester bonds. Each repeating unit is substituted in the C4 position with a D-glucoside and with an alkali-labile D-ala (Perkins 1963) in the C2 or C3 position, i.e. the point of attachment of D-ala on the ribitol unit has not yet been ascertained (Salton 1964).

The nature of the polyol-phosphate polymer and substitutions therein are subject to specific variations; for example, a glycerol phosphate polymer was isolated from Lactobacillus casei cell walls, an α-glucoside or an N-acetylglucosamine residue linked to the C4 of D-ribitol were detected in Lactobacillus arabinosus and Staphylococcus aureus respectively. A more detailed discussion of these and other examples may be found in Salton (1964).

Illustration taken from H.R. Perkins (1963).

ii) Biosynthesis of glycosaminopeptide

This topic has already been the subject of several reviews (Perkins 1963, Strominger and Tipper 1965, Ghuysen 1968, Osborn 1969, Rothfield and Romeo 1971) and will be briefly discussed here.

Since Park's (1952) first observation that cells of S. aureus, inhibited with penicillin, accumulated uridine nucleotides containing amino sugars and amino acids (figure 3), it has since been confirmed that uridine diphospho-N-acetylglucosamine (UDP Gl_cNA_c) and uridine diphospho-N-acetylmuramyl pentapeptide (UDP Mur NA_c pentapeptide - see figure 3) serve as precursors for the synthesis of glycosaminopeptides in both Gram positive and Gram negative organisms (Perkins 1963, Ghuysen 1968). Amino acids are added stepwise to UDP Mur NA_c, each step being under the control of enzymes specific for both the uridine nucleotide and the amino acid substrates; however, the last sequence, D-alanyl-D-ala, is added in one step to the UDP Mur NA_c tripeptide to form a pentapeptide similar to that in figure 3.

The synthesis of the nucleotide precursors is followed by a sequence of enzyme-catalyzed reactions which results in the formation of uncross-linked glycosaminopeptide strands. Three successive transfers are involved in this reaction cycle: attachment of the UDP Mur NA_c pentapeptide to a membrane-bound polyisoprenoid phosphate (Higashi, Strominger and Sweely 1967), transglycosylation of the lipid-

pentapeptide with UDP Gl_cNA_c (β-1,4 linkage) and transfer of the "lipid-disaccharide pentapeptide" complex to an endogenous acceptor, probably an incomplete glycosaminopeptide (Rothfield and Romeo 1971). The bridge-closing reaction, the last step in the synthesis of the glycosaminopeptide network, results from a penicillin-sensitive transpeptidation (Strominger and Tipper 1965, Tipper and Strominger 1965) in which the chemical potential of the terminal dipeptide (D-alanyl-D-ala) bond in the pentapeptide subunit (figure 3) is utilized to effect the closure of the peptide cross-link with the concomitant release of the terminal D-ala residue.

Ghuysen (1968) concluded that in spite of the variations in chemical composition and in structural details of bacterial glycosaminopeptides, they all possess the same type of network and appear to be synthesized by means of analogous mechanisms.

4. Cell Membranes

A. Morphology and Chemistry

Bacterial membranes became more accessible to direct investigation following the studies of protoplast formation by Weibull (1953) and the demonstration that membrane "ghosts" could be readily isolated by osmotic lysis of the protoplasts (Weibull 1953a). It has already been mentioned that protoplasting of Gram negative bacteria has met with limited success and for this reason, the isolation of homogeneous membrane fractions has been achieved only in a

few rare instances (Gray and Thurman 1967, Miura and Mizushima 1969, Martin and MacLeod 1971). The most notable exception in the Gram negative group is the membrane of Mycoplasma species which may be readily isolated because of the absence of an external cell wall (Morowitz and Terry 1969, Tillack, Carter and Razin 1970). Criteria such as density in linear density gradients, fine structure and the occurrence of membrane markers such as phospholipids, carotenoids, menaquinones, cytochromes and respiratory components (reviewed by Op den Kamp, van Deenen and Tomasi 1969, Salton 1971) have been used to assess the purity of membrane fractions. However, Salton (1971a) stressed the fact that although membrane markers are associated with isolated membrane, direct confirmation that they do indeed exist in the membranes of intact cells is generally lacking and very difficult to obtain.

In overall chemical composition, the bacterial membranes do not differ markedly from those of other types of cells; they are usually composed of 40-75% protein, 20-30% lipid and 0.2-20% carbohydrate (Salton 1967, Martin and MacLeod 1971). In addition to the major protein and lipid constituents, bacterial membranes usually contain varying amounts of RNA and lipid-soluble components such as carotenoids and menaquinones which account for 1% or less of the whole membrane structure (Salton and Schmitt 1967). The chemistry and distribution of bacterial membrane lipids is well documented (Kodicek 1962, Kates 1964, Salton 1967). It has also

been shown that the membrane lipid content can be varied experimentally; for example, the fatty acid composition of an E. coli mutant requiring unsaturated fatty acids for growth depends on the fatty acids added to the medium (Silbert, Ruch and Vagelos 1968) whereas glucosaminyl phosphatidylglycerol becomes a major constituent of B. subtilis when grown at acid pH (Op den Kamp, Iterson and Van Deenen 1967). In contrast, very little is known of the variety and nature of membrane proteins. From disc gel electrophoresis studies of membranes isolated from M. lysodeikticus, B. subtilis and S. lutea, Salton (1967a) concluded that bacterial membranes contain a complex variety of proteins differing from species to species. It is generally assumed that membranes consist of both structural and catalytic proteins although opinions vary as to which, if either, is essential to the integrity of the membrane (Korn 1969). Among others, many enzymes of the tricarboxylic acid cycle have been localized in the cell membranes of Gram positive organisms (compiled by Salton 1971a).

Thin sections of bacterial envelopes, first prepared by Kellenberger and Ryter (1958), show an underlying trilaminar cell membrane with an overall thickness of approximately 7.5 nm (Salton 1964 and see figure 1). It is now recognized that lipids are not essential for the trilaminar appearance of membranes (Korn 1969); for example, extraction with 90% acetone removed over 95% of labelled M. laidlawii membrane lipid but left sedimentable protein-rich membranous

material with unit membrane morphology (Morowitz and Terry 1969). Freeze-fractured and freeze-etched replicas of bacterial membranes have shown that the fracture faces are very similar to those obtained with erythrocytes and membranes from a variety of animal and plant sources (Salton 1971a). The globular particles on the freeze-fractured faces of bacterial membranes (figure 5) represent the internal globular structures revealed by cleavage of the internal region of the membrane as originally suggested by Branton (1966) and later confirmed in red blood cell membranes using a ferritin marker (Pinto da Silva and Branton 1970).

One of the first membrane models was presented by Danielli and Davson (1935) whereby cell membranes were conceived to be a bilayer of lipid molecules with their polar groups directed outwards and covered by monomolecular films of proteins; Robertson (1966) presented an excellent retrospective of the many membrane models that have since appeared. Since membranes consist mainly of lipid and protein, a number of models have been proposed based on studies of lipid-lipid, protein-protein and lipid-protein interactions. Of these, the lipid-lipid interactions are best understood and the reader is referred to the recent review by Henn and Thompson (1969) for a detailed discussion of lipid bilayer membranes; little is known about membrane protein-protein interactions. The lipid-protein interactions are the most actively debated aspect of membrane structure in spite of or because of the

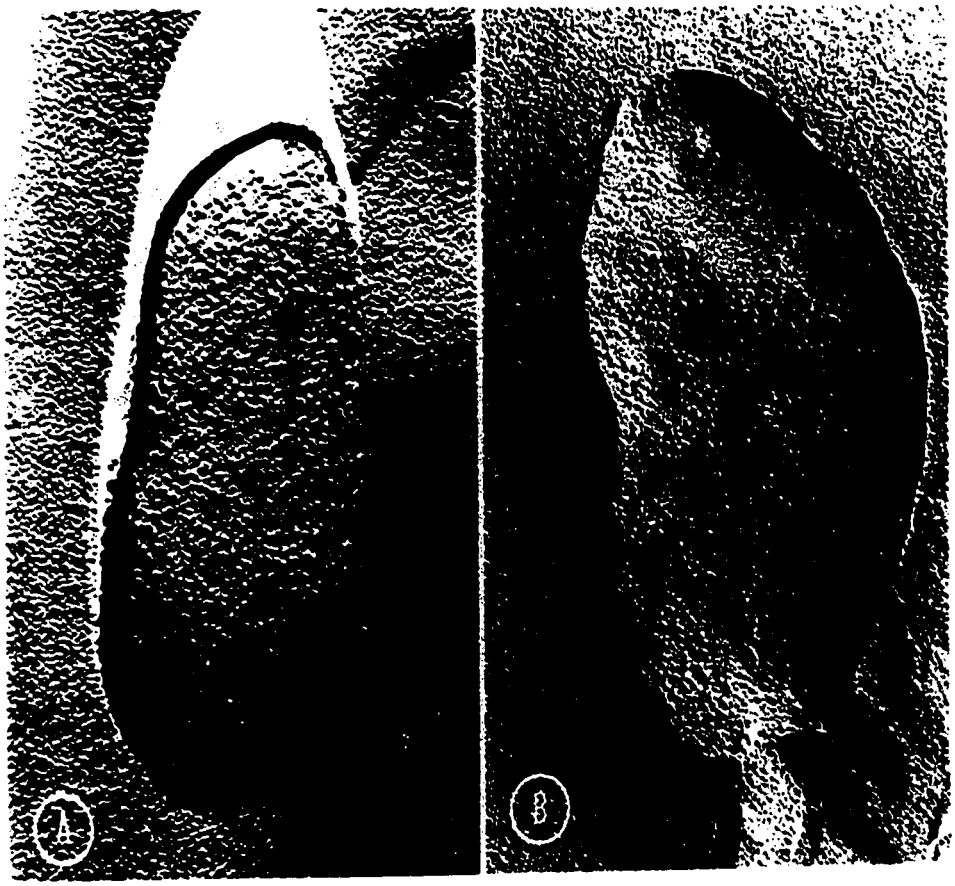
Figure 5. Fracture plane of cell membranes

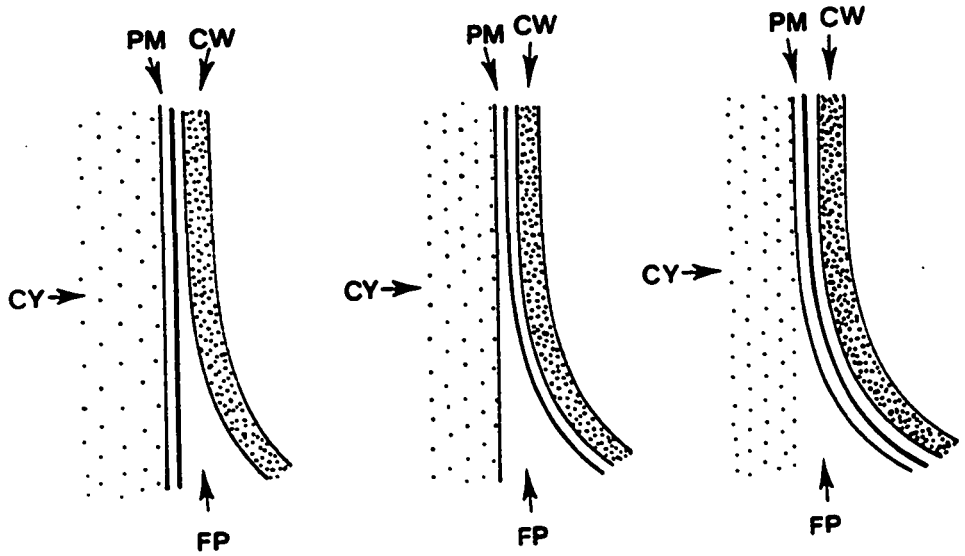
a and b. Platinum-carbon replica of the convex (a) and concave (b) fracture face of the membrane of freeze-fractured M. laidlawii cells. The observation that the convex face is always more densely packed with particles suggests the presence of a unique fracture plane of the cell membrane.

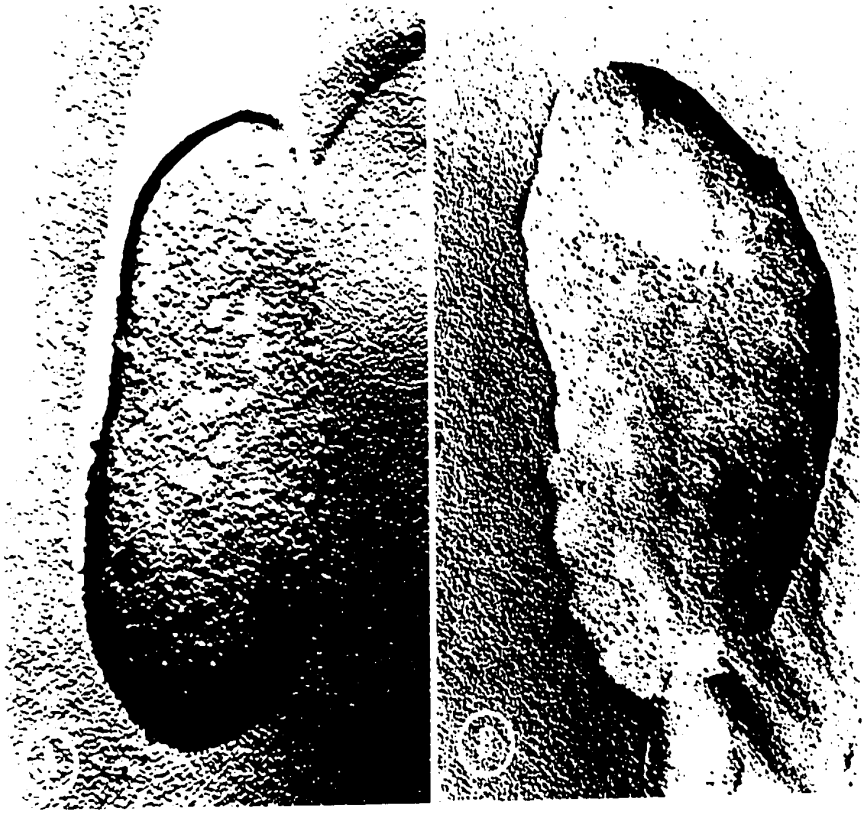
c. Of the three fracture possibilities represented here, complementary replicas and thin sections of fractured B. subtilis cells have shown that the membrane is fractured through its center (center sketch).

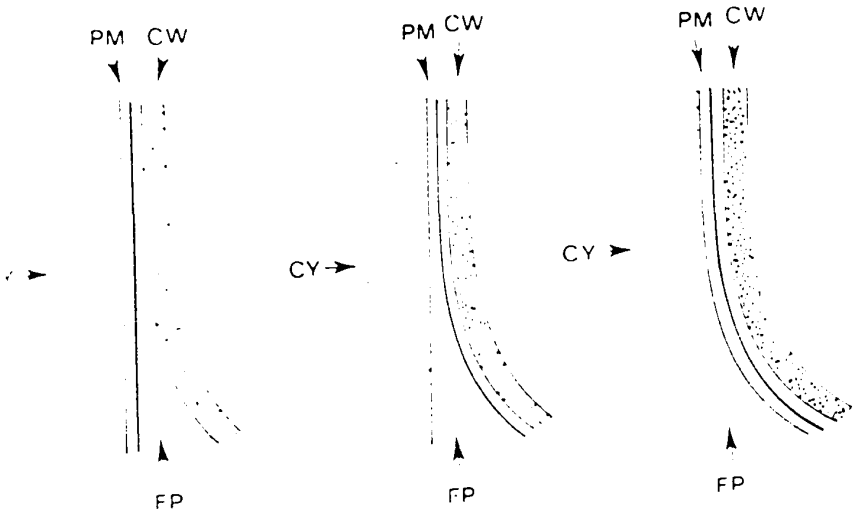
FP, fracture plane; CW, cell wall; PM, plasma membrane; CY, cytoplasm.

Figures a and b taken from Tillack, Carter and Razin (1970) and figure c taken from Nanninga (1971).








PM
CW
CY
FP
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lack of sound experimental data on the structure of lipoprotein complexes (Stoeckenius and Engelman 1969).

These same authors extensively discussed the evidence and arguments supporting or discrediting the two main models: the Danielli (lipid bilayer) and the subunit models. The subunit model proposes three classes of lipoprotein subunits in cell membranes; 'structural subunits' which determine the characteristic shape of membranes, 'functional subunits' capable of carrying out a given complex function of a membrane and 'assembly subunits' which would be used in membrane synthesis through a self-assembly process. Evidence for the subunit model, derived from ultrastructural studies, is confined to the demonstration of a globular substructure in the two dense layers of some thin-sectioned membrane preparations (reviewed by Stoeckenius and Engelman 1969) and perhaps to the globular particles on the fractured faces of cell membranes (figure 5). Reaggregation into membrane-like structures of disaggregated membrane fractions has often been used as an argument for subunit structure (Morowitz and Terry 1965, Butler, Smith and Grula 1967). Nevertheless, Stoeckenius and Engelman (1969) conclusively have shown that present evidence favors a modified Danielli model in which the central hydrocarbon region of the lipid bilayer is disordered (as opposed to the symmetrical arrangement in the original model) and in which the membrane continuum could conceivably be interrupted by the insertion of subunits in certain areas of the

membrane. (Hendler 1971, Singer and Nicolson 1972).

B. Mesosomes

Mesosomes, a term coined by Fitz-James (1960) are membranous structures which result from the invagination of the bacterial cell membrane. The mesosomes of Gram positive organisms usually consist of a membrane sac seemingly continuous with the cell membrane and containing internal membranes which have alternatively been described as lamellae, tubules or vesicles (Rogers 1970). These structures, whose number varies from one to many per cell, are widely distributed in Gram positive organisms whereas only a few reports describe membranous structures in Gram negative bacteria (Ryter 1968, Pontefract, Bergeron and Thatcher 1969, and see Chapter 3). Exposure of Gram positive bacteria to a hypertonic medium induces the extrusion of the mesosomes from the cytoplasm to the space between the cell membrane and the cell wall (Iterson 1961 and 1965); if the cell wall of these plasmolyzed cells is digested with lysozyme, protoplasts are liberated and mesosomal tubules are released into the surrounding medium (Fitz-James 1965). Partially purified mesosomal fractions obtained by a variety of techniques from a number of Gram positive bacteria (reviewed by Salton 1971a) have been subjected to chemical analyses. Mesosomes generally differ from the cytoplasmic membranes in composition, in the variety of protein bands recognizable on polyacrylamide electrophoresis gels and in their enzymic activities (Rogers 1970) although Salton (1971a) pointed

out that the difficulty of assessing the homogeneity of mesosome fractions is undoubtedly a factor in the conflicting reports on enzyme distribution between mesosomes and cell membranes. Evidence obtained from B. licheniformis suggests that the fatty acid composition of the mesosomes and cell membranes are identical (Rogers 1970).

A number of functions have been assigned to the mesosomes. Studies by Ellar, Lundgren and Slepecky (1967) have provided strong morphological evidence that mesosomes of B. megaterium are involved in septum formation. The contribution of mesosomes to cross-wall synthesis appears to be partly linked to their high content of C_{55} isoprenoid alcohol derivative (Thorne and Barker 1971), an essential component in glycosaminopeptide synthesis. The absence of mesosomes in cells whose division process is upset further supports the involvement of mesosomes in septation (Ryter, and Landman 1964, Rogers, McConnell and Burdett 1970). From their ultrastructural studies of the division cycle of B. subtilis, Ryter and Jacob (1964) found several mesosomes per cell and proposed a model whereby longitudinal division of mesosomes was necessary to effect nuclear segragation. With his more recent estimate of one mesosome per cell, Highton (1970) has pointed out the mechanical difficulties of a simple pulling apart of DNA by a single mesosome and called for a reassessment of the function of mesosomes in DNA separation. Cytochemical studies of Gram positive cells suggest that the mesosomes are also the loci of

respiratory activity. Tellurite and tetrazolium reduction is greater in the mesosomes than in the peripheral plasma membrane of a number of organisms (Iterson and Leene 1964, Kawata and Inoue 1965, Takagi, Abe and Ueda 1965, Sedar and Burde 1965). Cytological evidence also suggests that bacterial DNA may be connected to the cell membrane and/or the mesosomes (Fuhs 1965, Iterson 1965) although there is no definite answer as to whether the DNA is fixed to the membrane by a single or multiple points of attachment (Rogers 1970). As to the significance of this occurrence, Ryter (1968) commented that we do not know whether the point of attachment coincides with the point of replication or whether, on the contrary, DNA is anchored at a predetermined place where the site of replication travels along the length of the chromosome.

Chapter 2

Taxonomy of the marine psychrophile NRC 1004

Introduction

The marine psychrophile, a motile, Gram negative, rod-shaped bacterium contains a red pigment chromatographically similar to prodigiosin (Hagen, Kushner and Gibbons 1964). Because of its pigment, as well as its lipid composition (Kates and Hagen 1964), and its extracellular proteinases (McDonald and Chambers 1963, Nunokawa and McDonald 1968), the organism was once considered as possibly related to the genus Serratia. However, preliminary morphological studies showed the cell to be polarly flagellated (Chapter 3), a finding that did not support classification as a Serratia sp. The present chapter deals with the physiological and biochemical studies that led to the classification of the marine psychrophile NRC 1004 as a member of the genus Vibrio.

Materials and Methods

Growth medium

Cells were grown for 48-60 hr in shaken cultures at 10C in a medium containing 0.5M NaCl, 0.04M $MgCl_2$, 0.004M KH_2PO_4, 10^{-8}M $FeCl_2$, 1.5 x 10^{-4}M $CaCl_2$ and 0.8% (w/v) tryptone; pH 7.0. Agar plates were prepared by supplementing the growth medium with 1.5% (w/v) Bacto agar. This growth medium was used in all the experiments unless stated otherwise. The "all salts" washing solution contained all the components of the growth medium except tryptone.

Inoculum. Tubes and plates were inoculated with loopfuls of a 48-60 hr preculture (OD_{650} = 0.5 - 0.7).

Acid from carbohydrates

Acid production from carbohydrates, determined in standing tubes at 10C, were carried out in an "all salts" solution supplemented with 0.2% (w/v) tryptone, 1.0% carbohydrate and 0.003% bromthymol blue; final pH 7.4. Mono- and disaccharides were sterilized by filtration whereas a 10% aqueous solution of dextrin was autoclaved 7 min. All tests with carbohydrates were carried out in triplicate and checked daily. Aerobic and anaerobic utilization of glucose was determined by the Hugh and Leifson method (1953) modified so as to promote growth of the psychrophile. The medium contained "all salts" (in which the KH_2PO_4 was substituted for 0.03% K_2HPO_4), 0.2% tryptone, 1% glucose, 0.003% bromthymol blue and 0.3% agar; the pH was adjusted to 7.1 and the medium autoclaved for 15 min. Tubes of semi-solid medium, inoculated by stabbing, were checked daily.

Other physiological tests

Nitrate reduction, indole production and citrate utilization broths (Bradshaw 1963), made up in "all salts" solution, were carried out in standing tubes at 10C. The nitrate broth contained 0.1% KNO_3 and 0.2% peptone, the indole broth 1.0% tryptone and the citrate broth 0.57% Koser citrate (Difco); the final pH was adjusted to 7.0. Samples from the nitrate and indole tubes, assayed with sulfanilic acid and Kovacs reagents respectively (Bradshaw 1963), were checked daily until cell pigments interfered with the color reaction (4 days following inoculation).

Catalase, oxidase and starch hydrolysis (0.2% of soluble starch) tests were performed on 2-week-old cultures grown on agar plates of complete medium. Catalase activity was assayed by flooding a portion of the plate with 3.0% hydrogen peroxide whereas oxidase activity was detected using the p-aminodimethylanilino-HCl-α-naphthol reagent; liberation of gas and development of a blue color respectively, were taken as positive tests (Skerman 1967). Oxidase was also tested using Pathotec (Warner-Chilcott, N.J.) strips. Starch hydrolysis was checked by flooding the plates with Kopeloff's iodine (Bradshaw 1963).

Sensitivity to vibriostat 0/129

Vibriostat sensitivity was tested by placing filter paper discs (Schleicher and Schuell Co., Keene, N.H.) saturated with 2,4-diamino-6,7-diisopropyl pteridine solution (1 mg/ml in acetone) on seeded agar plates of complete medium (Bain and Shewan 1968); control discs had been soaked in acetone alone. Plates were examined after 12-15 days of incubation. Vibriostat sensitivity of the marine psychrophile was also compared to that of three strains of Vibrio parahemolyticus grown overnight at 37C on bromthymol blue-teepol agar plates; two of the strains were of human origin (K4 and Yana) and the third strain (T249) was isolated from Pacific shellfish. Zones of growth inhibition were measured from the edge of the impregnated discs.

Isolation, purification and thermal denaturation of deoxyribonucleic acid (DNA).

DNA was isolated according to the method of Marmur (1961) from a 60 hr culture washed in "all salts" solution. The washed cells were centrifuged and the resulting pellet suspended and lysed for 30-45 min. in cold one-tenth strength "standard saline citrate" (0.1x SSC) solution; standard saline citrate (1x SSC) contains 0.15M NaCl and 0.015M trisodium citrate, pH 7.0. As previously shown (Korngold and Kushner 1968), considerably higher concentrations of salts are needed to prevent lysis of this organism. The lysate, made 1x SSC with concentrated saline solution (10x SSC), was thoroughly mixed with an equal volume of cold chloroform-amyl alcohol (24:1 v/v) and centrifuged at 5,000 xg for 10 min. The turbid supernatant, removed by aspiration, was repeatedly extracted with chloroform-alcohol until clear and retained as a "crude DNA" extract. Crude DNA was precipitated with 2 volumes of cold 95% ethanol and collected by winding the DNA fibrils around a glass rod; the fibers were then solubilized in 0.1x SSC and stored in 1x SSC in the cold.

RNA'ase and pronase solutions (1 mg/ml in 1x SSC), used to purify the crude DNA preparation, were preincubated so as to remove possible contaminants; DNA'ase contaminants in the RNA'ase solution were heat denaturated by a 10 min. incubation in a 60C water bath (Marmur 1961) whereas incubation at 37C for 2 hr favored pronase digestion of contaminating

proteins. To purify the crude DNA solution, one-fifth volume of purified RNA'ase preparation was added and incubated at 37C for 30-40 min. followed by a 2 hr incubation with one-half volume of purified pronase solution. The resulting DNA solution was extracted with cold chloroform-amyl alcohol and the DNA precipitated with cold 95% ethanol (as previously described) to yield a pure DNA solution.

Thermal denaturation of DNA induces a helical to random coil change in configuration with a concomitant increase in optical density (hyperchromic shift) measured at 260nm (Marmur and Doty 1962). The T_m is that temperature corresponding to the midpoint of the shift and varies directly with the G + C content. The guanine plus cytosine base composition can be calculated directly by substituting the T_m value into the linear relation of Marmur and Doty (1962) i.e. $T_m = 69.3 + 0.41\ (G + C)$. T_m determinations were carried out on a Gilford recording spectrophotometer (model 2400) in which the cuvette chamber temperature, monitored by thermosensors, was automatically entered on a recorder chart; the chamber temperature was varied by means of a Haacke-ethylene glycol bath. Discrepancies between chamber and actual cuvette temperatures were corrected by means of a calibration curve. Cuvette temperatures were obtained by introducing the tip of a thermometer, held in a wooden block snugly fitted over the cuvette chamber opening, into a cuvette containing 3.0 ml of 1x SSC. As the chamber temperature was increased from 30 to 100 C, one degree in-

crements in cuvette temperature were read against the thermosensor temperature plot.

Simultaneous T_m determinations of the psychrophile's and standard B. subtilis DNA were carried in 1.0 cm path length, quartz suprasil cuvettes (Carl Zeiss, Germany) each filled with 3.0 ml of DNA solution (OD_{260} = 0.5) and sealed with plastic stoppers; 3.0 ml of 1x SSC was used as the blank. As the chamber temperature was increased from 30 to 100 C at an approximate rate of 40C degrees/hr, increases in optical density and chamber temperature were automatically plotted on the recorder chart; T_m values were read directly off the chart and the moles % G + C content of the psychrophile's and B. subtilis DNA calculated.

Per cent hyperchromicity, a measure of the double-strandedness of the purified DNA samples, was determined by acid denaturation. One drop (0.025 ml) of 6N HCl was added to 3.0 ml of DNA solution and the resulting increase in optical density, measured at 260nm on a Beckman DU spectrophotometer, was expressed as a per cent of the optical density of native DNA; reliable T_m values may be obtained from DNA samples exhibiting 30-40% hyperchromicity.

Results

Physiological tests:

Results of the physiological tests together with some morphological characteristics of the marine psychrophile are summarized in Table 1; corresponding characteristics of Vibrio marinus, taken from Colwell and Morita (1964),

are also included. The Hugh and Leifson test revealed that within 10 days, the marine psychrophile produces acid but no gas under both aerobic and anaerobic conditions. Under aerobic conditions, good growth occurred along the whole length of the stab; under anaerobic conditions, less growth occurred but a definite acid reaction was observed. Acid production from maltose and dextrin was observed 12 to 14 days following inoculation; no acid was produced from arabinose. Good growth occurred in all cases. Nitrate reduction to nitrite was detected 1 day following inoculation; no ammonia was produced during the non-pigmented phase of growth nor was any gas liberated following 14 days of incubation. Indole was not produced, nor was citrate utilized as sole carbon source. With the oxidase test, using naphtol-aminodimethylanilino-HCl reagent, most of the blue color developed along the edge of the 2-week-old colonies; Pathotec oxidase strips also gave a positive reaction. Catalase activity and starch hydrolysis were also observed on 12-14 day-old plates. The marine psychrophile was less sensitive to vibriostat 0/129 than Vibrio parahemolyticus; the zones of growth inhibition ranged from 1.0-2.0 mm for the psychrophile and from 3.0 to 7.0 mm for the three strains of Vibrio, the T249 strain being the most sensitive.

Table 1

Morphology and Physiology of the Marine Psychrophile NRC 1004

a. Data from Colwell and Morita 1964. For reactions of other vibrios, Davis and Park (1962) should be consulted.

b. NA = not available.

c. Data from McDonald and Chambers 1963.

Characteristic	Marine Psychrophile NRC 1004	Vibrio marinus[a]
Morphology	slightly curved, motile rods	slightly curved, motile rods
Colony	red, opaque with entire edge and smooth surface	grayish, opaque with entire edge and smooth surface
Temperature range for growth	0-19 C	0-20 C
Specific NaCl requirement for growth	2.75%	2.4%
Hugh-Leifson	acid from glucose aerobic and anaerobic	acid from glucose aerobic and anaerobic
Acid from		
maltose	+	+
dextrin	+	+
arabinose	-	NA[b]
Oxidase	+	+
Catalase	+	+
Starch hydrolysis	+	±
Casein hydrolysis	+[c]	+
Gelatin hydrolysis	+[c]	+
Citrate	-	-
Indole	-	-
Reduction of nitrate to nitrite	+	+
Vibriostat 0/129	+	+

Table 2

DNA G + C content by thermal denaturation

a. Data from this study. The standard deviation for the mean T_m of four or five separate determinations has been calculated. For references to literature values, see "Discussion".

Organism	Mean Tm[a]	Moles % G + C	
		Thermal denaturation[a]	Literature values
Marine psychrophile NRC 1004	85.7±0.40	40.0	
Marine Vibrio species			40.0-43.0
Bacillus subtilis	86.9±0.34	42.9	42.5-43.6

Figure 6

Thermal denaturation profiles

The profiles, constructed from data obtained in experiment 4, describe the hyperchromic shift resulting from the heat denaturation of DNA. Denaturation was pursued until no further optical density changes were observed. The abscissa corresponds to actual cuvette temperatures obtained from a calibration curve.

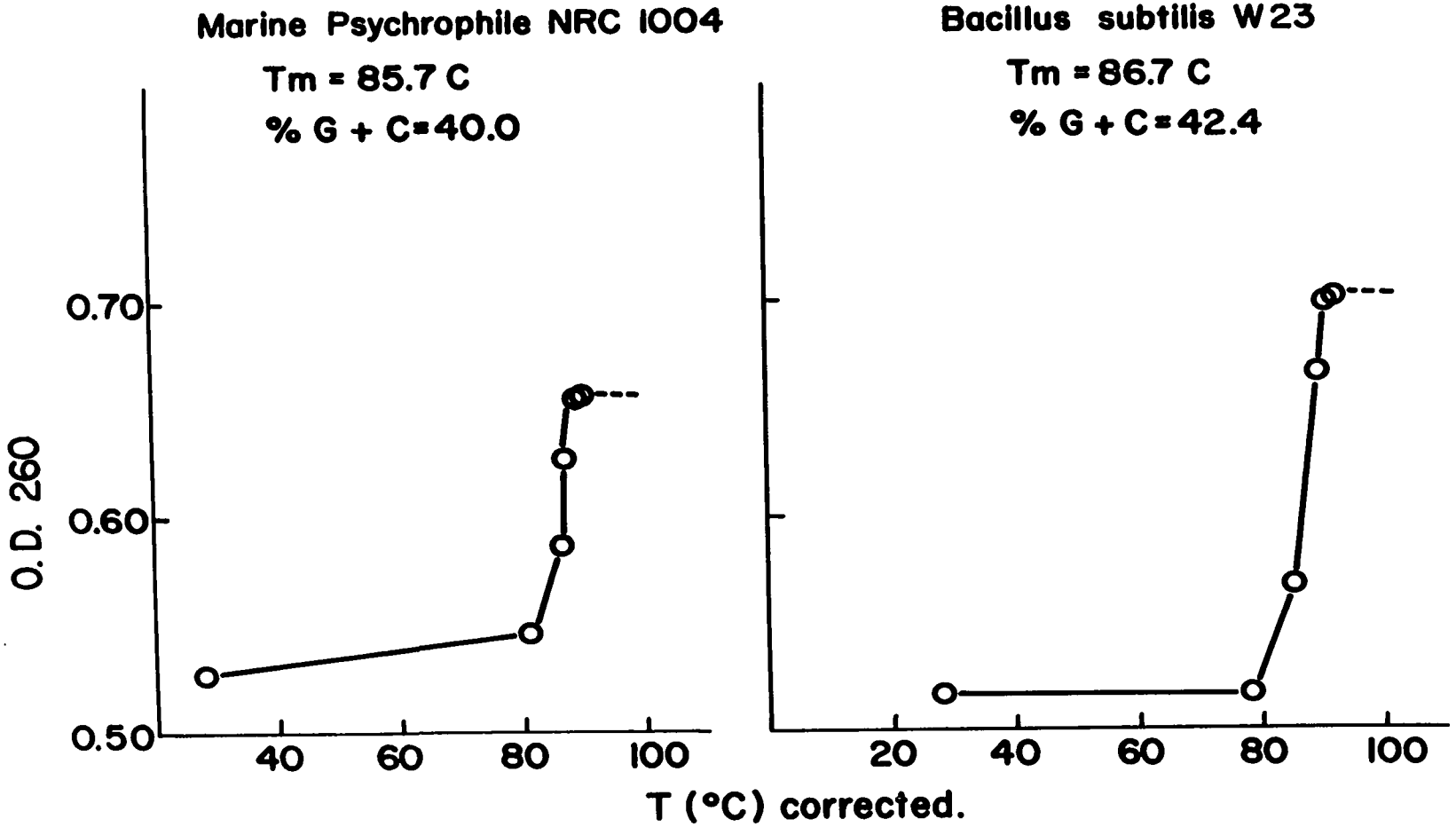


Table 3

Per cent hyperchromicity of DNA samples

a. The per cent hyperchromicity, determined by acid (6N HCl) denaturation, was obtained from the ratio of the increase in optical density to the optical density of native DNA and expressed as a %. Two batches of purified DNA were used in these studies.

Organism	Batch number	OD_{260}		% hyperchromicity	Mean % hyperchromicity
		Native	Denatured		
Marine psychrophile NRC 1004	1	0.015	0.019	27	30
	2	0.240	0.317	32	
Bacillus subtilis	1	0.179	0.255	42	39
	2	0.447	0.604	35	

Thermal denaturation studies:

Thermal denaturation studies (Table 2) showed that the DNA of the psychrophile contained 40.0% G + C. That of B. subtilis contained 42.9% G + C which agrees well with previously reported values. Typical thermal denaturation profiles, constructed from data obtained in experiment 4, are presented in figure 6. Thermosensor chamber temperatures were found to be higher than actual cuvette temperatures by Δ2C in the 30-35C range, gradually increasing to a Δ6C in the 35-75C range and tapering to a Δ7C in the 75-100C range. Mean per cent hyperchromicities of 30 and 39% were obtained for psychrophilic and B. subtilis DNA respectively (Table 3), values which are quite acceptable for thermal denaturation studies.

Discussion

As the data in Tables 1 and 2 show, the psychrophile shares many characteristics with V. marinus, notably vibriostat sensitivity, DNA G + C content and a specific salt requirement for growth which conforms with MacLeod's definition of a marine organism (1965). It differs from V. marinus, only in pigmentation and starch hydrolysis.

In many of the physiological tests carried out, the incubation period required to effect a noticeable change is remarkably long, usually approaching 14 days. Ingraham and Stokes (1959) commented that even in the absence of growth, biochemical changes of substrates due to psychrophiles may be detectable at temperatures below -10C if long enough

periods of incubation are used. Bacterial denitrification appears to take place readily in the sea but most marine microorganisms, including the psychrophile studied here, reduce nitrate only to nitrite (Brisou and Vargues 1963). The occurrence of a prodigiosin-related pigment in our psychrophile is not unique among marine bacteria for it has been identified in Serratia marinorubra, a species isolated originally from Pacific Ocean water and described by ZoBell and Upham (1944) and in two marine isolates, probably pseudomonads, described by Lewis and Corpe (1964). Despite its similarity in pigmentation and lipid composition to Serratia marcescens (Kates and Hagen 1964), the red psychrophile differs from this species in flagellation (Chapter 3), and in oxidase, starch hydrolysis and dextrin fermentation reactions (Colwell and Mandel 1965) as well as in DNA base composition where S. marcescens DNA contains from 56.2 - 58.4 moles % G + C (Marmur and Doty 1962, Colwell and Mandel 1965).

The marine psychrophile is clearly distinguished from other polarly flagellated rods. The taxonomic schemes of Hendrie and Shewan (1966) and of Bain and Shewan (1968) suggest that our organism belongs to the Vibrio rather than the Pseudomonas genus. Moreover, the pseudomonads oxidize but do not ferment glucose, produce acid from arabinose but not from maltose and dextrin, are insensitive to vibriostat 0/129 (Park 1962) and have a G + C DNA content of 61-66 moles % (Marmur and Doty 1962, Sebald et Véron 1963, Colwell

and Mandel 1964). Those in the genus Aeromonas are insensitive to vibriostat 0/129 (DeLey 1964, Bain and Shewan 1968) and have a G + C content of 56.5 - 59.0% (Colwell and Mandel 1964). Members of the genus Comamonas do not oxidize or ferment glucose or produce acid from a number of other carbohydrates (Park 1962, DeLey 1964). They are insensitive to vibriostat 0/129, and have a G + C content of 64.2% (Sebald et Véron 1963).

Sensitivity to vibriostat 0/129 has been recognized as an important taxonomic tool in distinguishing between vibrios and closely related Gram negative, polarly flagellated rods (DeLey 1964, Bain and Shewan 1968, Colwell and Gochnauer 1968). Except for the shigelloides strains recognized as Aeromonas by Ewing, Hugh and Johnson (1961), only vibrios, at present, are sensitive to this pteridine compound (Park 1962, Colwell and Gochnauer 1968).

The 40.0 moles % G + C DNA content of the psychrophile is well within the range of other marine Vibrio species studied by Colwell and Mandel (1964a) and Colwell and Gochnauer (1968). The G + C content of the B. subtilis standard also agrees well with literature values (Belozersky and Spirin 1960, Gause, Loshkauva, Zborsky and Gause 1964, and Marmur and Doty 1962).

The marine psychrophile is distinguished from other vibrios (Davis and Park 1962) by its obligately psychrophilic nature, its rapid death at temperatures above 20C, its red color and its inability to produce indole. From

the results obtained, I propose that the organism formerly known as marine psychrophile NRC 1004 be classified as Vibrio psychroerythrus.

The description of Vibrio psychroerythrus, a cold (psychro) requiring, red pigmented (erythrus) organism is extended as follows:

Gram negative asporogenous rods. Cells slightly bent or straight, single 2.5 to 3.5 μm long, 0.5 μm wide. Single, long polar flagellum (see Chapter 3).

Growth on synthetic seawater agar: NaCl, 2.75%; $MgCl_2$, 0.4%; KH_2PO_4, 0.05%; $CaCl_2$, 1.5×10^{-4}M; $FeCl_2$, 1×10^{-8}M; tryptone, 0.8%; agar, 1.5%; pH 7.0; incubated at 10-12C for 14 days. Colonies white at first appearance (7-8 days) becoming red within 10-12 days, opaque, raised, 3-5mm diameter, with entire edge. Non-diffusible pigment. Turbid growth with no pellicle formation. Cells grow in the temperature range 0-19C; cells lyse above 20C or in the absence of NaCl and divalent ions (Hagen, Kushner and Gibbons 1964, Korngold and Kushner 1968 and see Chapter 4).

Catalase-positive. Oxidase-positive. Sensitive to vibriostat 0/129. Casein hydrolyzed (McDonald and Chambers 1963).

Indole negative. Reduces nitrate to nitrite; no gas produced. No growth on citrate.

Acid from glucose under aerobic and anaerobic conditions without production of gas. Starch hydrolyzed. Acid produced from maltose and dextrin; no acid from arabinose.

Moles % G + C of DNA = 40.0.

The organism has been deposited as the type strain with the American Type Culture Collection and given the accession number ATCC 27364.

Chapter 3

Fine structure of *Vibrio psychroerythrus* and structural changes with varying growth conditions.

Introduction

Following the first report of pleomorphism in Spirillum and Vibrio sp. (Cornill and Babes 1890), Hallock's observations (1959 and 1960) that aging vibrios produce coccoid forms or round bodies prompted further investigations on the morphology of these peculiar cell forms. Williams and Rittenberg (1956) and Hallock (1959) suggested that round bodies represent a stage in the life cycle of these organisms. Felter, Colwell and Chapman (1969) found that these cell forms abound in the early and late stationary phase of growth of Vibrio marinus MP-1. They further suggested that the round bodies ("spheres"), 2 to 4 cell units enclosed within a common cell wall, result from aberrant cell division or cell wall synthesis.

It was thought that an ultrastructural study of V. psychroerythrus under various conditions of lysis would complement the existing data on the chemical changes occurring during its lysis (Hagen, Kushner and Gibbons 1964, Korngold and Kushner 1968). It was evident that such a study required knowledge of the structural variations in the normal cell. The present chapter describes studies on the general ultrastructure of V. psychroerythrus and the structural changes associated with culture age and growth conditions. Discussion of the results will be deferred in order to introduce some concepts on bacterial cell division and the mechanism of cell fixation.

In order to divide, a bacterial cell must increase its surface area, a problem that is compounded by the presence of an external, protective rigid wall. Unlike some forms of life which possess an exoskeleton that is continuously regrown while the older is shed, it is necessary for the procaryotic cell to maintain the protective nature of its coat while increasing its surface area. The manner in which this is done is not completely understood (Higgins and Shockman 1971) although it has often been suggested that growth of the bacterial surface requires some controlled degradation of the cell wall, probably by means of autolytic enzymes such as N-acetylmuramidase, N-acetyl-glucosaminidase and N-acetylmuramyl-L-alanine amidase (Ghuysen 1968) to open gaps where new wall material can be inserted. In his review, Rogers (1970) suggested that autolytic enzymes open the glycosaminopeptide "sacculus", allow realignment and rearrangement of existing glycosaminopeptides and are essential for the separation of daughter cells. The question of single or multiple sites of cell wall growth in coccal and rod forms has not been completely resolved. Immunofluorescent and ferritin-labelled antibody techniques (reviewed by Higgins and Shockman 1971) suggest that wall growth of streptococci is localized at the equator of the cell where newly synthesized wall is fed out both peripherally (increasing the surface area) and centripetally (forming a septum between daughter cells); from their ultrastructural studies, Higgins and Shockman (1970) presented a model em-

phasizing the localized cell wall growth of Streptococcus faecalis. Higgins and Shockman (1971) also presented a model for the localized wall growth of rod-shaped bacteria; the reader is referred to the original paper for a detailed discussion of the model. Recent reports on the multiple sites of wall growth in B. megaterium (Mauck, Chan, Glaser and Williamson 1972), in E. coli (van Tubergen and Setlow 1961) and B. cereus (Chung 1967) show that it is uncertain if wall growth in rod-shaped bacteria is localized or diffuse. The morphological characteristics of cell division in Gram positive and Gram negative bacteria are comparable (Steed and Murray 1966). In the Gram positive organisms, the cell membrane and the entire cell wall participate in septum formation whereas septation in Gram negative bacteria involves the cell membrane and the glycosaminopeptide layer, the outer membrane of the cell wall invaginating at a later stage. The role of mesosomes in septum formation was previously discussed (Chapter 1).

Since Kellenberger and Ryter's "standard" fixation of E. coli cells (1958), preparative methods for thin sectioning have been improved and much has been learned of the chemical action of fixatives. Kellenberger's (1958) procedure, a pre- and postfixation in buffered osmium tetroxide (OsO_4) followed by staining in an aqueous solution of uranyl acetate (UA_c), is widely used today although reports of aldehyde-OsO_4 fixation are steadily increasing. The quality of fixation has been appraised by comparing the fine struc-

ture of thin-sectioned and freeze-etched specimens (Remsen and Lundgren 1966); the loss of cell lipids has also been used to assess the quality of fixation-embedding procedures (Mitchell 1969). There is little doubt that comparison of X-ray diffraction patterns of intact and fixed specimens is a method of choice for evaluating the induced rearrangement of macromolecules in fixed specimens. For example, Moretz, Akers and Parsons (1969a and b) observed significant changes in the diffraction patterns of aldehyde or OsO_4 - fixed myelin membranes, changes which were accentuated following acetone or alcohol dehydration.

Aldehydes act by forming stable methylene bridges between protein (Bowes and Carter 1966, Habeeb and Hiramoto 1968, Hopwood 1969) or purine (Feldman 1967) NH_2 groups. Bridge formation proceeds in two stages (Feldman 1967); a labile aminomethylol, formed from a reversible addition reaction, is transformed to a stable methylene derivative i.e.

1) $R\text{-}NH_2 + CH_2O \rightleftharpoons R\text{-}NH\text{-}CH_2OH$

2) $R\text{-}NH\text{-}CH_2OH + R\text{-}NH_2 \longrightarrow R\text{-}NH\text{-}CH_2\text{-}NH\text{-}R + H_2O$

where R represents a purine or protein residue.

The efficiency in fixing biological materials differs with each aldehyde. For example, the glutaraldehyde rate of fixation of a fluorescein-conjugated albumin is greater than that of formaldehyde although more residual enzyme activity was detected in formaldehyde-fixed rat liver sections (Flitney 1966).

The mechanism of osmic acid (H_2OsO_4) fixation remains largely unknown. From his spectrophotometric studies on the interaction of fatty acids and phosphatides with OsO_4, Riemersma (1968) found that OsO_4 reacts in a proportion of approximately 1 mole OsO_4 per double bond and suggested that it immobilizes lipids by forming cyclic mono and/or diesters with the double bonds of fatty acid chains; evidence that OsO_4 reacts with lipid polar groups was also presented. The lipid monolayer studies of Dreher, Schulman, Anderson and Roels (1967) also showed an OsO_4 - double bond reaction but failed to provide evidence of an OsO_4 - lipid polar group interaction. Interest in the OsO_4 - lipid reactions stemmed from the "unit membrane" appearance of sectioned phosphatide-water preparations after osmium fixation, reminiscent of the trilaminar appearance of cell membranes. It was hoped that elucidation of the reaction mechanism of OsO_4 with lipids would provide an explanation for the electron transparent layer of thin-sectioned cell membranes; the finding that OsO_4 primarily reacts with fatty acid double bonds located in the hydrophobic region of a Danielli-type of membrane leaves this question very much open to speculation.

Since this chapter mainly deals with the fine structure of Vibrio psychroerythrus, it was thought that a brief introduction on the chemical action of fixatives used in our experiments was most appropriate. Current ideas on bacterial cell division in general were also discussed.

Materials and Methods

Negative staining. A loopful of young cells (36 hr culture) was spread on a precooled Formvar carbon-coated grid. After removal of excess liquid with filter paper, a loopful of cold aqueous 2% ammonium molybdate, pH 7.1, was applied to the grid and dried in air.

Fixation-embedding procedure. Cells grown and harvested as previously described (Chapter 2) were fixed, stained and dehydrated in the cold (10-15C) using precooled reagents. Washed cells were centrifuged at 8,000 xg, 10 min., and the resulting pellet suspended and fixed for 5 hr in an equal volume of 4% formalin made up in "all salts" solution. The fixed cell suspension, washed once with the "all salts" solution, was stabilized for 1 hr in buffered 1% uranyl acetate (Michaelis acetate-veronal buffer pH 6.1; final pH 4.4). Cells were washed once and dehydrated in a graded series of alcohols i.e. 1 hr in 50% alcohol and 30 min. each in 70, 90 and 100% alcohol. After each step of the fixation-dehydration procedure, 0.5ml samples were withdrawn for surface replication studies. The specimen was infiltrated at room temperature in a 1:1 ($^{w}/v$) mixture of Epon 812 epoxy resin and 100% acetone for 1 hr and polymerized at 55C for 2 to 3 days in freshly prepared resin. Specimen blocks were sectioned on a Porter Blum MT1 ultramicrotome and silver to gold sections (60-150nm thick) mounted on Formvar-coated copper grids (200 mesh). For additional contrast, mounted sections were floated for 15 min. on acetate-veronal

buffered 1% uranyl acetate, rinsed in a gentle stream of glass distilled water and dehydrated in 100% acetone. Specimens were viewed in an AEI 6B electron microscope operated at an accelerating voltage of 60KV.

Although several other combinations of fixatives and stains were tested, only the morphology of cells prepared by the standard glutaraldehyde-OsO_4 method will also be described. This method employs 4 hr fixation in 2% glutaraldehyde made up in "all salts" solution, 3 hr postfixation in 1% OsO_4 made up in Michaelis acetate-veronal buffer pH 6.3, 30 min. staining in 1% aqueous uranyl acetate pH 4.0 and dehydration in alcohols.

Surface replication. Preparation of samples for surface replication was carried out according to a technique elaborated by Kushner and Bayley (1963) with the following modifications. Samples withdrawn after each step of the fixation-dehydration procedure (see above) were smeared on clean glass slides, air dried and immediately shadowed with platinum-carbon at an incident angle of 20 degrees and strengthened with a film of evaporated carbon. Shadowing and replication were carried out on a Jeol vacuum evaporator model JVG-N1. The replica was floated onto a water surface, placed on a Formvar carbon-coated grid and permitted to air dry; occasionally the replicas were freed of organic matter before electron microscopic observation by treatment with H_2O_2.

A similar technique was used to demonstrate flagella in intact cells except that washed cells were smeared on pre-cooled glass slides and dried in the cold (10C) using an evacuated jar maintained at a residual pressure of 50μm Hg, measured with a McLeod gauge; dehydration was usually complete within 20 min. Leakage of UV-absorbing material resulting from the dehydration in vacuo was measured by rinsing each of triplicate 0.1 ml samples of dried cell suspension off the slides with 5.0 ml of cold "all salts" solution, centrifuging the rinse solutions at 15,000 xg, 10 min. and reading the absorbance (260nm) of the supernatants against an "all salts" solution blank. Leakage was expressed as a per cent of that released in cold distilled water ("100% leakage").

Osmotic activity of organic solutes. Intact cells were suspended in 0.5M solutions of sucrose and glycerol made up in "all salts" solution; changes in cell morphology were followed by phase contrast microscopy and cell lysis was measured by following the changes in absorbance of suspensions at 660 nm and of supernatants at 260 nm. The absorbance (660 nm) of duplicate 0.1 ml samples of a thick washed cell suspension suspended in 9.9 ml of cold sucrose or glycerol solution (0.5M) was read at regular intervals on a Coleman Junior spectrophotometer against an "all salts" solution blank; 0.1 ml of thick cell suspension made up to 10 ml with "all salts" solution was used as a control. After 2 hr of incubation, 0.1 ml samples were withdrawn from each cell

suspension and examined under phase contrast; the remaining cell suspensions were centrifuged at 15,000 xg, 10 min., and the supernatant read at 260 nm on a Beckman DU spectrophotometer. "100% leakage" was arbitrarily chosen as the absorbance (260 nm) of 0.1 ml of thick cell suspension lysed for 2 hr in 9.9 ml of cold distilled water.

Mitomycin C induction. Mitomycin C (50 µg/ml) in regular growth medium was added to 100 ml cultures of young (23 hr; OD_{660} = 0.13) and old 73 hr; OD_{660} = 0.83) cells to a final concentration of 0.5 µg/ml. The mitomycin-cell suspension, incubated on ice for 15-20 min., was centrifuged at 8,000 xg, 10 min., the pellet washed once in "all salts" solution and suspended in 100 ml of sterile growth medium. The absorbance (660 nm) was read for 6 hr at hourly intervals. Ten ml of the original culture, washed in "all salts" solution and suspended in 10 ml of sterile growth medium, was used as the control.

Morphological changes with increasing culture age. Two 1 liter flasks containing 300 ml of regular medium and 300 ml of regular medium supplemented with 0.01 M $CaCl_2$ were each inoculated with 4 ml of preculture (see Chapter 2). Ten ml samples were periodically withdrawn from each culture and their absorbance read at 660 nm; a few drops of each sample were examined by phase contrast microscopy and the remainder processed for thin section studies.

Morphology of cells grown on enriched solid media. Agar plates of 1% (w/v) proteose peptone (PP), 0.5% proteose peptone plus 0.5% tryptone (PPT), or regular medium, all made up in "all salts" solution pH 7.0, were inoculated with a loopful of preculture. After 3 weeks of incubation at 10C, cells washed off the agar surfaces with cold "all salts" solution were centrifuged at 8,000 xg, 10 min. and the pellets processed for thin section studies.

Results

Motility

Motility of Vibrio psychroerythrus depends on a single long polar flagellum (plate 1). About 30% of the cells investigated by this method had such a flagellum which could also be demonstrated in a much lower proportion of cell replicas. From the observation that cells dehydrated in vacuo released 50% of their UV-absorbing material (i.e. mean absorbance (260 nm) of "100% leakage" and dehydrated samples was 1.03 and 0.5 respectively), it is likely that flagella were lost during this treatment.

Osmotic activity of organic solutes

Studies on the possible osmotic activity of solutes showed that after 2 hr, the absorbance (660 nm) of cells incubated in 0.5M solutions of sucrose and glycerol decreased by less than 10% with respect to the control and little if any morphological changes could be detected by phase contrast microscopy. The mean per cent release of UV-absorbing material from cells suspended in sucrose and gly-

cerol was 16% and 18% respectively whereas the control lost 17% of its UV-absorbing material.

Fine structure

Thin sections of formalin-fixed cells showed that these were surrounded by two triple-layered structures, each 6.0 to 7.5 nm wide (Plate 2), which we consider to be the outer membrane and cell membrane; no structure which could be identified with the glycosaminopeptide (R) layer was observed. The densely staining ribonucleoprotein particles were loosely packed throughout the periphery of the cytoplasmic matrix in a seemingly random fashion. Fine fibrils of nucleic acids were preferentially oriented along the long axis of the cell. Mesosomes, coiled infoldings of the cytoplasmic membrane, were observed on occasion (Plate 2B). The standard glytaraldehyde-OsO_4 fixation failed to give satisfactory resolution of the cell envelope layers (Plate 3).

Groups of 2 to 4 uniformly dense rod-shaped organelles 10-15 nm wide and varying length, usually aligned along the long axis of the cell, were frequently seen crossing the septum of dividing cells (Plate 4A); rod-shaped structures 20 nm wide exhibiting 4 equally spaced (6 nm), densely staining layers were occasionally seen (Plate 4B). Electron dense circular particles, 15 nm in diameter, usually occuring in groups of 2 to 4, were frequently observed in cross sections of intact cells (Plate 5); at higher magnification (Plate 6), 2 concentric densely staining layers

5 nm apart could be resolved with some suggestion of subunit structure. The organelle also appears to be rigid as it is seen pushing against (Plate 7) and protruding through (Plate 8) the cell membrane which adheres closely to the organelle. However, the organelle is not necessarily rigid; plate 17 suggests that a protruding organelle may arise following the separation of daughter cells.

Circular electron transparent structures (Plates 13, 18 and 21) ranging from 100 to 130 nm in diameter are bounded by a densely staining layer (Plates 21 and 28); these structures, designated as inclusion bodies, are seemingly real since they occur regularly in intact cells and structures of similar shape and size (75 to 100 nm in diameter) were observed in negatively stained preparations of intact cells (Plate 1).

Surface replicas

Surface changes occurring during the preparative steps for thin sectioning were followed by surface replication. The matted surface and regular contour of unfixed intact cells (Plate 9) was relatively unaffected by formalin fixation (Plate 10). In addition to the prominant undulations of the cell surface, a central furrow was occasionally observed in replicas of formalin-fixed, uranyl acetate-stained cells (Plate 11); alcohol dehydration accentuated the rough appearance of the cell surface (Plate 12).

Mitomycin C induction

The possibility that the rod-shaped organelle was of viral origin was investigated using mitomycin induction. The antibiotic failed to generate any lytic particles as no significant decrease in optical density (660 nm) was measured.

Cell division

Cell division in V. psychroerythrus proceeds with an initial constriction and centripetal growth of the cell membrane (Plates 13 and 14) where cell membrane septation proceeds to completion (Plate 15) before the outer membrane begins to participate in the division process. In the final stages of cell division, the outer membrane invaginates (Plates 16 and 17) ultimately liberating 2 daughter cells.

Morphological changes with increasing culture age.

The growth curves of V. psychroerythrus grown in regular and Ca^{+2} - supplemented medium are presented in figure 7. A greater amount of Ca^{+2} in the medium does not alter the growth rate, both curves exhibiting a similar slope, although a slightly greater cell yield is obtained from a culture grown at the higher Ca^{+2} concentration.

Phase contrast studies revealed marked differences between cells grown in regular and Ca^{+2} - supplemented medium (Figure 7). After 61 hr of growth in regular medium, at least 20% of the cells were phase pale, deformed or fragmented, the proportion of which rapidly increased with

increasing culture age. In contrast, cells grown for 61 hr in the Ca^{+2} - supplemented medium were all phase dense rods showing little if any deformation. The onset of significant morphological changes in cells grown in the Ca^{+2} - supplemented medium occurred 159 hr following inoculation; after approximately 9 days of growth, the number of bent phase dense rods increased to 60%, the remaining cells apparently still intact. The high Ca^{+2} concentration (0.01M) also had a marked effect on motility and pigmentation. The proportion of motile cells in log phase cultures (40 hr) grown in regular and Ca^{+2} - supplemented media was approximately 20% and 40% respectively. Very few motile cells were observed in the 91 hr culture grown in regular medium whereas 10-15% of the cells in the 9 day-old culture grown in the Ca^{+2} - supplemented medium were still motile. At all the sampling times (except 20 hr where both cultures are non-pigmented), cell pellets from the Ca^{+2} - supplemented medium were always less pigmented than the corresponding pellet from the regular medium; for example, 75% of the pellet (lower portion) from the 9 day-old culture grown in the high Ca^{+2} medium was non-pigmented.

Ultrastructural studies have shown that the high concentration of Ca^{+2} (0.01M) tends to maintain the morphological integrity of aging cells. Cells grown for 39 hr in regular medium (Plate 18) exhibit an undulate often discontinuous outer membrane with short triple-layered segments; densely staining ribosomes and fine nucleic fibrils are

contained within a taut cell membrane. In the cells shown, rod-shaped organelles and a prominent inclusion body are also present. The fine structure of cells grown for 39 hr at the high Ca^{+2} concentration (Plate 19) is similar to that shown in Plate 18. After 49 hr of growth, the stabilizing effect of the Ca^{+2} supplement on the morphological integrity of intact cells becomes apparent. Cells grown in regular medium show fewer nucleic acid fibrils and some loss in the resolution of the outer and cytoplasmic membranes as unit structures (Plate 20); the morphology of cells grown at the higher Ca^{+2} concentration (Plate 21) does not differ significantly from that of younger cells (Plate 19) exhibiting triple-layered outer and cytoplasmic membranes and a nucleoid densely filled with fibers. Approximately 60% of the cells taken from a 91 hr culture grown in regular medium exhibited discontinuous and poorly preserved outer and cell membranes (Plate 22), the remaining 40% being made up of lysed cells and cell fragments. The proportion of lysed cells and cell fragments rapidly increased with prolonged incubation although the morphology of a few better preserved cells in the 9 day-old culture (Plate 24) compares well with that seen in the 91 hr culture (Plate 22). The structural integrity of cells grown for 91 hr (Plate 23) and as long as 9 days (Plate 25) in the Ca^{+2} - supplemented medium was remarkably well preserved, surpassing that obtained in cells grown for equivalent periods of time in the regular medium; although the separation between the tripartite outer membrane

and the remaining cell body tends to increase with increasing culture age (Plates 23 and 25), a taut triple-layered cell membrane frequently enclosed an apparently full complement of ribosomes and nucleic acid fibrils.

Morphology of cells grown on enriched solid media

Growth on agar plates of regular medium preserves cellular fine structure. In spite of the 3 week period of incubation, long segments of triple-layered outer and cytoplasmic membranes were frequently observed in addition to a good preservation of cytoplasmic organelles (Plate 26). These morphological characteristics are comparable to that found in 2-3 day-old cultures grown in regular medium (Plate 20). The fine structure of cells grown on proteose peptone agar medium (Plates 27 and 28) was similar to that of cells grown on regular agar medium (Plate 26) differing however in the greater number of rod-shaped organelles (Plate 27) and membranous vesicles (Plate 28). Distinct tripartite outer and cell membranes were frequently observed in cells grown on proteose peptone-tryptone medium (Plate 29); intracytoplasmic membranes occurred as closed vesicles (Plate 30) and invaginating membranes, or occasionally as concentric layers (Plate 31). A rare occurrence of a membranous organelle apparently associated with the cell membrane in dividing cells is shown in Plate 32.

PLATE 1

Polar flagellum of V. psychroerythrus

Negatively stained preparation of intact cells dried in "all salts" solution showing a long single polar flagellum. Electron transparent inclusion bodies are also seen (arrow). X 25,000.

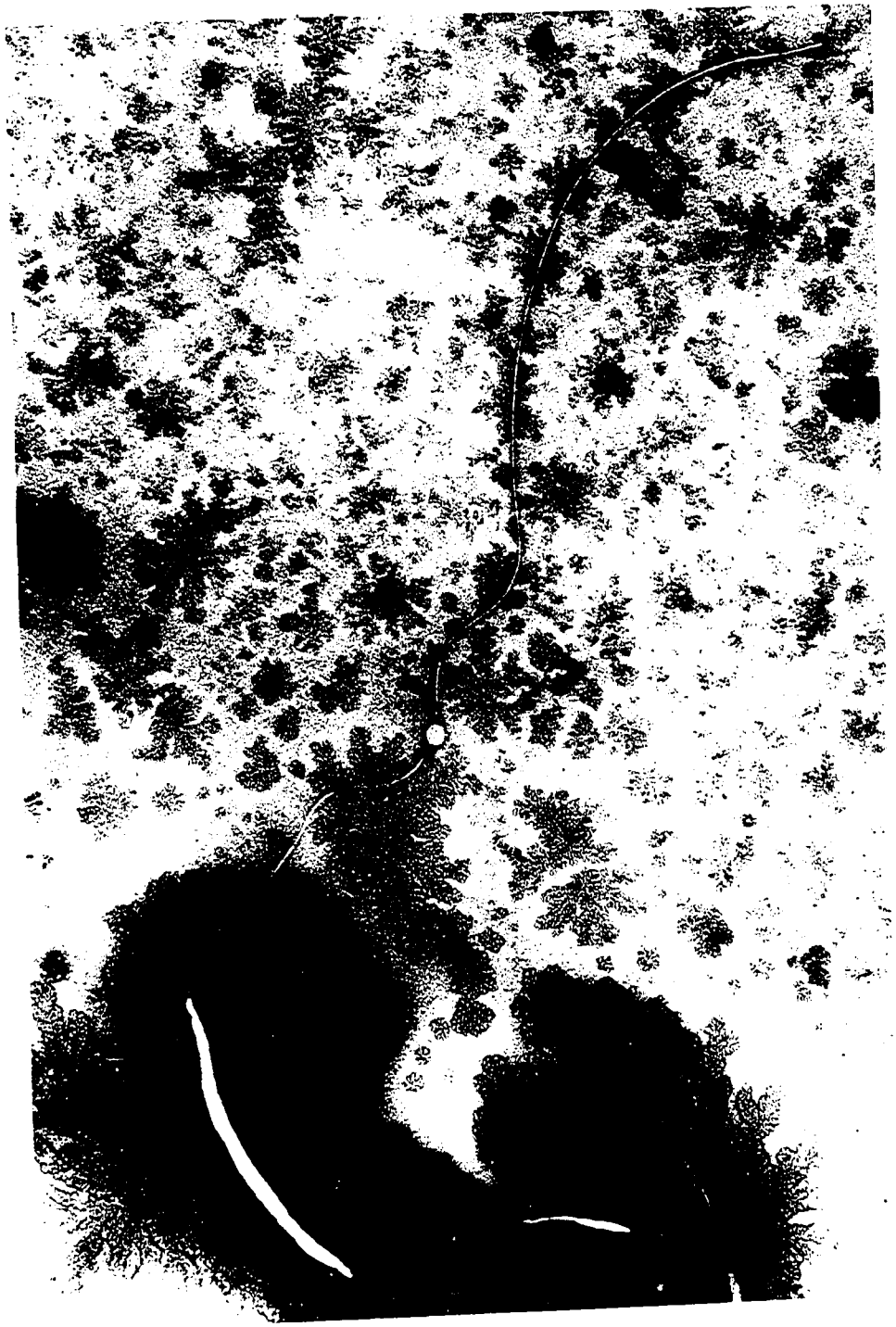


Plate 2

Fine structure of *V. psychroerythrus*

A. Longitudinal section showing the triple-layered outer membrane (om) and cytoplasmic membrane (cm), dense ribonucleoprotein particles (rnp) and fine fibrils of nucleic acid (nf). X90,000.

B. Section showing a mesosome, a coiled infolding of the cell membrane. X180,000.

Plate 3

V. psychroerythrus fixed in glutaraldehyde - OsO_4

Short segments of triple-layered outer membrane and cell membrane were occasionally seen. X96,000.

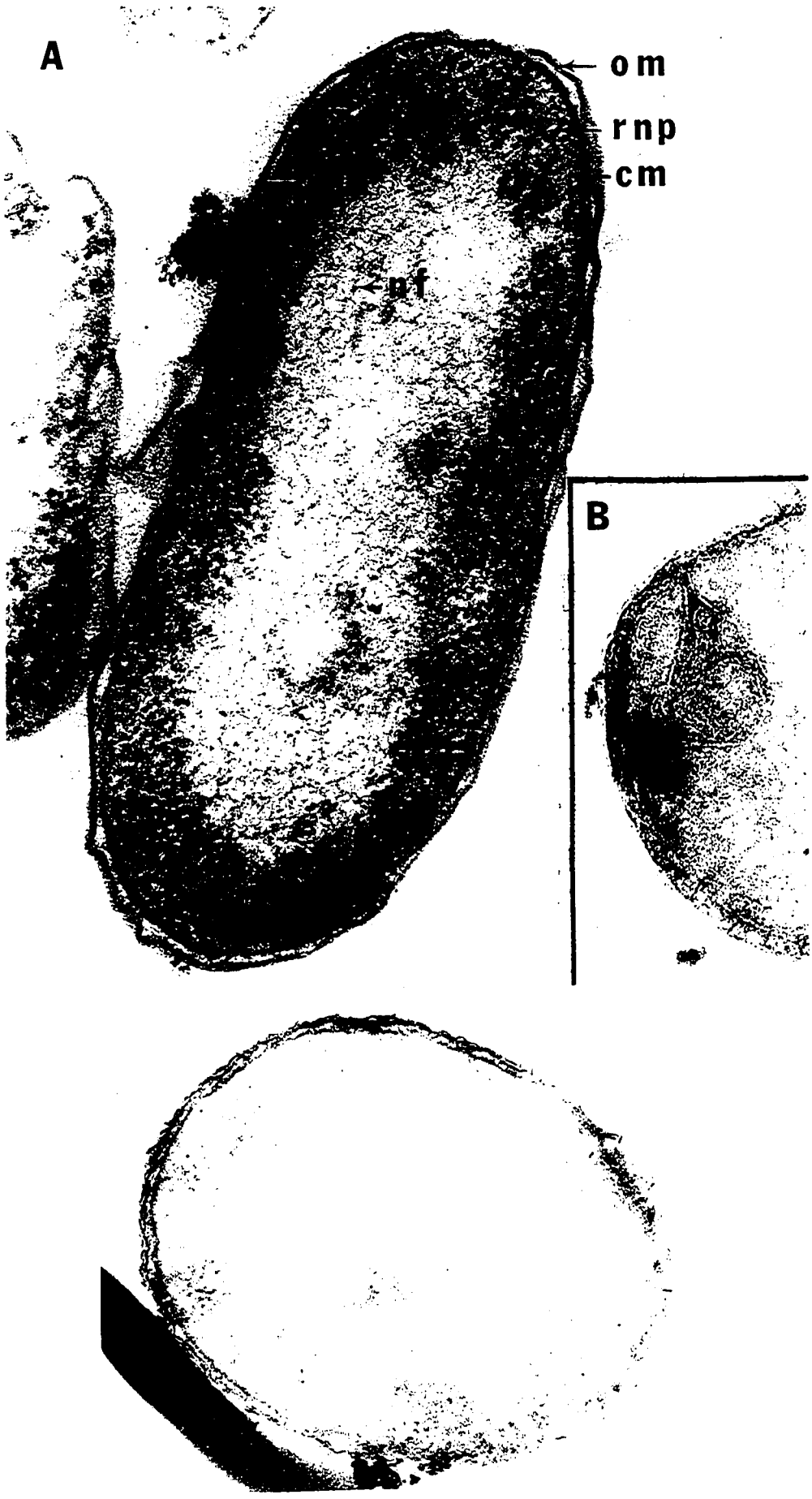
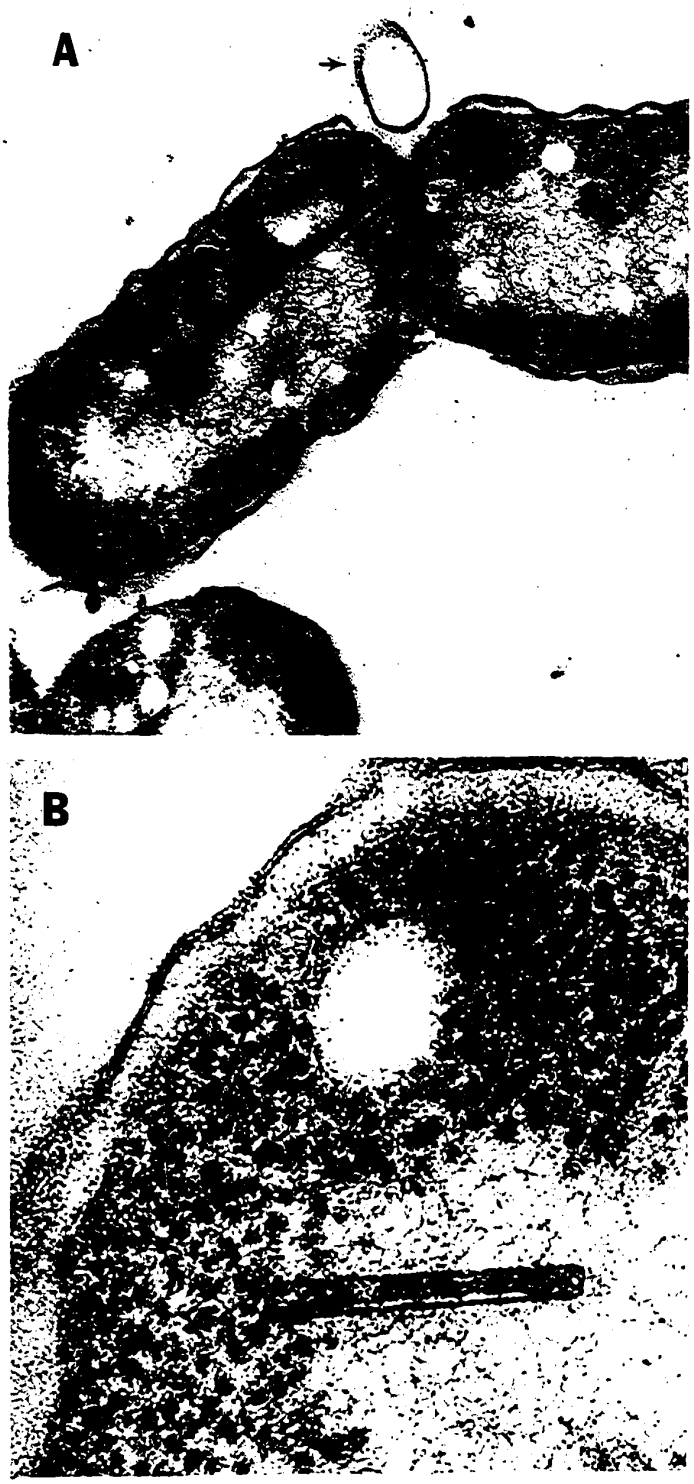

A
om
rnp
cm
pf
B

Plate 4

Longitudinal sections of a rod-shaped organelle

A. Group of 3 organelles of uniform density crossing the septum of dividing cells. A detached outer membrane vesicle (arrow) is also seen. X64,000.

B. A rod-shaped structure of similar width exhibiting four densely staining layers. X230,000.


A
B

Plates 5 and 6

Cross-sections of the rod-shaped organelle

Plate 5 (top). Electron dense particles of uniform diameter usually occurred in groups of 2 to 4 (arrow). X144,000.

Plate 6 (bottom). High magnification of the dense particles showing 2 concentric densely staining layers with some suggestion of subunit structure. X600,000.

Plates 7 and 8

Rigidity of the rod-shaped organelle

Plate 7 (top). Longitudinal section of an intact cell showing 2 rod-shaped organelles pushing against the cell membrane. X96,000.

Plate 8 (bottom). Two organelles protrude through the cell membrane which remains closely apposed to the organelle. X70,000.

Plates 9 to 12

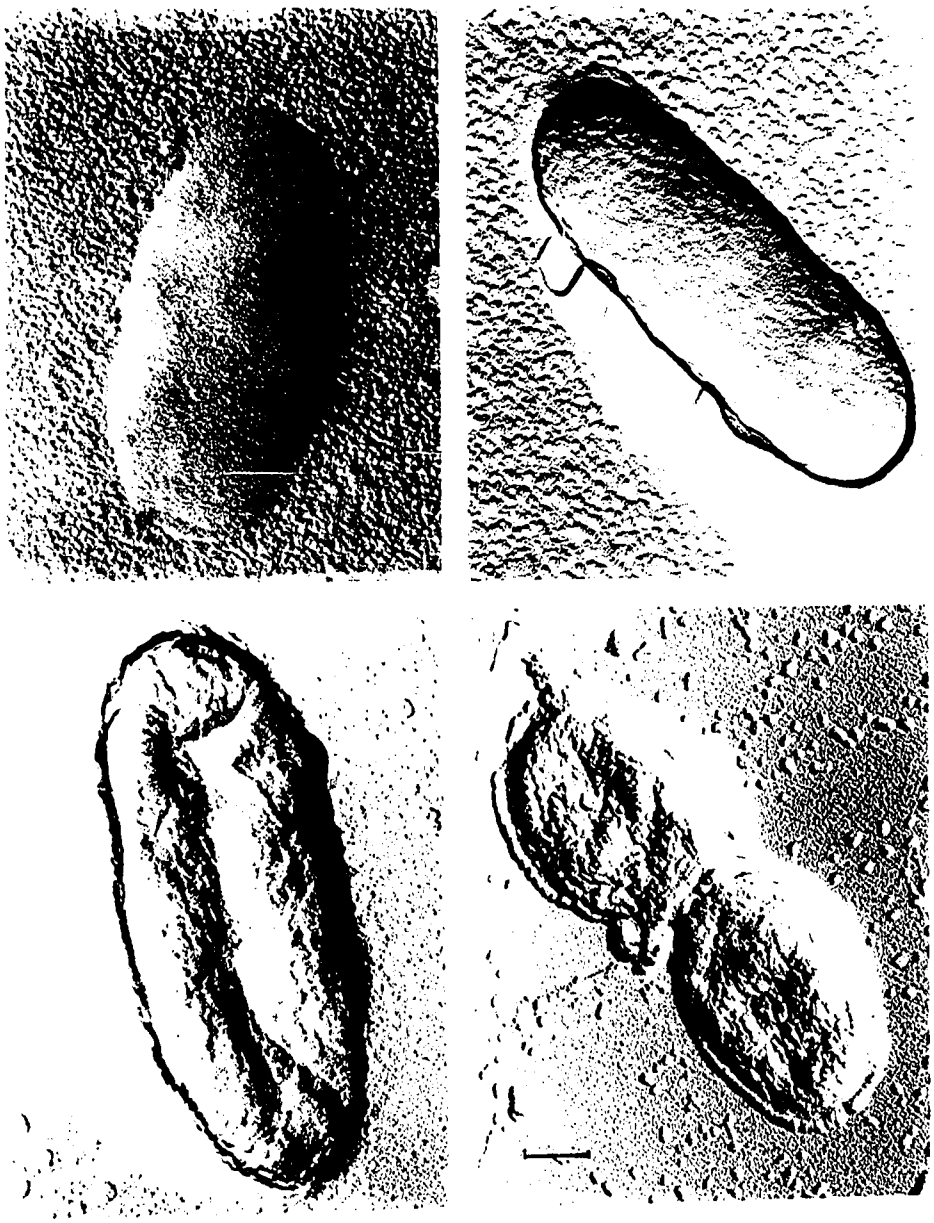
Surface replicas of *V. psychroerythrus*

Plate 9 (top-left). Intact cell showing a matted surface and a regular contour. X30,000.

Plate 10 (top-right). Formalin-fixed cell showing a regular contour and minor deterioration of the cell surface. X40,000.

Plate 11 (bottom-left). Formalin-fixed cell stained with uranyl acetate showing an undulated surface. A central furrow was occasionally seen. X30,000.

Plate 12 (bottom-right). Increased undulations were observed on the surface of formalin-fixed cells stained with uranyl acetate and dehydrated in 50% alcohol. X20,000.



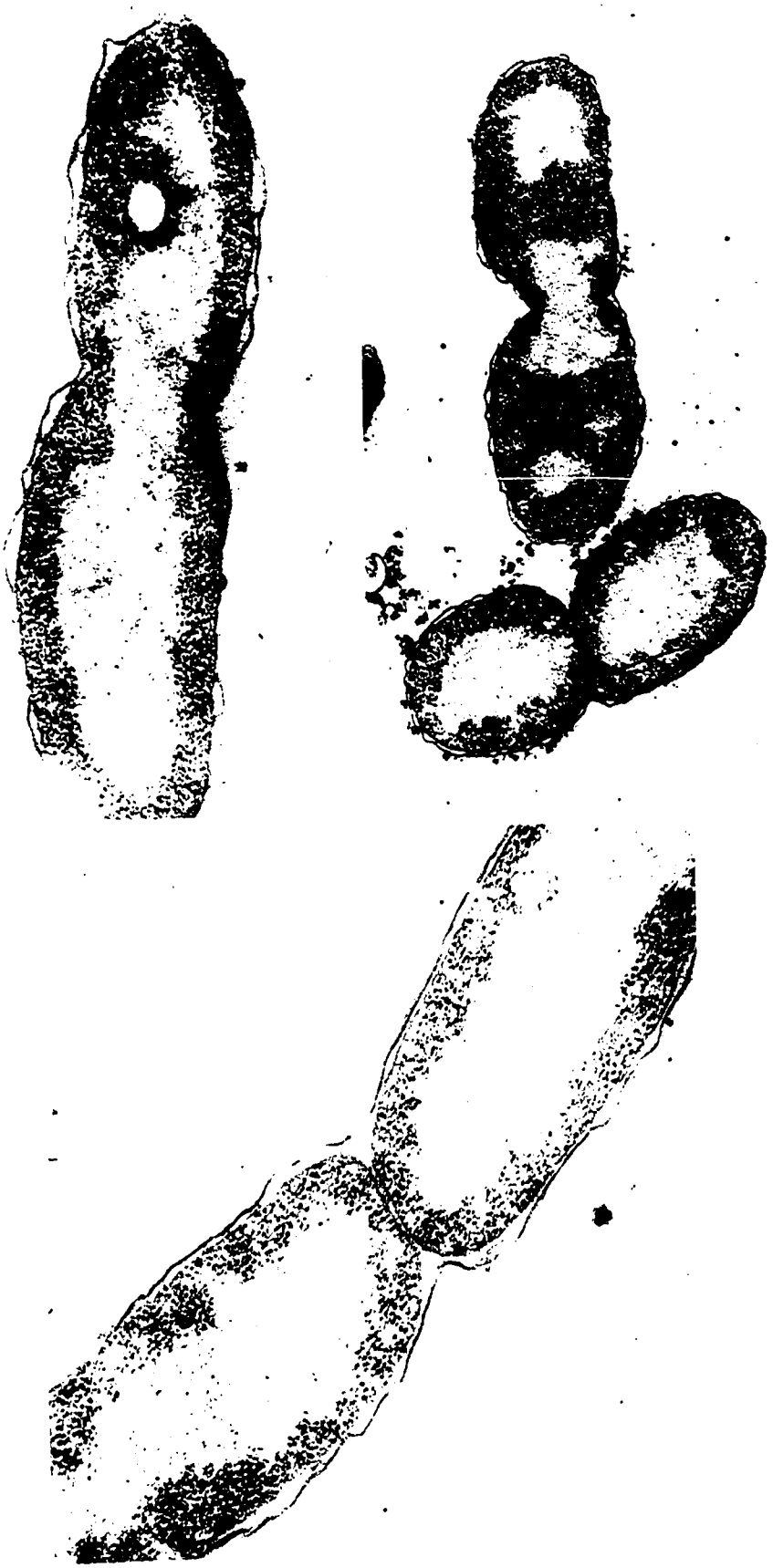
Plates 13 to 17

Cell division in V. psychroerythrus

Dividing cells taken from 20 to 40 hr cultures grown in regular medium supplemented with 0.01 M $CaCl_2$.

Plates 13 (top-left) and 14 (top-right). Cell division proceeds with an initial constriction of the cell membrane. Magnification is X45,000 and X24,000 respectively.

Plate 15 (bottom). Following the initial constriction, a cell membrane septum is formed. X60,000.



Plates 16 and 17

Plates 16 (top) and 17 (bottom). The outer membrane of the cell wall invaginates ultimately liberating two daughter cells. X32,000.

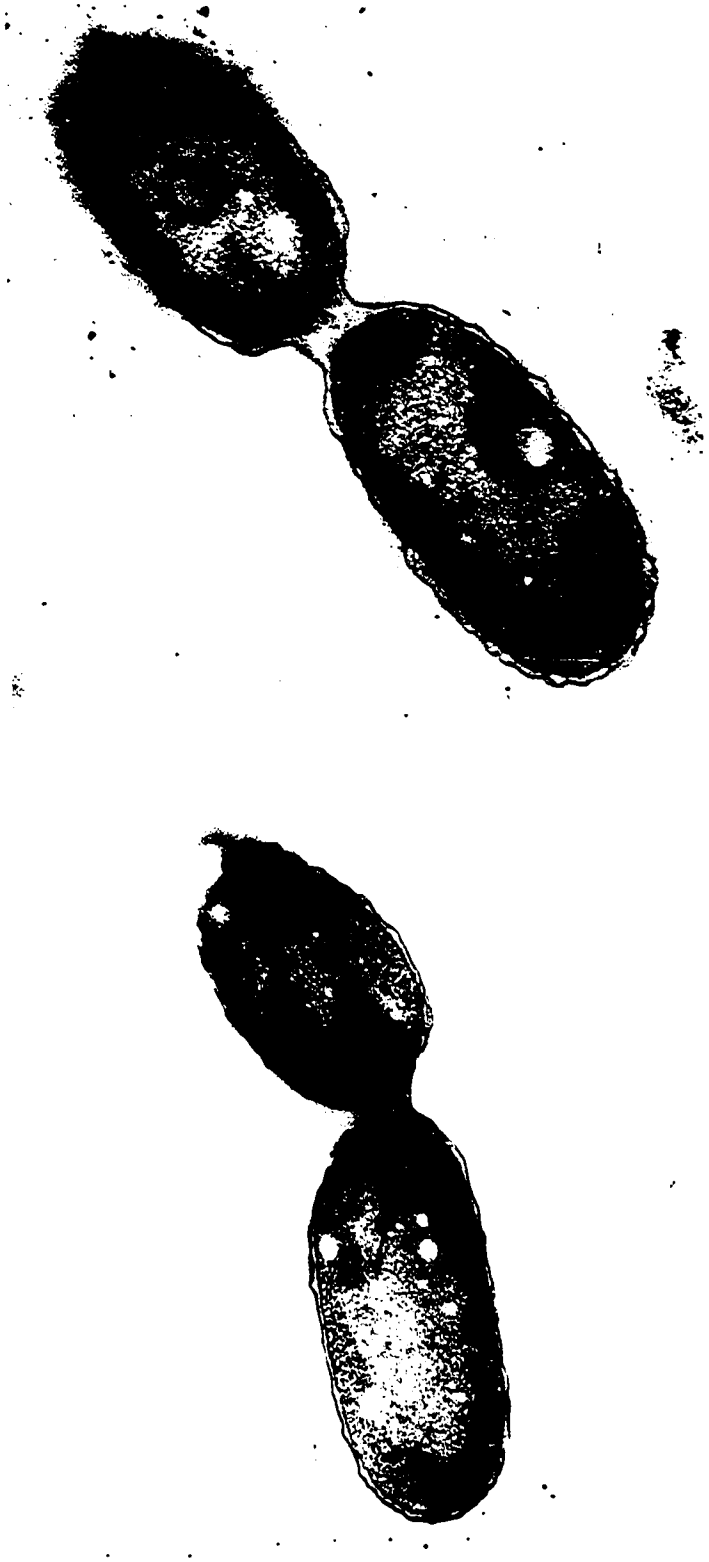
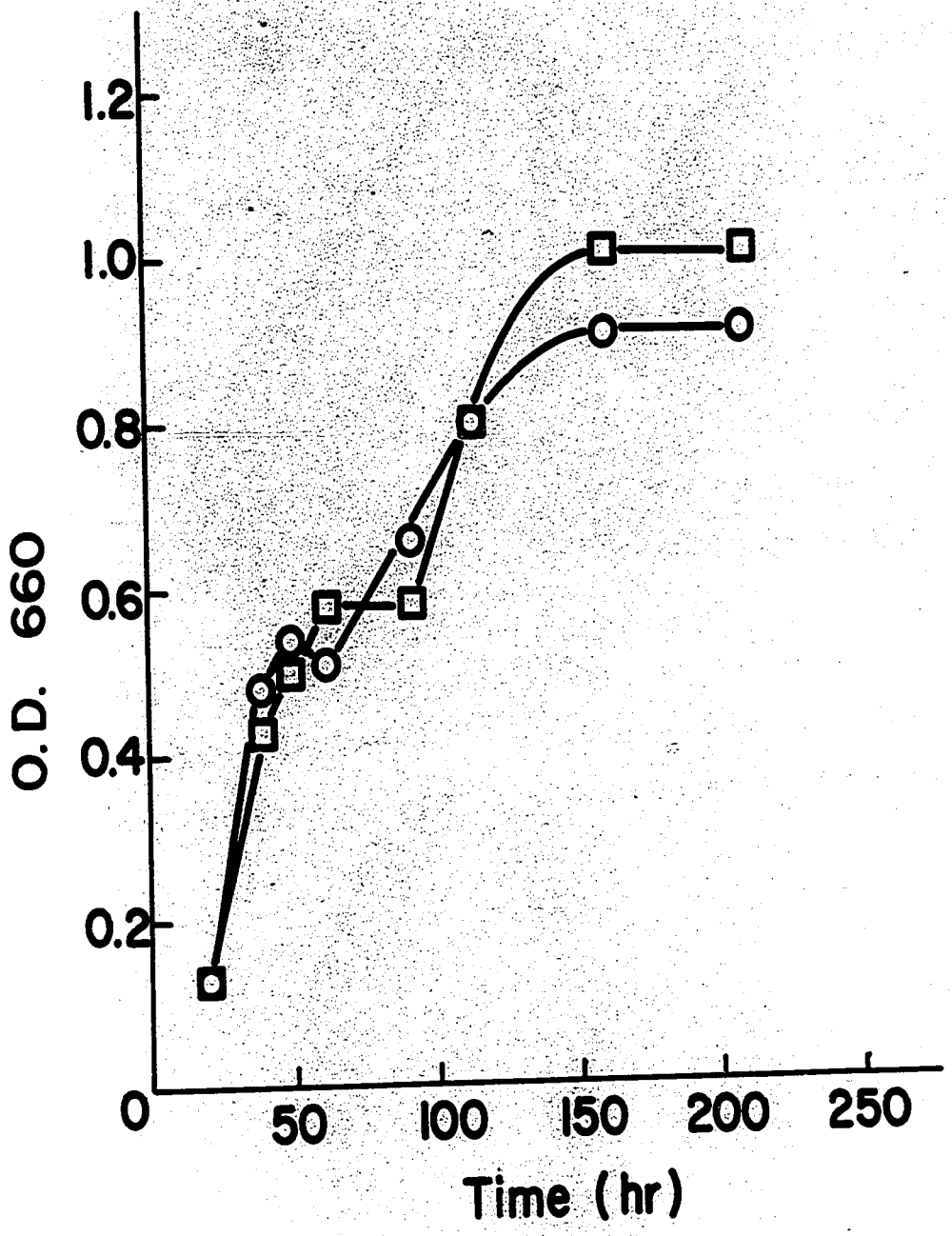


Figure 7

Growth curves of V. psychroerythrus

Growth of the organism in regular (O———O) and in Ca^{+2} - supplemented (□———□) medium was measured by following the changes in absorbance (660nm).



Plates 18 to 25

Fine structure of V. psychroerythrus with increasing culture age.

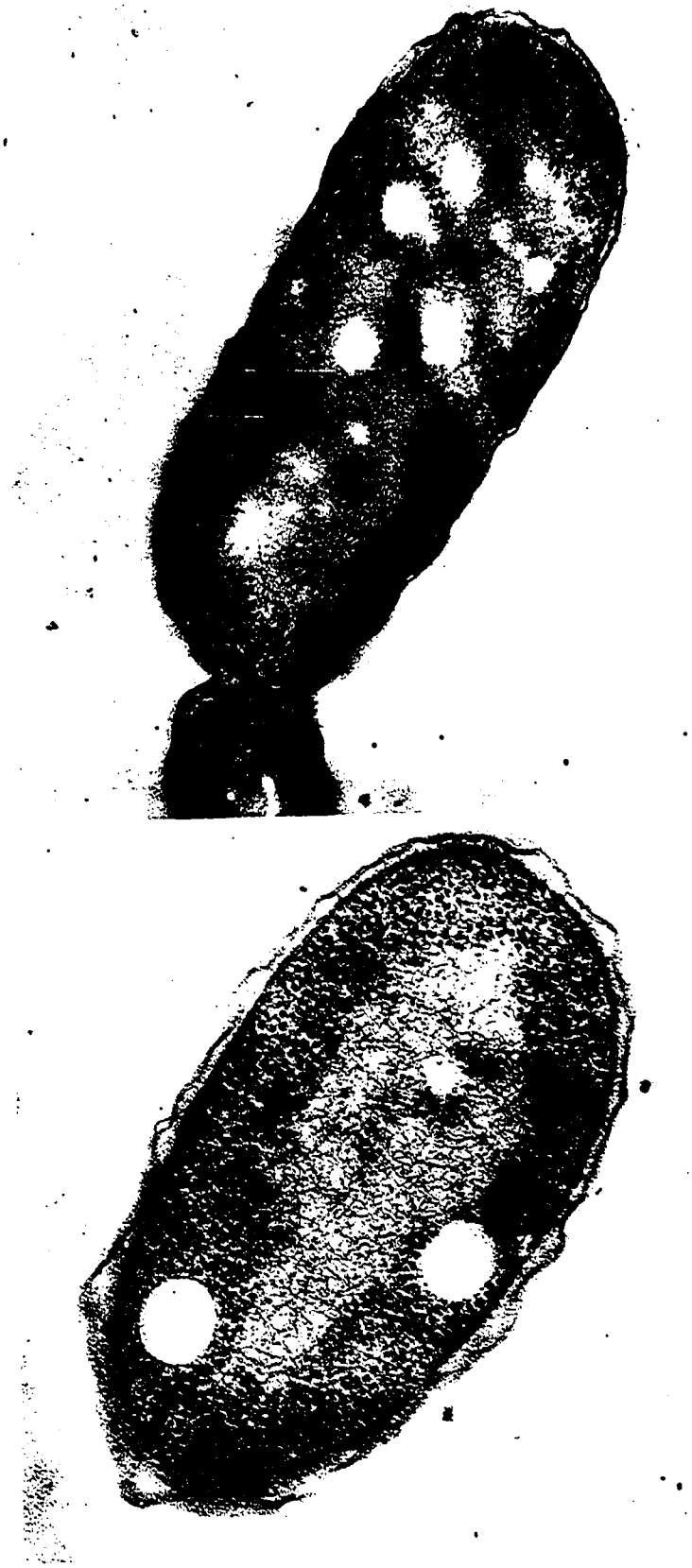
Plate 18 (top). Thin section of cells taken from a 39 hr culture grown in regular medium. In addition to an undulate outer membrane and a taut cell membrane, the cytoplasmic components are well preserved. X32,000.

Plate 19 (bottom). Thin section of a cell taken from a 39 hr culture grown in Ca^{+2} - supplemented medium. The morphology is similar to that shown in Plate 18. X48,000.

Plates 20 and 21

Plate 20 (top). Thin section of a cell taken from a 49 hr culture grown in regular medium. Cells showed fewer nucleic fibrils and some loss in the resolution of the outer and cytoplasmic membranes as unit membrane structures, X64,000.

Plate 21 (bottom). Thin section of a cell taken from a 49 hr culture grown in Ca^{+2} - supplemented medium. The envelope layers are distinctly triple-layered and the nucleoid appears to contain a full complement of fibrils. Inclusion bodies are particularly prominent. X95,000.



Plates 22 and 23

Plate 22 (top). Thin section of cells taken from a 91 hr culture grown in regular medium. The envelope layers are more discontinuous and poorly resolved than that found in cells grown for 49 hr in the same medium (Plate 20). X 32,000.

Plate 23 (bottom). Thin section of a cell taken from a 91 hr culture grown in Ca^{+2} - supplemented medium. Although undulation of the outer membrane increases with increasing culture age (compare with Plate 21), preservation of fine structure is better than that obtained for cells grown in regular medium (Plate 22). X125,000.

Plates 24 and 25

Plate 24 (top). Thin section of cells taken from a 9 day old culture grown in regular medium. The culture mostly contained lysed cells and cell fragment. The morphology of a few better preserved cells (shown here) compares well with that shown in Plate 22. X60,000.

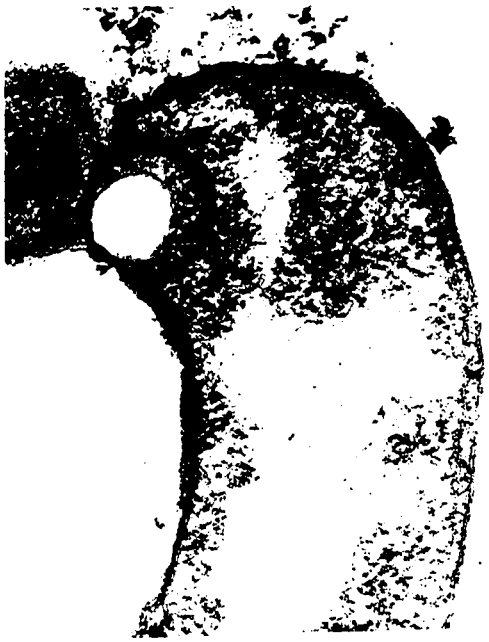
Plate 25 (bottom). Thin section of cells taken from a 9 day old culture grown in Ca^{+2} - supplemented medium. Although the tripartite outer membrane is greatly distended, a taut triple-layered cell membrane is usually present. X96,000.

Plates 26 to 32

Fine structure of V. psychroerythrus grown on solid media.

Plate 26 (top). Thin section of a cell grown on agar plates of regular medium. Long segments of triple-layered outer and cytoplasmic membranes were frequently observed. X 96,000.

Plates 27 (bottom-left) and 28 (bottom-right). Thin section of cells grown on proteose peptone (PP) agar medium. Rod-shaped organelles (Plate 27) and membranous vesicles (Plate 28) were observed more frequently in cells grown on PP medium than in cells grown on regular agar medium. Magnification of plates 27 and 28 is X 135,000 and X 96,000 respectively.



Plates 29 and 30

Plate 29 (top). Thin section of a cell grown on proteose peptone tryptone (PPT) agar medium. The outer and cytoplasmic membranes of better preserved cells usually occurred as distinct tripartite structures. Note that, in this preparation, the outer membrane is closely allied to the cell membrane and shows no undulations. X96,000.

Plate 30 (bottom). Thin section of a cell grown on PPT agar medium. Intracytoplasmic membranes occur as closed vesicles (arrows) or as invaginating membranes (im). X96,000.


im

Plates 31 and 32

Plate 31 (top). Thin section of a cell grown on PPT agar medium showing a concentric arrangement of intracytoplasmic membranes. X160,000.

Plate 32 (bottom). Thin section of cells grown on PPT agar medium. A membranous organelle (arrow) is associated with the cell membrane septum in dividing cells. X51,000.

Discussion

The sensitivity of V. psychroerythrus to changes in its environment (Korngold and Kushner 1968) may be the cause of the difficulties experienced in fixing cells for thin-section examination. To demonstrate fine structure, formaldehyde was used as the fixative because the more standard glutaraldehyde-OsO_4 fixation seldom yielded a satisfactory resolution of wall and membrane structure (Plate 3). Although OsO_4, a strong oxidizing agent, has been widely used as a fixative, it may not always be suitable. Costerton et al (1967) experienced difficulties in preserving the fine structure of pseudomonad protoplasts fixed by the Kellenberger-Ryter (1958) OsO_4 method. A few isolated cases of OsO_4 - induced artefacts have also been reported; OsO_4 fixation alters the X-ray diffraction pattern of native myelin membranes (Moretz, Akers and Parsons 1969a), disrupts the organized structure of negatively stained assemblies of lipid micelles (Glauert and Lucy 1969) and induces new configurations in the membrane of ciliated epithelial cells (Tormey 1964).

Since a rather high concentration of formaldehyde (0.5M) was used to fix cells, it seemed possible that formaldehyde might also be exerting an osmotic effect. It was previously found, however, that although cells were stable in 0.5M NaCl plus 0.1M $MgCl_2$, raising the NaCl concentration to 1.0 M did not lead to any discernible morphological changes (Korngold and Kushner 1968). Experiments reported

here in which 0.5 M sucrose or glycerol were added to cells suspended in "all salts" solution showed no change in turbidity (OD_{660}), release of UV-absorbing materials (OD_{260}) or appearance under phase contrast. It seems very unlikely, therefore, that formalin acts in any way except as a fixative, a finding that is further supported by the unaltered surface of formalin-fixed cells (Plates 9 and 10).

The ultrastructure of the polarly flagellated V. psychroerythrus is typically that of Gram negative bacteria exhibiting triple-layered outer and cytoplasmic membranes. Thin sections of intact V. psychroerythrus usually show an undulated cell wall (Plate 2) characteristic of many marine and non-marine Gram negative bacteria; these undulations have been attributed to the inherent flexibility of their outer membrane (reviewed by Glauert and Thornley 1969). The occurrence of a wavy outer membrane in freeze-etched preparations of intact Ferrobacillus ferrooxidans, a Gram negative bacterium, supports this view. Our surface replica studies (Plates 11 and 12) suggest that certain preparative steps in thin sectioning techniques are at least partly responsible for the undulations observed in the cell wall of V. psychroerythrus and other Gram negative bacteria (Glauert and Thornley 1969).

In Gram negative bacteria, the glycosaminopeptide (R) layer is localized in the periplasmic space, an electron transparent region of variable thickness bounded by the cell membrane and the outer layer of the cell wall. A thin

R layer, 3 to 8 nm wide (Glauert and Thornley 1969) has been observed in certain bacteria by staining with lead, uranyl, or lanthanum salts (Murray, Steed and Elson 1965, DePetris 1967, Buckmire and Murray 1970) although other species known to contain significant amounts of glycosaminopeptide fail to give this staining reaction (Birdsell and Cota-Robles 1967, Hofschneider and Martin 1968). Concerted efforts to demonstrate the R layer in thin sections of the marine pseudomonad NCMB 19 had proved unsuccessful (Buckmire and MacLeod 1965, Costerton et al 1967); very recently, a rigid (R) layer was found to be closely allied to the outer face of the cell membrane of plasmolyzed cells and mureinoplasts (Forsberg, Rayman, Costerton and MacLeod 1972). The amount of lysozyme-sensitive material in the marine pseudomonad is particularly low (1% of the cell dry weight) compared to 5-10% in other Gram negative bacteria (Rogers and Perkins 1968), a finding which partially explains the difficulty in demonstrating such a layer. An analogous situation may exist in V. psychroerythrus which fails to show an R layer in thin sections of cells grown in liquid (Plate 2) and solid media (Plates 27 and 29) even though the presence of muramic acid has been confirmed (see Chapter 5). It does not appear that the absence of an R layer in thin sections is due to the formalin fixation since such a layer could not be identified in thin sections of cells fixed by the glutaraldehyde - OsO_4 method (Plate 3) nor has such a layer been described in thin sections of OsO_4 - fixed (Kellenberger-

Ryter 1958) cells of the closely related V. marinus (Felter, Colwell and Chapman 1969, Kennedy, Colwell and Chapman 1970, Felter, Kennedy, Colwell and Chapman 1970).

Mesosomes, coiled infoldings of the cell membrane (Plate 2B) do not appear to be as numerous nor as complex as those encountered in Gram positive organisms (Ryter 1968, Rogers 1970). As pointed out earlier (Ryter and Jacob 1966) mesosomes in E. coli occur as delicate folds of the cell membrane that can only be seen if the section is cut in a plane exactly perpendicular to the fine folds of the mesosome; the few and simple structured mesosomes of V. psychroerythrus suggest that they are equally delicate. The membranous vesicles (Plates 28 and 30) are considered to be rudimentary mesosomes; similar forms of mesosomes have been described in E. coli (Kaye and Chapman 1963, Valentine and Chapman 1966) and in a marine pseudomonad (Wiebe and Chapman 1968a). Demonstration of mesosomes (Pontefract, Bergeron and Thatcher 1969) and intracytoplasmic membranes (Fischman and Weinbaum 1967) in E. coli was found to be nutrient dependent; more abundant and fully developed mesosomes were also observed in a marine Achromobacter sp. when grown in a high (1.0% peptone) rather than a low (0.1% peptone) nutrient medium (Wiebe and Chapman 1968a). Our results show that growth of V. psychroerythrus on enriched agar medium also stimulates the production of intracytoplasmic membranous figures. The occurrence of mesosomal-like structures in the vicinity of the cell mem-

brane septum and in direct contact with the nucleoid (Plate 32) suggests that septum formation and nuclear segregation in V. psychroerythrus is probably mesosome dependent.

The nature of the rod-shaped organelles is not certain. The apparently rigid tubular structures, 10-15 nm wide, are homogeneously dense (Plates 4A, 7 and 8) although on a few occasions (Plate 4B) an organelle 20 nm wide, exhibiting 4 densely staining layers 6 nm apart, was observed. In cross section, double cylinders are seen, their densely staining layers separated by 5 nm (Plate 6). From these considerations it appears that Plates 4A, 4B, 6 and 8 pertain to the same organelle; it is also suggested that the organelle, most prominent in young cells and often seen crossing the septum of dividing cells (Plates 4 and 17), is synthesized and distributed in synchrony with cell division processes. The rod-shaped organelle is not of membrane origin; the absence of a triple-layered appearance (Plate 4), the double cylinder profiles of uniform diameter (Plates 5 and 6) and the apparent rigidity (Plates 7 and 8) of this organelle are not consistent with properties of "unit membranes". Bacteria are known to harbor a variety of latent particles ranging in complexity between bacteriocins and bacteriophages; the marine bacteria are well represented in this respect (Spencer 1955, Valentine and Chapman 1966, Delisle and Levin 1969 and 1972). Our experience with V. psychroerythrus never led us to suspect the presence of a lytic particle in this organism; however, in view of the

morphological features of the rod-shaped organelle, the possibility that it might be a prophage or bacteriocin particle could not be overlooked. Mitomycin C, which acts by cross-linking the guanine and cytosine bases of complementary DNA chains (Iyer and Szybalski 1964), has been used to induce prophages in lysogenic strains of B. subtilis (Seaman, Tarmy and Marmur 1964, Haas and Yoshikawa 1969) and bacteriocins in Vibrio comma (Farkas-Himsley, Kormendy and Jayawardene 1971). Our inability to induce lytic activity in mitomycin-treated cells is by no means conclusive and does not eliminate the possibility that the structure is of viral origin. Possibly the phage genome (assuming that the rod-shaped organelle is a phage) is defective in coding for proteins essential for the production of lytic particles, a condition which would not be altered by mitomycin.

The rod-shaped organelle also bears a striking structural resemblance to the "microtubules" of Proteus mirabilis (Iterson et al 1967) and the rhapidosomes of Pseudomonas, Photobacterium, Proteus and Saprospira species (Yamamoto 1967); later the microtubules of Proteus sp. were tentatively identified as tubular variants of bacteriophages (Coetzee, Klerk and Coetzee 1968). Bradley (1967) referred to the rhapidosomes as perhaps one of the most interesting phage-like particles since they are in the form of headless contracted tails. Rhapidosomes and microtubules are synonymous in that they refer to long, smooth and rigid tubular structures approximately 20 nm in diameter; however, these struc-

tures differ chemically in that rhapidosomes contain RNA (Bradley 1967, Yamamoto 1967) whereas true microtubules, probably confined to eucaryotic cells (Kushner 1969), contain protein with a molecular weight of 60,000 (Stephens 1968). Other "microtubular-like" structures have been found in bacteria. Long tubular "polysheaths" observed in purified preparations of T4 phages are considered to be polymerized tail sheaths of the T4 phage (Kellenberger and Boy de la Tour 1964); purified bacteriocin (pyocin) preparations from Ps. aeruginosa also contain polysheaths (Higerd, Baechler and Berk 1967). Some bacteriocins, particles that absorb to and lyse sensitive cells but that are not propagated by infection, morphologically resemble headless phages (see Bradley 1967 for a critical review). It is then clear that there exists considerable morphological overlap between rhapidosomes, polysheaths and some bacteriocins and that further investigation on the nature of these structures may show that they correspond to abortive viral particles exhibiting varying degrees of organization.

The rod-shaped organelle of V. psychroerythrus does share a number of common features with rhapidosomes of other bacterial species (Yamamoto 1967) in that it is a long, rigid tube with a smooth contour differing only in its smaller width; the double cylinder arrangement (Plates 5 and 6) was also observed in the negatively stained rhapidosomes of Saprospira grandis (Yamamoto 1967). It then seems possible that the rod-shaped organelle of V. psychro-

erythrus is also a rhapidosome. Tubular structures have also been reported in the closely related psychrophilic MP-1 strain of V. marinus; "rows of circular profiles" 21 nm in diameter were occasionally seen in thin section preparations and considered to be cross sections through tubular evaginations of the cell membrane or microtubules (Felter, Kennedy, Colwell and Chapman 1970). The significance of rhapidosomes crossing the septum (Plates 4A and 17) is not clear although these organelles, usually seen in direct contact with the nucleoid, may participate in the replication and segregation of the bacterial nucleoid (Yamamoto 1967). It is also interesting to note that the flagellum (Plate 1) and the rhapidosome are of similar width.

The presence of inclusion bodies in our marine organism is not unqiue for they have been observed in marine Achromobacter sp. (Wiebe and Chapman 1968a). The structure appears to be bounded by a densely staining layer (Plates 21 and 28) apparently not of unit membrane structure; the electron transparent appearance of the bodies may be due to a lack of specificity of the uranyl salts for the enclosed material or more probably due to extraction of material during the fixation-embedding procedure.

Cell division in V. psychroerythrus proceeds with an initial constriction and centripetal deposition of the cell membrane (septation) followed by the invagination of wall material releasing 2 daughter cells (Plates 13 to 17). A

similar mechanism has been described in V. marinus (Felter, Colwell and Chapman 1969, Kennedy, Colwell and Chapman 1970) where the formation of a cell membrane septum was considered to be somewhat unique having been demonstrated only on one other occasion in an unidentified bacillus (Chapman 1959). According to Steed and Murray (1966), the mode of cell division in both Gram positive and Gram negative bacteria is similar, involving the simultaneous invagination of the cell wall and cell membrane in Gram positive bacteria and the cell membrane plus the glycosaminopeptide (R) layer in Gram negative bacteria. The apparently unusual division process in V. marinus (Felter, Colwell and Chapman 1969) and our vibrio may rest on the inability to demonstrate the R layer in thin sections of intact cells. Assuming that the R layer invaginates with the cell membrane, cell division in these vibrios would be similar to that described for other Gram negative organisms such as E. coli (Conti and Gettner 1961, Steed and Murray 1966), Spirillum serpens (Steed and Murray 1966) and Achromobacter sp. (Wiebe and Chapman 1968).

Phase contrast and electron microscope studies have shown that after 50 hr of growth (late log phase) in regular medium, cell structure begins to deteriorate. With increasing culture age, cells become deformed and/or phase transparent with a concomitant decrease in motility. Ultrastructural studies of aging cells show that the outer membrane progressively becomes more undulate (similarly observed

in V. marinus by Kennedy, Colwell and Chapman 1970) and discontinuous exhibiting less and less of its tripartite structure; an increasing number of outer membrane vesicles of the type shown in Plate 4 were also observed. The number of rhapidosomes and inclusion bodies did not change significantly with increasing culture age. The significance of these morphological changes is difficult to evaluate since comparable studies with other marine bacteria are generally lacking.

Growth in the Ca^{+2} - supplemented medium had a marked effect on the motility, pigmentation and morphology of V. psychroerythrus. The prolonged motility of cells grown in this medium may result from a Ca^{+2} stabilization of flagellar proteins through salt linkages. Although Gerber and Noguchi (1967) showed that the flagellin subunits of the non-marine Salmonella abortus equi contain a high proportion of nonpolar amino acid residues, it is conceivable that V. psychroerythrus has adapted to its native marine environment by incorporating a greater proportion of acidic proteins in its flagella, as it did in its cell envelope (see Chapter 5).

That high concentrations (0.01M) of $CaCl_2$ apparently inhibit pigment production in V. psychroerythrus was rather unexpected in view of the Ca^{+2} - dependent prodigiosin production in the marine isolate 169R, probably a member of the Pseudomonas genus (Lewis and Corpe 1964). In 1968, Nunokawa and McDonald isolated a non-pigmented variant of V. psychro-

erythrus on a L-leucine supplemented medium. We recently isolated a white variant from our stock cultures of V. psychroerythrus grown on regular agar medium. This suggests that lack of pigmentation is not solely Ca^{+2} dependent.

The fine structure of log phase cells grown in regular and Ca^{+2} - supplemented medium was similar (Plates 18 to 21) although the morphology of cells in both cultures differed greatly with increasing culture age. Cells taken from a stationary phase culture grown in the Ca^{+2} - supplemented medium showed distended and/or broken outer membranes with long segments of tripartite structure and taut unit cell membranes (Plates 23 and 25); such good preservation of the envelope layers was seldom encountered in cells grown for equivalent periods of time in regular medium (Plates 22 and 24). These results suggest that Ca^{+2} ions are involved in maintaining the structural integrity of the cell envelope layers, especially that of the cell membrane. Evidence that Ca^{+2} ions specifically maintain membrane structure was presented by Salton (1971a) who found that protoplasts of Micrococcus lysodeikticus formed in the presence of 10 mM Ca^{+2} were exceedingly difficult to lyse by osmotic shock. Tobias, Agin and Pawlowski (1962) also observed an increase in ohmic resistance of synthetic cholesterol-phosphatidyl serine membranes with increasing Ca^{+2} concentrations; they suggested that the Ca^{+2} effect was due to its combination with acidic groups of the phospholipid. As pointed out earlier, divalent cations can be

expected to stiffen lipoprotein membranes by forming salt linkages between neighboring negatively-charged groups (Brown 1964). The effectiveness of divalent cations in preventing the lysis of the marine pseudomonad B-16 (MacLeod and Matula 1961) and the release of soluble non-dialyzable material from isolated cell envelopes (Buckmire and MacLeod 1971) has been associated with a cation-shielding of electronegative groups in the envelope layers.

Small amounts of agar (0.1%) have been used to delay the onset of lysis in cultures of V. psychroerythrus grown in regular medium (R.R. Korngold - personal communication). We have also found that the presence of agar helps maintain cell structure (Plate 26). Enrichment of the agar medium (Plates 27 to 32) further improves the general morphology of cells and stimulates the production of intracytoplasmic membrane structures. Similar membrane structures were observed in the closely related V. marinus grown for 18 to 72 hr. in liquid medium (Felter, Kennedy, Colwell and Chapman 1970). It appears that membranous organelles of V. psychroerythrus are particularly sensitive to growth conditions in that they are poorly preserved in cells grown in regular liquid medium and are best seen in cells grown on enriched solid medium; the presence of 0.01M $CaCl_2$ in the regular liquid medium also preserves membrane structure (Plates 21, 23 and 25). The occurrence of cytomembranes in E. coli (Fischman and Weinbaum 1967) and in marine Pseudomonas and Achromobacter sp. (Wiebe and Chapman 1968a)

was also found to be dependent primarily on the nutrient level of the medium. The enriched medium possibly promotes an increased rate of membrane synthesis prior to cell division (Fischman and Weinbaum 1967).

It was stated earlier that knowledge of structural variations in normal cells was a prerequisite to the morphological study of V. psychroerythrus under various conditions of lysis. With this prerequisite now satisfied, we then propose to study the structural changes accompanying lysis of V. psychroerythrus.

Chapter 4

Structural changes during lysis of *Vibrio psychroerythrus*

Introduction

Several reports, briefly reviewed in the preface, have dealt with the physiology of V. psychroerythrus, its stability in salt solutions and the chemical changes accompanying lysis. The work of Korngold and Kushner (1968) showed that the red psychrophile requires both NaCl and $MgCl_2$ for growth and stability and that divalent salts, some in very low concentrations, could maintain turbidity of cell suspensions. They also found that intact cells lysed in distilled water or broken mechanically in cold seawater or psychrophile salt medium (PSM) lost more than half their lipid phosphorus and hexosamine in a form not sedimentable at 15,000 x g for 30 min. We pursued further this line of investigation by studying the structural changes occurring under different lytic conditions. We thought that such a study would complement the existing chemical data and possibly reconcile the ionic requirement of V. psychroerythrus for growth and stability with a specific structural role of mono- and divalent cations. The possibility that K^+ ions played a structural role was also investigated.

Materials and Methods

Growth conditions. Cells were grown and harvested as previously described (See Chapter 2).

Lysis of intact cells. Experiment 1. To test the effects of ions on lysis and cell ultrastructure, 0.1 ml samples of a thick, washed cell suspension were suspended in 9.9 ml of cold (10C) water or aqueous solutions of salts at specified

concentrations. An equal volume of thick cell suspension made up to 10.0 ml with "all salts" solution was used as a control. The cells were permitted to equilibrate to a constant turbidity (absorbance at 660 nm); 2 hr was usually required. An aliquot was then removed for phase contrast examination and the remaining cell suspension sedimented at 15,000 x g for 15 min. Cell pellets were fixed and embedded as previously described (see Chapter 3) and the absorbance of the supernatant fluid was read at 260 nm against a water blank.

Experiment 2. Chemical changes accompanying lysis under different conditions (see Table 4) were also studied. Ten ml samples of a washed cell suspension were sedimented at 8,000 x g for 10 min. Pellets were suspended in an equal volume of salts solution or water and the suspensions equilibrated till the turbidity (absorbance at 660 nm) became constant. Cells were then sedimented at 15,000 x g for 30 min.; the pellets were immediately extracted with cold 10% trichloroacetic acid (TCA) and the 15,000 x g supernatant read at 260 nm against a water blank.

Lysis was also induced by exposing washed cells to slightly acidic pH ("all salts" solution, pH 5.0, 7C) and high temperature ("all salts" solution, pH 7.0, 37C). After suspensions had reached constant turbidity (usually 2 hr.), suspensions were centrifuged as above, and pellets were fixed and embedded.

Particles released on lysis. Particles released from a thick cell suspension lysed in cold distilled water were separated by differential centrifugation. After the cell suspension had reached constant turbidity, it was sedimented at 15,000 x g for 20 min. and the pellet fixed and embedded. Pellets obtained from the 15,000 x g supernatant following 30,000 x g (20 min.) and 65,000 x g (60 min.) centrifugations were suspended and stained for 1 hr. in a minimal volume of cold 1% aqueous uranyl acetate, applied to a formvar-C coated grid and permitted to air dry before electron microscope examination.

A similar method was used to study the fine structure of particles released from cells lysed in 0.5M NaCl, 0.1M $MgCl_2$ and 0.1M NaCl plus 0.02M $MgCl_2$.

Structural role of K^+ cations. The possibility that K^+ ions contributed to the preservation of cell membrane structure was studied using envelopes prepared in K^+ - supplemented and K^+ - deficient salt solutions. Washed cells from a 50 hr culture grown in regular medium were separated into 6 lots and sedimented at 8,000 x g for 10 min. The pellets were suspended in 10 ml of buffered (Michaelis acetate-veronal buffer pH 7.0) or unbuffered salt solution containing 0.5M NaCl plus 0.1M $MgCl_2$ and KCl at specified concentrations. The cell suspensions were then broken mechanically in a Mickle disintegrator for 15 min. at 10C using ballotini No.12 glass beads; intact cells were removed by two 10 min. centrifugations at 2,000 x g. Envelopes and envelope frag-

ments were separated by differential centrifugation (fig. 8). Samples (4 ml) for lipid-P determinations were taken from the 2,000 x g and 15,000 x g supernatants and immediately extracted with chloroform-methanol (see below). The envelopes (pellet 2) were fixed and embedded as previously described (see Chapter 3) with some modifications; for each pellet, the salt composition of the fixing and washing solutions was identical to that used in preparing the envelopes. Pellets 3 and 4 (see fig. 8) were suspended and stained for 1 hr. (in the cold) in a minimal volume of 1% uranyl acetate made up in the "appropriate" salt solution; samples of stained material were applied to a formvar-C coated grid and dried in the cold before electron microscope examination.

Dry weight of intact cells. Dry weight of cells was measured turbidimetrically by using a standard curve relating salt-free dry weight to absorbance at 660 nm. Cells from a 60 hr culture grown in regular medium were washed and suspended in "all salts" solution as a thick suspension. Salt-free dry-weight was determined by drying duplicate 10 ml samples of thick cell suspension to constant weight at 105C and subtracting the weight of separately dried 10 ml samples of "all salts" solution.

Nucleic acid determination. Intact cells from a 60 hr culture grown in regular medium or pellets remaining after lysis (see Experiment 2 above) were extracted with cold and then with hot trichloroacetic acid as described by Schneider

(1945). The intermediate lipid extraction could be omitted without affecting nucleic acid measurements. Deoxyribonucleic acid (DNA) was measured in hot trichloroacetic acid extracts with the diphenylamine reagent (Burton 1956), by using a highly polymerized calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) as standard. Ribonucleic acid (RNA) was measured in hot trichloroacetic acid extracts by the orcinol reagent (Albaum and Umbreit 1947) with a yeast RNA (Worthington Biochemical Corp.) used as a standard.

Lipid phosphorus determination. Lipids from mechanically prepared envelopes (see fig. 8) were extracted with chloroform-methanol as described by Bligh and Dyer (1959). Lipid phosphorus was determined according to the method of Chen et al (1956) using ascorbic acid-molybdate reagents. An aliquot of the chloroform layer was evaporated to dryness in a 40-50C water bath and the lipid residue ashed in the presence of 0.1 ml of concentrated sulphuric acid (H_2SO_4). The digest was then clarified with 0.05 ml of 30% hydrogen peroxide (H_2O_2) and dissolved in a known volume of distilled water. Aliquots were then taken for lipid phosphorus determination and read at 800 nm against a reagent blank. Standards were prepared from a stock solution of K_2HPO_4 (20 µg P/ml).

Results

Lysis of intact cells

Effects of environmental changes on cell stability and chemical composition are shown in Table 4. As observed earlier (Hagen et al 1964, Korngold and Kushner 1968), the

bacterium was stable at low temperatures in "all salts" solution or in 0.5M NaCl plus 0.1M $MgCl_2$. The turbidity remained constant, and only small amounts of ultraviolet (UV)-absorbing material were released from the cells. When the NaCl plus $MgCl_2$ was diluted, turbidity fell, and more UV-absorbing materials appeared in the supernatant fluid. In 0.5M NaCl alone, turbidity fell slowly, and the final release of UV-absorbing substances approached that found in distilled water. At lower NaCl concentrations, turbidity fell more rapidly, and the release of UV-absorbing substances increased. Cells were not stable in $MgCl_2$ solutions of 0.1M or lower. However, $MgCl_2$ solutions were better able to prevent release of UV-absorbing substances than fivefold greater concentrations of NaCl.

Even after extensive lysis, substantial amounts of DNA and RNA remained associated with the residual cellular material. Solutions of 0.5M NaCl or of 0.1M $MgCl_2$ were better able to prevent loss of cellular DNA than loss of RNA. Extensive loss of nucleic acids took place after lysis at 37C.

Almost all cells in "all salts" solution and in 0.5M NaCl plus 0.1M $MgCl_2$ were rod-shaped and dense under phase contrast. In 0.3M NaCl plus 0.06M $MgCl_2$, about 75% were rod-shaped and the rest spherical; in 0.1M NaCl plus 0.02M $MgCl_2$, at least 90% of the cells became phase-pale spheres. In 0.1M $MgCl_2$ all cells became spherical in a few minutes. Cells in 0.5M NaCl, examined after 2 hr, were still rod-

shaped but less phase-dense than intact cells.

The fine structure of cells treated as in experiment 1, Table 4, was examined. Though the characteristic outer membrane and inner membrane structure of intact cells was retained in 0.5M NaCl plus 0.1M $MgCl_2$ (Plate 33), suspension in more dilute solutions led to a loss of definition of the cell membrane as a triple-layered structure (Plate 34), followed by distortion and fragmentation of the outer membrane (Plate 35).

When cells were suspended in 0.5M NaCl alone, the outer membrane appeared as a detached but distinct triple layer, and only a faint suggestion of the cell membrane remained (Plate 36). The nuclear region was poorly defined. Cells suspended in 0.1M NaCl were transformed to a collection of fragments and vesicles (Plate 37). These are very similar to those obtained from cells suspended in distilled water (Plate 40A) except that the latter had a smaller proportion of outer membranes or outer membrane fragments with continuous smooth structure. Few traces of the cell membrane remained in the material sedimented after lysis in 0.1M NaCl or distilled water.

In 0.1M $MgCl_2$ (Plate 38), a better preservation of the cell membrane was obtained than in 0.5M NaCl. Even in 0.02M $MgCl_2$ (Plate 39), cellular integrity was better preserved than in 0.1M NaCl alone (Plate 37).

Particles released on lysis

It was observed earlier that, when cells were lysed in distilled water, a good deal of cellular material was released in a form not sedimentable by centrifuging for 10 min. at 8,000 x g but sedimentable after 60 min. at 100,000 x g (Korngold and Kushner 1968). Debris from cells lysed in distilled water (Plate 40A) were obtained by centrifuging 20 min. at 15,000 x g. On centrifuging at 27,000 to 30,000 x g for 20 min., more material sedimented, similar in appearance to that shown in Plate 40A. After 60 min. at 65,000 x g, an assortment of particles sedimented (Plates 40B to 40D). No further material sedimented after 60 min. at 100,000 x g.

The 65,000 x g sediment included debris and different-sized vesicles. The larger vesicles were 150 to 250 nm in diameter. Many vesicles only 12.5 to 15 nm in diameter were also observed. A few of these (shown especially in Plate 40B) had a doughnut appearance. In others of the same size, only a suggestion of such a structure could be seen. Though the "doughnuts" are perhaps the most interesting structures found, they occur rarely (usually only a few per sample), and we have not succeeded in obtaining large numbers of them regularly from each batch of cells.

As noted above (Plates 35, 36 and 38), exposure to 0.5M NaCl alone, 0.1M $MgCl_2$ alone, or 0.1M NaCl plus 0.02M $MgCl_2$ led to characteristic changes in the structure of cells sedimenting at 15,000 x g. When supernatant fractions

from the first two treatments were centrifuged at 27,000 x g for 20 min., no material sedimented. Centrifuging at 65,000 x g for 60 min. sedimented very small amounts of material resembling the fragments and vesicles shown in Plate 40D. No material could be sedimented from the 15,000 x g supernatant fraction remaining after treatment with 0.1M NaCl plus 0.02M $MgCl_2$.

Temperature- and acid-induced lysis

When cells were held for 2 hr at 37C in "all salts" solution, considerable structural deterioration took place, as suggested by the fall in turbidity (Table 4) and by the appearance of cells in thin section (Plate 41). Even in cells that retained their shape, the outer membrane was degraded, and much of the outer membrane material was liberated as vesicles. The cell membrane was rarely observed as a three-layered structure.

Cells lysed at pH 5.0 in "all salts" solution retained their rod-shape. In thin section, the outer membrane was distorted, and the cytoplasmic membrane was less well defined than that of the intact cell, though some of the tripartite structure remained (Plate 42).

Cell surface structure and its changes during lysis

It was previously shown (Plate 9) that replicas of unfixed cells dried in the cold revealed a matted surface texture. On lysis in distilled water, the surface was considerably disrupted (Plate 43), a result to be expected considering the appearance of such cells in thin section

Figure 8

Separation of envelopes and envelope fragments by differential centrifugation

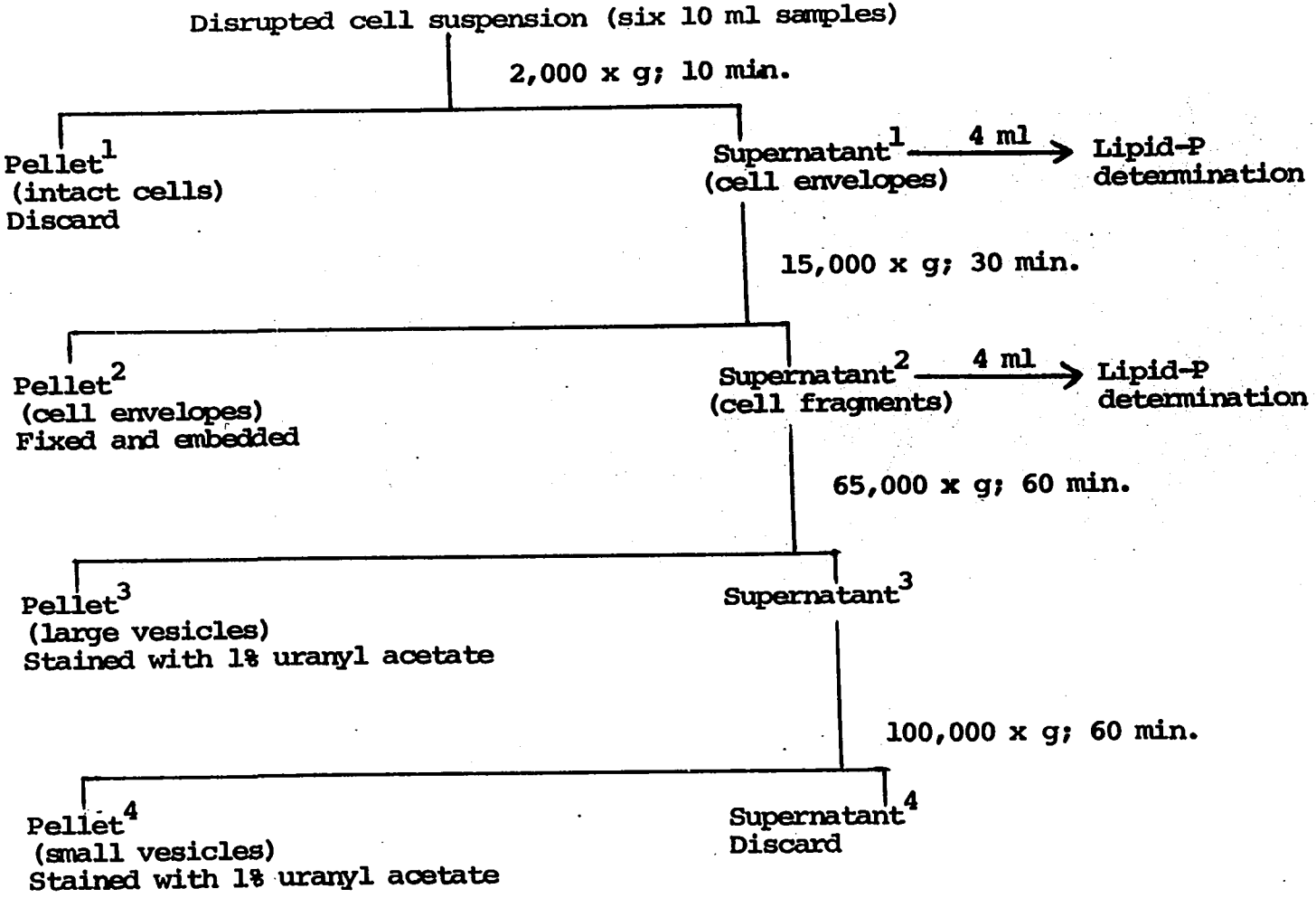

Disrupted cell suspension (six 10 ml samples)
2,000 x g; 10 min.
Pellet 1
(intact cells)
Discard
Supernatant 1
(cell envelopes)
4 ml
Lipid-P
determination
15,000 x g; 30 min.
Pellet 2
(cell envelopes)
Fixed and embedded
Supernatant 2
(cell fragments)
4 ml
Lipid-P
determination
65,000 x g; 60 min.
Pellet 3
(large vesicles)
Stained with 1% uranyl acetate
Supernatant 3
100,000 x g; 60 min.
Pellet 4
(small vesicles)
Stained with 1% uranyl acetate
Supernatant 4
Discard

Table 4

Changes accompanying lysis of *V. psychroerythrus*

a. All incubations at 0C unless stated otherwise. A 40 hr culture was used for experiment 1 and a 60 hr culture for experiment 2.

b. "One-hundred per cent turbidity" equals that found in "all salts" solution. The turbidity (absorbance at 660 nm) for experiment 1 was 0.590 (corresponding to 0.86 mg of bacteria per ml) and for experiment 2, 0.480 (0.68 mg of bacteria per ml).

c. Expressed as absorbance of supernatant fractions at 260 nm per mg of bacteria per ml (read after four-to-five-fold dilutions).

d. The amounts of DNA and RNA in intact cells (in "all salts" solution) is taken as 100%. Cells contained 0.300 mg of RNA per mg (dry weight) of cells and 0.070 mg of DNA per mg (dry weight) of cells.

Experimental conditions[a]	Turbidity (%)[b] after				Release of ultraviolet-absorbing substances[c]	DNA remaining (%)[d]	RNA remaining (%)[d]
	15 min	60 min	120 min	180 min			
Experiment 1							
"All salts"	100	100	100		0.24		
0.5 M NaCl plus 0.1 M $MgCl_2$	108	106	106		0.30		
0.4 M NaCl plus 0.08 M $MgCl_2$	83	83	81		0.27		
0.3 M NaCl plus 0.06 M $MgCl_2$	86	86	86		0.49		
0.1 M NaCl plus 0.02 M $MgCl_2$	55	38	32		2.56		
0.5 M NaCl	81	71	30		2.38		
0.3 M NaCl	65	40	13		2.51		
0.1 M NaCl	22	12	10		2.89		
0.1 M $MgCl_2$	51	49	46		1.22		
0.06 M $MgCl_2$	51	46	42		1.67		
0.02 M $MgCl_2$	42	30	27		2.13		
Water	15	10	10		3.00		
"All salts," 37 C	88	49	46				
Experiment 2							
"All salts"	100	100	98	96	0.28	100	100
0.5 M NaCl plus 0.1 M $MgCl_2$	107	106	106	104	0.43	94	94
0.5 M NaCl	96	91	81	47	2.65	80	57
0.1 M $MgCl_2$	50	46	46	45	2.41	93	67
Water	24	19	19	18	3.95	53	47
"All salts," 37 C	88	61	60	60	4.55	43	25

Table 5

Effect of KCl on phospholipid retention in mechanically-prepared envelopes.

a. The salt solutions contained 0.5M NaCl plus 0.1M $MgCl_2$ and specified amounts of KCl. The pH of the unbuffered salt solutions was adjusted to 7.0.

b. The lipid phosphorus content of supernatant 1, expressed as µg lipid-$P/_{ml}$, was taken as 100%.

c. The lipid phosphorus content of supernatant 2 corresponds to that associated with envelope fragments not sedimentable at 15,000 xg for 30 min.

Suspending medium[a]	Supernatant 1[b] (µg P/ml)	Supernatant 2[c]	Lipid retention in envelopes (%)
no KCl	4.9	2.9	40
0.1M KCl	5.4	2.9	46
0.3M KCl	5.3	2.9	45
Buffered; no KCl	4.0	1.9	52
Buffered; 0.1M KCl	5.9	3.0	49
Buffered; 0.3M KCl	6.2	3.0	50

Plates 33 to 35

Fine structure of cells suspended in NaCl plus $MgCl_2$.

Plate 33 (top-left). Thin section of cells suspended in 0.5M NaCl plus 0.1M $MgCl_2$, showing characteristic triple layered outer and cytoplasmic membrane structure. X128,000.

Plate 34 (top-right). Thin section of cells suspended in 0.3M NaCl plus 0.06M $MgCl_2$. The cell membrane can be faintly distinguished as a tripartite structure and fewer fibrils remain in the nucleoid. X64,000.

Plate 35 (bottom). Thin section of cells suspended in 0.1M NaCl plus 0.02M $MgCl_2$. Tripartite membrane structure has disappeared. Outer membranes are broken and fragments (f) and vesicles (V) thought to originate from the outer membranes are seen. X60,000.


V

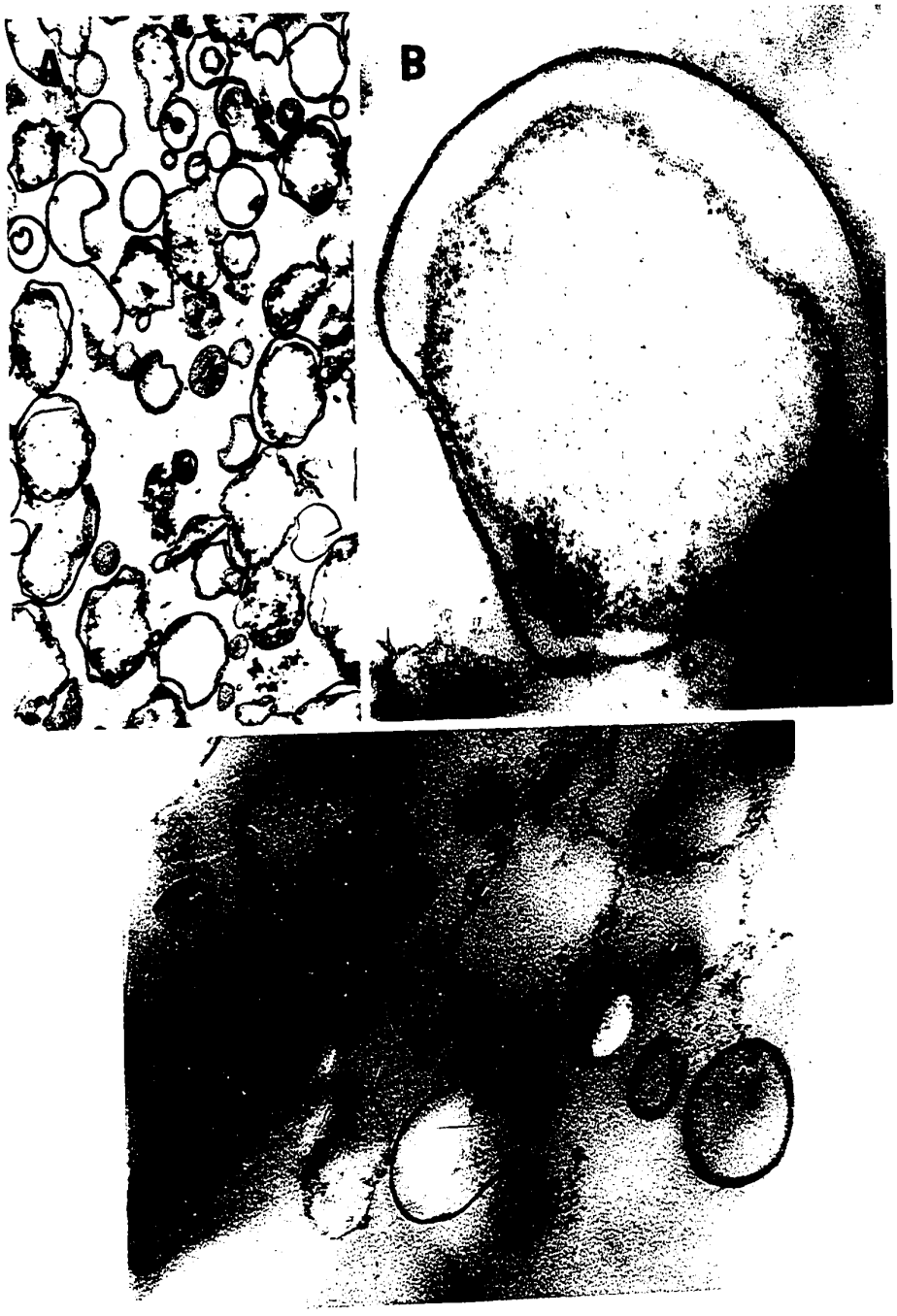
Plates 36 and 37

Fine structure of cells suspended in NaCl alone.

Plate 36. Fine structure of cells suspended in 0.5M NaCl. A (top-left). Approximately 40% of the preparation consisted of clean outer membrane vesicles, free of cytoplasmic material. X12,000.

B (top-right). Cell at higher magnification, showing a continuous triple-layered outer membrane and short segments of the cell membrane. X90,000.

Plate 37 (bottom). Cells suspended in 0.1M NaCl showing fragments with a continuous smooth structure. X64,000.


B

Plates 38 and 39

Fine structure of cells suspended in $MgCl_2$ alone.

Plate 38 (top). Thin section of a cell suspended in 0.1M $MgCl_2$. The cell membrane has retained almost all of its tripartite structure (compare with cells in 0.5M NaCl, Plate 36). X96,000.

Plate 39 (bottom). Thin section of cells suspended in 0.02M $MgCl_2$. Structure is better preserved than in a fivefold greater Na^+ concentration (Plate 37). X31,000.

Plate 40

Effect of distilled water on cell structure

Plate 40A. Thin section of lysed cells sedimented at 15,000 xg. Outer membrane fragments seem to be reforming into vesicles (arrows). Little if any suggestion of the cell membrane remains. X65,000.

Plates 40B to 40D. Material sedimenting at 65,000 xg and stained in cold 1% aqueous uranyl acetate. B. 12.5 to 15.0 nm vesicles exhibiting a "doughnut" shape. X300,000. C and D. Larger vesicles (150 to 250 nm), including a few "doughnut-shaped" vesicles (arrow). Magnification is X100,000 and X96,000 respectively.

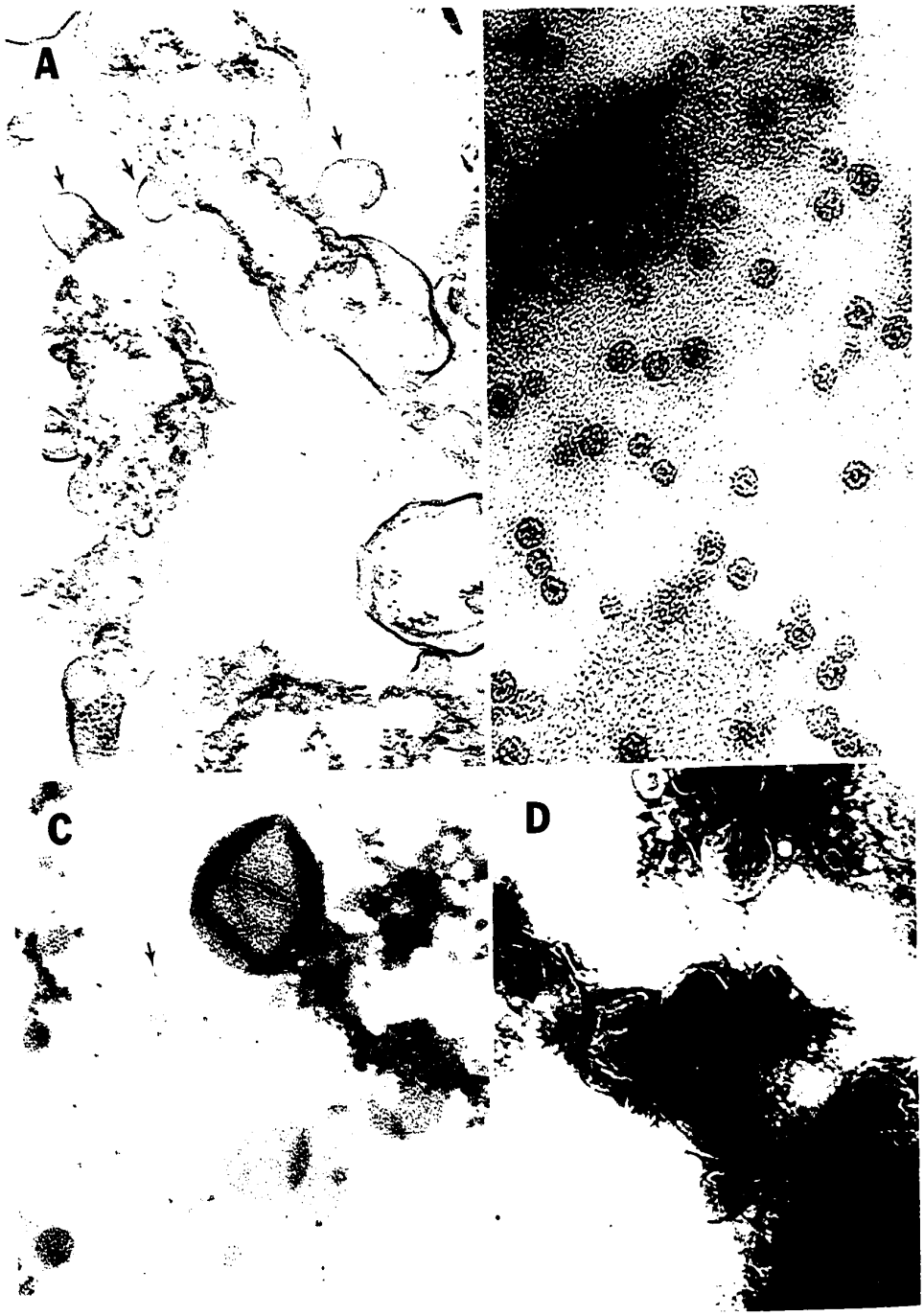

A
C
D

Plate 41

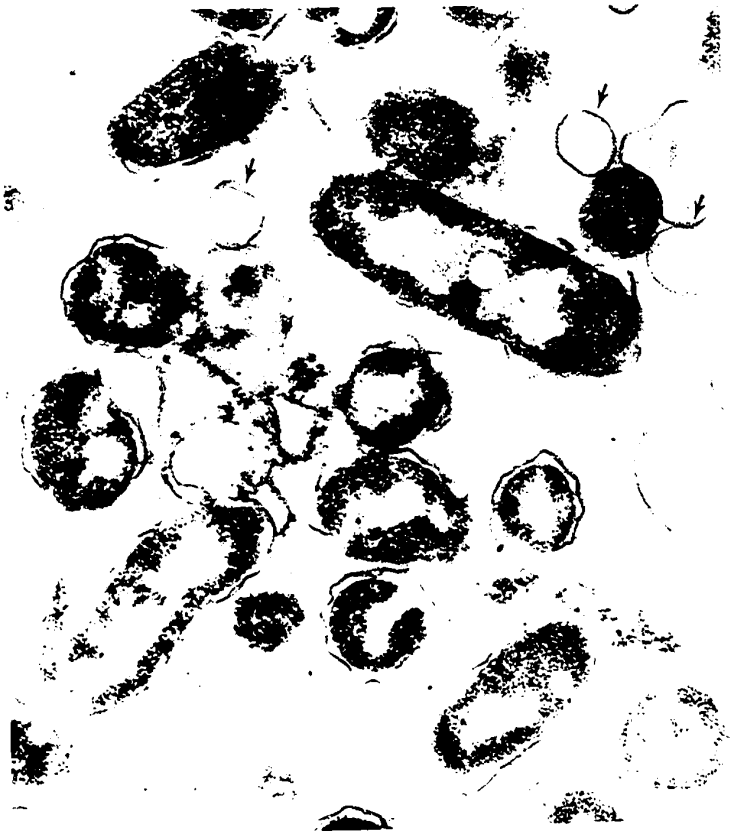
Fine structure of temperature-lysed cells.

Thin section of cells suspended in "all salts" solution and held at 37C for 2 hr, during which time considerable structural deterioration occurred. Some vesicles (arrows), possibly from outer membranes, appear. Even in cells that do not appear extensively degraded, most of the tripartite structure of the cytoplasmic membrane is lost. X30,000.

Plate 42

Fine structure of acid-lysed cells.

Thin section of cell lysed at pH 5.0 in "all salts" solution. Breaks appear in the distorted outer membrane, and a faint suggestion of the triple-layered cell membrane remains. X60,000.



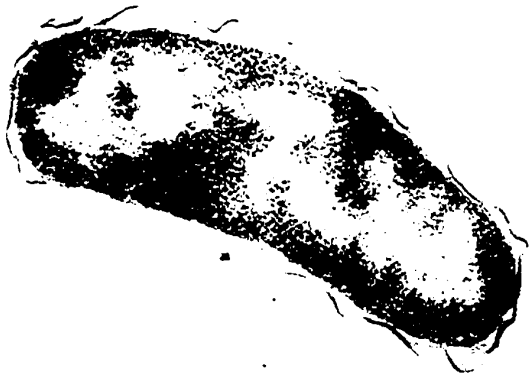


Plate 43

Surface replica of water-lysed cells.

Surface replica of cells lysed in cold distilled water. Blebs (arrows), approximately 200 nm in diameter, were particularly prominent. X35,000.

Plate 44

Surface replica of temperature-lysed cells.

Surface replica of cells suspended in "all salts" solution and held at 37C for 2 hr. Cells usually showed an irregular contour and a rugged surface. X40,000.

Plate 45

Fine structure of cell envelopes prepared in KCl.

Thin section of cell envelopes prepared in 0.5M NaCl plus 0.1M $MgCl_2$ and 0.1M KCl. The outer and cytoplasmic membranes were rarely observed as tripartite structures. X36,000.

Plate 46

Particles released from mechanically-prepared envelopes.

The material released from intact cells broken mechanically in 0.5M NaCl plus 0.1M $MgCl_2$ and sedimented at 65,000 xg (pellet 3) consisted of different-sized vesicles. X70,000.

(Plate 40A). Blebs, about 200 nm in diameter, appeared on the cell surface. Lysis at pH 5.0 caused some wrinkling of the surface, which seemed otherwise intact. Lysis at 37C (Plate 44) led to quite irregular contours and a rugged surface.

A structural role of K^+ ions

Envelopes prepared in a K^+-deficient or K^+-supplemented salt solution differed by less than 10% in their lipid retention (Table 5) and all usually showed distorted and poorly preserved outer membranes and cytoplasmic membranes (Plate 45). The material sedimenting at 65,000 and 100,000 x g (Pellets 3 and 4 - fig. 8) was morphologically similar and consisted of vesicles approximately 30 to 140 nm in diameter (Plate 46); a greater number of large vesicles was observed in pellet 3. However, there was in general no evidence that K^+ had any structural role.

Discussion

For stability, the red psychrophile needs low temperatures (Hagen et al 1964), pH values near neutrality (Madeley et al 1967) and both monovalent and divalent cations (Korngold and Kushner 1968). In contrast to other marine bacteria (MacLeod 1965, Buckmire and MacLeod 1970), its structure is poorly maintained by monovalent salts and even more poorly by nonionic solutes (Korngold and Kushner 1968). Our studies showed that the red psychrophile is stable in 0.5M NaCl plus 0.1M $MgCl_2$ and that dilution of this salt solution leads to increased leakage (Table 4) and deteriora-

tion of cellular fine structure (Plates 33 to 35). The envelope layers of the marine pseudomonad NCMB 19 (Buckmire and MacLeod 1965 and 1971), the marine isolate C-Al (DeVoe and Oginsky 1969) and our psychrophile (Plates 33 to 35) are equally sensitive to solutions of low ionic strength. The separation between the outer membrane and cytoplasmic membrane of these organisms increases significantly with decreasing salt concentration and different-sized fragments of the outer membranes are liberated in the medium (Plates 37 and 40).

Although both Na^{+} and Mg^{+2} are needed for cell stability, these ions appear to act differently on different layers of the cell envelope. Thin sections show that both ions are capable of maintaining the integrity of the outer membrane whereas the cytoplasmic membrane is best stabilized by Mg^{+2} and loses its characteristic tripartite structure in NaCl alone (Plates 36 and 38). The fact that $MgCl_2$ is better able to prevent leakage of UV-absorbing compounds and loss of nucleic acids than a fivefold greater concentration of NaCl (Table 4) further supports our interpretation of a structural role of Mg^{+2} ions on the cell membrane. The greater efficiency of divalent cations than of monovalent cations in maintaining cell structure has been reported in other marine bacteria. For example, Buckmire and MacLeod (1965 and 1971) found that divalent cations were more effective in preventing lysis and release of soluble non-dialyzable material from isolated cell envelopes of the

NCMB 19 pseudomonad. Korngold and Kushner (1968) also found that V. psychroerythrus was stable in low concentrations (10^{-3}M) of divalent salts such as $CuSO_4$, $ZnCl_2$ and $NiCl_2$, releasing less than 30% of its UV-absorbing material.

The rapid transition of intact V. psychroerythrus from a rod to a sphere when suspended in 0.1M $MgCl_2$ alone suggests that Na^+ cations preserve the integrity of the R layer. Buckmire and MacLeod (1965) came to similar conclusions from their study of the effects of monovalent salts on the stability of the marine pseudomonad envelope; they suggested that Na^+ cations shield the negative charges on adjacent units of the R layer. The NCMB 19 pseudomonad and our red psychrophile differ in their ionic requirement for cell membrane preservation. Buckmire and MacLeod (1965) showed distinct triple-layered cell membranes in pseudomonad envelopes prepared in 0.5M NaCl alone. In contrast, intact cells of V. psychroerythrus suspended in 0.5M NaCl (Plate 36) or broken mechanically in 0.5M NaCl plus 0.1M $MgCl_2$ and 0.1M KCl (Plate 45) seldom showed a tripartite cell membrane. Recent studies on the cation-dependent retention of α-aminoisobutyric acid (Wong, Thompson and MacLeod 1969, DeVoe et al 1970) and the K^+-dependent deplasmolysis of the NCMB 19 marine pseudomonad (Thompson, Costerton and MacLeod 1970) suggest that Na^+, in addition to its previously established role in active transport, controls the porosity of the cell membrane.

It is generally accepted that cations shield or form bridges between electronegative groups in the surface layers of salt-requiring bacteria (see Chapter 1). The isoelectric point of V. psychroerythrus lies between pH 2.0 and 2.5 (Madeley et al 1967), indicating that its cell wall contains excess acidic groups. Conceivably, the ability of Na^+ and Mg^{+2} cations to stabilize the wall rests on their capacity to mask mutually repulsive negative charges. However, such a cooperative relationship between Na^+ and Mg^{+2} cations is not necessarily true for all marine bacteria. For example, the c-Al marine bacterium, pre-exposed to 0.05M $MgCl_2$, underwent more extensive lysis when transferred to low concentrations of NaCl than when transferred to distilled water; it was also shown that the degree of disruption of cells transferred to distilled water from mixtures of 0.05M $MgCl_2$ and NaCl (0 to 1.0M) was dependent on the NaCl concentration. Such susceptibility to lysis has been ascribed to a competition between monovalent (Na^+ and Li^+) and divalent (Mg^{+2}) cations for electronegative sites in the cell envelope of this organism (DeVoe and Oginsky 1969).

Previous work showed that, when intact cells of the red psychrophile were lysed in distilled water, particles containing most of the cell lipid phosphorus and hexosamine were released (Korngold and Kushner 1968). The morphology of these particles has now been studied. In addition to amorphous debris, different-sized vesicles were found. The larger ones (Plates 40C and 40D), 150 to 250 nm in diameter,

were as large as parts of the wall pinched off from water-lysed cells (Plate 40A). They also compared well with the blebs on the surface of such cells (Plate 43) and are believed to originate in the outer membrane. Many vesicles only 12.5 to 15 nm in diameter were also observed (Plate 40B). Since much of the outer membrane remained unbroken or formed large vesicles, the smaller vesicles are thought to have originated in the cell membrane.

Particles containing hexosamine and lipid phosphorus are also released when cells are broken mechanically in cold seawater or psychrophile salt medium (Korngold and Kushner 1968). An electron microscope study of these (Plate 46) revealed that they resemble the collection of particles released on distilled water lysis (Plates 40C and 40D). Similar particles were released on lysis in 0.5M NaCl or in 0.1M $MgCl_2$ alone. These results show that disruption of cellular integrity, however induced, leads to extensive fragmentation of the outer layers of the cell.

Intact cells broken mechanically in cold seawater or psychrophile salt medium (0.5M NaCl, 0.1M $MgCl_2$ and 0.01M Tris buffer pH 7.0) lose more than half their lipid phosphorus in a form not sedimentable at 15,000 x g for 30 min. (Korngold and Kushner 1968). Assuming that most of the lipid phosphorus comes from the cell membrane and that the red psychrophile actively concentrates K^+ ions, it appeared that this extensive loss of lipid phosphorus may have resulted from a K^+-dependent preservation of the cell mem-

brane. As the cells broke open, the intracytoplasmic K^+ would have been diluted to a concentration no longer capable of maintaining cell membrane structure. If this hypothesis was correct, envelopes prepared in a K^+-supplemented salt solution should retain more lipid phosphorus and show a better preservation of the cell membrane than envelopes prepared in a K^+-deficient salt solution. Our results show, however, that lipid retention in envelopes prepared in the presence and absence of K^+ differs by less than 10% (Table 5), a finding that is not consistent with a K^+-dependent preservation of the cell membrane. Thin section studies (Plate 45) also fail to support a structural role for K^+ ions.

Lysis of V. psychroerythrus at elevated temperatures releases small amounts of hexosamine and most of the lipid phosphorus is broken down, presumably through the action of a phosphatidase (Hagen et al 1964). Our results show that lysis at 37C induces considerable structural deterioration (Plates 41 and 44) and heavy leakage of cellular constituents (Table 4). The mechanism responsible for the lysis of V. psychroerythrus at elevated temperatures is uncertain although activation of autolytic enzymes has been proposed (Hagen et al 1964). Mattingly and Best (1971 and 1972) have shown that lysis of Bacillus psychrophilus at temperatures above its maximum growth temperature (28C) results from the physical dissociation of a heat labile wall and not from the activity of autolytic enzymes. These conclu-

sions were partly derived from the observations that lysis at 45C released few N-terminal amino groups and that inhibition of autolytic enzymes with 10M LiCl had little effect on the lysis of isolated walls. They also found that at temperatures above the maximum growth temperature, the rate of lysis was temperature-dependent i.e. rate of lysis increased with increasing temperature. A similar phenomenon has been reported in V. psychroerythrus (Hagen et al 1964). Possibly, the obligate psychrophilic nature of V. psychroerythrus partly rests on a heat labile cell envelope.

We have shown that the fine structure of the outer layers of V. psychroerythrus are extraordinarily susceptible to environmental change. Mitochondrial and other cell membranes retain their "unit membrane" (tripartite) structure even after most of the lipid is extracted with acetone (Fleischer, Fleischer and Stockenius 1967). The triple-layered structure of red blood cell membranes remains after digestion with phosphatidase C (Ottolenghi and Bowman 1970). In contrast, much more gentle treatment of the red psychrophile can lead to the disappearance of tripartite structure (Plates 35, 36 and 41). Quite possibly, the structure of these membranes depends on a delicate balance of charges between protein and lipid molecules. Such a balance could be disturbed either by a change in the ionic environment or removal of the charged portions of phosphatides by enzymic action. These changes could lead not only to disappearance of tripartite structure but also to fragmentation and release of parts of the outer and cytoplasmic membranes as vesicles.

Chapter 5

Preliminary attempts at isolating and characterizing envelope layers.

Introduction

Fine structural studies of V. psychroerythrus (Chapter 3) failed to show a densely staining cell wall layer which could be identified with the glycosaminopeptide (R) layer, typical of other Gram negative bacteria (Glauert and Thornley 1969). Difficulties in demonstrating an R layer in marine bacteria is quite common (Wiebe and Chapman 1968, DeVoe and Oginsky 1969a, Felter et al 1969, Kennedy et al 1970) and is believed to result from the low glycosaminopeptide content in their cell envelopes (Forsberg et al 1972). Although we believe that an analogous situation exists in our red psychrophile, some of the work presented in this chapter will attempt to demonstrate that V. psychroerythrus does contain components of "typical R layers". We also suggested that cations stabilize the envelope of V. psychroerythrus by shielding negative charges localized on the cell's surface (Chapter 4); demonstration that the cell envelope contains an excess of acidic amino acids will support such a "cation-shielding" hypothesis.

A number of studies on the lytic susceptibility of V. psychroerythrus to elevated temperatures, acidic pH and solutions of low ionic strength (Hagen et al 1964, Madeley et al 1967, Korngold and Kushner 1968 and this thesis - Chapter 4) have suggested that the determinants of its lytic behavior are to a large extent localized in the cell envelope. Our work (Chapter 4) and that of Korngold and Kushner (1968) showed that the red psychrophile specifically requires

mono- and divalent cations to maintain envelope structure. Hagen et al (1964) suggested that temperature-induced lysis may be caused by a particle-bound phosphatidase, possibly one bound to the cell membrane itself. However, evidence that a thermolabile cell envelope may be the determinant of psychrophily in B. psychrophilus (Mattingly and Best 1971 and 1972) and possibly in our red psychrophile has already been discussed (Chapter 4). A question which has emerged from these studies is whether the cell wall or the cell membrane (or both) are responsible for the lytic behavior of V. psychroerythrus. Such studies would require pure cell wall and cell membrane preparations; results of a preliminary fractionation of the cell envelope of V. psychroerythrus will be presented in this chapter.

Materials and Methods

A. Chemical characterization of cell envelopes

Preparation of cell envelopes. Cell envelopes were prepared from 50 to 60 hr cultures grown in regular liquid medium. Washed intact cells suspended in "all salts" solution were broken mechanically at 10C with a Mickle disintegrator (15 min) or a wrist-arm shaker (10 cm displacement - 60 min) using ballotini No. 12 glass beads. Envelope pellets obtained by differential centrifugation (figure 8) were suspended in a minimal volume of "all salts" solution.

Amino acid analysis. Samples of washed intact cells and cell envelopes were hydrolyzed in 6N HCl at 105C for 18 hr in sealed glass ampoules. The HCl was then evaporated in

a boiling water bath and the residue taken up in 1.0 ml of acetate-sucrose buffer. Samples (0.2 ml) were analyzed in an amino acid analyzer (Technicon Instrument Corp., Ardsley, N.Y.) and read against a set of amino acid standards of known concentration.

Protein determination. Samples of washed intact cells and cell envelopes were digested overnight in 1N NaOH at room temperature. Protein, measured according to the method of Lowry et al (1951) was read at 500 nm against a reagent blank. Standards were prepared from a stock solution (1 mg/ml) of crystalline bovine serum albumin (General Biochemicals, Chagrin Falls, Ohio).

Preparation of envelope hydrolyzate for chromatography. A suspension of washed cell envelopes was dialyzed 4 days at 10C against distilled water (8 changes). The dialyzate was sedimented at 15,000 x g for 30 min. and approximately 30 mg (wet weight) of the pellet was hydrolyzed in 6N HCl (1 ml) at 105C for 18 hr in a sealed glass tube. The HCl was then evaporated in a boiling water bath and the residue taken up in distilled water, filtered and the filtrate stored in the cold. This material was used for chromatographic and colorimetric determination of muramic and diaminopimelic acids.

Muramic acid chromatography. Samples of envelope hydrolyzate were separated by ascending chromatography (22 hr) on Whatman No. 1 paper with n-butanol-acetic acid-water (57:14:29 v/v/v) as solvent. Aqueous solutions (1 mg/ml)

of muramic acid (Sigma Chemicals Co., St. Louis, Missouri) and D-glucosamine hydrochloride (Eastman Organic Chemicals, Rochester, N.Y.) were used as standards. Possible retardation of muramic acid by the envelope hydrolyzate was checked by spotting a mixture (equal volumes) of muramic standard and envelope hydrolyzate. The envelope hydrolyzate was also separated with 75% aqueous phenol and ethyl acetate-acetic acid-water (140:30:30 v/v/v) on Whatman No. 1 paper. All chromatograms were developed with 0.2% (w/v) ninhydrin in acetone.

Muramic acid assay. Muramic acid was determined by the method of Stewart-Tull (1968), a modification of an earlier method by Cessi and Piliego (1960). Under alkaline conditions, muramic acid reacts with acetyl acetone to form a non-volatile chromogen which, when reacted with p-dimethylaminobenzaldehyde reagent, produces a chromophore that absorbs maximally at 510 nm after 24 hr. The non-volatile chromophores of the envelope hydrolyzate and of the muramic acid standard (50 µg) were scanned between 400 and 600 nm against a reagent blank using a Beckman DB recording spectrophotometer.

Diaminopimelic acid chromatography. Samples of envelope hydrolyzate were chromatographed on Whatman No. 1 paper against standard solutions (1 mg/ml) of DL-α, ε-diaminopimelic acid (Sigma Chemicals Co.) and L-lysine monohydrochloride (Nutritional Biochemical Corp., Cleveland, Ohio). Descending chromatograms were run for 6-10 hr in n-butanol-acetic acid-

water (50:25:25 v/v/v) and methanol-water-HCl(10N)-pyridine (80:17.5:2.5:10 v/v/v/v) solvent systems (Rhuland et al 1955). Chromatograms were developed with 0.2% (w/v) ninhydrin in acetone.

Diaminopimelic acid assay. Diaminopimelic acid (DAP) was measured colorimetrically at 440 nm according to the method of Work (1957) using an acidic (pH 0.9) ninhydrin reagent. Samples of envelope hydrolyzate and DAP standard (0.1 mg/ml) were incubated with equal volumes of glacial acetic acid and acidic ninhydrin reagent at 37C for 90 min. According to Work (1957), absorption of contaminating amino acids such as lysine and tryptophan is minimal under these reaction conditions. The reaction mixtures were then made up to 5.0 ml with glacial acetic acid and scanned between 300 and 600 nm against a reagent blank using a Beckman DB recording spectrophotometer.

B. Isolation and Chemistry of cell envelope layers.

In these studies we attempted to separate by a non-degradative procedure the cell envelope layers into homogeneous fractions. Lysis in 0.5M NaCl appeared particularly suitable since previous studies had shown that the outer membrane of the cell wall separated from the remaining cell body or formed vesicles (Plate 36).

Preparation of ficoll density gradients. Discontinuous ficoll density gradients were prepared by carefully layering 7.0 ml of 15, 10 and 5% (w/v) ficoll (Pharmacia Chemicals, Uppsala, Sweden) made up in 0.5M NaCl in 30 ml polycarbonate

tubes; the gradient tubes were then equilibrated overnight in the cold before use.

Continuous 5 to 15% (w/v) ficoll density gradients were prepared in the cold (10C) using a Buchler (Fort Lee, N.J.) gradient preparator; the 25 ml gradients were equilibrated in the cold before use. Linearity of the continuous ficoll density gradient was measured using a methylene blue indicator. One drop (0.025 ml) of 0.5% (w/v) methylene blue in 0.5M NaCl was added to the 5% ficoll chamber and the gradient prepared as described above. The gradient tube was perforated and 30 drop fractions, collected in 10 ml Kimax tubes, were diluted with 3.0 ml of 0.5M NaCl. The absorbance of each fraction was read at 700 nm on a Coleman Junior II spectrophotometer against a 0.5M NaCl blank.

Fractionation of cell lysate on discontinuous density gradients. A 500 ml culture (in 2.8 liter flask) was grown for 60 hr in regular medium. Cells were harvested by a 15 min. centrifugation at 8,000 x g and the pellet washed twice in "all salts" solution. The resulting pellet, equal to a salt-free dry weight of 1.48 gm was lysed for 2 hr in 100 ml of cold 0.5M NaCl; lysis was carried out on a reciprocating shaker so as to promote separation of the outer membrane from the remaining cell body. The lysed cell suspension was then stabilized by a 30 min fixation with 2% (w/v) p-formaldehyde in 0.5M NaCl; the final formaldehyde concentration was 0.2%. The fixed lysate was then separated

into six lots and sedimented at 100,000 x g for 1 hr (10C) in a IEC (International Equipment Co., Needham, Mass.) B60 centrifuge. Each pellet was suspended in 5.0 ml of cold 0.5M NaCl and layered on separate 5-10-15% (w/v) ficoll discontinuous density gradients. The cell lysates were fractionated by centrifuging the gradient tubes held in a swinging bucket rotor (IEC-SW 110) at 105,000 x g for 18 hr (10C). Red-pigmented bands (A, B and C) were collected with a J-shaped pipette and pooled. The pooled material was diluted with 0.5M NaCl and sedimented at 105,000 x g for 1 hr; the resulting pellets (A, B and C) were suspended in 5.0 ml of 0.5M NaCl and subfractionated on continuous ficoll density gradients.

Subfractionation of A, B and C fractions on continuous gradients. Bands A, B and C obtained by fractionation on discontinuous gradients (see above) were subfractionated on continuous ficoll density gradients by centrifuging at 105,000 x g for 20 hr (figure 18). The position of bands A_2, B_2 and C_2 in the linear gradient was measured directly (having corrected for the volume occupied by the sample layer) and expressed in terms of ficoll concentration (w/v); the ficoll concentration value for each band was then converted to a buoyant density value (gm/ml) using a standard curve supplied by the manufacturer (Pharmacia Chemicals). Bands A_2, B_2 and C_2 were then collected with a J-shaped pipette, diluted with 0.5M NaCl and sedimented at 105,000 x g for 1 hr. The resulting pellets, washed 3x in 0.5M NaCl,

into six lots and sedimented at 100,000 x g for 1 hr (10C) in a IEC (International Equipment Co., Needham, Mass.) B60 centrifuge. Each pellet was suspended in 5.0 ml of cold 0.5M NaCl and layered on separate 5-10-15% (w/v) ficoll discontinuous density gradients. The cell lysates were fractionated by centrifuging the gradient tubes held in a swinging bucket rotor (IEC-SW 110) at 105,000 x g for 18 hr (10C). Red-pigmented bands (A, B and C) were collected with a J-shaped pipette and pooled. The pooled material was diluted with 0.5M NaCl and sedimented at 105,000 x g for 1 hr; the resulting pellets (A, B and C) were suspended in 5.0 ml of 0.5M NaCl and subfractionated on continuous ficoll density gradients.

Subfractionation of A, B anc C fractions on continuous gradients. Bands A, B and C obtained by fractionation on discontinuous gradients (see above) were subfractionated on continuous ficoll density gradients by centrifuging at 105,000 x g for 20 hr (figure 18). The position of bands A_2, B_2 and C_2 in the linear gradient was measured directly (having corrected for the volume occupied by the sample layer) and expressed in terms of ficoll concentration (w/v); the ficoll concentration value for each band was then converted to a buoyant density value (gm/ml) using a standard curve supplied by the manufacturer (Pharmacia Chemicals). Bands A_2, B_2 and C_2 were then collected with a J-shaped pipette, diluted with 0.5M NaCl and sedimented at 105,000 x g for 1 hr. The resulting pellets, washed 3x in 0.5M NaCl,

were suspended in a minimal volume of 0.5M NaCl and dialyzed in the cold against 0.5M NaCl for 2 days (2 changes). The dialyzates were made up to known volumes with 0.5M NaCl and stored in the cold.

Morphology and chemistry of fractions A_2, B_2 and C_2.

1. Fine structure. For thin section studies, samples of fractions B_2 and C_2 previously exposed 0.2% formaldehyde were fixed for 4 hr in cold 2% p-formaldehyde made up in 0.5M NaCl. The fixed material was sedimented at 30,000 x g for 20 min, washed in 0.5M NaCl and stained for 1 hr in cold buffered 1% uranyl acetate (Michaelis acetate-veronal buffer pH 6.1; final pH 4.4). After 2 washings with 0.5M NaCl, the material was dehydrated and embedded as previously described (Chapter 3). Negatively stained preparations of fractions B_2 and C_2 were prepared by spreading loopsfull of B_2 and C_2 on formvar-carbon coated grids. After removing excess liquid with filter paper, a loopfull of cold 1% phosphotungstic acid, pH 6.1, was applied to each grid and air dried.

2. Chemical composition.

Protein assay. The protein content of fractions A_2, B_2 and C_2 was measured by the Lowry (1951) method (see 'Protein determination' above).

Lipid phosphorus assay. Lipids from fractions A_2, B_2 and C_2 were extracted with chloroform-methanol (Bligh and Dyer 1959) and lipid phosphorus was determined according to the method of Chen et al (1956), described in Chapter 4.

Carbohydrate assay. Carbohydrates in fractions A_2, B_2 and C_2 were determined by the phenol-sulphuric acid reaction of Dubois et al (1956). The absorbance of the reaction mixtures was read at 490 nm against a reagent blank. Standards were prepared from a 1 mg/ml stock solution of D(+) glucose (British Drug House). Experiments on the capacity of ficoll solutions (1 to 1000 µg/ml in 0.5M NaCl) to react with the phenol-sulphuric reagent showed that ficoll gave 0.7 the absorbance reading of an equivalent amount of glucose. In order to obtain true carbohydrate values for A_2, B_2 and C_2, the three fractions were washed 3 X with 0.5M NaCl before analysis.

3. Lipid chromatography.

Sample preparation. The phosphatide composition of Bligh and Dyer (1959) lipid extracts of intact cells (60 hr culture grown in regular medium) and fractions B_2 and C_2 was determined by thin layer and paper chromatography. Water in the Bligh and Dyer (1959) chloroform phase was removed as a water-benzene azeotrope under a stream of nitrogen gas. The lipid residue was suspended in a minimal volume of chloroform and stored in a glass stoppered tube in the cold.

Thin layer chromatography (TLC). A 0.25 mm layer of silica gel H (Brinkman Instruments, Rexdale, Ontario) was spread on 20 x 20 cm glass plates with a Desaga (Germany) spreader. Plates, dried in air, were washed in chloroform-methanol (1:1 v/v) and activated for 12 hr in a 120C oven.

Lipid extracts (4-6 µg lipid phosphorus) were spotted 2.0 cm from the bottom edge of the plates and separated by ascending chromatography (45-60 min) in chloroform-methanol-ammonium hydroxide (conc.) (65:35:5 v/v/v) solvent system. A mixture of authentic phosphatidyl ethanolamine, phosphatidyl choline (kindly donated by Dr. Kates) and phosphatidyl serine (Pierce Chemicals Co., Rockford, Ill.) were used as standard. Spots were detected by spraying with 0.2% ninhydrin in acetone-lutidine (9:1 v/v) as described by Marinetti (1962) and by the phosphate stain of Dittmer and Lester (1964). Other lipid components were localized by charring the TLC plates on a hot plate.

Paper chromatography. Lipid extracts were also separated on Whatman SG 81 silica gel paper (W & R Balston Ltd., England) with diisobutyl ketone-acetic acid-water (40:25:5 v/v/v) as solvent. Spots were detected with Rhodamine 6G and 0.2% ninhydrin as described by Marinetti (1962).

Results

A. Chemical characterization of cell envelopes.

Amino acid analyses

As shown in Table 6, intact cells and cell envelopes contained all the amino acids normally found in proteins and were particularly rich in aspartic and glutamic acids, alanine, glycine and leucine. Envelopes contained twice as much dicarboxylic (glutamic and aspartic) as basic (histidine, arginine, lysine) amino acids; a similar ratio was observed in intact cells.

Muramic acid chromatography

The presence of muramic acid in the envelope hydrolyzate of V. psychroerythrus was demonstrated by paper chromatography. With the butanol-acetic acid-water solvent system (figure 9), a ninhydrin-positive spot with an R_f value of 0.33 coincided with the muramic standard; the R_f value of the glucosamine standard was 0.22. Our results also show that the hydrolyzate does not affect the rate of migration of authentic muramic acid (figure 10); muramic acid alone (C) or mixed with an equal volume of hydrolyzate (C*) migrated the same distance from the origin. Good separation of the envelope hydrolyzate was also obtained with 75% phenol (figure 11) and ethyl acetate-acetic acid-water (figure 12) as solvents. Both systems separated a ninhydrin-positive spot whose R_f value compared well with authentic muramic acid.

Muramic acid assay

Although the spectrum of the muramic acid standard showed a peak at 500-510 nm, no such peak was observed in the envelope hydrolyzate (figure 13). As the envelope hydrolyzate sample was scanned from 600 to 400 nm, an initial increase in absorbance was observed at 520 nm attaining a maximum and levelling off in the 510 to 500 nm range. At lower wavelengths, the absorbance gradually increased giving a peak at 400 nm.

Diaminopimelic acid chromatography

No components of the envelope hydrolyzate, separated by descending chromatography with methanol-water-hydrochloric acid-pyridine (figure 15) or butanol-acetic acid-water (figure 16) as solvents, could be identified with diaminopimelic acid (DAP). Considerable trailing of the DAP standard and envelope hydrolyzate was observed with the former solvent system (figure 15). Better separation was obtained with the butanol-acetic acid-water system (figure 16); the violet spot nearest the origin is tentatively identified as lysine.

Diaminopimelic acid assay

The spectrum of the standard DAP-ninhydrin reaction product read against an undiluted reagent blank showed one characteristic peak at 440 nm. When the DAP standard and reagent blank were diluted ten fold with glacial acetic acid, two additional peaks (310 and 350 nm) were obtained (figure 14). Work (1957) reported that the acidic ninhydrin reagent did not absorb above 430 nm although it showed very strong absorption at 320-340 nm; they also found that the DAP-ninhydrin reaction product gave a peak at 340 nm. It would appear that dilution of the reagent blank with acetic acid reduces the amount of ninhydrin absorption resulting in an increased resolution of the standard DAP spectrum. The spectrum of the envelope hydrolyzate (figure 14) read against a ten fold diluted reagent blank failed to show a DAP-specific peak at 440 nm although a peak at 340 nm was observed.

B. Isolation and chemistry of cell envelope layers.

Fractionation of lysate on discontinuous density gradients.

We previously reported (Chapter 4) that cells lysed in 0.5M NaCl for 2 hr in standing tubes occur as phase pale rods. We found, however, that on shaking in 0.5M NaCl, at least 50% of the cells are converted to phase pale spheres. The 0.5M NaCl lysate consistently separated into three red-pigmented bands (A, B and C) when fractionated on 5-10-15% discontinuous ficoll density gradients; a pellet (P) was also obtained (figure 18). The three bands appeared to form at the interfaces of the ficoll layers where A and C occurred as the least and most dense bands respectively. Preliminary experiments also showed that formaldehyde fixation did not affect the density of the lysed material; fixed and unfixed lysates prepared from the same cell batch separated into three pigmented bands with similar buoyant density characteristics.

Subfractionation of lysate on continuous density gradients.

Experiments with methylene blue showed mixing of the ficoll gradient to be linear throughout most of the tube (figure 17). The last five fractions failed to show a proportional increase in absorbance and is perhaps due to the reduced rate of flow and increased mixing of the last 2-3 ml of ficoll solution in the gradient preparator. When fractions A, B and C were subfractionated on continuous 5-15% ficoll

gradients according to the scheme outlined in figure 18, three bands (A_2, B_2 and C_2), with approximate buoyant densities of 1.017, 1.027 and 1.038 gm/ml respectively, were obtained. The small pellets (P_1 and P_2) were discarded.

Morphology and chemistry of fractions A_2, B_2 and C_2.

1. Fine structure

Vesicles free of cytoplasmic debris and exhibiting tripartite walls approximately 7.0 nm wide and some membranous fragments were observed in thin section preparations of B_2 and C_2; the diameter of the vesicles ranged from 0.2-0.4 μm for B_2 and 0.3-0.5 μm for C_2 (Plates 47 and 48). Rod-shaped ghosts free of cytoplasmic contaminants and exhibiting long segments of tripartite structure were occasionally seen in C_2. Structures morphologically similar to membrane sheets (Salton 1971a) were observed in negatively stained preparations of both fractions (Plate 49). Thin section preparations of pellet (P) showed cell debris and lysed cells similar to that shown in Plate 36. The small amount of material collected as the A_2 fraction was used for chemical rather than morphological characterization. However, vesicles (0.2-0.3 μm in diameter) and membranous fragments similar to those shown in Plate 48 were observed in thin section preparations of a homogeneous band obtained from a different fractionation procedure (10,000 x g for 2 hr on a continuous 3-10% ficoll density gradient); the buoyant density of this band was 1.015 gm/ml.

2. Chemical composition

Studies on the chemical composition of fractions A_2, B_2 and C_2 showed that all three fractions contained protein, phospholipid and carbohydrate and that the total amount of material collected in the three fractions accounted for less than 2% of that found in intact cells (Table 7). The phospholipid-protein ratio of A_2 was similar to that of B_2 but significantly higher than that of C_2. The sugar-protein ratio was fairly constant in the three fractions.

3. Lipid chromatography

Chromatographic separation of lipid extracts of B_2 and C_2 on TLC plates suggests that their phosphatide composition is identical (figure 19). Both fractions showed a ninhydrin and phosphate positive spot (R_f = 0.42) which could be identified with authentic phosphatidyl ethanolamine (R_f = 0.45). The remaining two spots (R_f values of 0.53 and 0.61), visualized by charring, were not identified. In addition to these three components, lipid extracts of intact cells (I) contained a fourth component (ninhydrin and phosphate positive) that separated at an R_f value (0.19) comparable to that of phosphatidyl serine (0.17).

Lipid extracts of fractions B_2 and C_2 separated into 3 components on silica-impregnated paper (figure 20). The fastest running spot (R_f = 0.54) which stained yellow with Rhodamine 6G and gave a positive ninhydrin reaction migrated the same distance from the origin as standard phosphatidyl ethanolamine (PE). The other two ninhydrin negative

Table 6

Amino acid analyses of intact cells and cell envelopes.

a. The amino acid content is expressed as μmoles/mg protein.

b. The amount of amino acid exceeded instrument calibration.

Amino Acid	Intact cells[a]	Cell Envelopes[a]
Aspartic acid	0.67	0.66
Threonine	0.33	0.25
Serine	0.37	0.28
Glutamic acid	0.90	0.63
Proline	0.31	0.17
Glycine	>0.74[b]	0.38
Alanine	>0.74[b]	0.54
Valine	0.46	0.42
Methionine	0.013	0.024
Isoleucine	0.35	0.28
Leucine	0.56	0.54
Tyrosine	0.13	0.13
Phenylalanine	0.24	0.21
Lysine	0.41	0.31
Histidine	0.12	0.11
Arginine	0.30	0.25

Figures 9 to 12

Muramic acid chromatography

Figure 9 (left). Ascending chromatography in n-butanol-acetic acid-water (57:14:29) for 22 hr. The R_f values of authentic muramic acid (M) and glucosamine (G) were 0.33 and 0.22 respectively. A ninhydrin-positive spot in the envelope hydrolyzate (S) coincided with authentic muramic acid.

Figure 10 (right). Ascending chromatography in n-butanol-acetic acid-water (57:14:29) for 22 hr. Authentic muramic acid (C) was not retarded when run in the envelope hydrolyzate (C*). A ninhydrin-positive spot in the envelope hydrolyzate (S) partitioned at an equal distance from the origin as the muramic acid standard (C).

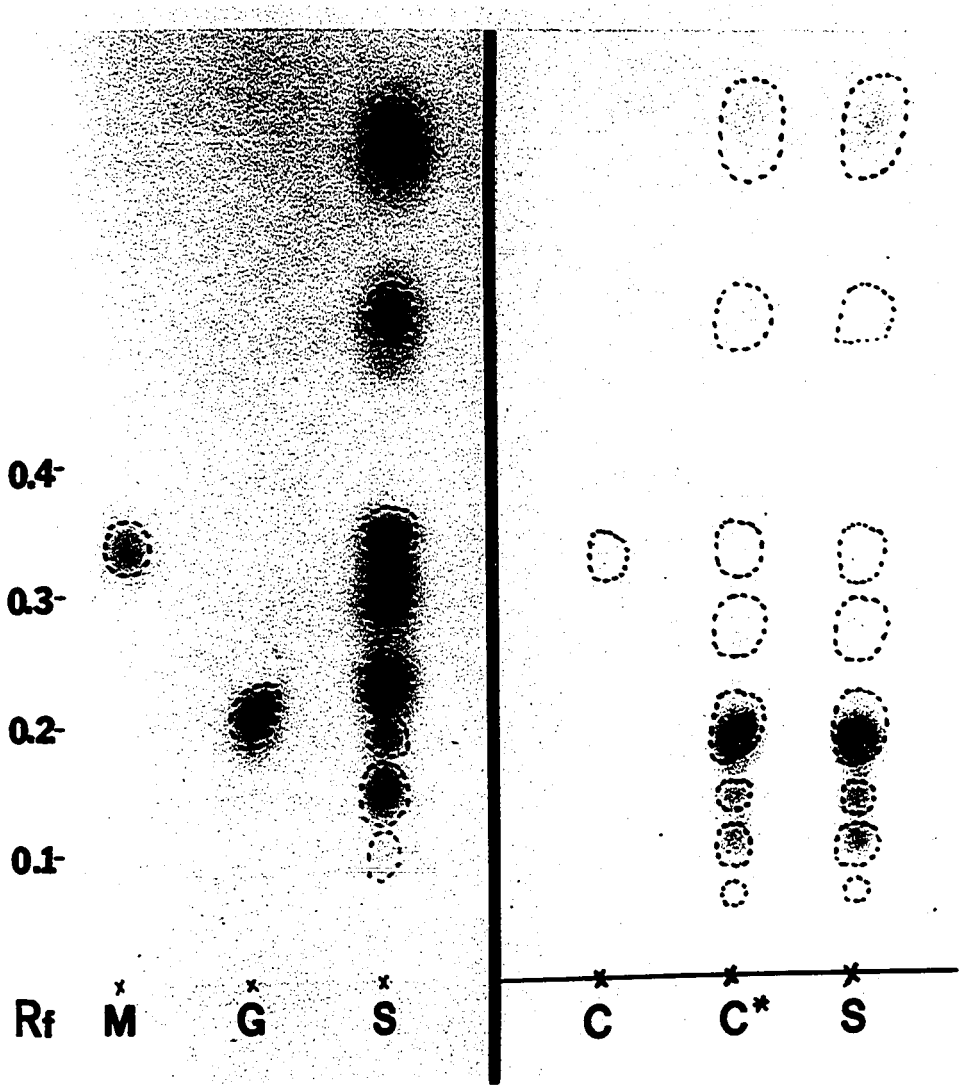

0.4
0.3
0.2
0.1
Rf
M
G
S
C
C*
S

Figure 11 (left). Ascending chromatography in 75% phenol for 25 hr. Authentic muramic acid (C) migrated to an R_f value of 0.50 and was not retarded when run with the envelope hydrolyzate (C*). Good separation of the envelope hydrolyzate (S) was obtained; one spot gave an orange (O) reaction with ninhydrin.

Figure 12 (right). Descending chromatography in ethyl acetate-acetic acid-water (140:30:30) for 14 hr. A ninhydrin-positive spot in the envelope hydrolyzate (S) coincided with authentic muramic acid (C).

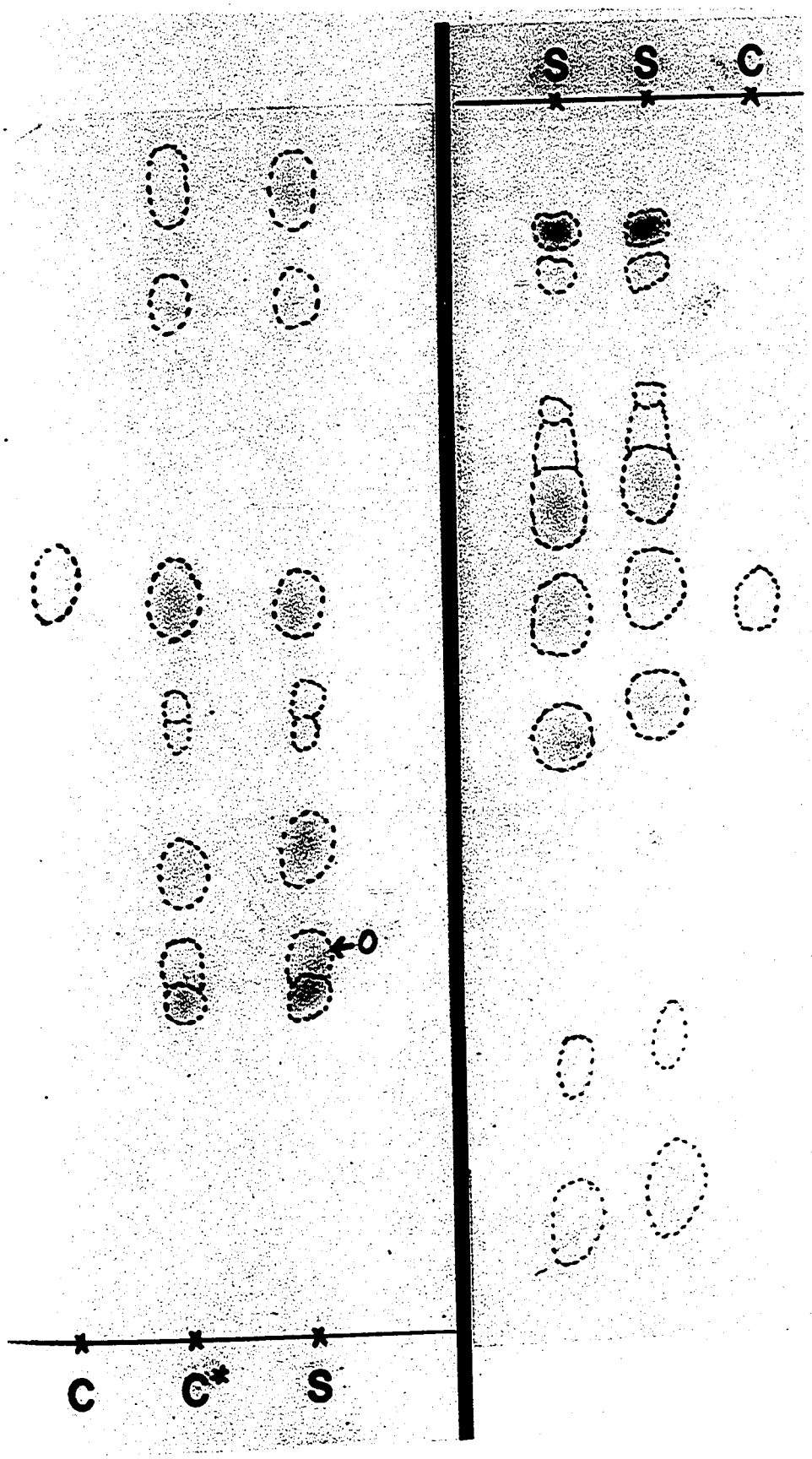

S
S
C
O
C
C*
S

Figure 13

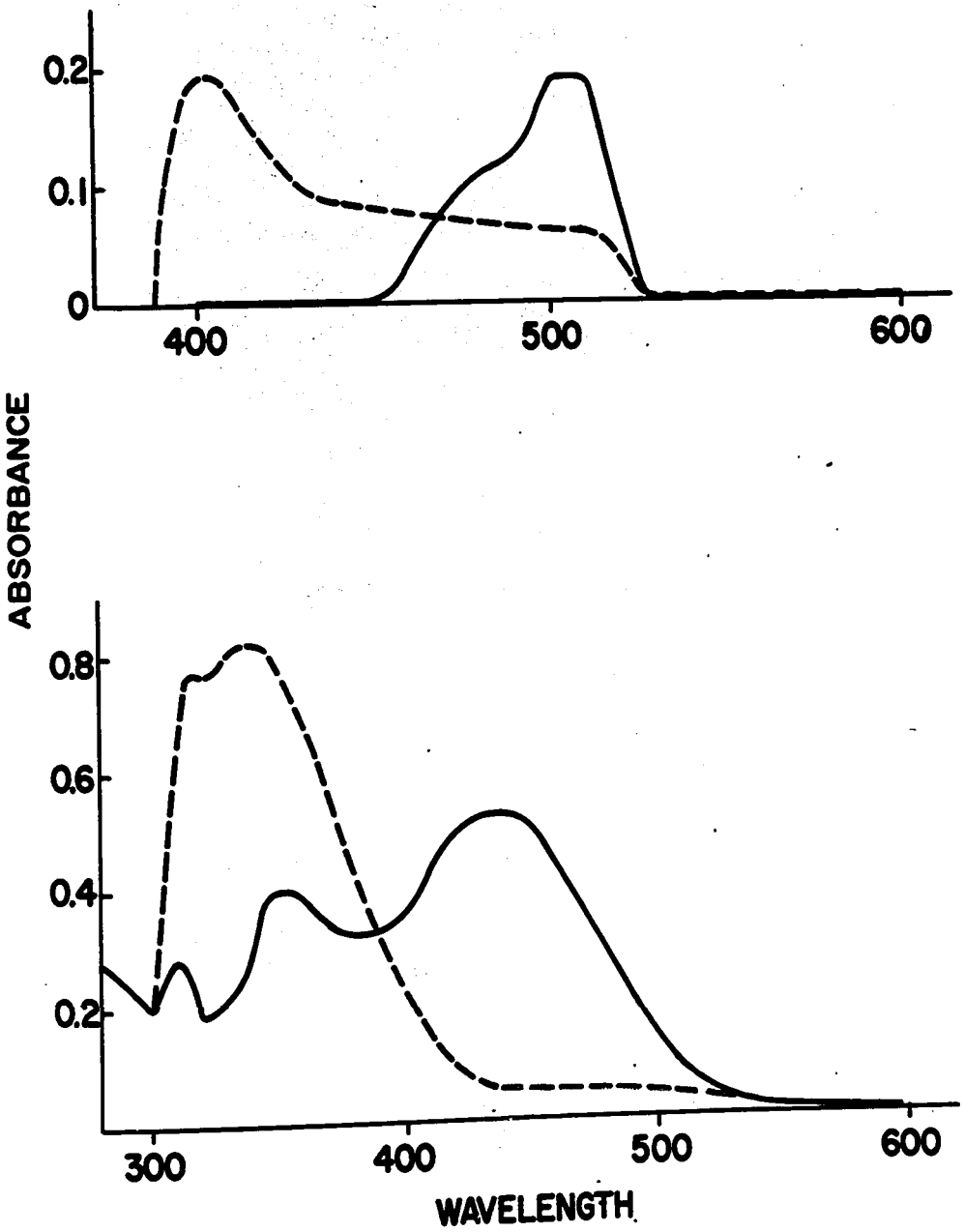
Muramic acid assay

Spectra of muramic acid (solid line) and envelope hydrolyzate (dotted line) read against a reagent blank.

Figure 14

Diaminopimelic acid assay

Spectra of diaminopimelic acid (solid line) and envelope hydrolyzate (dotted line) read against a ten-fold diluted reagent blank.


0.2
0.1
0
400
500
600
ABSORBANCE
0.8
0.6
0.4
0.2
300
400
500
600
WAVELENGTH

Figures 15 and 16

Diaminopimelic acid chromatography

Figure 15 (left). Descending chromatography in methanol-water-hydrochloric acid-pyridine for 6 hr. Considerable trailing of the DAP standard (C) was observed. (L) = lysine standard and (S) = envelope hydrolyzate.

Figure 16 (right). Descending chromatography in n-butanol-acetic acid-water for 10 hr. Components of the envelope hydrolyzate (S) were well separated; the violet spot nearest the origin is tentatively identified as lysine. (C) = DAP standard and (L) = lysine standard.

Color symbols: P(pink); V(violet); B(blue); Br(brownish); G(green).

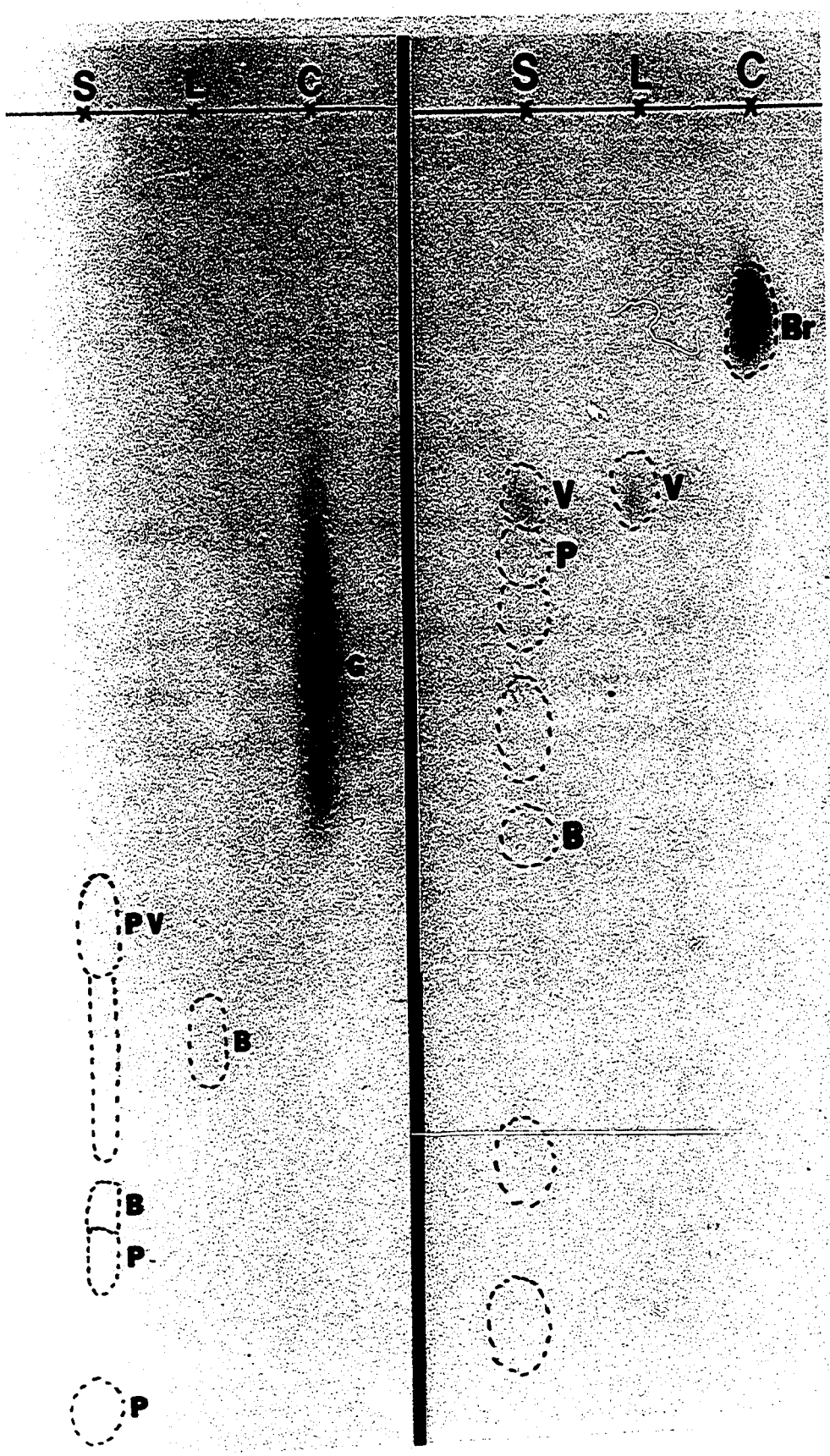

S
L
C
S
L
C
Br
V
V
P
G
B
PV
B
B
P
P

Figure 17

Linearity of ficoll continuous density gradient

Linearity of a 5 to 15% ficoll continuous density gradient was measured using a methylene blue indicator. The gradient tube was perforated and the absorbance of 30 drop fractions was read at 700 nm.

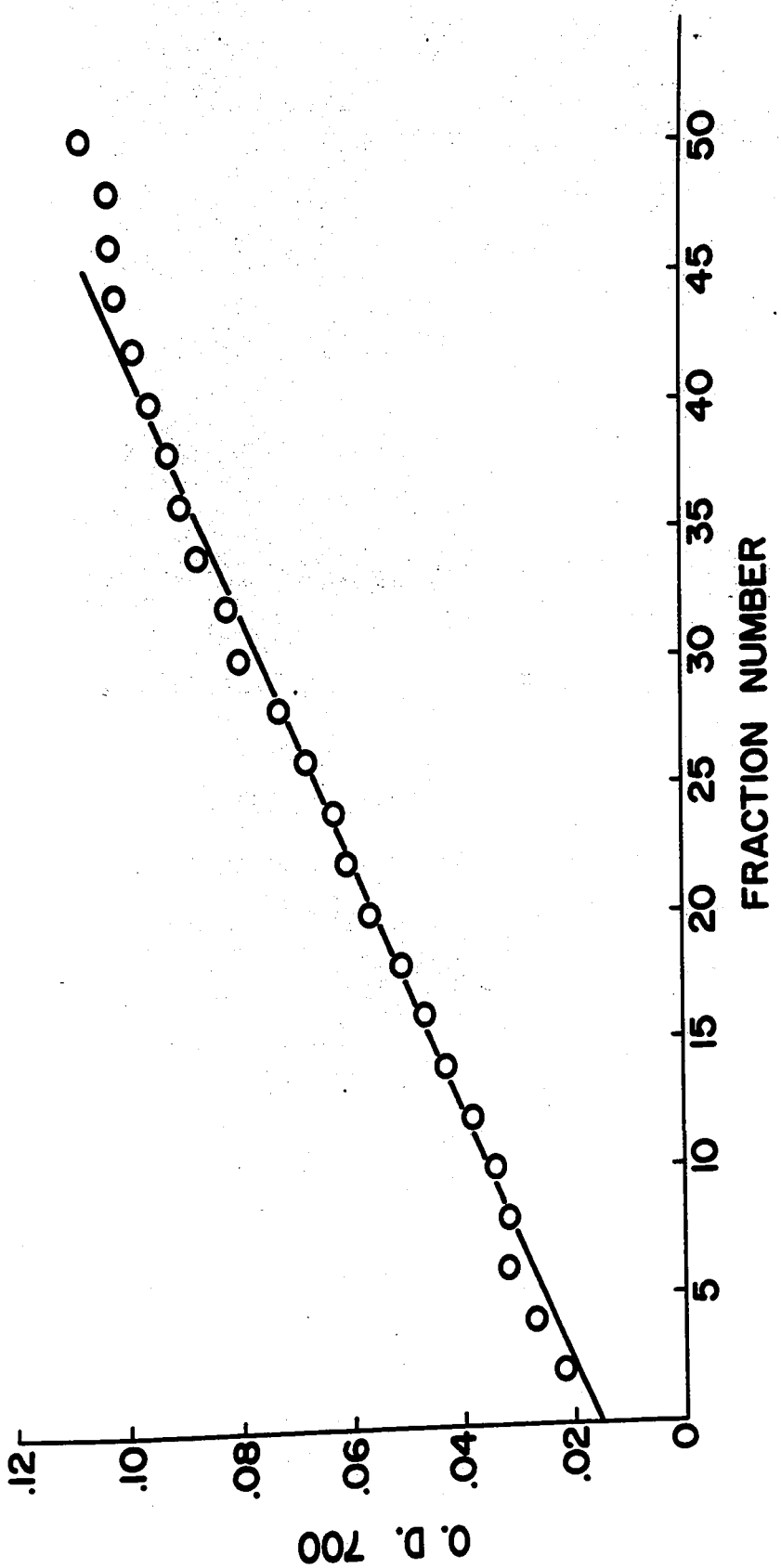
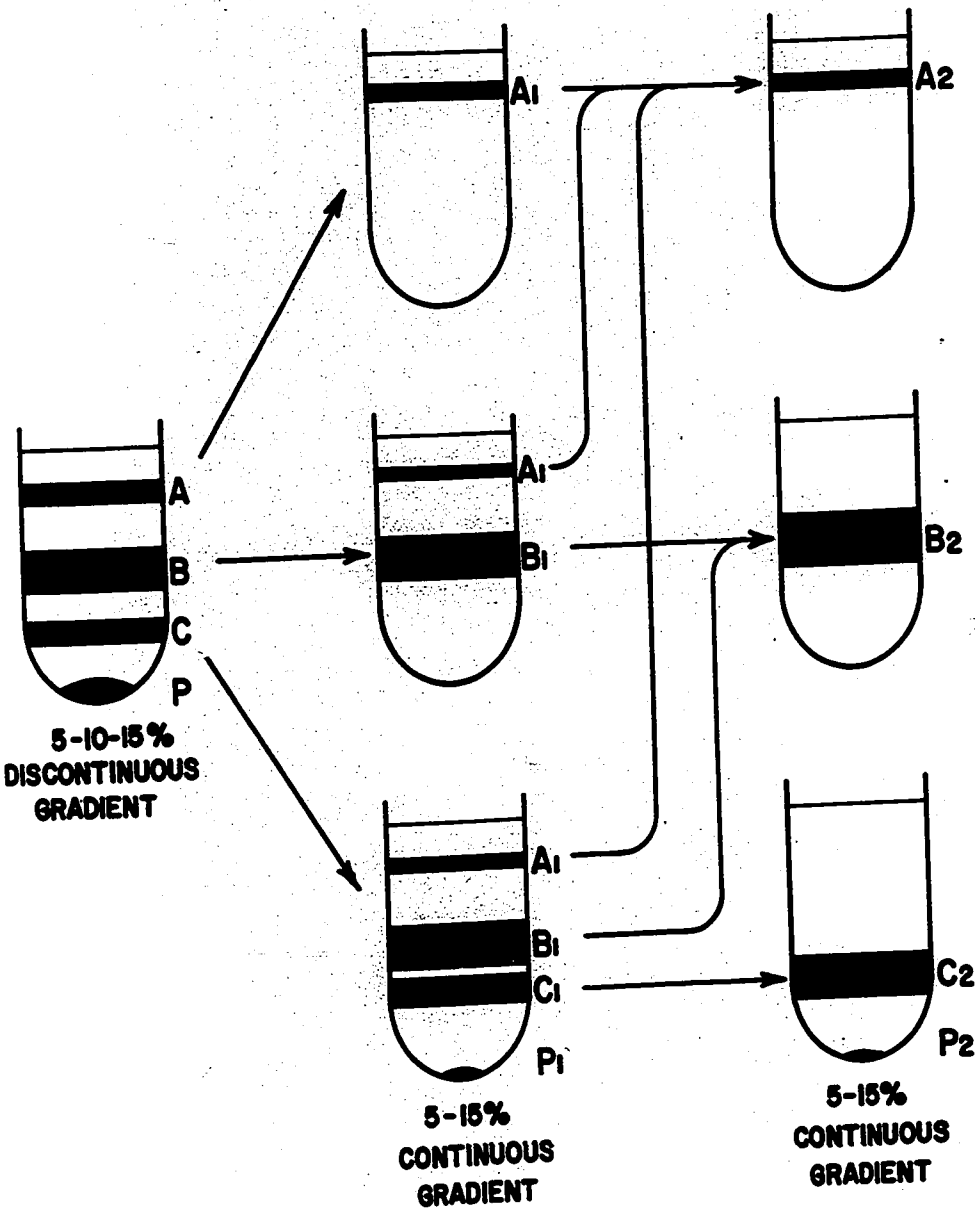

O. D. 700
.12
.10
.08
.06
.04
.02
0
5
10
15
20
25
30
35
40
45
50
FRACTION NUMBER

Figure 18

Fractionation scheme of cell lysate on ficoll density gradients.

Bands A, B and C were obtained when cell lysates were fractionated on discontinuous 5-10-15% ficoll density gradients (105,000 x g, 18 hr). Each of these fractions was further separated by centrifuging at 105,000 x g for 20 hr on continuous 5-15% ficoll density gradients to yield A_1, B_1 and C_1. Equivalent bands were pooled and fractionated again on continuous ficoll gradients to yield A_2, B_2 and C_2.


A
B
C
P
5-10-15%
DISCONTINUOUS
GRADIENT
A1
A1
B1
A1
B1
C1
P1
5-15%
CONTINUOUS
GRADIENT
A2
B2
C2
P2
5-15%
CONTINUOUS
GRADIENT

Plates 47 and 48

Fine structure of B_2 and C_2 fractions

Plate 47 (top). Thin section preparation of the B_2 fraction showing a closed vesicle approximately 0.4 μm in diameter. The tripartite structure is particularly prominent. X170,000.

Plate 48 (bottom). Thin section preparation of the C_2 fraction showing vesicles and membranous fragments (arrow). X120,000.

Plate 49

Material from the C_2 fraction negatively stained with 1% PTA. The structures of different shapes and sizes are morphologically similar to membrane sheets. X93,000.

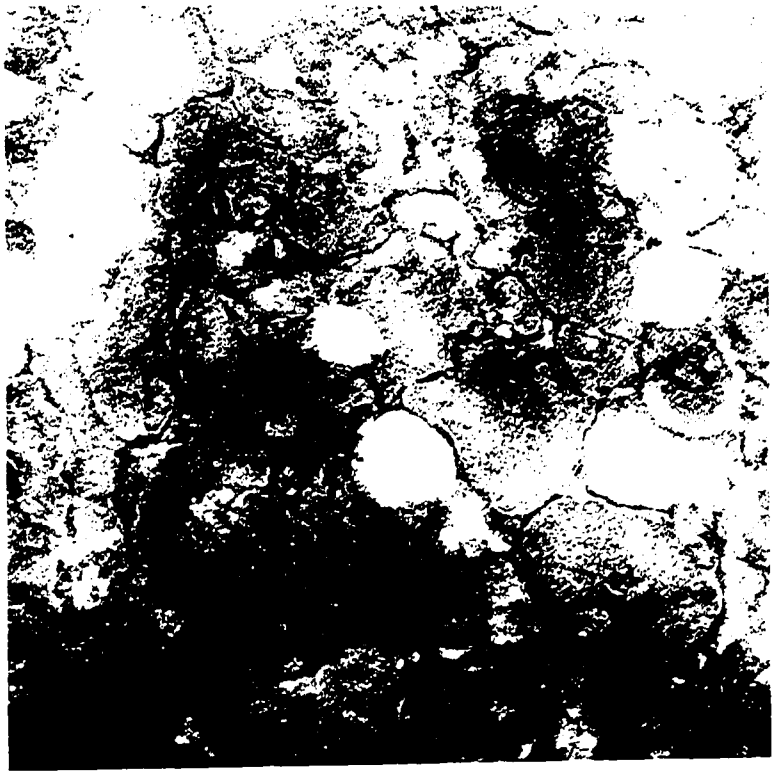


Table 7

Composition of fractionated lysate

1. Chemical composition of intact cells was obtained from separate experiments where cells were grown for 60 hr in regular medium, washed in "all salts" solution and aliquots analyzed for protein, lipid phosphorus and carbohydrate. Composition of the samples is expressed as mg/gm dry weight intact cells.
2. Phospholipid = lipid phosphorus X 25.5.

SAMPLE[1]	PROTEIN	PHOSPHOLIPID[2]	CARBOHYDRATE	PHOSPHOLIPID / PROTEIN	CARBOHYDRATE / PROTEIN
A_2	0.073	0.13	0.032	1.8	0.44
B_2	2.10	3.57	1.27	1.7	0.60
C_2	2.13	2.42	1.13	1.1	0.53
Intact cells	595.0	108.0	100.0	0.18	0.17

Figure 19 (left)

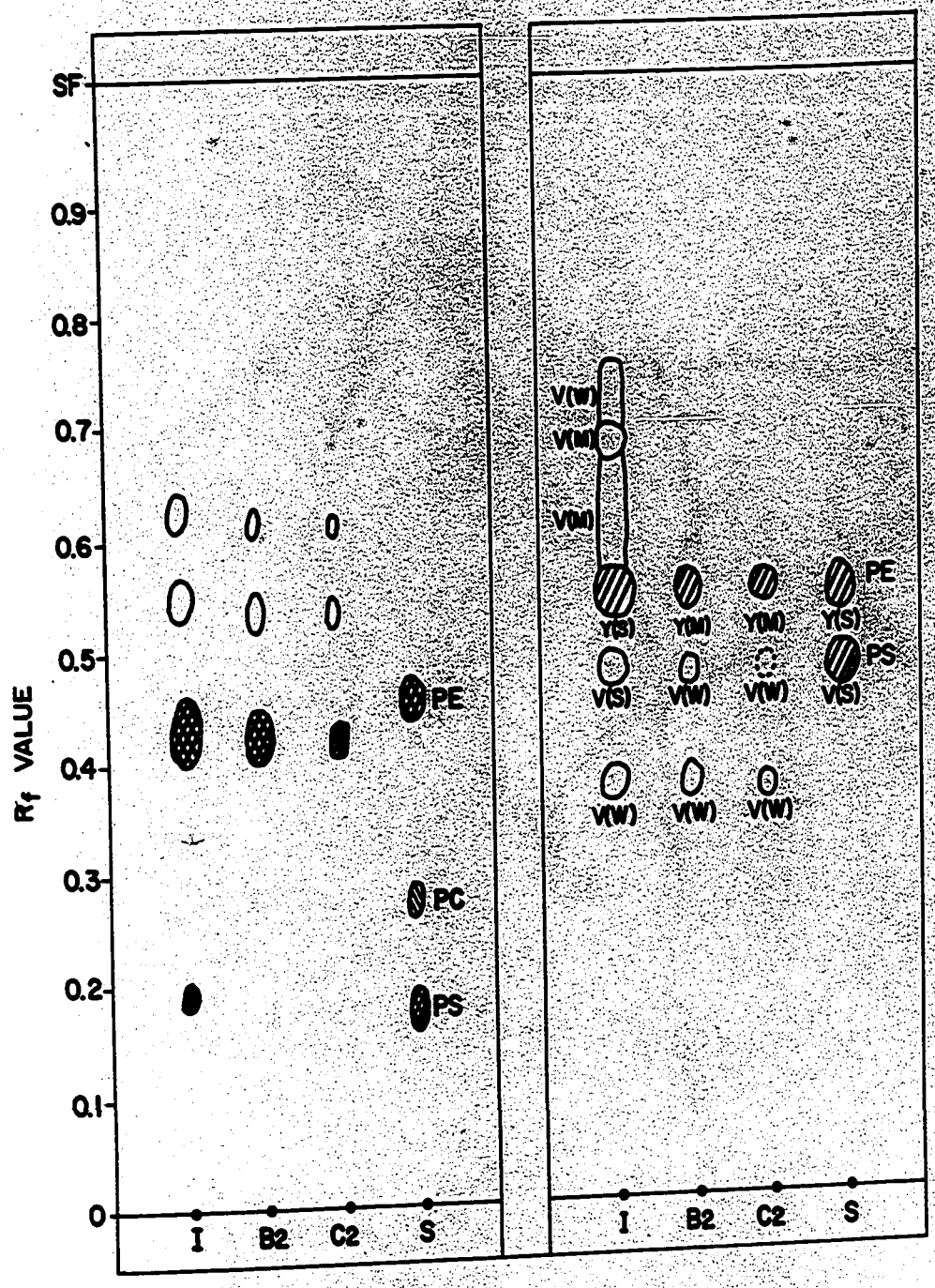
Phospholipid chromatography on TLC plates.

Chromatogram of total lipids from intact cells (I) and B_2 and C_2 fractions run in chloroform-methanol-ammonium hydroxide solvent system against a standard mixture of phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl choline (PC). Spots were detected with ninhydrin (◍), phosphate stain (◍) and by charring (O). Cell pigments migrated with the solvent front (SF).

Figure 20 (right)

Phospholipid chromatography on silica impregnated paper.

Chromatogram of total lipids from intact cells (I) and B_2 and C_2 fractions run in diisobutyl ketone-acetic acid-water solvent system against a standard mixture of phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). Spots were detected with Rhodamine 6G (O) and with ninhydrin (◍). Reaction of the separated material with Rhodamine 6G gave violet (V) and yellow (Y) spots of strong (S), moderate (M) or weak (W) intensity.


SF
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
R_f VALUE
I
B2
C2
S
PE
PC
PS
V(W)
V(M)
V(M)
Y(S)
Y(M)
Y(M)
Y(S)
V(S)
V(W)
V(W)
V(S)
V(W)
V(W)
V(W)
PE
PS
I
B2
C2
S

spots (R_f values of 0.37 and 0.47) stained violet with Rhodamine 6G. In addition to these three components, lipid extracts of intact cells (I) showed a poorly separated component (R_f = 0.68) that stained violet with Rhodamine 6G but no component which could be identified with phosphatidyl serine.

Discussion

The specific salt requirement for the growth and stability of extremely halophilic (Kushner 1968) and marine (MacLeod 1965) bacteria has been attributed partly to the capacity of mono- and divalent cations to shield or form bridges between mutually repulsive negative charges in the cell envelope layers of these organisms. A more detailed discussion of this topic was presented in Chapter 1. Amino acid analyses have been used to measure the electronegativity of cell envelopes and to provide grounds for the "cation-shielding" hypothesis; for example, Kushner et al (1964) found four times more dicarboxylic (aspartic, glutamic) than basic (lysine, histidine, arginine) amino acids in mechanically-prepared envelopes of the extremely halophilic H. cutirubrum. The cell envelopes of marine bacteria contain at least twice as much dicarboxylic as basic amino acids (DeVoe and Oginsky 1969a and Forsberg et al 1970) whereas those of terrestrial Gram negative bacteria (Salton 1964, DeVoe and Oginsky 1969a) are less acidic. The excess of acidic over basic amino acids in envelopes of V. psychroerythrus (Table 6) concurs with its acidic isoelectric point

(Madeley et al 1967) and is not inconsistent with the hypothesis that Na^{+} and Mg^{+2} ions stabilize the wall of the red psychrophile by shielding surface negative charges (Chapter 4).

Chromatographic separation with different solvent systems confirmed the presence of muramic acid in the envelope hydrolyzate of V. psychroerythrus (figures 9 to 12). With 75% phenol and butanol-acetic acid-water as solvents, the R_f values of muramic acid was 0.50 (figure 11) and 0.33 (figure 9) respectively; these values compared well with those reported by Cohen and Panos (1971). Spectrophotometric studies further confirmed the presence of muramic acid in the envelope hydrolyzate of V. psychroerythrus. Although the hydrolyzate failed to give a sharp muramic acid peak at 510 nm, it absorbed significantly at this wavelength (figure 13). Presumably, this absorption is due to muramic acid since the chromophores of contaminating sugars and amino acids fade rapidly and those of muramic acid attain an absorption maximum after 24 hr (Stewart-Tull 1968). However, the spectrum of the envelope hydrolyzate does show non-specific absorption at lower wavelengths, a finding that partially explains the absence of a distinct muramic acid peak. The presence of muramic acid in the cell envelope of V. psychroerythrus suggests that a glycosaminopeptide (R) layer, of the type found in other Gram negative bacteria (Glauert and Thornley 1969), maintains the shape of the red psychrophile. The absence of a densely staining layer in

different thin section preparations of intact cells (Plates 2 and 3) further suggests that only small amounts of this material occur in the cell wall. Costerton et al (1967) also experienced difficulties in demonstrating an R layer in thin sections of the NCMB 19 marine pseudomonad. Recent work (Forsberg et al 1972) showed that the R layer of this organism is closely allied to the outer face of the cell membrane and accounts for 1% of the cell dry weight, a value which is very low compared to the 5-10% reported for other Gram negative bacteria (Rogers and Perkins 1968).

Rhuland et al (1955) showed that single-dimensional chromatography with the methanol-water-HCl-pyridine solvent system (figure 15) separates the LL- but not the DD-isomer of DAP from meso-DAP; LL-DAP ran slightly ahead of the meso- and DD-isomers but behind L-lysine. They also found that all isomeric forms of DAP gave a green reaction with the ninhydrin reagent. Our results (figure 15) suggest that DAP isomers are absent in the envelope hydrolyzate of V. psychroerythrus although significant amounts of lysine were detected (figure 16 and Table 6). The spectrum of the hydrolyzate-ninhydrin reaction mixture also failed to show a DAP-specific peak at 440 nm (figure 14); the strong absorption at 340 nm is most probably due to contaminating amino acids such as histidine, proline and tyrosine (Work 1957) known to occur in the envelope hydrolyzate of V. psychroerythrus (Table 6). Although the envelope hydrolyzate does not appear to contain any DAP, trace amounts may have escaped chromatographic and

spectrophotometric detection. The apparent lack of DAP in the envelope of V. psychroerythrus was rather unexpected since significant amounts of this compound have been detected in other vibrios (Winter et al 1971) and in the cell wall of marine (Forsberg et al 1972) and terrestrial (Key, Gray and Wilkinson 1970) pseudomonads.

Phase contrast studies suggest that lysis of intact cells of V. psychroerythrus in shaken 0.5M NaCl solutions damages the R layer since previous studies (Chapter 4) showed that intact cells lysed in 0.5M NaCl (standing tubes) occurred as phase pale rods. Fractionation of the 0.5M NaCl lysate on discontinuous and continuous ficoll density gradients yielded three distinct bands (figure 18) containing membranous vesicles and fragments (Plates 47 and 48); the vesicles were morphologically similar to the detached outer membranes of the NCMB 19 marine pseudomonad (Costerton et al 1967). It was previously shown (Chapter 4) that lysis of V. psychroerythrus in 0.5M NaCl disrupts the triple layered structure of the cytoplasmic membrane but has little effect on the structural integrity of the outer membrane (Plate 36). It follows that the large membranous vesicles and fragments observed in the three fractions (Plates 47 and 48) most probably originate from the outer membrane rather than the cytoplasmic membrane. However, this evidence does not exclude the possibility that some of the vesicles or fragments are of cytoplasmic membrane origin.

Miura and Mizushima (1968) were able to separate spheroplast membranes of E. coli into cell wall and cell membrane fractions on sucrose density gradients. Identification of the three fractions obtained was based on their protein, phospholipid and carbohydrate content and distribution of some respiratory enzymes and cytochromes. A more recent immunological study confirmed the identity of the cell wall fraction (Miura and Mizushima 1969). The chemical data presented in Table 7 does not permit us to ascertain the origin of fractions A_2, B_2 and C_2 i.e. cell wall or cell membrane material. Assuming that most of the carbohydrates originate from the cell wall, the carbohydrate-protein ratios (Table 7) together with thin section studies (Plates 47 and 48) suggest that the three fractions are equally rich in cell wall material. The significance of the lower phospholipid-protein ratio of C_2 (Table 7) is not clear. The densities of bands A_2, B_2 and C_2 do not provide any additional information on the origin of the fractionated material. For example, the density of native (Morowitz and Terry 1969) and reformed (Engelman and Morowitz 1968, Razin et al 1969) Mycoplasma membranes on sucrose density gradients ranges from 1.16 to 1.22 gm/ml, values which differ significantly from those of A_2, B_2 and C_2.

Thin layer and paper chromatography showed that the phosphatide composition of B_2 and C_2 was identical (figures 19 and 20). Lipid extracts of intact V. psychroerythrus could be resolved into at least five components (figure 20),

a finding which agrees with that previously reported by Kates and Hagen (1964). The only ninhydrin-positive spot, tentatively identified as phosphatidyl ethanolamine (PE) occurred as the major component of intact cells and B_2 and C_2 lipid extracts. PE is also a major lipid component in intact cells (Kates 1964, Ikawa 1967) and walls (Wilkinson 1970) of Gram negative bacteria. The ninhydrin and phosphate positive spot (R_f = 0.19) in lipid extracts of intact cells (figure 19) does not appear to be PS since an equivalent spot could not be resolved by paper chromatography (figure 20). The absence of PS in V. psychroerythrus concurs with that previously reported by Kates and Hagen (1964).

In summary, we were confident that the 0.5M NaCl lysate could be separated into a major cell wall and minor cell membrane fraction since previous studies (Chapter 4) had shown that, under these lytic conditions, the outer membrane detached from the cell body and maintained its tripartite structure whereas most of the cell membrane structure was disrupted. The observations that each fraction is composed of large tripartite vesicles and that the carbohydrate-protein ratio of the three fractions is similar suggest that A_2, B_2 and C_2 are mainly composed of cell wall material. The significance of the lower phospholipid-protein ratio of C_2 is not clear; more detailed biochemical studies would be required to clarify this point.

Chapter 6

The regular surface pattern of *Halobacterium cutirubrum*: a honeycomb network.

Introduction

The physiology of the halobacteria, Gram negative organisms requiring at least 15% NaCl for their growth and stability has been described in detail in several reviews (Larsen 1962 and 1967, Brown 1964a, Kushner 1968). Since Houwink's (1956) first electron microscope study of the flagella, gas vacuoles and cell wall structure in Halobacterium halobium, more recent papers have dealt with the general ultrastructure of halobacteria (Cho, Doy and Mercer 1967, Steensland and Larsen 1969, Larsen, Omang and Steensland 1967), structural transformations under different lytic conditions (Mohr and Larsen 1963, Kushner and Bayley 1963, Kushner et al 1964), fractionation-characterization of cell envelope lysates (Stoeckenius and Rowen 1967, Stoeckenius and Kunau 1968) and characterization of the H. halobium purple membrane (Oesterhelt and Stoeckenius 1971, Blaurock and Stoeckenius 1971).

In this chapter, we present the results of an ultrastructural study on the surface pattern of Halobacterium cutirubrum, a red-pigmented motile rod. The surface pattern of H. halobium was first described from shadowed intact cells as a single layer of hexagonally arranged globular particles (Houwink 1956) and later shown to consist mainly of protein (Marshall, Wicken and Brown 1969). Regular hexagonal arrays of globular particles have also been observed in shadowed envelopes or envelope fragments of H. cutirubrum (Kushner and Bayley 1963, Kushner et al 1964) and H. salinarium (Mohr

and Larsen 1963). Although negative staining is a method of choice for revealing fine structural details, it has not been possible to apply this method to halobacteria because of their high salt requirement for stability (Glauert and Thornley 1969). Using a low beam intensity technique, we have been able to show new structural details of the surface pattern of unfixed and unstained H. cutirubrum.

Materials and Methods

Organism and growth conditions. Cells of H. cutirubrum were grown 60-70 hr in shaken cultures at 37C in a modified Sehgal and Gibbons (1960) complex medium (Kushner and Bayley 1963). Cells were harvested by centrifuging 20 min. at 8,000 x g, washed once and suspended in an "all salts" solution (growth medium without yeast extract or amino acids).

Negative staining. An aliquot of unwashed intact cells was mixed and stained 5 min. with 1% (w/v) aqueous uranyl acetate pH 5.5 (final concentration = 0.25% uranyl acetate in 18% (w/v) NaCl). A drop of the stained cell suspension was applied to a formvar-carbon coated grid and permitted to air dry.

Surface replication. Sample preparation for surface replication was carried out according to the technique of Kushner and Bayley (1963). A slide mount of washed intact cells was shadowed with platinum-carbon at an angle of 20^{o} with respect to the platinum-carbon source and strengthened with a film of evaporated carbon. The replicas were floated onto a water surface, placed on a formvar-carbon coated grid and dried in air.

Low beam intensity studies. Cells washed in "all salts" or in 25% NaCl were placed on formvar-carbon coated grids, the excess of liquid removed with the edge of a filter paper and the samples dried in air. Scanning and focusing were carried out at low beam intensities on an AEI 6B electron microscope at an accelerating voltage of 60KV.

Thin section preparations. Washed intact cells were fixed with 5% glutaraldehyde (in 0.1M Na cacodylate-HCl pH 7.1, 0.01M $CaCl_2$, 4.3M NaCl), stained with 1% (w/v) osmium tetroxide and uranyl acetate (in 4.3M NaCl) and dehydrated in a graded series of alcohol saturated with NaCl as described by Steensland and Larsen (1969). Pellets were infiltrated for 1 hr in a 1:1 (v/v) mixture of acetone and epoxy (Epon 812) resin, embedded in freshly prepared resin and polymerized at 55C for 2-3 days.

Results

Flagellation

As previously reported for other halobacteria (Houwink 1956), motility of H. cutirubrum is dependent on a single polar tuft of three to five flagella (Plates 50 and 51). Tufts containing as many as eight flagella were observed on occasion.

Surface Replication

Replicas showed what appears to be a hexagonal array of particles or subunits with a center-to-center spacing of 15.0 to 16.5 nm arranged along three principal axes of symmetry mutually set at 120° (Plate 52).

Thin section preparations

In addition to fine nucleic fibrils and densely staining ribonucleoprotein particles, short segments of a dented surface were occasionally observed in thin sections of intact cells (Plate 53). The periodicity of the protruding units was approximately 13.0 nm.

Low beam intensity studies

When unfixed intact cells dried from an "all salts" or 25% NaCl solution were examined under low beam intensity, a honeycomb network (Plate 54) gradually appeared. The holes of the network, many of which are hexagonally shaped (Plates 54A and 55), are 9.5 to 11.0 nm in diameter with a center-to-center repeat distance of 15.5 to 17.0 nm and are aligned along three axes of symmetry set at 120°. Occasionally, blebs with a periodicity of 15 nm were observed along the edge of some cells (Plate 54B). This network was stable for about 1 min after which it progressively deteriorated (Plate 55). The network was most clearly seen in those cells that were less electron-dense than the rest.

When cells were dried from 0.2% phosphotungstic acid (PTA) in 20% NaCl (pH 6.0), no surface pattern was seen. Reasons for this apparently disruptive effect of PTA are not clear. Prefixation for 1 hr with 4% formalin in "all salts" solution increased the stability of the network by a factor of two, but did not change the resolution obtained.

Plates 50 and 51

Flagella of H. cutirubrum

Plate 50 (top). Intact cell negatively stained with 1% uranyl acetate showing a polar tuft of five flagella. X32,000.

Plate 51 (bottom). Shadowed preparation of an intact cell showing three intertwined flagella. The background is laden with salt crystals. X48,000.

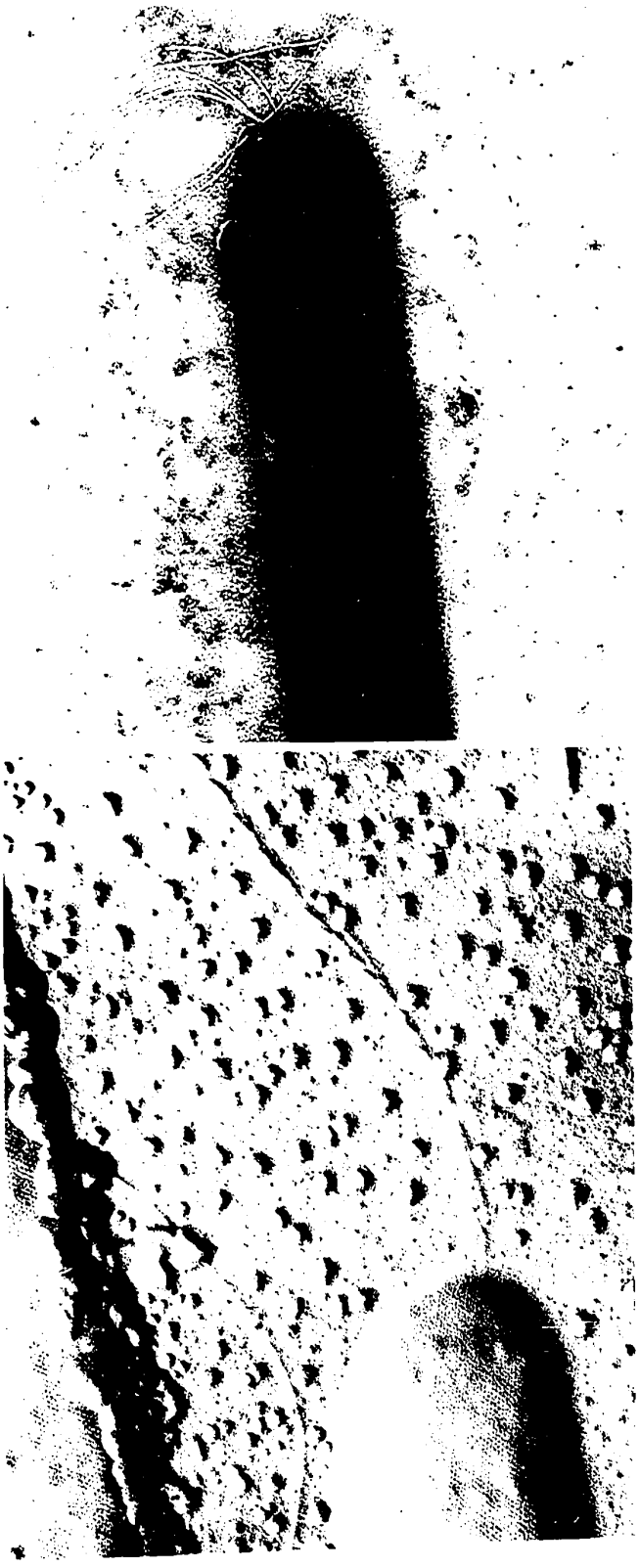


Plate 52

Hexagonal pattern by surface replication

Regular hexagonal pattern of particles or subunits on the surface of intact cells shadowed with Pt-carbon. Flagellar fragments and salt crystals can be seen in the background. X45,000.

Plate 53

H. cutirubrum in thin section

Short segments of a dented surface (arrow) were occasionally observed in thin sections of intact cells. X176,000.

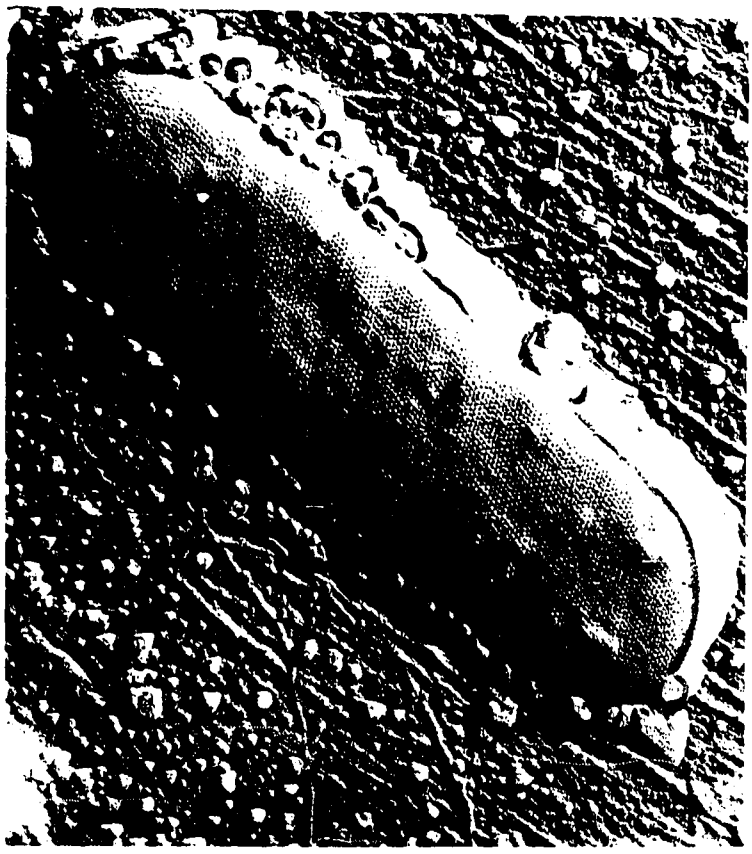


Plate 54

Honeycomb network of H. cutirubrum

Unfixed cells dried down from an "all salts" solution; similar structures were seen in cells dried from 25% NaCl.

A. Honeycomb network exhibiting a regular hexagonal array of holes; some of the holes are hexagonally shaped. X180,000.

B. Blebs which apparently originate from an "edge-view" of the network as it rolls over the edge of the cell (arrow). X120,000.

A

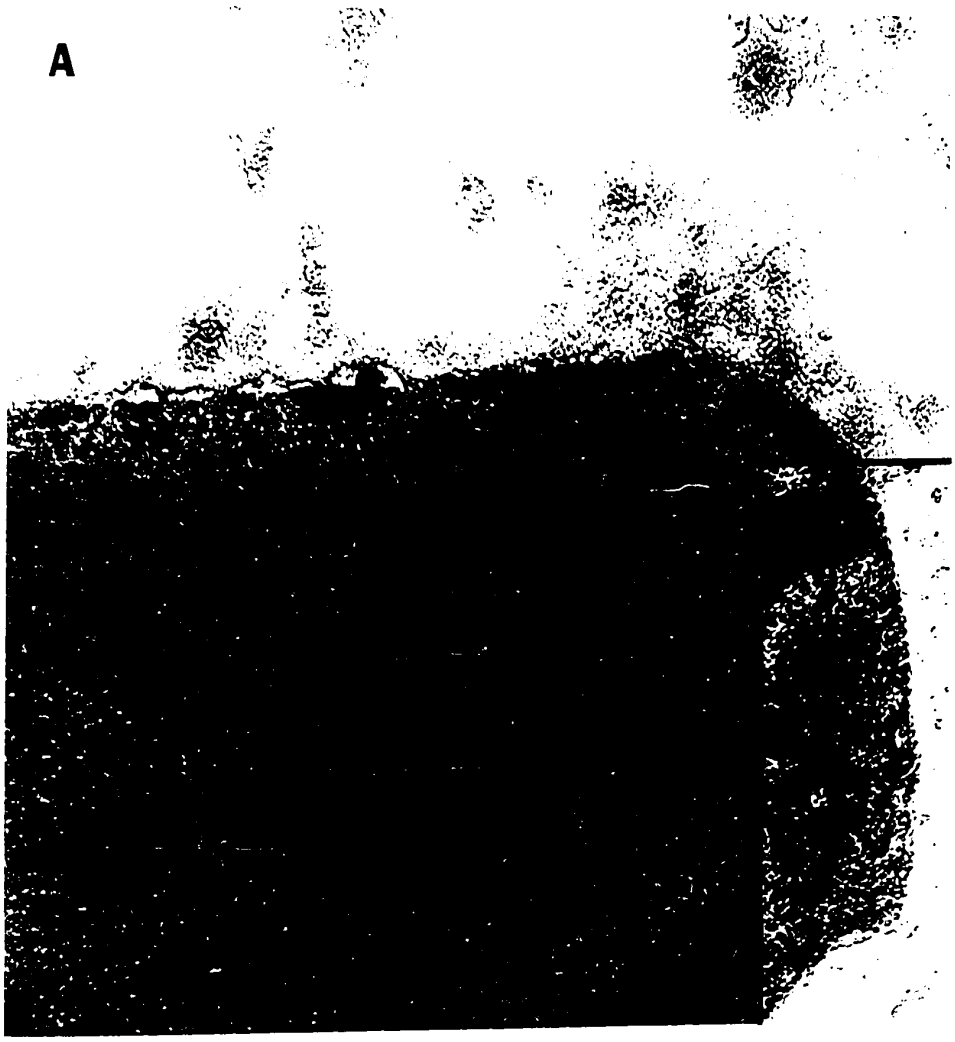
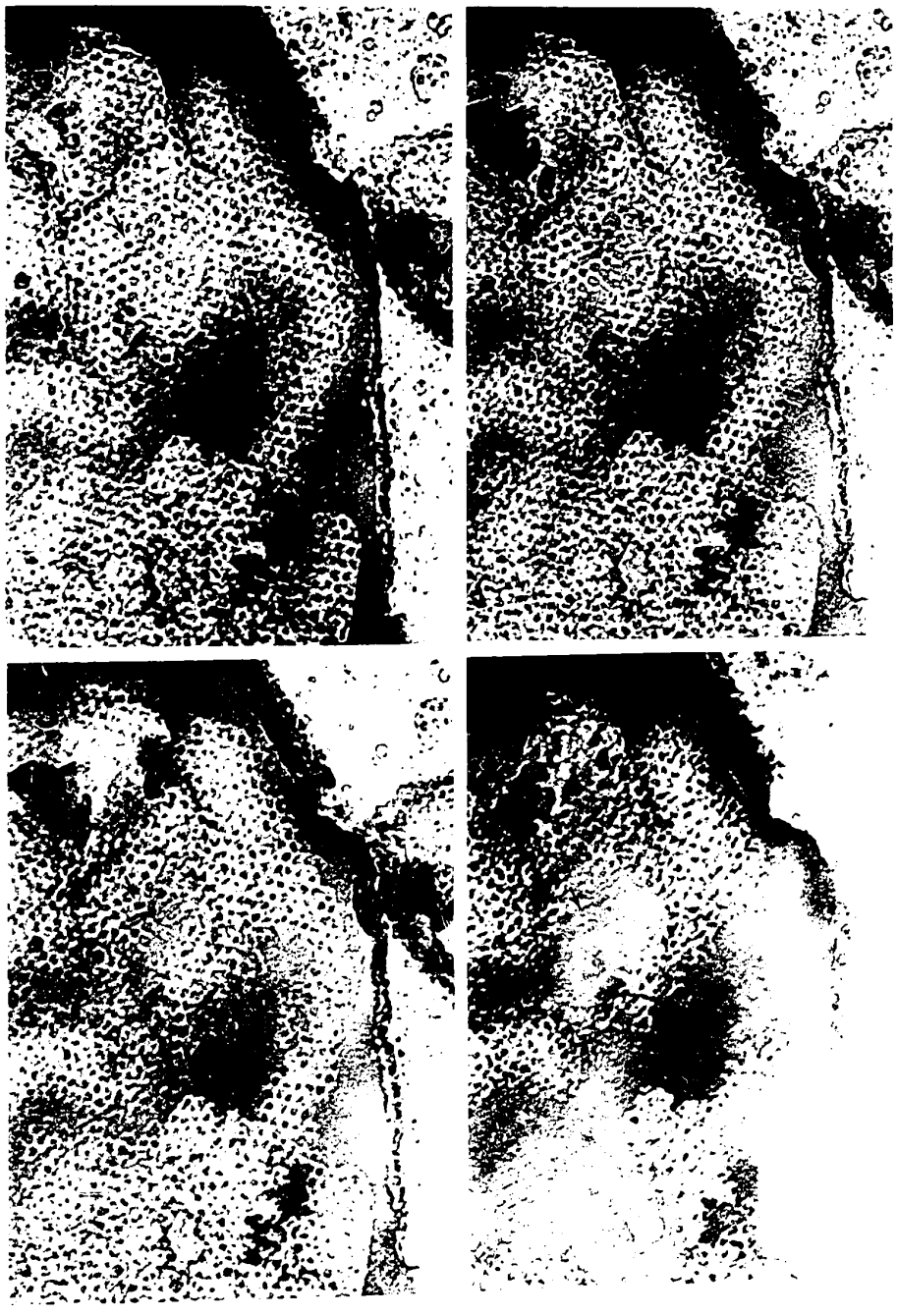


Plate 55

Stability of the honeycomb network

Micrographs of the unfixed network taken at 30-S intervals showing the deterioration of the network. Note the changes in the selected area (arrow). Patches of the honeycomb network, sloughed off the cell surface during manipulations, probably make up the beam-sensitive material in the background. X128,000.



Discussion

The regular hexagonal surface pattern of halobacteria is not unique, for similarly structured surface layers have been observed in other bacterial genera. For example, an outer layer of subunits in hexagonal array was reported in the cell walls of Spirillum serpens (Murray 1963), Micrococcus radiodurans (Work and Griffiths 1968), Lampropedia hyalina (Chapman, Murray and Salton 1963) and Chromatium sp. (Hageage and Gherna 1971). For a comprehensive review on the regular surface pattern in Gram negative bacteria, the reader is referred to the excellent paper by Glauert and Thornley (1969).

Insight as to the spacing and arrangement of the surface units in Halobacterium species has been obtained from surface replica studies of intact cells and direct viewing of shadowed envelopes (Houwink 1956, Kushner and Bayley 1963 and Plate 52). Since intact cells and envelopes require high salt concentrations for their stability, drying probably deposits salt crystals and cell debris between adjacent surface units thus adversely affecting the resolution of the units themselves. The reason why resolution under low beam intensity is not adversley affected by such material may be partly due to a cleaning action by the electron beam itself, i.e. the pattern appears gradually and is stable for only a short time (Plate 55).

Since the honeycomb network (Plate 54), the only structure exhibiting symmetry, was electron transparent, it

appeared that it might be negatively stained. However, the nature of the material responsible for this staining reaction is not certain. Cells washed in NaCl alone show the network, as well as those washed in "all salts" solution; although NaCl is known to act as a negative stain (Valentine and Horne 1962), it is doubtful that this salt could give the degree of resolution obtained. Another possibility is that these cells have picked up traces of heavy cations from the complex growth medium. Such ions might bind readily to the acidic proteins of the outer layer (Kushner 1968).

The agreement in the periodicity and symmetry of the pattern of replicated and directly-viewed preparations suggests that both structures are in fact the same and that the unfixed honeycomb network did not deteriorate on short exposure to the electron beam.

It is interesting to note the similarities of the honeycomb layer in terms of axes of symmetry, repeat distance and diameter of the units with the "perforate layer" of Lampropedia hyalina, studied by Chapman, Murray and Salton (1963); in fact, their model coincides well with Plate 54. According to this model, the blebs (Plate 54B) would then be the honeycomb network as it turns over the cell's edge and correspond to the "castellated edge" in L. hyalina. The occurrence of a dented surface in thin sections of H. cutirubrum (Plate 53) and in Halobacterium sp. strain 5 (Steensland and Larsen 1969) may well correspond to a sectional view

of the blebs. These findings support the view that the honeycomb network occupies the outermost layer of the cell envelope and that its symmetry, as suggested for L. hyalina (Chapman, Murray and Salton 1963), corresponds to a two-dimensional crystal lattice.

Thesis summary and concluding remarks

Our studies have dealt with two Gram negative, rod-shaped, red-pigmented organisms that live under more or less extreme conditions and that exhibit specific requirements for growth and stability. The extreme halophile, H. cutirubrum, needs very high salt concentrations for its growth (reviewed in Chapter 1) and becomes deformed in acidic salt solutions (Kushner and Bayley 1963). The marine psychrophile, V. psychroerythrus, also unstable in acidic salt solutions (Madeley et al 1967), specifically requires salts (Korngold and Kushner 1968) and low temperatures (Hagen et al 1964) for growth. The specific salt requirements of both organisms appear to be partly linked to the capacity of mono- and divalent cations to shield or bridge repulsive electronegative groups in their envelopes. Exposure to solutions of reduced ionic strength leads to extensive leakage, cell deformation and/or extensive fragmentation of their envelope layers (see Chapters 1 and 4). The salt requirement of these two organisms is quantitatively, but probably not qualitatively different from that of most other Gram negative bacteria. Most cells probably need divalent cations for maintaining their structure as shown by the general efficacy of EDTA and its ability to potentiate the effects of lysozyme. The need of marine bacteria for salt and of many non-marine bacteria for small amounts of mono- and divalent cations was discussed in Chapter 1.

Taxonomical studies (Chapter 2) have shown that the marine psychrophile, formerly designated as NRC 1004, is a member of the genus Vibrio. Because of its red pigment and its obligate psychrophilic nature, the organism was identified as V. psychroerythrus sp. n. The organism has been deposited as the type strain with the American Type Culture Collection, Rockville Maryland, and given the accession number ATCC 27364.

The fine structure of V. psychroerythrus (Chapter 3) is typically that of other Gram negative bacteria exhibiting triple layered outer and cytoplasmic membranes each 6.0 to 7.5 nm wide. Although muramic acid was detected in mechanically-prepared envelopes, the absence of a rigid (R) layer in thin section preparations suggests that only small amounts of glycosaminopeptide material occurs in its cell wall. In addition to mesosomes and inclusion bodies, rod-shaped organelles tentatively identified as rhapidosomes were observed in the cytoplasmic matrix. Cell division proceeds with the formation of a cell membrane septum followed by a centripetal growth of the outer membrane ultimately resulting in the release of daughter cells; such a mechanism conforms with that described for other Gram negative bacteria. It was also shown that growth on enriched solid media and in Ca^{+2}-supplemented liquid medium help preserve cellular fine structure.

Morphological and chemical studies of the lytic susceptibility of V. psychroerythrus in solutions of low ionic strength (Chapter 4) showed that both Na^+ and Mg^{+2} preserve the fine structure of the outer membrane whereas the cytoplasmic membrane is best stabilized by Mg^{+2}. These results strongly support an earlier report (Korngold and Kushner 1968) of a cation-dependent preservation of cell envelope structure in this organism. Our results also suggest that Ca^{+2} ions stabilize cytoplasmic membrane structure. When exposed to low salt concentrations or elevated temperature (37C), cells of V. psychroerythrus undergo considerable structural deterioration as seen in the release of different-sized vesicles and fragments in the medium. Significant losses of nucleic acids were also measured.

For growth, the red psychrophile specifically requires Na^+ and Mg^{+2} ions and traces of other ions (Korngold and Kushner 1968). These authors also found the organism to be stable in 0.1M Mg^{+2} plus 0.5M NaCl whereas Li^+ and Rb^+ could less effectively replace Na^+. Divalent salts, some in very low concentrations, could also maintain cells intact. Our results suggest that Na^+ and Ca^{+2} specifically contribute to the structural integrity of the outer membrane and cytoplasmic membrane respectively; Mg^{+2} appears to stabilize both structures. It became clear that a more rigid demonstration of these effects would require pure membrane preparations. Attempts at isolating the cell envelope layers of V. psychroerythrus (Chapter 5) yielded three homogeneous,

red-pigmented bands on ficoll density gradients. However, morphological and chemical characterization of the fractionated material did not permit us to identify the fractions as cell wall or cytoplasmic membrane material. The technique, nevertheless, does show great potential in view of cell wall isolation.

Low beam intensity studies (Chapter 6) showed that the surface pattern of H. cutirubrum, seen as a regular hexagonal array of particles in shadowed preparations, occurs as a regular honeycomb network containing hexagonally-shaped holes. The network appeared to be negatively stained by NaCl or heavy cations absorbed from the growth medium. The contribution of this surface layer to the structure and/or physiology of halobacteria has yet to be determined.

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Vibrio psychroerythrus sp. n.: Classification of the Psychrophilic Marine Bacterium, NRC 1004

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A red-pigmented organism, formerly known as marine psychrophile NRC 1004, has been classified as *Vibrio psychroerythrus* sp. n. Classification was mainly based on morphology, the ability of the organism to oxidize and ferment glucose, its sensitivity to vibriostat 0/129, and its deoxyribonucleic acid base composition of 40.0 moles % guanine plus cytosine, determined by thermal denaturation. The organism gave positive reactions for catalase, oxidase, and starch hydrolysis and produced acid from maltose and dextrin but not from arabinose. It was indole- and citrate-negative and reduced nitrate to nitrite without producing gas.

A red, motile, gram-negative, rod-shaped bacterium, originally isolated from flounder eggs by K. Eimhjellen of Trondheim, Norway, is of special interest because of its obligately psychrophilic nature and its exacting salt requirements. This organism grows at 0 to 19 C but dies and rapidly lyses at 21 C or higher (13). Unlike several other marine bacteria, whose stability can be supported by NaCl alone, or even by sucrose (17), this organism needs relatively high concentrations of both Na^+ and Mg^{2+} ions for stability (9, 16). Electron microscope studies have shown that either ion will support cell wall structure, whereas Mg^{2+} is needed for cytoplasmic membrane stability (9).

Because of its red pigment, which behaved in preliminary chromatographic studies as did prodigiosin, as well as its fatty acid composition and its extracellular proteinase, this organism was once considered as possibly related to the genus *Serratia* (15, 20). However, no systematic taxonomic studies were undertaken. It has been shown to possess a single polar flagellum (9), and hence the designation as a *Serratia* species does not appear to be suitable. We report work that provides assignment of this organism to the genus *Vibrio*.

MATERIALS AND METHODS

Growth conditions were similar to those previously described (9). Acid production from carbohydrates, determined in standing tubes at 10 C, was carried out in an "all salts" solution (9) supplemented with 0.2% (w/v) tryptone, 1.0% carbohydrate, and 0.003% bromothymol blue; final *p*H 7.4. Aerobic and anaerobic utilization of glucose was determined by the method of Hugh and Leifson (14) using semisolid media in tubes; final *p*H 7.1. Mono- and disaccharides were sterilized by filtration; a 10% aqueous solution of dextrin was autoclaved for 7 min. All tests with carbohydrates were carried out in triplicate. Nitrate reduction, indole production, and citrate utilization broths (3) made up in an "all salts" solution, were carried out in standing tubes at 10 C. The nitrate broth contained 0.1% KNO_3 and 0.2% peptone, the indole broth 1.0% tryptone, and the citrate broth 0.57% Koser citrate (Difco); the final *p*H was adjusted to 7.0. Samples from the nitrate and indole tubes, assayed with sulfanilic acid and Kovacs reagents, respectively (3), were checked daily until cell pigments interfered with the color reactions (4 days after inoculation). Catalase (23), oxidase (23), and starch hydrolysis (3) (0.2% soluble starch) tests were performed on 2-week-old cultures grown on agar plates of "all salts" solution plus 0.8% tryptone (9). Oxidase was also tested by using Pathotec (Warner-Chilcott, N.J.) strips. Sensitivity to vibriostat 0/129 was tested by placing filter paper discs (Schleicher and Schuell Co., Keene, N.H.) saturated with 2,4-diamino-6,7-diisopropyl pteridine solution (1 mg/ml in acetone) on seeded agar plates (1). Control discs had been soaked in acetone alone. Plates were examined after 12 to 15 days of incubation.

Deoxyribonucleic acid (DNA) was obtained from intact cells lysed for 1 hr in one-tenth strength "standard saline citrate" (18). As previously shown (9, 16), considerably higher concentrations of salts are needed to prevent lysis of this organism. DNA was purified by the method of Marmur (18). From

thermal denaturation curves obtained with a Gilford automatic spectrophotometer (model 2400); T_m values were determined and moles % guanine plus cytosine (G + C) was calculated according to the linear relation of Marmur and Doty (19). A purified DNA from *Bacillus subtilis* strain W23 was used as standard and run simultaneously with the psychrophilic DNA.

RESULTS

Results of the physiological tests are summarized in Table 1. The Hugh and Leifson test revealed that within 10 days the marine psychrophile produces acid but no gas under both aerobic and anaerobic conditions. Under aerobic conditions, good growth occurred along the whole length of the stab; under anaerobic conditions, less growth occurred but a definite acid reaction was observed. Acid production from maltose and dextrin was observed 12 to 14 days after inoculation; no acid was produced from arabinose. Good growth occurred in all cases. Nitrate reduction to nitrite was detected 1 day after inoculation; no ammonia was produced during the nonpigmented phase of growth nor was any gas liberated after 14 days of incubation. Indole was not produced, nor was citrate utilized as sole carbon source. With the oxidase test, using naphtholaminodimethyl anilino HCl reagent, most of the blue color developed along the edge of the 2-week-old colonies; Pathotec oxidase strips also gave a positive reaction. Catalase activity and starch hydrolysis were also observed in 12- to 14-day-old plates. Vibriostat 0/129 inhibited the growth of the marine psychrophile. Thermal denaturation studies (Table 2) showed that the DNA of the psychrophile contained 40.0% G + C. *B. subtilis* DNA contained 42.9% G + C which agrees well with previously reported values.

TABLE 1. *Morphology and physiology of the marine psychrophile NRC 1004*

Characteristic	Marine psychrophile NRC 1004	*Vibrio marinus*[a]
Morphology	Slightly curved, motile rods	Slightly curved, motile rods
Colony	Red, opaque with entire edge and smooth surface	Grayish, opaque with entire edge and smooth surface
Temp range for growth	0–19 C	0–20 C
Specific NaCl requirement for growth	2.75%	2.4%
Hugh-Leifson	Acid from glucose aerobic and anaerobic	Acid from glucose aerobic and anaerobic
Acid from:		
Maltose	+	+
Dextrin	+	+
Arabinose	–	NA[b]
Oxidase	+	+
Catalase	+	+
Starch hydrolysis	+	±
Casein hydrolysis	+[c]	+
Gelatin liquefaction	+[c]	+
Citrate	–	–
Indole	–	–
Reduction of nitrate to nitrite	+	+
Vibriostat 0/129	+	+

[a] Data from reference 8. For reactions of other vibrios, reference 10 should be consulted.
[b] NA = not available.
[c] From reference 20.

DISCUSSION

As the data in Tables 1 and 2 show, the psychrophile shares many characteristics with *Vibrio marinus* although our organism differs in pigmentation and starch hydrolysis. Despite its similarity in pigmentation to *Serratia marcescens* (15), the red psychrophile differs from this species in flagellation (9), and in oxidase, starch hydrolysis, and dextrin fermentation reactions (7), as well as in DNA composition (56.2–58.4 moles % G + C for *S. marcescens*) (7, 19). It is clearly distinguished from other polarly flagellated rods. The pseudomonads oxidize but do not ferment glucose, produce acid from arabinose but not from maltose and dextrin, are insensitive to vibriostat 0/129 (21),

TABLE 2. *G + C content of DNA by thermal denaturation*

Organism	Mean T_m[a]	Moles % G + C	
		Thermal denaturation[a]	Literature values
Marine psychrophile NRC 1004	85.7 ± 0.40	40.0	
Marine *Vibrio* species			40.0–43.0 (4, 6)
Bacillus subtilis	86.9 ± 0.34	42.9	42.5 (2) 43.6 (12) 43.0 (19)

[a] Data from the present study. The optical density (260 nm) of DNA for thermal denaturation studies was 0.5. The mean percent hyperchromicity values of the psychrophilic and *B. subtilis* DNA, as determined by acid denaturation (6 N HCl), were 30.0 and 38.8%, respectively. The standard deviation for the mean T_m of four determinations has been calculated.

and have a G + C DNA content of 61 to 66 moles % (5, 19, 22). Those in the genus *Aeromonas* are insensitive to vibriostat 0/129 (1, 11) and have a G + C content of 56.5 to 59.0% (5). Members of the genus *Comamonas* do not oxidize or ferment glucose or produce acid from a number of other carbohydrates (11, 21). They are insensitive to vibriostat 0/129 and have a G + C content of 64.2% (22).

This organism is distinguished by its obligately psychrophilic nature, its rapid death at temperatures above 20 C, its red color, and its specific requirement for divalent as well as monovalent ions for stability. We propose the name *Vibrio psychroerythrus*.

The description of *Vibrio psychroerythrus*, a cold (psychro)-requiring, red-pigmented (erythrus) organism, is extended as follows:

Gram-negative asporogenous rods. Cells slightly bent or straight, single, 2.5 to 3.5 μm long, 0.5 μm wide. Single, long polar flagellum.

Growth on synthetic seawater agar: NaCl, 2.75%; $MgCl_2$, 0.4%; KH_2PO_4, 0.05%; $CaCl_2$, 1.5×10^{-4} M; $FeCl_2$, 1×10^{-8} M; tryptone, 0.8%; agar, 1.5%; pH 7.0; incubated at 10 to 12 C for 14 days. Colonies white at first appearance (7–8 days) becoming red within 10 to 12 days, opaque, raised, 3 to 5 mm diameter, with entire edge. Nondiffusible pigment. Turbid growth with no pellicle formation. Cells grow in the temperature range 0 to 19 C; cells lyse above 20 C or in the absence of NaCl and divalent ions (9, 13, 16).

Catalase-positive. Oxidase-positive. Sensitive to vibriostat 0/129. Casein hydrolyzed (20).

Indole-negative. Reduces nitrate to nitrite; no gas produced. No growth on citrate.

Acid from glucose under aerobic and anaerobic conditions without production of gas. Starch hydrolyzed. Acid produced from maltose and dextrin; no acid from arabinose. Moles % G + C of DNA = 40.0.

The organism has been deposited as the type strain with the American Type Culture Collection and given the accession number ATCC 27364.

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Structural Changes During Lysis of a Psychrophilic Marine Bacterium[1]

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The marine psychrophile, a red, gram-negative motile rod with a single polar flagellum, is stable when suspended in 0.1 M Mg^{2+} plus 0.5 M NaCl at 0 C and neutral *p*H but lyses if the salt composition of the medium is changed, the temperature raised above 20 C, or the *p*H lowered. Lysis is accompanied by a fall in turbidity, a release of ultraviolet-absorbing substances, and a loss of deoxyribonucleic acid and ribonucleic acid. Ultrastructural changes accompanying lysis were studied. Thin sections of cells fixed while intact showed a triple-layered cell wall and cytoplasmic membrane, each 6.0 to 7.5 nm thick. Mesosomes were also observed. Either Na^+ or Mg^{2+} could maintain wall integrity, whereas Mg^{2+} was needed for membrane integrity. In distilled water, lysis was very extensive, and much material was released as wall fragments and as vesicles which probably came from the wall and cytoplasmic membrane. Lysis at 37 C resulted in degradation of the wall and liberation of wall fragments. The cell membrane was rarely observed as a triple-layered structure in such temperature-lysed cells. After lysis at *p*H 5.0, the cell wall was distorted, and only a suggestion of the cell membrane remained. Replicas showed that this organism had a matted surface which was distorted under different conditions of lysis.

Previous work from our group was concerned with the physiology of a red-pigmented psychrophilic marine bacterium, NRC 1004 (11, 14, 18). This short, gram-negative motile rod grows in a temperature range of 0 to 19 C. At higher temperatures, death and lysis occur rapidly (11), accompanied by a release of hexosamines and a degradation of lipid phosphorus, presumably through the action of a cell-bound phosphatidase (11, 14). Cells are also lysed by low ionic strength or acid *p*H values. The surface effects of different kinds of lysis were studied by microelectrophoresis (18).

This organism differs from certain other marine bacteria in its ionic requirements for stability. Monovalent salts or sucrose can maintain the stability of other marine bacteria, whereas the red psychrophile also needs divalent cations. Though certain divalent cations can maintain the stability of this organism, it needs both NaCl (0.5 M) and Mg^{2+} ions (0.1 M) for growth (14, 17).

We present here the results of an electron microscope study of the relationships between ionic requirements for stability, chemical changes occurring during lysis, and alterations in cellular fine structure.

MATERIALS AND METHODS

Cells were grown in shaken cultures at 10 C in a medium containing 0.5 M NaCl, 0.04 M $MgCl_2$, 0.04 M KH_2PO_4, 10^{-6} M $FeCl_2$, 1.5×10^{-4} M $CaCl_2$, and 0.8% (w/v) tryptone; *p*H 7.0. After 48 to 60 hr of growth, the bacteria were harvested by centrifugation, washed once, and suspended in an "all salts" solution (growth medium without the nutrients).

Fixation in 2% glutaraldehyde (made up in "all salts" solution) and postfixation in buffered 1% OsO_4 (Michaelis acetate-Veronal buffer, *p*H 6.3) followed by staining in 1% aqueous uranyl acetate did not satisfactorily show cell wall or cytoplasmic membrane morphology. Several combinations of fixatives, stains, and buffering systems were tested; best results were obtained when washed cells were fixed with 4% formalin, (made up in "all salts" solution) for 5 hr at 10 C. Cells were then stabilized with 1% buffered uranyl acetate (Michaelis acetate-Veronal buffer, *p*H 6.1) for 1 hr at 10 C. Dehydration was carried out in a graded series of alcohols. The specimen was then infiltrated in a 1:1 (w/v) mixture of Epon 812 epoxy resin and 100% acetone for 1 hr and subsequently polymerized at 55 C for 2 to 3 days in freshly prepared resin.

Preparation of samples for surface replication was carried out according to a technique elaborated by

[1] A preliminary report of some of these findings was presented at the 71st Annual Meeting of the American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

Kushner and Bayley (15), with the following modifications. The cell sample was smeared on a precooled glass slide and dried in vacuo at 10 C. The slide mount was immediately shadowed with platinum-carbon and strengthened by a film of evaporated carbon. The replica was floated onto a water surface, placed on a Formvar-carbon-coated grid and permitted to air dry. Addition of hydrofluoric acid was not needed to float replicas off slides (15); occasionally the replicas were freed of organic matter before electron microscopic observation by treatment with H_2O_2.

For negative staining, a loopful of young cells (36-hr culture) was spread on a precooled Formvar-carbon-coated grid. After removal of excess liquid with filter paper, a loopful of cold aqueous 2% ammonium molybdate, *p*H 7.1, was applied to the grid and dried in air.

To test the effects of ions on lysis and cell ultrastructure, 0.1-ml samples of a thick cell suspension were suspended in 9.9 ml of water or aqueous solutions of salts at specified concentrations. The cells were permitted to equilibrate to a constant turbidity (absorbance at 660 nm); 2 hr was usually required. The cells were then sedimented at 15,000 × *g* for 15 min, and the absorbance of the supernatant fluid was read at 260 nm. Cell pellets were fixed and embedded as described above. Lysis was also induced by exposing washed cells to acidic *p*H ("all salts" solution, *p*H 5.0, 7 C) and high temperature ("all salts" solution, *p*H 7.0, 37 C). After suspensions had reached constant turbidity (usually 2 hr), suspensions were centrifuged as above, and pellets were fixed and embedded.

Studies were also made of the effects of distilled water, high temperature, and low *p*H on surface structure. After the turbidity became constant, samples for surface replication were taken directly from the treated suspension without centrifuging.

Analytical methods. Dry weight of cells was measured turbidimetrically by using a standard curve relating salt-free dry weight to absorbance at 660 nm. Salt-free dry weight was determined by drying a thick suspension in "all salts" solution to constant weight at 105 C and subtracting the weight of a separately dried sample of "all salts" solution.

For nucleic acid determinations, cells or pellets remaining after lysis were extracted with cold and then with hot trichloroacetic acid as described by Schneider (22). The intermediate lipid extractions could be omitted without affecting nucleic acid measurements. Deoxyribonucleic acid (DNA) was measured in hot trichloroacetic acid extracts with the diphenylamine reagent (5), by using a highly polymerized calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) as standard. Ribonucleic acid (RNA) was measured in hot trichloroacetic acid extract by the orcinol reagent (3), with a yeast RNA (Worthington Biochemical Corp.) used as standard.

RESULTS

Motility of the marine psychrophile depends on a single long polar flagellum, which can be seen in negatively stained preparations (Fig. 1). About 30% of the cells investigated by this method had such a flagellum, which could also be demonstrated in a much lower proportion of cell replicas. Possibly, flagella were lost when cells were dried in vacuo.

Ultrastructure of intact cells. Examination of thin sections of cells fixed with formalin in "all salts" solution showed that these were surrounded by two triple-layered structures, each 6.0 to 7.5 nm wide (Fig. 2), which we consider to be the cell wall and cytoplasmic membrane. Mesosomes, presumably originating as invaginations of the cytoplasmic membrane (7, 21, 22), were seen in several preparations (Fig. 2B). Thin sections of cells suspended in 0.5 M NaCl plus 0.1 M $MgCl_2$, which preserves cells stability (reference 14 and Table 1), were similar to sections of cells in "all salts" solution.

Chemical and structural changes accompanying lysis. Effects of environmental changes on cell stability and chemical composition are shown in Table 1. As observed earlier (11, 14), the bacterium was stable at low temperatures in "all salts" solution or in 0.5 M NaCl plus 0.1 M $MgCl_2$. The turbidity remained constant, and only small amounts of ultraviolet (UV)-absorbing material were released from the cells. When the NaCl plus $MgCl_2$ was diluted, turbidity fell, and more UV-absorbing materials appeared in the supernatant fluid. In 0.5 M NaCl alone, turbidity fell slowly, and the final release of UV-absorbing substances approached that found in distilled water. At lower NaCl concentrations, turbidity fell more rapidly, and the release of UV-absorbing substances increased. Cells were not stable in $MgCl_2$ solutions of 0.1 M or lower. However, $MgCl_2$ solutions were better able to prevent release of UV-absorbing substances than fivefold greater concentrations of NaCl.

Even after extensive lysis, substantial amounts of DNA and RNA remained associated with the residual cellular material. Solutions of 0.5 M NaCl or of 0.1 M $MgCl_2$ were better able to prevent loss of cellular DNA than loss of RNA. Extensive loss of nucleic acids took place after lysis at 37 C.

Almost all cells in "all salts" solution and in 0.5 M NaCl plus 0.1 M $MgCl_2$ were rod-shaped and dense under phase contrast. In 0.3 M NaCl plus 0.06 M $MgCl_2$, about 75% were rod-shaped and the rest spherical; in 0.1 M NaCl plus 0.02 M $MgCl_2$, at least 90% of the cells became phase-pale spheres. In 0.1 M $MgCl_2$, all cells became spherical in a few minutes. Cells in 0.5 M NaCl, examined after 2 hr, were still rod-shaped but less phase-dense than intact cells.

The fine structure of cells treated as in experiment 1, Table 1, was examined. Though the

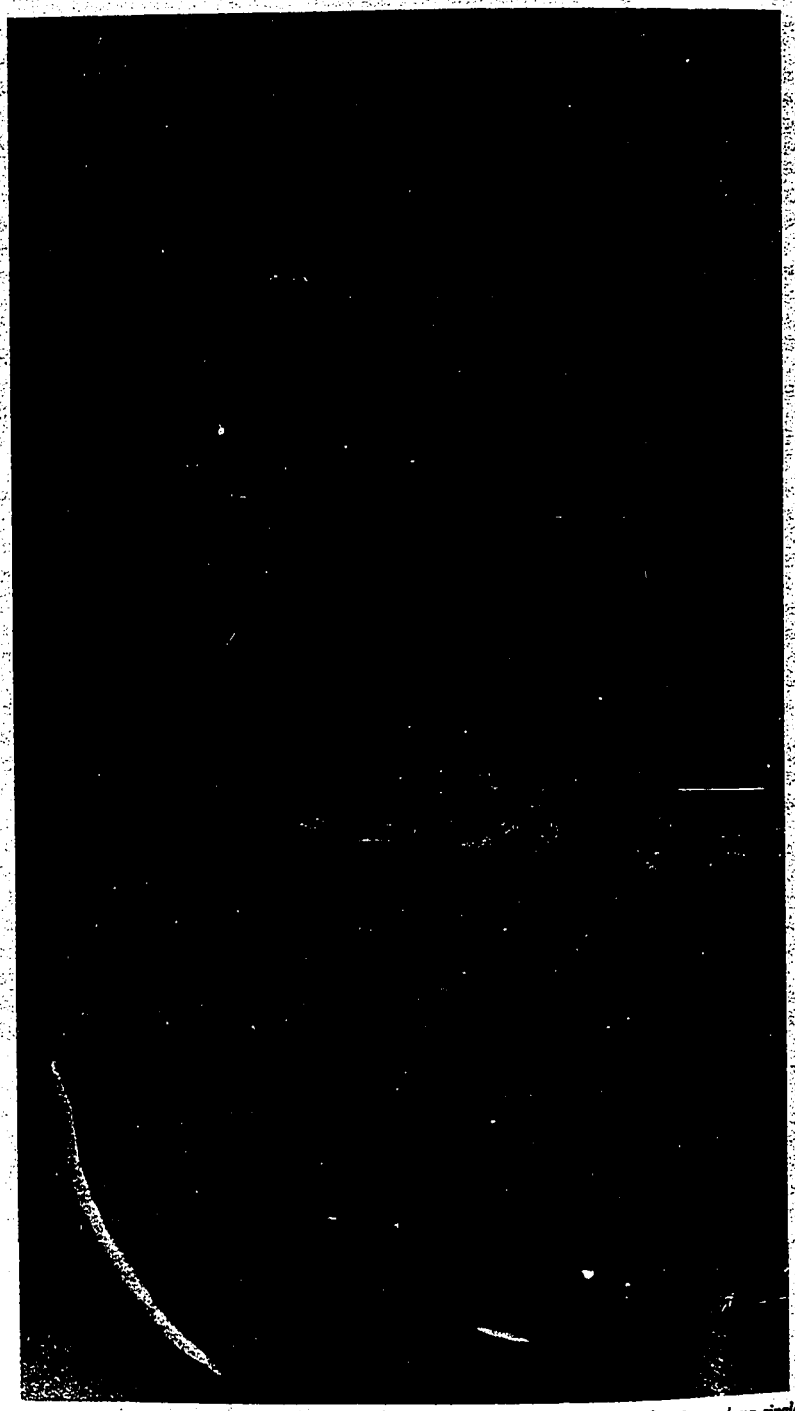

FIG. 1. *Negatively stained preparation of intact cell dried in "all salts" solution showing a long single polar flagellum. Bar is 0.5 μm.*

characteristic wall and membrane structure of intact cells was retained in 0.5 M NaCl plus 0.1 M $MgCl_2$ (Fig. 3A), suspension in more dilute solutions led to a loss of definition of the cell membrane as a triple-layered structure (Fig. 3B), followed by distortion and fragmentation of the wall (Fig. 3C).

When cells were suspended in 0.5 M NaCl

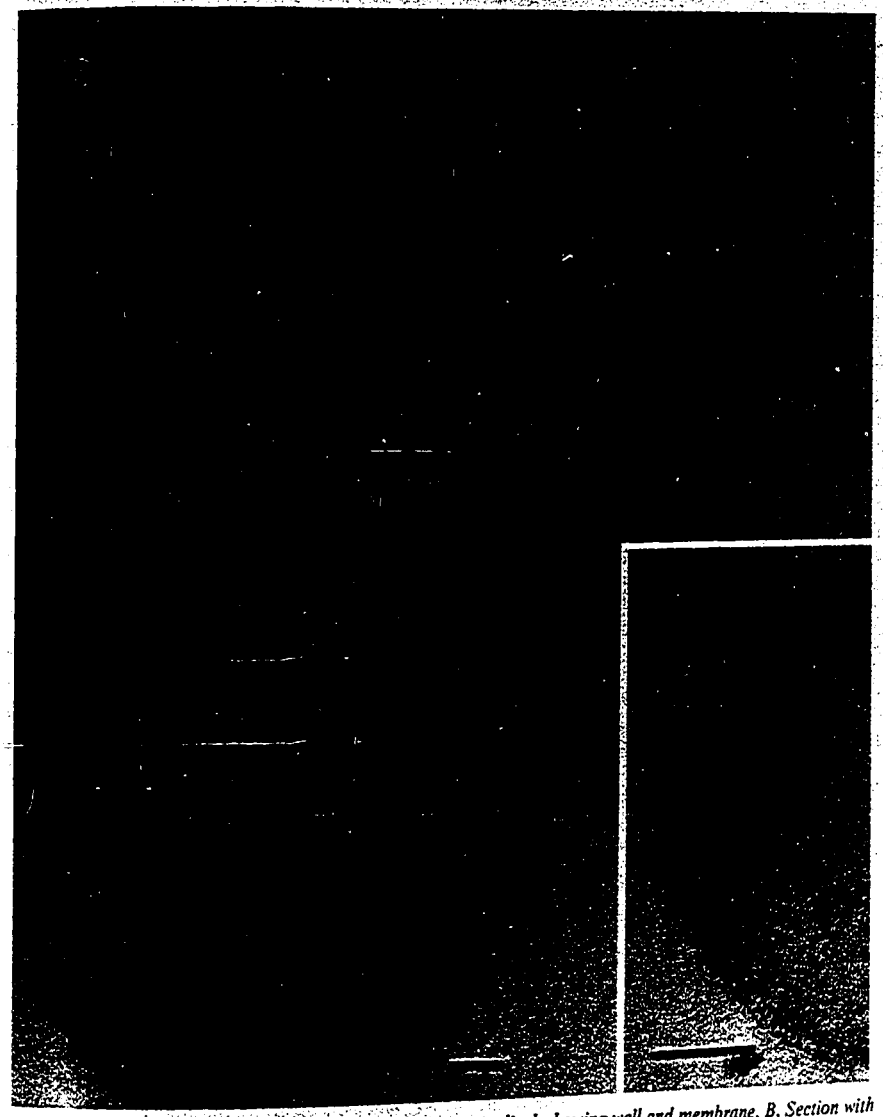

FIG. 2. *Thin sections of the marine psychrophile. A, Longitudinal, showing wall and membrane. B. Section with a mesosome. Transverse strands between wall and membrane were seen in some preparations. Bars are 0.1 μm.*

alone, the cell wall appeared as a detached but distinct triple layer, and only a faint suggestion of the cytoplasmic membrane remained (Fig. 4A and 4B). The nuclear region was poorly defined. Cells suspended in 0.1 M NaCl were transformed to a collection of fragments and vesicles (Fig. 4C). These are very similar to those obtained from cells suspended in distilled water (Fig. 6A), except that the latter had a smaller proportion of walls or wall fragments with continuous smooth structure. Few traces of the cytoplasmic membrane remained in the material sedimented after lysis in 0.1 M NaCl or distilled water.

In 0.1 M $MgCl_2$, a better preservation of the cell membrane was obtained than in 0.5 M NaCl (Fig. 5A). Even in 0.02 M $MgCl_2$, cellular integrity was better preserved than in 0.1 M NaCl alone (Fig. 4C and 5B).

Particles released on lysis. It was observed earlier that, when cells were lysed in distilled water, a good deal of cellular material was released in a form not sedimentable by centrifuging for 10 min at 8,000 × *g* but sedimentable after 60 min at 100,000 × *g* (14). Debris from cells lysed in distilled water (Fig. 6A) sedimented at 15,000 × *g* for 20 min. On centrifuging at 27,000 to 30,000 × *g* for 20 min, more material sedimented, similar in appearance to that shown in Fig. 6A.

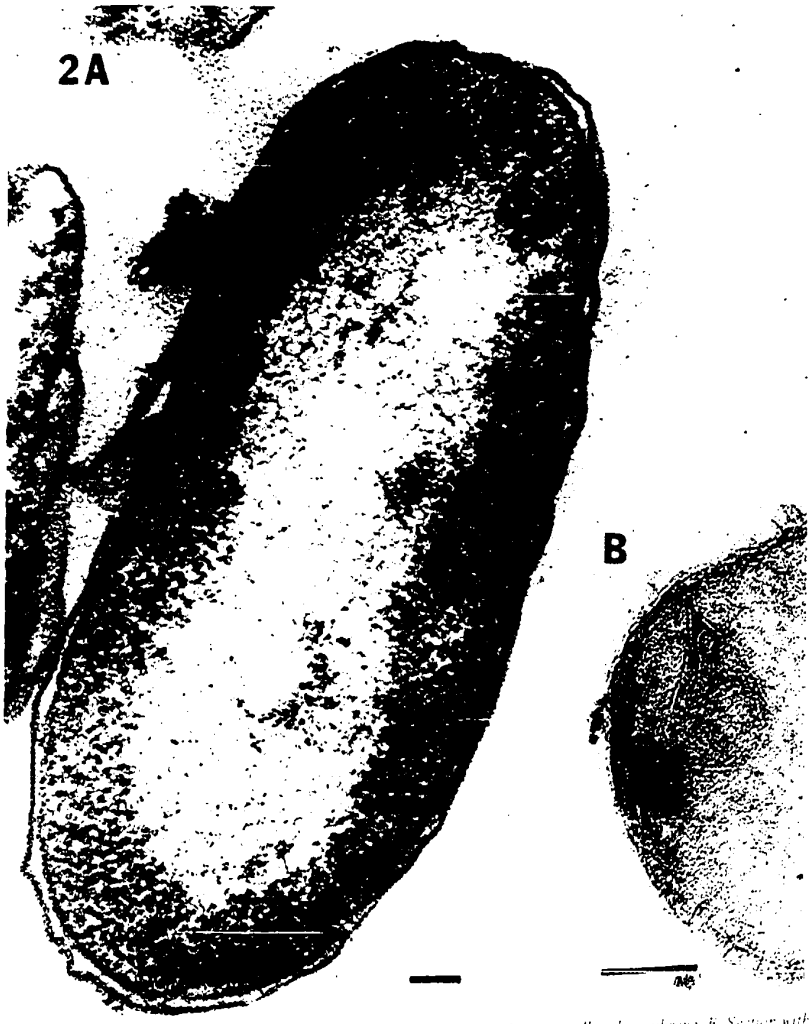

FIG. 2. *Thin sections of the marine psychrophile. A, Longitudinal, showing wall and membrane. B, Section with a mesosome. Transverse strands between wall and membrane were seen in some preparations. Bars are 0.2 μm.*

alone the cell wall appeared as a detached but distinct triple layer, and only a faint suggestion of the cytoplasmic membrane remained (Fig. 4A and B). The nuclear region was poorly defined. Cells suspended in 0.1 M NaCl were transformed to a collection of fragments and vesicles (Fig. 4C). These are very similar to those obtained from cells suspended in distilled water (Fig. 6A), except that the latter had a smaller proportion of whole wall fragments with continuous smooth surface. Few traces of the cytoplasmic membrane remained in the material sedimented after lysis in 0.1 M NaCl or distilled water.

In 0.1 M $MgCl_2$, a better preservation of the cell membrane was obtained than in 0.5 M NaCl (Fig. 5A). Even in 0.02 M $MgCl_2$, cell envelope integrity was better preserved than in 0.1 M NaCl alone (Fig. 4C and 5B).

Particles released on lysis. It was observed earlier that, when cells were lysed in distilled water, a good deal of cellular material was released in a form not sedimentable by centrifuging for 1 hr at 8,000 × *g* but sedimentable after 1 hr at 100,000 × *g* (14). Debris from cells lysed in distilled water (Fig. 6A) sedimented at 15,000 × *g* for 20 min. On centrifuging at 27,000 to 31,000 × *g* for 20 min, more material sedimented, similar in appearance to that shown in Fig. 6A

TABLE 1. *Changes accompanying lysis of the marine psychrophile*

Experimental conditions[a]	Turbidity (%)[b] after				Release of ultraviolet-absorbing substances[c]	DNA remaining (%)[d]	RNA remaining (%)[d]
	15 min	60 min	120 min	180 min			
Experiment 1							
"All salts"	100	100	100		0.24		
0.5 M NaCl plus 0.1 M $MgCl_2$	108	106	106		0.30		
0.4 M NaCl plus 0.08 M $MgCl_2$	83	83	81		0.27		
0.3 M NaCl plus 0.06 M $MgCl_2$	86	86	86		0.49		
0.1 M NaCl plus 0.02 M $MgCl_2$	55	38	32		2.56		
0.5 M NaCl	81	71	30		2.38		
0.3 M NaCl	65	40	13		2.51		
0.1 M NaCl	22	12	10		2.89		
0.1 M $MgCl_2$	51	49	46		1.22		
0.06 M $MgCl_2$	51	46	42		1.67		
0.02 M $MgCl_2$	42	30	27		2.13		
Water	15	10	10		3.00		
"All salts," 37 C	88	49	46				
Experiment 2							
"All salts"	100	100	98	96	0.28	100	100
0.5 M NaCl plus 0.1 M $MgCl_2$	107	106	106	104	0.43	94	94
0.5 M NaCl	96	91	81	47	2.65	80	57
0.1 M $MgCl_2$	50	46	46	45	2.41	93	67
Water	24	19	19	18	3.95	53	47
"All salts," 37 C	88	61	60	60	4.55	43	25

[a] All incubations at 0 C unless otherwise stated. A 40-hr culture was used for experiment 1 and a 60-hr culture for experiment 2.

[b] "One-hundred per cent turbidity" equals that found in "all salts" solutions. The turbidity (absorbance at 660 nm) for experiment 1 was 0.590 (corresponding to 0.86 mg of bacteria per ml) and for experiment 2, 0.480 (0.68 mg of bacteria per ml).

[c] Expressed as absorbance of supernatant fractions at 260 nm per mg of bacteria per ml (read after four- to five-fold dilutions).

[d] The amounts of DNA and RNA in intact cells (in "all salts" solution) is taken as 100%. Cells contained 0.300 mg of RNA per mg (dry weight) of cells and 0.070 mg of DNA per mg (dry weight) of cells.

After 60 min at 65,000 × *g*, an assortment of particles sedimented (Fig. 6B–6D). No further material sedimented after 60 min at 100,000 × *g*.

The 65,000 × *g* sediment included debris and different-sized vesicles. The larger vesicles were 150 to 250 nm in diameter. Many vesicles only 12.5 to 15 nm in diameter were also observed. A few of these (shown especially in Fig. 6B) had a "doughnut" appearance. In others of the same size, only a suggestion of such a structure could be seen. Though the "doughnuts" are perhaps the most interesting structures found, they occur rarely (usually only a few per sample), and we have still not succeeded in obtaining large numbers of them regularly from each batch of cells.

As noted above (Fig. 3C, 4A, and 5A), exposure to 0.5 M NaCl alone, 0.1 M $MgCl_2$ alone, or 0.1 M NaCl plus 0.02 M $MgCl_2$ led to characteristic changes in the structure of cells sedimenting at 15,000 × *g*. When supernatant fractions from the first two treatments were centrifuged at 27,000 × *g* for 20 min, no material sedimented. Centrifuging at 65,000 × *g* for 60 min sedimented very small amounts of material resembling the fragments and vesicles shown in Fig. 6D. No material could be sedimented from the 15,000 × *g* supernatant fraction remaining after treatment with 0.1 M NaCl plus 0.02 M $MgCl_2$.

Temperature- and acid-induced lysis. When cells were held for 2 hr at 37 C in "all salts" solution, considerable structural deterioration took place, as suggested by the fall in turbidity (Table 1) and by the appearance of cells in thin section (Fig. 7). Even in cells that retained their shape, the wall was degraded, and much of the wall material was liberated as vesicles. The cytoplasmic membrane was rarely observed as a three-layered structure.

Cells lysed at *p*H 5.0 in "all salts" solution retained their rod shape. In thin sections the cell wall was distorted, and the cell membrane was less well defined than that of the intact cell, though some of the trilaminar structure remained (Fig. 8).

Cell surface structure and its changes during lysis. Replicas of unfixed cells dried in the cold

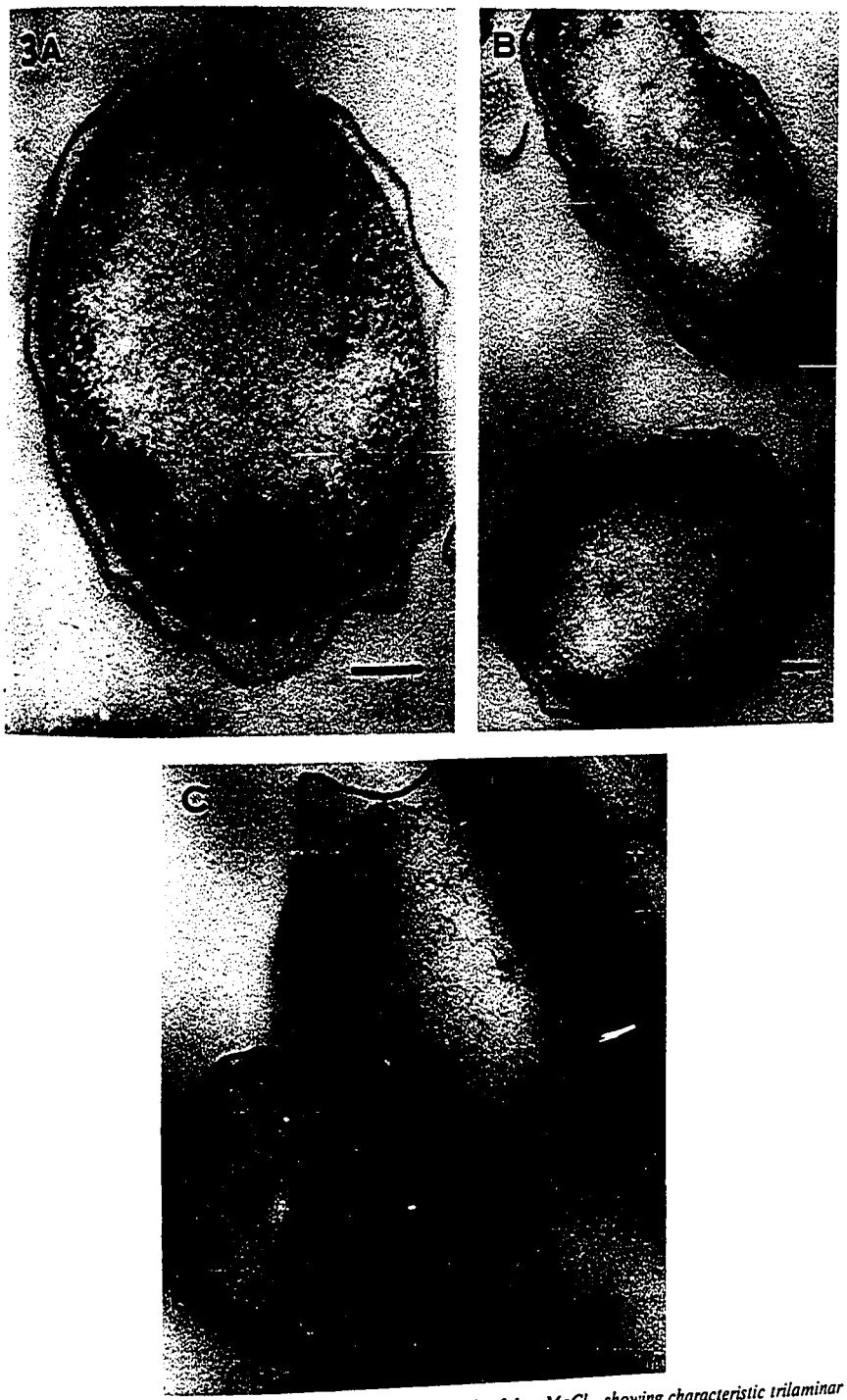

FIG. 3. *Thin sections of cells. A, Suspended in 0.5 M NaCl plus 0.1 M $MgCl_2$, showing characteristic trilaminar wall a[illegible] membrane structure. B, Suspended in 0.3 M NaCl plus 0.06 M $MgCl_2$. Cell membrane can be faintly dis-tingui[illegible]d as a trilaminar structure, and fewer fibrils remain in the nucleoid. C, Suspended in 0.1 M NaCl plus 0.02 [illegible] $MgCl_2$. Trilaminar membrane structure has disappeared. Walls are broken and fragments (f) and vesicles (v) th[illegible]ght to originate from the walls, are seen. Bars are 0.1 μm.*

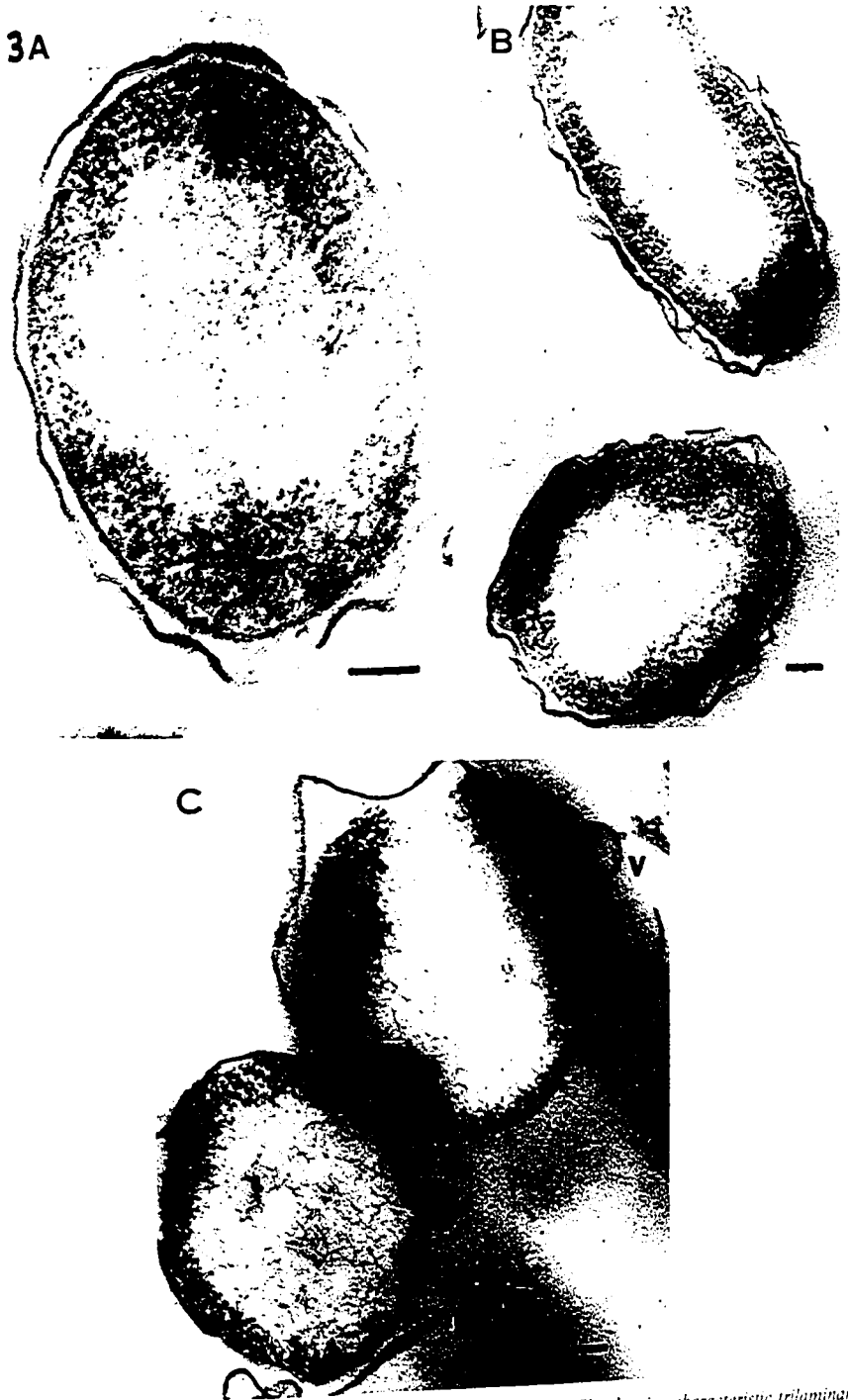

FIG. 3. *Thin sections of cells. A. Suspended in 0.5 M NaCl plus 0.1 M $MgCl_2$, showing characteristic trilaminar cell membrane structure. B. Suspended in 0.3 M NaCl plus 0.06 M $MgCl_2$. Cell membrane can be faintly discerned as a trilaminar structure, and fewer fibrils remain in the nucleoid. C. Suspended in 0.1 M NaCl plus 0.0... $MgCl_2$. Trilaminar membrane structure has disappeared. Walls are broken and fragments (f) and vesicles (v) ...ght to originate from the walls, are seen. Bars are 0.1 μm.*

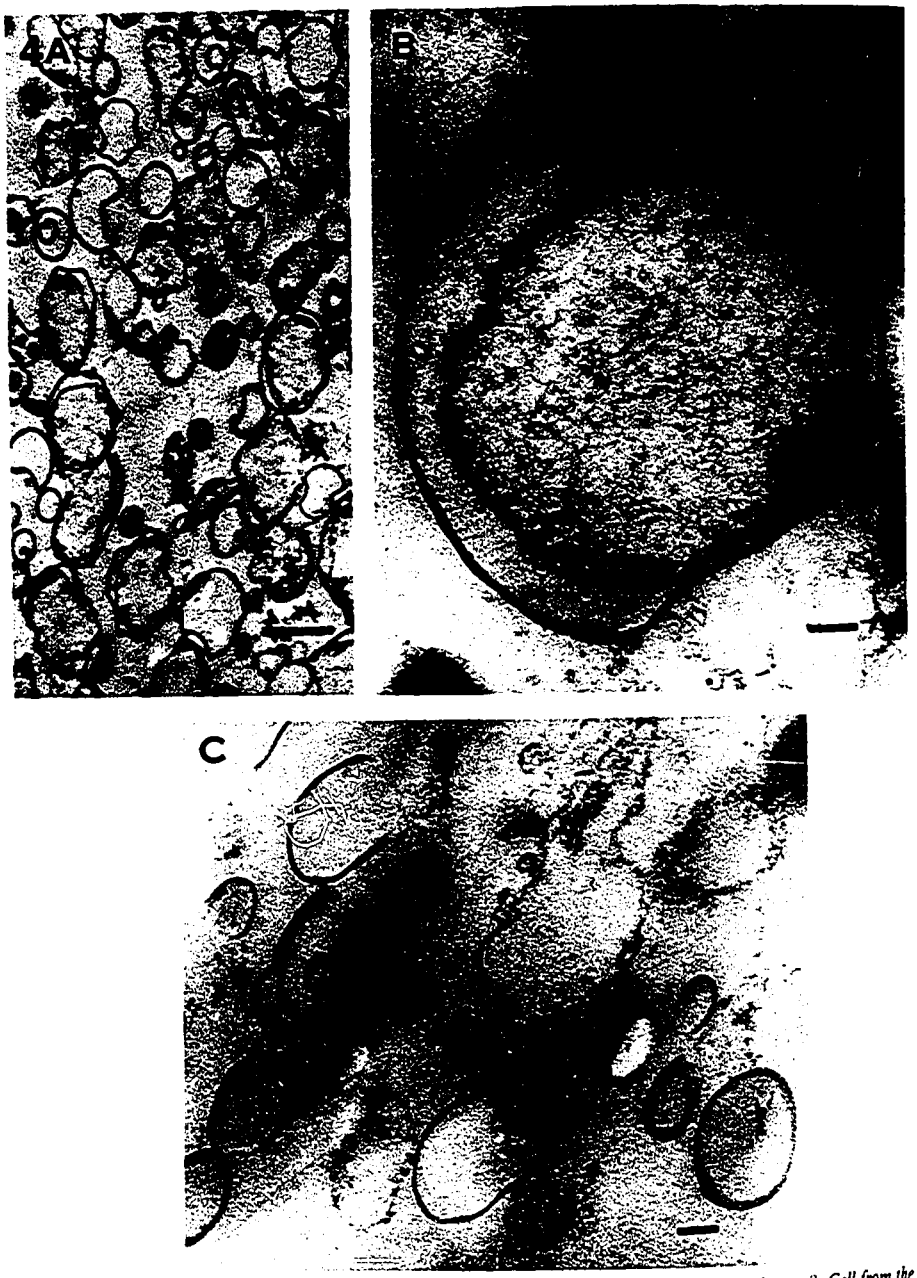

FIG. 4. *Thin sections of cells suspended in NaCl alone. A, Cells in 0.5 M NaCl. Bar is 1.0 μm. B, Cell from the 0.5 M NaCl preparation at higher magnification, showing a continuous trilaminar cell wall and short segments of the cell membrane. Bar is 0.1 μm. C, Cells suspended in 0.1 M NaCl showing fragments with a continuous smooth structure. Bar is 0.1 μm.*

revealed a matted surface texture. On lysis in distilled water, the surface was considerably disrupted, a result to be expected considering the appearance of such cells in thin section (Fig. 6A). Blebs, about 200 nm in diameter, appeared on the cell surface. Lysis at *p*H 5 caused some wrinkling of the surface, which seemed otherwise intact. Lysis at 37 C led to quite irregular contours and a rugged surface.

DISCUSSION

It was suggested earlier that organism should be classified as a *Serratia* es because of its pigmentation, fatty acid, osphatide

FIG. 4. *Thin sections of cells suspended in NaCl alone. A, Cells in 0.5 M NaCl. Bar is* [illegible] *μm. B, Cell from the 0.5 M NaCl preparation at higher magnification, showing a continuous trilaminar cell wall and short segments of the cell membrane. Bar is 0.1 μm. C, Cells suspended in 0.1 M NaCl showing fragments of a continuous smooth structure. Bar is 0.1 μm.*

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DISCUS[illegible]

It was suggested earl[illegible] that this organism should be classified as a [illegible] species because of its pigmentation, fatt[illegible] and phosphatide

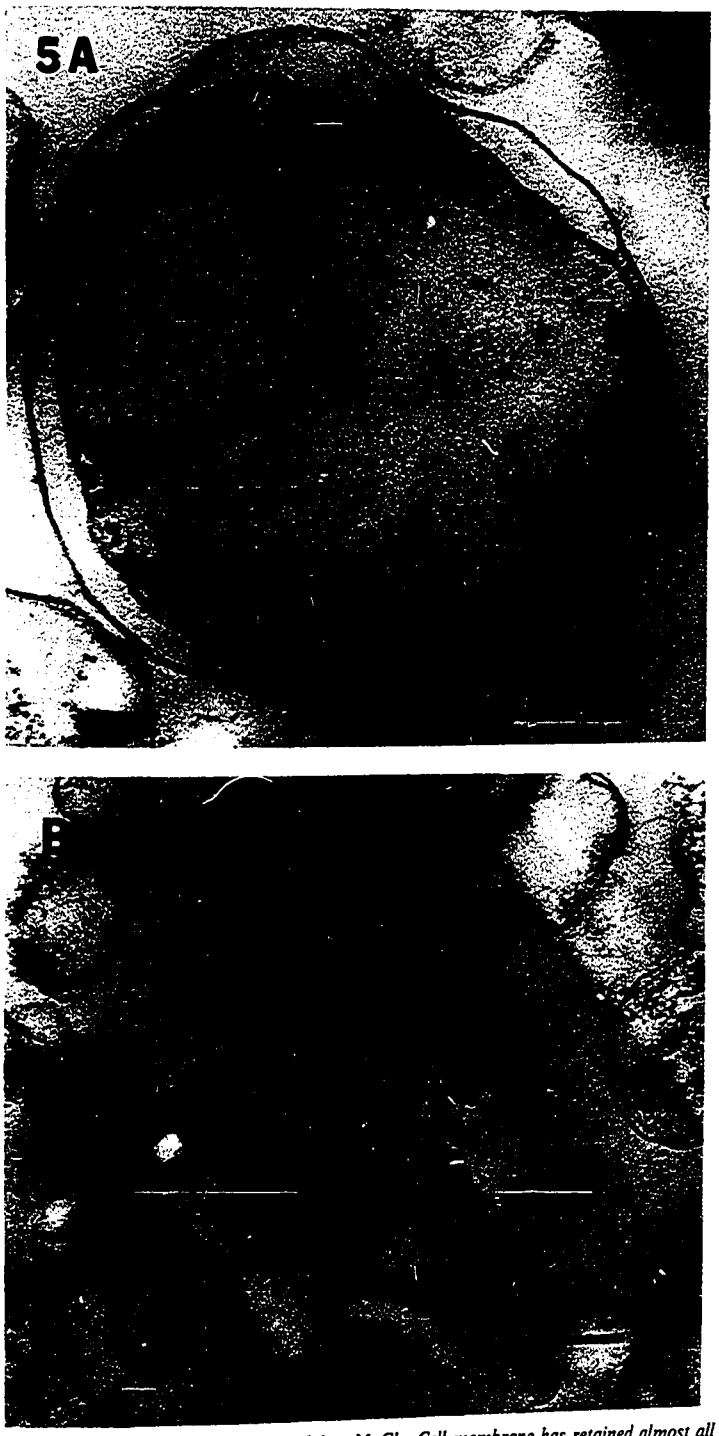
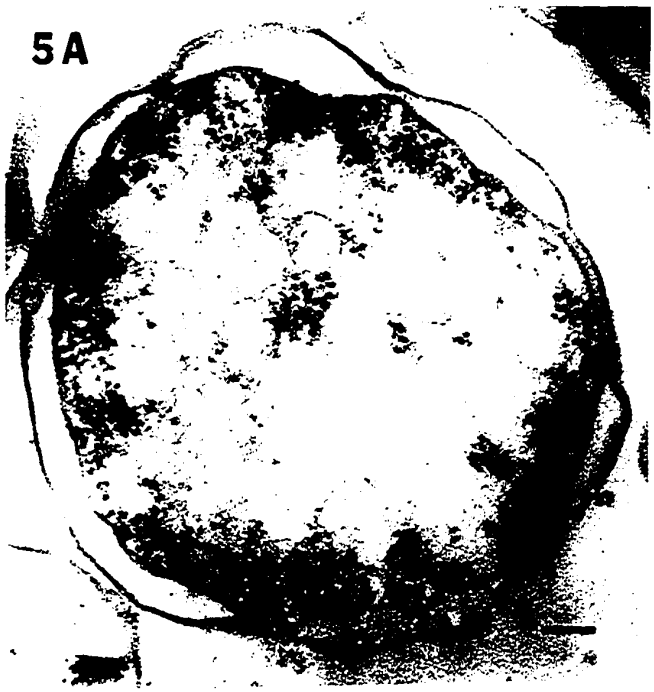


FIG. 5. *A, Thin section of a cell suspended in 0.1 M $MgCl_2$. Cell membrane has retained almost all of its trilaminar structure (compare with cells in 0.5 M NaCl, Fig. 4B). Bar is 0.1 μm. B, Thin section of cells suspended in 0.02 $MgCl_2$. Structure is better preserved than in a fivefold greater Na^+ concentration (Fig. 4C). Bar is 0.5 μm.*



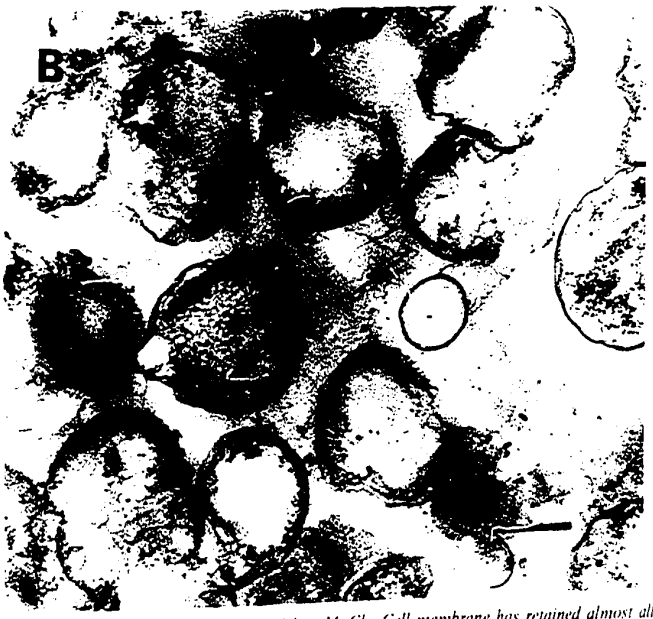


FIG. 5. A. Thin section of a cell suspended in 0.1 M $MgCl_2$. Cell membrane has retained almost all of its trilaminar structure (compare with cells in 0.5 M NaCl, Fig. 4B). Bar is 0.1 μm. B. Thin section of cells suspended in 0.05 M $MgCl_2$. Structure is better preserved than in a fivefold greater Na^+ concentration (Fig. 4C). Bar is 0.5 μm.

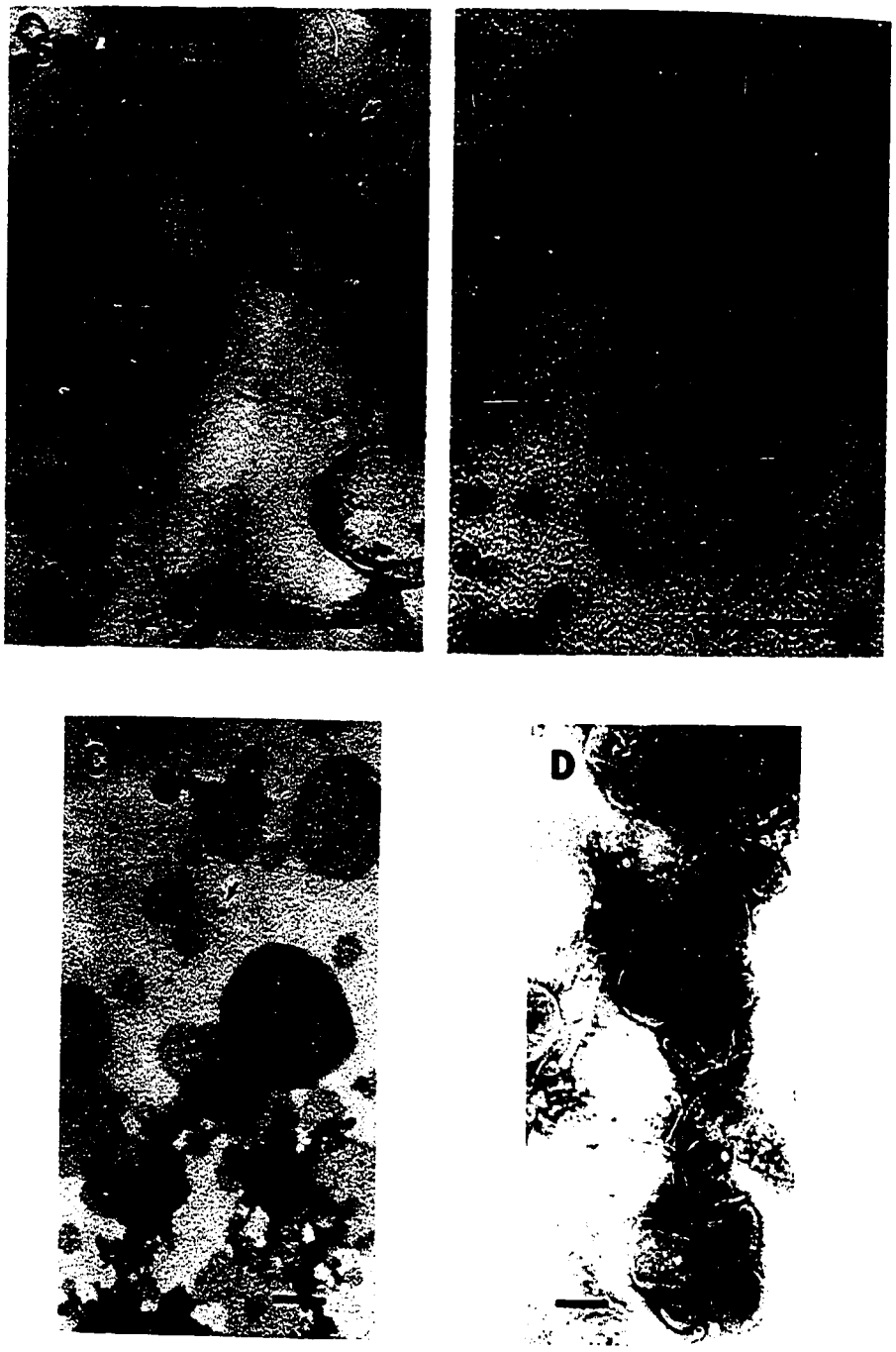


FIG. 6. *Effect of distilled water on cell structure. A, Thin section of lysed cells sedimented at 15,000 × g; cell wall fragments seem to be re-forming into vesicles (arrows). Little if any suggestion of the cell membrane remains. B to D, Material sedimenting at 65,000 × g and stained in cold aqueous 1% uranyl acetate. B, 12.5 to 15.0 nm vesicles exhibiting a "doughnut" shape; C and D, larger vesicles (150 to 250 nm), including a few "doughnut-shaped" vesicles (arrow). Bars are 0.1 μm.*

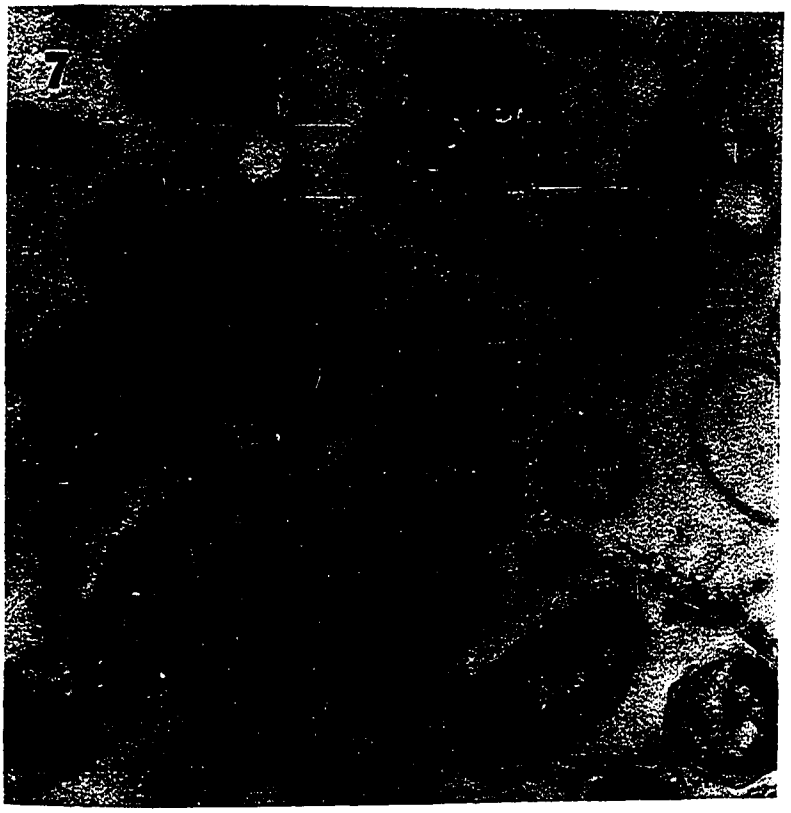


FIG. 7. *Thin section of cells suspended in "all salts" solution and held at 37 C for 2 hr, during which time considerable structural deterioration occurred. Some vesicles (arrows), possibly from cell walls, appear. Even in cells that do not appear extensively degraded, most of the trilaminar membrane structure is lost. Bar is 0.5 μm.*

composition (11, 12). Because it has only a single polar flagellum, this classification is unsuitable.

For stability, the red psychrophile needs low temperatures, *p*H values near neutrality, and both monovalent and divalent cations (11, 14, 18). In contrast to other marine bacteria, its structure is poorly maintained by monovalent salts and even more poorly by nonionic solutes (14, 17). Its sensitivity to changes in the environment may be the cause of the difficulties we experienced in fixing cells for thin-section examination. To demonstrate fine structure, we used formaldehyde as a fixative, because the more standard glutaraldehyde plus OsO_4 fixation seldom yielded a satisfactory resolution of wall and membrane structure.

Since a rather high concentration of formaldehyde (0.5 M) was used to fix cells, it seemed possible that formaldehyde might also be exerting an osmotic effect. It was found previously, however, that, although cells were stable in 0.1 M $MgCl_2$ plus 0.5 M NaCl, raising the NaCl concentration to 1.0 M did not lead to any discernible morphological changes (14). Experiments were also carried out in which 0.5 M sucrose or glycerol was added to cells suspended in "all salts" solution. No changes in turbidity, release

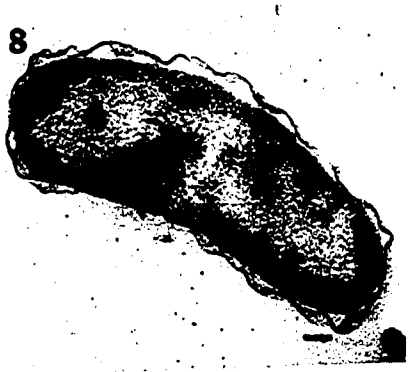


FIG. 8. *Thin section of cell lysed at pH 5.0 in "all salts" solution. A few breaks appear in the distorted cell wall, and a faint suggestion of the trilaminar cell membrane remains. Bar is 0.1 μm.*

of UV-absorbing material, or appearance under phase contrast were observed. We think it very unlikely, therefore, that formalin acts in any way except as a fixative.

Cells are surrounded by a triple-layered cell wall and a well-defined cytoplasmic membrane, characteristic of certain other gram-negative terrestrial and marine bacteria (6, 7, 10). No structures appeared in electron micrographs that could be regarded as specific to psychrophilic bacteria. Recent work has shown that psychrophilic and nonpsychrophilic marine vibrios are structurally very similar to each other (13).

In many gram-negative bacteria, a densely stained layer, probably containing the peptidoglycan components of the cell, can be observed between the wall and cytoplasmic membrane (9, 19). Such a layer could not be demonstrated by staining the B-16 marine pseudomonad extensively studied by MacLeod et al. (6, 7), and it does not appear in our red psychrophile. Possibly this is due to the unusual fixing method used. However, in parts of a few cells fixed and stained with glutaraldehyde-OsO_4-uranyl acetate, walls and membranes could be clearly seen, but no dense intermediate layer was seen.

Though both Na^+ and Mg^{2+} are needed for cell stability, these ions appear to act differently on different layers of the cell. Thin sections show that both ions are capable of maintaining wall integrity, whereas the cell membrane is best stabilized by Mg^{2+} and loses its characteristic trilaminar structure in NaCl alone. The fact that $MgCl_2$ is better able to prevent leakage of UV-absorbing compounds and loss of nucleic acids than a fivefold greater concentration of NaCl (Table 1) further supports our interpretation of a structural role of Mg^{2+} ions on the cell membrane. In contrast, Mg^{2+} ions were previously thought to be involved in wall rather than in membrane structure in marine and terrestrial gram-negative bacteria (6, 14).

The isoelectric point of this bacterium lies between *p*H 2.0 and 2.5 (18), indicating that its cell walls contain excess acidic groups. The ability of Na^+ and Mg^{2+} to stabilize the wall could be due to their masking mutually repulsive negative charges and conferring greater stability to the cell wall. This masking action has been suggested to account in part for the ionic requirements of extremely halophilic bacteria (1, 2, 4, 16).

Previous work showed that, when cells were lysed in distilled water, particles containing most of the cell lipid phosphorus and hexosamine were released. The morphology of these particles has now been studied. In addition to amorphous debris, different-sized vesicles were found. The larger ones (150 to 250 nm in diameter) were as large as parts of the wall pinched off from water-lysed cells (Fig. 6A). They also compared well with the blebs on the surface of such cells and are believed to originate in the cell wall. Many vesicles only 12.5 to 15 nm in diameter were also observed. Since much of the cell wall remained unbroken or formed large vesicles, the smaller vesicles are thought to have originated in the cytoplasmic membranes.

Particles containing hexosamine and lipid phosphorus are also released when cells are broken mechanically in NaCl plus $MgCl_2$ medium (14). Preliminary electron microscopy of these revealed that they resemble the collection of particles released on distilled water lysis. Either method of breaking down cellular structure seems to lead to extensive fragmentation of the outer layers of the cell.

Our results show that the fine structure of the outer layers of these bacteria is extraordinarily susceptible to environmental change. Mitochondrial and other cell membranes retain their "unit-membrane" (trilaminar) structure even after most of the lipid is extracted with acetone (9). The trilaminar structure of red blood cell membranes remains after digestion with phosphatidase C (20). In contrast, much more gentle treatment of the red psychrophile can lead to the disappearance of trilaminar structure (Fig. 3C, 4A, 4B, and 7). Quite possibly, the structure of these membranes depends on a delicate balance of charges between protein and lipid molecules. Such a balance could be disturbed either by a change in the ionic environment or removal of the charged portions of phosphatides by enzymic action. These changes could lead not only to disappearance of trilaminar structure but also to fragmentation and release of parts of the walls and membranes as vesicles.

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The regular hexagonal surface layer of *Halobacterium cutirubrum*: a honeycomb network[1]

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D'AOUST, J.-Y., and D. J. KUSHNER. 1972. The regular hexagonal surface layer of *Halobacterium cutirubrum*: a honeycomb network. Can. J. Microbiol. 18: 1767–1768.

The regular surface pattern of *Halobacterium cutirubrum*, as studied from replicas, appears to be made up of spherical subunits in hexagonal array. Low-beam intensity studies of cells dried from salt solutions revealed a regular honeycomb network containing hexagonal holes 9.5 to 11.0 nm in diameter with a center-to-center repeat distance of 15.5 to 17.0 nm. These structures appeared to be negatively stained by NaCl or heavy cations adsorbed from the growth medium. This network shares a number of common features with the perforate outer layer of *Lampropedia hyalina*.

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La surface ombragée de *Halobacterium cutirubrum* semble être formée d'un réseau hexagonal de sous-unités sphériques. Les études à rayon de faible intensité de cellules séchées à partir de solutions salines révèlent un réseau régulier en forme de nid d'abeilles; les trous hexagonaux, 9.5 à 11.0 nm de diamètre, se répètent centre à centre tous les 15.5 à 17.0 nm. Ces structures semblent être teintes négativement par NaCl ou par des cations de poids atomique élevé provenant du milieu de croissance. Ce réseau partage un nombre de caractéristiques communes avec la couche extérieure perforée de *Lampropedia hyalina*.

Halobacterium cutirubrum and other *Halobacterium* species require at least 15% NaCl for growth and stability (5, 7). Surface replicas of intact cells and envelope preparations have revealed a regular hexagonal array of subunits with a center-to-center spacing of 13 to 15 nm (4, 6); such arrangements of subunits are found on the surfaces of several other bacterial genera (3, 9, 13). The subunits of *H. halobium* (8) and *Spirillum serpens* (1) have been isolated and shown to consist mainly or entirely of proteins.

The high salt concentrations required for stability in the extreme halophiles make studies of their ultrastructure difficult. So far, good resolution of surface structures has not been obtained. This report shows that direct observation with low electron-beam intensity of unfixed low electron-density cells dried from their suspending salt solution is a valuable method for revealing new details of the surface pattern of *H. cutirubrum*.

Cells were grown 60 h in shaken cultures at 37°C in a modified Sehgal and Gibbons (10) complex medium (6), washed once, and resuspended in an "all salts" solution (growth medium without yeast extract or amino acids).

Sample preparation for surface replication with a platinum–carbon layer was carried out as previously described (6) except that water instead of hydrofluoric acid was used to detach the replicas from the slide. Replicas showed, as before (6), what appears to be a hexagonal array of particles or subunits with a center-to-center spacing of 15.0 to 16.5 nm arranged along three principal axes of symmetry mutually set at 120° (Fig. 1).

For direct observations, cells washed in "all salts" solution or in 25% NaCl were placed on formvar-carbon coated grids, the excess of liquid removed with filter paper, and the samples dried in air. Scanning and focussing were carried out at low beam intensities. A honeycomb network (Fig. 2A) gradually appeared. The holes of the network, many of which are hexagonally shaped (Figs. 2A and 3), are 9.5 to 11.0 nm in diameter with a center-to-center repeat distance of 15.5 to 17.0 nm and are aligned along three axes of symmetry set at 120°. Occasionally, blebs with a periodicity of 15 nm were observed

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along the edge of some cells (Fig. 2B). This network was stable for about 1 min after which it progressively deteriorated (Fig. 3). The network was most clearly seen in those cells that were less electron-dense than the rest.

When cells were dried from 0.2% phosphotungstic acid (PTA) in 20% NaCl (pH 6.0), no surface pattern was seen. Reasons for this apparently disruptive effect of PTA are not clear. Prefixation for 1 h with 4% formalin in "all salts" solution increased the stability of the network by a factor of two, but did not change the resolution obtained.

These results show that a much more detailed picture of surface structure can be obtained by direct viewing of cells than by replica techniques. With the latter technique, drying probably deposits salts crystals and cell debris between adjacent surface units, thus adversely affecting the resolution of the units themselves. Why is the resolution under direct viewing not adversely affected by such material? This may be partly due to a cleaning action by the electron beam itself. The pattern appears gradually and is stable for only a short time. Indeed, this instability presents one of the greatest disadvantages of the method.

Since the network (the only structure exhibiting symmetry) was electron transparent, it appeared that it might be negatively stained. If so, what is the staining material? Cells washed in NaCl alone show the network as well as those washed in "all salts" solution, and NaCl is known to act as a negative stain (12) though it is doubtful that this salt could give the degree of resolution obtained. Another possibility is that these cells have picked up traces of heavy cations from the complex growth medium. Such ions might bind readily to the acidic proteins of the outer layer (5).

The agreement in the periodicity and symmetry of the patterns of replicated and directly-viewed preparations suggests that both structures are in fact the same and that the unfixed honeycomb network did not deteriorate on short exposure to the electron beam.

It is interesting to note the similarities of the honeycomb layer in terms of axes of symmetry, repeat distance, and diameter of the units with the "perforate layer" of *Lampropedia hyalina* as studied by Chapman, Murray, and Salton (2); in fact, their model coincides well with Fig. 2. According to this model, the blebs (Fig. 2B) would then be the honeycomb network as it turns over the cell's edge and corresponds to the "castellated edge" in *L. hyalina*. The occurrence of a dented surface in thin sections of our organism and in *Halobacterium* sp. strain 5 (11) may well correspond to a sectional view of the blebs. These findings support the view that the honeycomb network occupies the outermost layer of the cell envelope and that its symmetry, as suggested for *L. hyalina* (2), corresponds to a two-dimensional crystal lattice.

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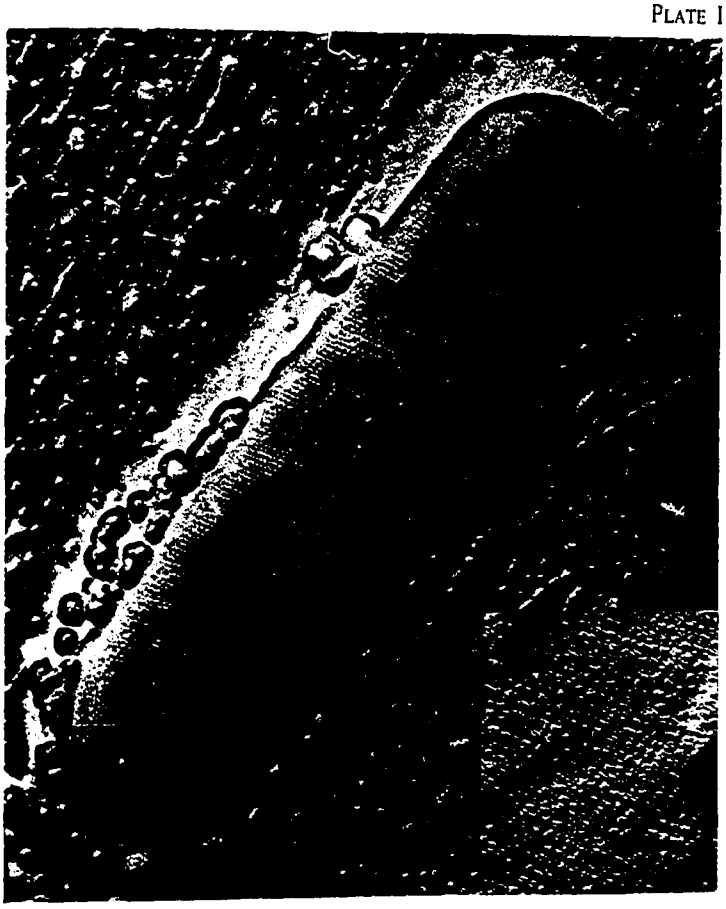
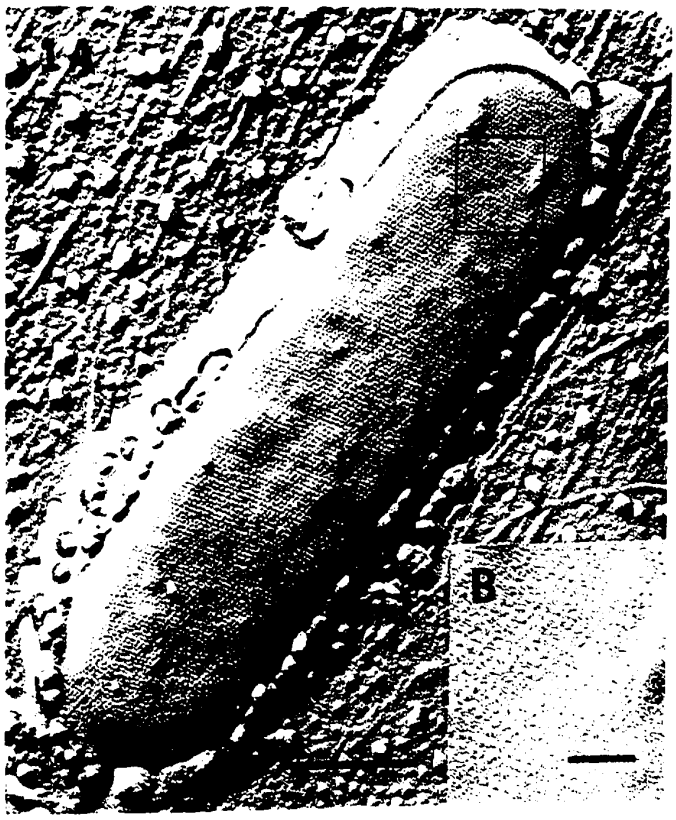


FIG. 1. Surface replica of an intact cell. (A). Showing the regular hexagonal array of "particles." The background is laden with salt crystals and flagellar fragments. (B). Higher magnification of the "particles." Bars are 0.5 μ and 0.1 μ respectively.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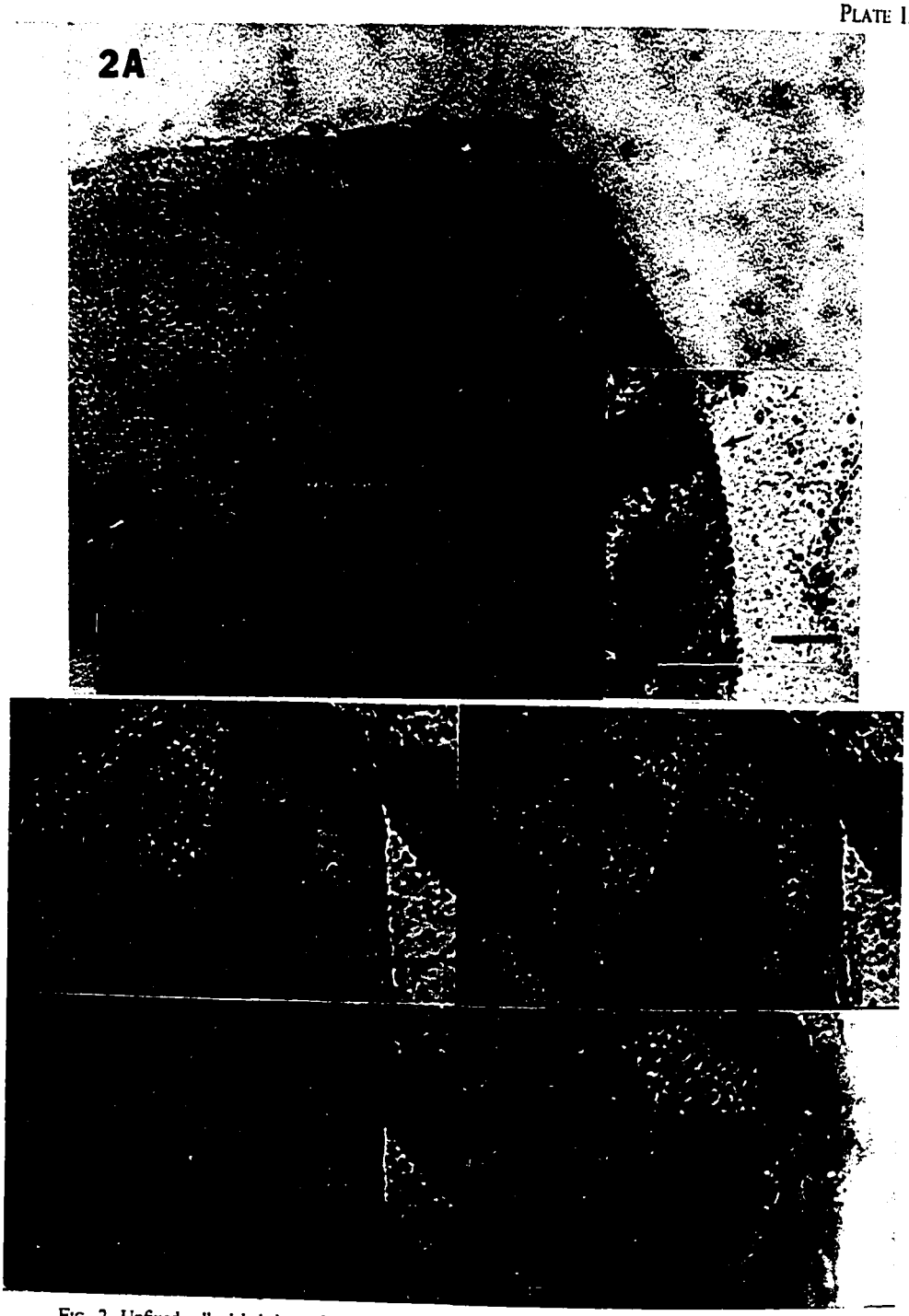

FIG. 2. Unfixed cells dried down from an "all salts" solution; similar structures were seen in cells d down from 25% NaCl. (A). Honeycomb network exhibiting a regular hexagonal array of holes. (B). F which apparently originate from an 'edge-view' of the network as it rolls over the edge of the cell (arr Bars are 0.1 μ. FIG. 3. Instability of the honeycomb network under low electron beam intensity. (A t Micrographs of the unfixed network taken at 30-s intervals showing the deterioration of the network. the changes in the selected area (arrow). Patches of the honeycomb network, sloughed off the cell su during manipulations, probably make up the beam-sensitive material in the background. Bar is 0.1 μ.

2A