A COMPARATIVE STUDY OF THE CELL ENVELOPES OF A MARINE AND A TERRESTRIAL PSEUDOMONAD

by

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STATEMENT OF THE PROBLEM

This thesis is concerned with an experimental study of a bacterium isolated from the ocean. The study was designed to answer the following questions:

a. What are the specific ions normally found in the ocean which are required by this organism for growth and for maintenance of cellular integrity?

b. Is this marine bacterium so basically different in its properties from its terrestrial counterparts that its survival outside the ocean environment would be tenuous? What are the primary lesions resulting from removal of the bacterium from the ocean environment? What are the causes of these lesions?

c. In what ways does this marine bacterium differ biochemically, structurally, and/or physiologically from a similar terrestrial organism? Why should such differences make this marine organism better fit for survival and propagation in the ocean environment?

INTRODUCTION

A. General.

Over the past sixty years, there has accumulated a vast quantity of information concerned with the biochemistry and physiology of bacteria from non-marine sources, e.g. fresh water, soil, animals, and plants; however, relatively few comparable studies have been carried out on bacteria from the ocean. Most of the investigations on such organisms have been concerned with the identification and classification of various bacteria from the many marine environments, such as tidal areas, sediments, marine marshes, and various depths and conditions of the deep sea(1,2,3).

One of the reasons that bacteria from the marine environments have not been studied as extensively as terrestrial forms is that there has been some doubt as to whether specific marine bacteria exist(4). It is possible to suppose that bacteria isolated from the ocean are merely the progeny of contaminants from land or fresh water sources, which could survive and grow in the sea. If no fundamental differences exist between terrestrial forms and the forms found in the ocean, or if the differences are readily lost by subculturing in the laboratory, then there would be little point in studying bacteria from the ocean.

As a result of the paucity of biochemical and physiologi-

cal information on bacteria from the ocean, one is hard put to supply a single definition which would set bacteria indigenous to the ocean apart from those from other sources. From the early comparisons between bacteria from marine and non-marine sources, it was determined that bacteria from the sea generally require sea water for growth(1). Some controversy has developed, however, concerning this sea water requirement. Zobell and Rittenberg(2) noted that on initial isolation, chitinoclastic bacteria from the sea demonstrated a requirement for sea water for growth. However, after prolonged laboratory cultivation, these bacteria developed the ability to grow in fresh water media. MacLeod and Onofrey(5) noted this same phenomenon with a pseudomonad isolated from a marine source. The organism in this case was "trained" to grow on a protein hydrolysate medium without sea water. An analysis of this medium by flame photometry indicated that the medium contained 0.025 M Na^+ (the concentration of Na^+ in sea water is approximately 0.46 M). This same organism would not grow initially, nor could it be trained to grow without Na^+. Cases in which bacteria have "lost" their sea water requirement have, for the most part, been concerned with bacteria grown in complex media, such as nutrient broth, peptone, fish broth, or trypticase(4). These media could be expected to be contaminated with inorganic ions at levels sufficiently high to support the growth of organisms requiring these ions. Other investigators have reported extreme difficulty in "training" sea water-requiring bacteria to grow in fresh water media(6,7).

In one investigation, ninety-six separate isolates taken from the coastal waters of Florida were found to have a specific Na^+ requirement(8). Payne(9) also demonstrated a requirement for Na^+ in a pseudomonad from a marine source. Neither Rb^+, Cs^+, nor Li^+ could substitute for Na^+ in the growth medium of this organism. Sea water-requiring bacteria, which have been studied for their ionic requirements, have been found to require Na^+, among other ions, for growth(4).

As more information accumulates on the ionic requirements of bacteria from the ocean, it may become evident that, generally, bacteria indigenous to the ocean are obligate halophiles. The information presently available suggests that this may be true. If this is so, then one may define marine bacteria, tentatively, as bacteria from the ocean that demonstrate a requirement for sea water, or more specifically for certain ions from the sea water including Na^+. Bacteria which require Na^+ for growth fall into the general classification of halophilic bacteria. Studies of halophilic bacteria indicate that the organisms of this group have many common characteristics, especially concerning their response to their salt environment. To better understand the marine bacteria, it is useful to consider this group of halophiles as a whole.

B. The halophilic bacteria.

Halophilic bacteria have been defined as those bacteria which are favorably influenced by certain concentrations of NaCl in their aqueous environments(10). This definition includes those bacteria which can grow in the absence of NaCl

but whose growth is "favorably" influenced by the presence of NaCl: the facultative halophiles. The facultative halophiles will not be considered here. Those bacteria that will be considered are the obligately halophilic bacteria, which require the presence of Na^+ in their growth medium.

I. Classification of halophilic bacteria.

The obligately halophilic bacteria can be divided into three main groups depending on their level of Na^+ requirement: (a) the extreme halophiles which require Na^+ in the medium at levels from 20 to 30%(3.4 to 5.1 M), i.e. just below the saturation concentration of 31%; (b) the moderate halophiles which grow best in media containing NaCl from 5 to 20%(0.85 to 3.4 M); and (c) the slight halophiles which require Na^+ in their growth medium at levels from 2 to 5%(0.34 to 0.85 M). The non-halophilic bacteria are those organisms which grow best below 2%(0.34 M) NaCl(10).

The extreme halophiles are composed of two distinct groups: the Halobacterium group and the Sarcina-Micrococcus group. Due to the presence of carotenoid pigments, both groups are characteristically red.

The moderate halophiles include both gram-negative and gram-positive organisms, sporeformers and non-sporeformers, motile and non-motile organisms, rods, cocci, and sarcina. Most are colorless in contrast to the red-pigmented extreme halophiles. Both the extreme and moderate halophiles inhabit salt brines, salted fish, salted hides, salted beans, and bacon-curing brines. These organisms are very probably intro-

duced into the brines and other habitats when solar salt is added. Solar salt is often contaminated with as many as 10^5 to 10^6 organisms per gram of salt(10).

The slight halophiles include the marine bacteria from the ocean and surrounding marine environments. These organisms are distributed throughout the oceans(11). One or more representatives of more than sixty of the well-defined bacterial genera have been isolated from sea water or marine mud(1,10,11,12). In spite of the abundance of marine bacteria, relatively few investigations have been carried out on these organisms.

Before the field of marine bacteriology can advance beyond its present formative stage, many problems concerning the mechanisms of survival and propagation of bacteria in the ocean environment need to be considered. The salt requirement of these organisms is only one of the factors basic to the study of marine bacteriology. Morita and his co-workers have studied other aspects of marine microbiology including the psychrophilic nature of the marine bacterium Vibrio marinus(3,13,14), as well as temperature-salinity interactions(15). The effects of hydrostatic pressure on marine organisms have been studied only superficially; some observations on hydrostatic pressure on marine organisms have been presented by Morita(16).

Although this thesis is concerned with studies on a slight halophile isolated from the ocean environment, all three groups of halophiles will be considered in the following sections. The reason for this becomes apparent when one

compares the similarities between the three groups, especially concerning the specific aspects of their inorganic ion requirements.

II. Physiology of halophiles in response to salt environment.

The requirement of halophiles for NaCl indicates that a basic difference exists between these salt-requiring bacteria and the non-halophilic bacteria. To understand this difference, one must look in some detail at the inorganic salt requirement at the biochemical and physiological level.

(a) Intracellular Na^+ concentration.

By what mechanism are some organisms capable of living in NaCl concentrations up to saturation level? Why do some organisms require relatively high concentrations of NaCl in their medium in order to grow? Cells growing in a high salt environment must either exclude the salt from inside the cells by some mechanical means, i.e. a membrane impermeable to such salt, or have internal proteins which are adapted to withstand salt concentrations which would be expected to salt out most proteins.

The internal salt (NaCl) concentration of bacteria growing in high salt environments is a factor that should be considered when determining those properties which might make these organisms better fit for survival in such environments. If internal salt concentrations of halophiles are high, relative to those of non-halophilic bacteria, then one might expect to find enzyme proteins which are capable of resisting the salting out effects of high salt levels. Several investi-

gations on the internal concentration of NaCl have been carried out. The data from these investigations are summarized in Table 1.

The data in Table 1 indicate that the ratios of internal/external salt vary greatly from one organism to the next, even among those of the same group. However, in the group of extreme halophiles, the internal salt concentration was at least 1.37 M, and in some cases approached 4.0 M. These high internal salt concentrations suggest that intracellular proteins may be structurally different from those of organisms having lower internal salt concentrations. Such structural differences could provide the mechanism for survival of these organisms in high salt concentrations and may also account for the requirement of these organisms for high salt concentrations; the physiological significance of such different protein structure will be discussed in the following sections.

There are inconsistencies (Table 1) in the findings involving the moderate halophile Micrococcus halodenitrificans. Christian and Ingram found that the internal salt concentration was approximately the same as the external over a range of 1.0 to 2.0 M NaCl(17). However, with the same organism, Gibbons and Baxter found that an intracellular salt concentration of 0.85 M was maintained even when external concentrations were raised above 1.4 M to as high as 3.7 M(18). The ability to maintain this internal salt concentration was found to involve an energy-dependent mechanism.

A number of studies on animal tissues from non-halo-

Table 1

Internal/external salt concentration of bacteria.

Group of halophile and organism	Internal salt (Molar)	External salt (Molar)	Ratio of internal/external	†	Ref.
Extreme					
Sarcina litoralis	≈4.0	4.0	≈1.0	β	17
	1.7-3.2	3.1-4.6	0.55-0.69	α	18
Sarcina morrhuae	3.17	4.0	0.79	γ	19
Halobacterium halobium	≈4.0	4.0	≈1.0	β	17
Halobacterium salinarium	1.37	4.0	0.34	γ	19
Moderate					
Micrococcus halodenitrificans	0.25	0.34-0.68	0.74-0.37	α	18
	0.85	1.4-3.7	0.61-0.23	α	18
	0.3	1.0	0.30	γ	19
	1.0-2.0	1.0-2.0	1.0	β	17
Vibrio costicolus	0.68	1.0	0.68	γ	19
	1.0-2.0	1.0-2.0	1.0	β	17
Slight					
Marine pseudomonad	0-1.0	0-1.0	1.0	δ	20
Non-halophiles					
Staphylococcus aureus	0.098	0.15	0.65	γ	19
Salmonella oranienburg	0.13	0.15	0.87	γ	19

†α Calculated as internal Cl^- concentration.
β Calculated as freezing point differences.
γ Flame photometric studies of trichloroacetic acid extracts.
δ Calculated from uptake of Na^{22}.

philic organisms including nerve, muscle, frog skin, red blood cells, and cells from other tissues have provided evidence that Na^+ is maintained at lower levels inside cells than outside by an energy-requiring mechanism(21). Of the non-halophilic bacteria, Escherichia coli K12 has also been shown to possess an energy-linked mechanism for extruding Na^+(22). Whether the same is true for Staphylococcus aureus and Salmonella oranienburg is not known (Table 1).

Another factor that must be considered in a discussion of intracellular salt concentrations, is the internal concentration of K^+. If the internal K^+ concentration is also maintained at high levels, then the resultant ionic strength inside the organisms could, in some cases, be extremely high. In the halophilic bacteria studied for their internal K^+ concentrations, such internal concentrations have been found to be greater than the external in all three classes of halophiles (19,20). Furthermore, of those organisms studied by Christian and Waltho(19), only two had internal K^+ levels lower than that of Na^+: Vibrio costicolus and Sarcina morrhuae. In the case of Halobacterium salinarium the internal K^+ level was close to the limit of solubility of KCl, approaching 4.6 M.

(b) Metabolic activity.

As described above, the obligate halophiles are distinguished from other bacteria in their specific requirements for the cation Na^+. The results of investigations into the nature of this requirement present some interesting findings. Baxter and Gibbons(23) investigated the effects of NaCl and

KCl on selected enzymes of H. salinarium and M. halodenitrificans. The enzymes investigated were glycerol dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, cysteine desulfhydrase, glutamate-aspartate transaminase, cytochrome oxidase and catalase. As one might expect, in view of the internal Na^+ and K^+ levels, all enzymes of the extreme halophile, H. salinarium, demonstrated maximum activity at NaCl concentrations above 1.0 M with the exceptions of cysteine desulfhydrase, which was progressively inhibited as the NaCl concentration increased, and catalase, which reached maximum activity at 0.7 M NaCl. Succinate dehydrogenase and lactate dehydrogenase exhibited the highest activity in 4.0 M NaCl, the highest concentration tested. For maximum activity of these two enzymes, higher concentrations of NaCl may be required. The activity of enzymes from the moderate halophile, M. halodenitrificans, were generally depressed by the presence of NaCl above 0.2 M; however, lactate dehydrogenase reached maximum activity in 1.5 M NaCl. An interesting point is that all the above enzymes were more active in K^+ than in Na^+ solutions(10). Both ions may act together in maintaining enzyme activity, or high levels of K^+ may be required to compete effectively with Na^+ for sites on the enzyme.

The high concentrations of salts required for activity by some enzymes of halophiles may be needed to maintain the active configuration of the enzymes. It may be that the enzymes of halophiles differ from those of non-halophiles in be-

ing less firmly held in their catalytically-active conformation(24). At low salt concentrations, the electrostatic repulsion between ionized groups on the enzyme may change the structure of the protein to a form which is not catalytically active. Furthermore, this change in form at sufficiently low salt concentrations may progress to a stage at which the protein is irreversibly denatured(24).

If a non-halophilic cell could be injected with NaCl at concentrations encountered by the enzymes of the extreme and moderate halophiles, one might expect the internal metabolic process would be drastically influenced(10). Proteins would be denatured, and to some extent precipitated. Ingram(25) suggested in 1938 that protein molecules of halophiles might be smaller than those of non-halophiles, thereby allowing them to resist "salting-out" effects. Direct evidence to support this hypothesis has, to my knowledge, not been presented.

Investigations on the effect of NaCl concentration on enzymatic activity have also been carried out with slight halophiles. In one marine pseudomonad 50 mM Na^+ was required by whole cells for the maximum oxidation rate of acetate, butyrate, propionate, glucose, or galactose(26). For the maximum oxidation rate of malate, citrate, or fumarate, three to four times this concentration of Na^+ was required. However, when cell-free extracts were tested using the same substrates, in all cases the Na^+ level required by whole cells for maximum oxidation rate was inhibitory to cell-free enzyme

activity. When the enzymes of the tricarboxylic acid cycle from the same organism were tested for their response to inorganic salts, there was no specific stimulation of activity in any case by Na^+(27). Aconitase activity was increased by the presence of a number of salts, but not specifically by Na^+. In fact, isocitrate dehydrogenase activity was higher in the absence of those salts tested.

The localization of a specific requirement for Na^+ has been demonstrated in two marine bacteria. In each case Na^+ was required for the transport of substances across the membrane. In one instance, gluconate uptake was dependent on the presence of Na^+, and no other ion tested could replace it(9). In another marine bacterium a Na^+-dependent active transport of the labelled amino acid analogue α-aminoisobutyric acid (AIB) was demonstrated. AIB was concentrated in the cell up to three thousand times that of the outside concentration(28). The maintenance of this high internal concentration was also dependent on the presence of Na^+; although both Li^+ and K^+ were found to substitute for Na^+ in the latter case, neither was as effective.

The Na^+ has also been shown to play a similar role in animal tissues. Several workers have shown a Na^+ requirement for the absorption of various sugars by intestinal tissue(29,30,31).

(c) Requirement for ions in the maintenance of cellular integrity of halophilic bacteria.

Cellular integrity is maintained when the envelope of the cell is sufficiently intact to prevent the leakage of

cytoplasmic components from the cell.

On the other hand, lysis of whole cells is here defined as the disruption of the envelope of the cell accompanied by leakage of cytoplasmic material, and, when measured spectrophotometrically, is also accompanied by a decrease in optical density of cell suspensions. Such a decrease in optical density of cell suspensions is not, in itself, sufficient evidence for lysis, so therefore, optical density measurements must be accompanied by other tests to detect cell leakage materials or cell envelope degradation. Lysis of isolated envelopes refers to the disaggregation of the envelope resulting in a decrease in optical density of envelope suspensions, or in some cases, in a loss of envelope protein.

The instantaneous lysis of the extreme halophile Halobacterium in distilled water is characteristic for all the species of this genus(10). The other group of the extreme halophiles, Sarcina-Micrococcus, does not appear to be much affected morphologically by hypotonic environments(10). The morphology of Halobacterium cutirubrum, Halobacterium halobium, and Halobacterium salinarium at various concentrations of NaCl was studied(32). Cells in 4.5 M NaCl were normal rods, while at 3.6 M irregular cell forms were observed. At 2.8 M the morphology of the cells was bizarre, club-shaped, and swollen. At 1.8 M the cells became spheres; at lower concentration cells underwent a sudden lysis resulting in a rapid decrease in optical density. This lysis was attributed to osmotic factors and possibly to cell envelope denaturation. The envelopes of H. cutirubrum were found to disintegrate in

low ionic strength solutions(33). The rate of cell envelope disintegration was rapid but measurable, whereas whole cell disintegration was complete at "zero time", apparently as the result of both electrostatic interactions and osmotic factors. Disintegration of cell envelopes was measured as the amount of protein in the pellet after centrifugation at 15,000 g for 30 min. At 33 C, 75% of the disintegration had taken place in the first 6 sec in distilled water, and the rate of disintegration decreased thereafter. By 40 sec a stable residue, consisting of approximately 15% of the original protein, remained in the pellet. The disintegration thus occurred in two stages.

Certain monovalent cation chloride salts have been more effective than others in preventing lysis of extreme halophiles. The chloride salts of Na^+ and Li^+ were found to maintain cell integrity of H. halobium at lower concentrations than did those salts of K^+ or NH_4^+(34). It has been proposed that the ions that provide the greatest protection against lysis (Na^+ and Li^+) are those with greater hydrated volumes and, therefore, these ions are unable to penetrate the cell as easily as those with smaller hydrated volumes (K^+ and NH_4^+)(34). Therefore, Li^+ and Na^+ presumably provide greater osmotic protection for H. halobium than do K^+ or NH_4^+. A similar situation was observed with H. cutirubrum, in which NaCl was much more effective than KCl or NH_4Cl in preserving cell integrity(35).

As has been the case in all investigations on lysis of

halophiles, enzymes could not be ruled out as the agent of lysis. Osmotic factors were considered not to act in envelope disintegration. When the temperature was raised the rate and degree of disintegration of the envelopes increased, apparently the result of increased molecular activity. No intact envelopes or envelope fragments could be detected microscopically following the disintegration.

Available information indicates that the mechanism of lysis in low ionic environments may be the same for moderate halophilic bacteria as for the extreme halophilic bacteria. V. costicolus, a moderate halophile, also lyses immediately upon transfer to distilled water. This organism has been observed to lyse at NaCl concentrations which were constant fractions of the NaCl concentrations in the growth medium (36). The lysis was therefore interpreted as an osmotic phenomenon brought about by high internal osmotic pressure. However, on further investigation V. costicolus was found to respond differently to various salts, in that lysis occurred in KCl and NH_4Cl solutions but not in NaCl or LiCl solutions at identical concentrations(36). There was thus specificity for particular ions to maintain the integrity of the cell, and lysis could not be explained solely in osmotic terms.

The pattern of lysis observed in studies of marine bacteria, or slight halophiles, is similar to that of both the extreme and moderate halophilic bacteria. This conclusion is based on experiments with a variety of marine bacteria, some examples of which are described below.

As early as 1915, Harvey(37) noted that marine luminous bacteria failed to produce light when the sea water in which they were suspended was diluted with distilled water. This effect was attributed to cytolysis brought about by lowered osmotic pressure, since light production could be maintained when the sea water was replaced by 1.0 M sucrose. When ninety-six gram-negative marine bacteria were tested for their behavior in distilled water, in all cases, an optical density decrease occurred(8). Five other marine bacteria were tested for their lytic susceptibility in low ionic environments(38). One of the organisms belonged to the genus Cytophaga, two were members of the genus Pseudomonas, and two were different strains of Achromobacter. These organisms were transferred from 1.0 M NaCl to solutions of various concentrations of NaCl, distilled water, or other monovalent cation salt solutions. Even though lysis occurred - as determined by optical density decreases of cell suspensions, ultraviolet absorption spectra of cell supernates, and morphology studies of cells in the light microscope - no changes in cell shape, such as swelling, were observed for any of the five organisms as the solute concentration was decreased. Although the lytic susceptibilities varied greatly among the five organisms, the patterns within each genus appeared similar.

As was noted with the extreme and moderate halophiles, NaCl and LiCl were more effective in maintaining cell integrity of the marine bacteria than NH_4Cl or KCl(38). In the case of the pseudomonads, 0.5 M sucrose was found to main-

tain the optical densities of cell suspensions(38); however, later data indicate that cell envelopes had undergone lesions that did not affect the optical densities(39). These lesions were evidenced by a separation of the layers of the bacterial envelope.

The lysis of envelopes of another marine pseudomonad was studied by Brown(40). The pattern of lysis for this organism appears much the same as that of the other halophiles above, i.e. optical densities of envelope suspensions in low solute concentrations decreased with time. This optical density decrease was accompanied by the loss of a number of soluble components from the envelope, such as protein degradation products and hexosamines. The nature of this envelope degradation led Brown to conclude that envelope disintegration was due to a lytic enzyme or enzymes. He proposed that this enzyme was active in low solute concentrations, and that inorganic ions produced conformational changes in the lytic enzyme in the cell envelope which resulted in an inactive enzyme molecule. The lysis then was considered to be an autolysis, and the direct result of conformational changes of endogenous lytic enzyme at low concentrations of inorganic ions.

The studies of Buckmire and MacLeod(41) on the degradation of bacterial envelopes of a marine pseudomonad revealed that a separation of inner and outer envelope layers occurred when envelopes were placed in 0.01 M NaCl. This separation was accompanied by a release of both dialyzable and non-dialyzable components from the envelope. The non-dialyzable

fraction contained components of the rigid mucopeptide or murein layer, which is described later in this Introduction. It was suggested that the murein layer might be made up of units which form a continuous layer only if their negative charges are screened by cations. Buckmire and MacLeod (41) proposed that the loss of this murein component from whole cells resulted in weakening of the envelope structure to a point where osmotic forces caused the envelope to rupture.

Neither Brown(40) nor Buckmire and MacLeod(41) observed complete disintegration of envelopes of marine pseudomonads, as was observed with the extremely halophilic bacteria. However, certain soluble components were released from the envelopes during degradation in low ionic environments. The argument by Brown(40) for enzymatic degradation of envelopes in low ionic environments is not convincing, as sufficient evidence, e.g. kinetic or enzyme purification studies, was not presented to confirm the presence of an active lytic enzyme. The evidence presented by Brown(40) can also be interpreted in terms of envelope degradation due to electrostatic repulsion between polyanionic groups in the envelope. Conversely, no direct evidence has been presented in any of the studies on lysis of halophiles or on degradation of their envelopes in low ionic environments, which rules out lytic enzymes as the causative agent. Autolytic enzymes are known to operate in envelopes of other gram-negative bacteria(42, 43). So, until such time as evidence is presented for or against the presence of a lytic enzyme(s), the problem re-

mains unanswered.

It is quite evident that cell disruption of halophiles in low ionic environments is intimately associated with the biochemistry, physiology, and structure of the bacterial envelopes. A discussion of the envelope structure in halophilic bacteria would not be meaningful at this point without first discussing bacterial envelopes in general. For this reason, a discussion of the envelopes of halophiles is presented at the end of the following section.

C. General characteristics of bacterial cell envelopes.

I. General.

The envelope consists of those structures external to the cytoplasm which constitute a "wall" surrounding the bacterial cell. Bacterial envelopes are generally obtained by violently shaking the suspension of organisms in a container containing small glass beads, resulting in physical rupture of the organism. The envelopes are then washed free of cytoplasmic debris.

The material isolated from gram-positive bacteria in this manner is relatively free of the cytoplasmic membrane, and consists primarily of a mucopeptide polymer, known as murein(42). In some cases, murein is linked to teichoic acid polymers of ribitol or glycerol phosphate. In gram-negative bacteria, the cytoplasmic membrane remains attached to envelopes following isolation procedures, and with present techniques, cannot be selectively detached from the remainder of the envelope. The gram-positive envelope is relatively simple in nature when compared to the gram-negative

bacterial envelope, which consists of cytoplasmic membrane, murein layer, lipopolysaccharide, lipids and proteins. Because this thesis problem is concerned with gram-negative bacteria, only the gram-negative bacterial envelope will be discussed in any great detail.

II. The biochemistry of the gram-negative envelope.

(a) The cytoplasmic membrane.

A well-defined, separate cytoplasmic membrane in gram-negative bacteria may perhaps be demonstrable only in electron micrographs(44). Until very recently, it has been impossible to obtain protoplasts of gram-negative bacteria, i.e. organisms from which the layers of the envelope external to the cytoplasmic membrane have been removed. The isolation of protoplasts makes it possible to study the physiological processes of the cytoplasmic membrane separate from the remainder of the envelope. Costerton et al(39) claim to have succeeded in removing the external layers from the envelopes of marine bacteria by exposing these cells to 0.5 M sucrose with the addition of ethylenediamine tetraacetic acid (EDTA) and lysozyme. However, confirmatory results by other investigators with other gram-negative bacteria have not yet been obtained. Therefore, to date little is known of the specific biochemical make-up or the physiology of the cytoplasmic membrane of gram-negative bacteria due to a lack of techniques for isolating this membrane from the other components of the envelope.

Information on the composition of the cytoplasmic membrane of gram-positive bacteria is now available. The cyto-

plasmic membrane of these organisms can be isolated by digesting away the wall of the cell enzymatically with lysozyme(45). This leaves a protoplast, which can be gently lysed in hypotonic solutions allowing the isolation of the cytoplasmic membrane(46).

Based on a study of the cytoplasmic membranes of Micrococcus lysodeikticus, Sarcina lutea, Bacillus licheniformis, and Bacillus stearothermophilus, Salton and Freer(47) found that the membranes are comprised mainly of protein (53-75%) and lipid (20-30%) with small amounts of RNA and polysaccharide which may be contaminants from the cytoplasm. Bacterial membranes from M. lysodeikticus and S. lutea, when subjected to non-ionic surface-active agents, disaggregated into "sub-units" which were rather homogeneous in size as determined in the ultracentrifuge(48). Ultrasonic treatment was also capable of breaking the membrane into small particles of relatively low sedimentation coefficients of 4S to 5S. Vorbeck and Marinetti(49) analyzed the cytoplasmic membrane of Streptococcus faecalis for lipids; these consisted primarily of phosphatidyl glycerol, diphosphatidyl glycerol, and lipoamino acids (O-amino acid esters of phosphatidyl glycerol). Other studies have shown that major lipid constituents of bacterial membranes may vary from strain to strain(50).

In cells from plants and animals there are several sources of membranes: "surface" plasma membrane, the endoplasmic reticulum, nucleus, lysosomes, golgi, mitochondria, and chloroplasts. In bacteria, internal membrane systems

have been seen in thin sections, but are not always seen in the gram-negative bacteria grown under usual growth conditions(50). The mesosome, an invagination of the cytoplasmic membrane, was first seen in fine detail by Ryter and Kellenberger(51). Mesosomes are only rarely seen in strict anaerobes, but appear to develop normally when facultative organisms are grown under anaerobic conditions (50).

It has been suggested that the mesosomes carry out the functions in bacteria that the mitochondria carry out in higher forms(50). Van Iterson and Leene(52) observed that tellurite was reduced at the site of the mesosome in Bacillus subtilis, suggesting that these structures are the locale of the electron transport system. However, more direct evidence has been presented in support of this argument(53). On plasmolysis, the mesosome apparently evaginates and can be separated from the remainder of the cytoplasmic membrane. All the cytochromes known to be present in B. subtilis were found in these evaginated mesosomes.

Other functions of bacterial membranes are not completely understood. The mesosomes appear to be involved in division of the nuclear material(50). Sites for transport mechanisms (permeases) and coordinated protein synthesis may also be located in or on the cytoplasmic membrane(50).

(b) Murein layer.

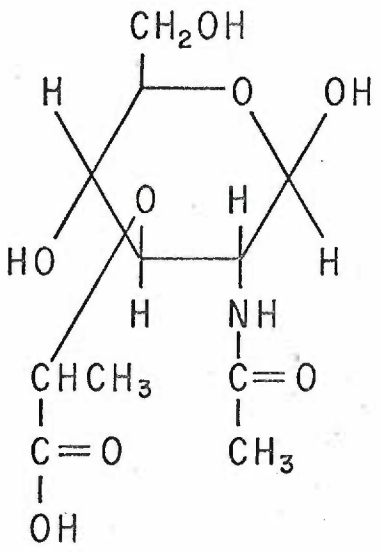
Located externally to the cytoplasmic membrane is a

layer called the murein layer(42). The function of this layer is the maintenance of the shape and rigidity of the cell envelope, thereby allowing the cell to maintain its integrity in adverse osmotic environments. This component of the walls of bacteria consists mainly of a polymer containing N-acetyl glucosamine and N-acetyl muramic acid (3-lactyl glucosamine) linked in a β-1,4 linkage(54). Side chains attached to the lactyl moiety of muramic acid contain D-glutamic acid, D- and L-alanine, either lysine or diaminopimelic acid (DAP), and variably glycine, serine, and/or threonine(55,56,57). Due to the complex nature of the gram-negative envelope, the determination of the components of the murein layer has been rather difficult. Mandelstam(58,59) purified this layer from Escherichia coli, Proteus vulgaris, Klebsiella pneumoniae, Citrobacter freundii, Serratia marcescens, and Pseudomonas fluorescens by extracting lipids of the isolated envelopes with ethanol-ether, digesting the envelopes with pepsin, and further extracting with phenol. Analysis of the residue indicated that the composition of the murein layer of gram-negative bacteria was much like that of the gram-positive bacteria. The components present were N-acetylmuramic acid and N-acetylglucosamine (Figure 1), plus glutamic acid, alanine, DAP, lysine and glycine.

The structure shown in Figure 2 is bonded to other identical structures, forming a complex network or "bag-shaped macromolecule"(42).

Figure 1. Structure of amino sugars in the murein layer: (a) N-acetylmuramic acid; (b) N-acetylglucosamine.

(a)


CH2OH
H
O
OH
O
H
HO
H
H
NH
CHCH3
C=O
C=O
CH3
OH

(b)

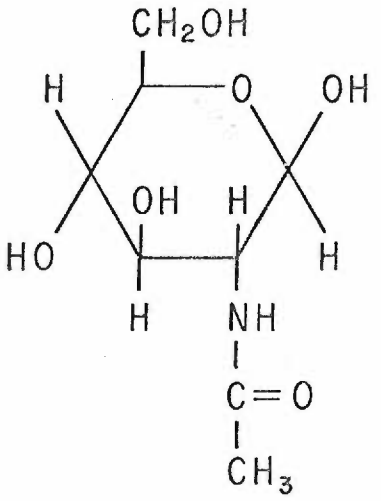
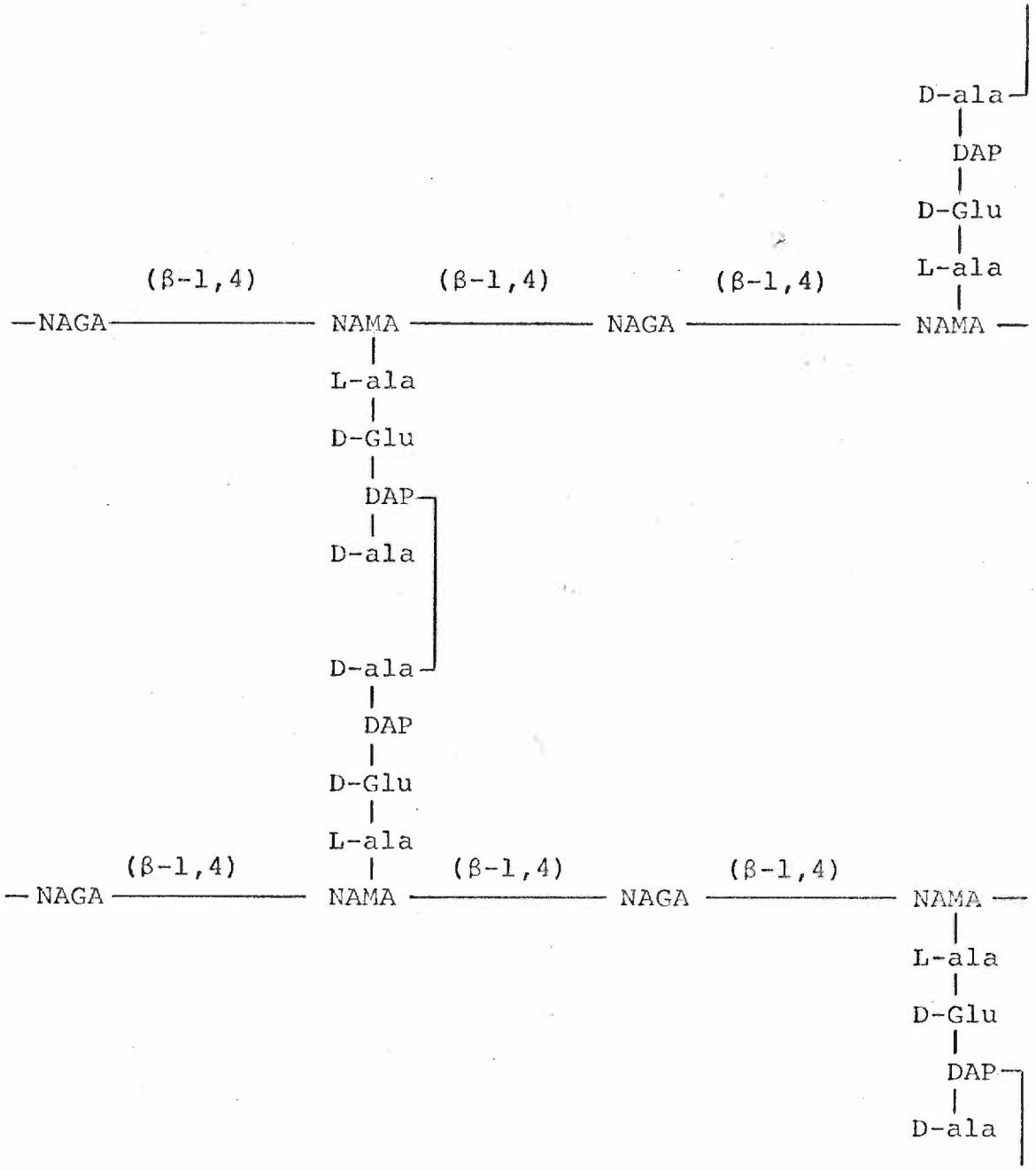

CH2OH
H
O
OH
OH
H
HO
H
H
NH
C=O
CH3

Figure 2. Structure of the murein layer from Escherichia coli(42,54). NAGA, N-acetylglucosamine; DAP, diaminopimelic acid; NAMA, N-acetylmuramic acid.


(β-1,4)
(β-1,4)
(β-1,4)
NAGA
NAMA
NAGA
NAMA
L-ala
D-Glu
DAP
D-ala
D-ala
DAP
D-Glu
L-ala
D-ala
DAP
D-Glu
L-ala
(β-1,4)
(β-1,4)
(β-1,4)
NAGA
NAMA
NAGA
NAMA
L-ala
D-Glu
DAP
D-ala

(c) Lipopolysaccharide component of the cell envelope.

Another component of the cell envelope of gram-negative bacteria, especially abundant in the enteric bacteria, is lipopolysaccharide (lps). Lipopolysaccharide is readily extractable from the envelopes of gram-negative bacteria with hot phenol by the method of Westphal and Luderitz(60). This extracted lps is water soluble. The hydrolysis of lps from E. coli 0111(61) and Salmonella typhimurium(62) yielded several components: (1) a water-insoluble Lipid A fraction, consisting of long-chain fatty acids, glucosamine and phosphate; (2) a water-soluble fraction containing heptose, phosphate, 2-keto-3-deoxyoctanoate, and ethanolamine; (3) a backbone fraction of polysaccharide with as many as seven different sugars.

The Lipid A fraction from E. coli 0111 has been analyzed quite thoroughly(61). The purified Lipid A, as obtained by silicic acid chromatography, was shown to have a molecular weight of 1700 and to contain long-chain fatty acyl groups. An analysis of these fatty acids indicated that they were β-hydroxy acids, primarily β-hydroxymyristic acid. The Lipid A fraction contained almost no unsaturated or cyclopropane fatty acids. Of the bacterial lipids of E. coli, β-hydroxymyristic appeared to be unique to the lps, since it is not a component of the hot ethanol, chloroform-methanol extractable lipids of E. coli(63). A tentative structure for the E. coli Lipid A has been proposed in which N-acetylglucosamine molecules, with esterified fatty acid side chains, are joined together by phosphate groups

to form a polymer(61).

The composition of the lps from different bacteria, and even from different strains of the same species, may differ greatly, especially in the polysaccharide fraction. Lipopolysaccharide is water-soluble, yet contains lipid material which itself is water-insoluble. Therefore, the infrared spectrum has a unique pattern which is characteristic for lps(64).

(d) Lipids of gram-negative bacterial cell envelopes.

The principal lipid types found in bacteria are phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidic acid, and glycolipids. Two other species of lipids also found, although rarely, are phosphatidylcholine and phosphatidylinositol. Triglycerides, sterols, sphingolipids, and polyunsaturated fatty acids are not found in bacteria(65).

Kaneshiro and Marr(63) analyzed the lipids of three gram-negative bacteria, E. coli, Azotobacter agilis, and Agrobacterium tumefaciens extracted with ethanol and chloroform-methanol. The principal phospholipid found was phosphatidylethanolamine. A. tumefaciens was the only one of the three bacteria which contained detectable amounts of phosphatidylcholine, which is found only rarely in bacteria.

The fatty acid composition of these three bacterial species is representative of many gram-negative bacteria (66). Palmitic acid was the predominant straight chain,

saturated fatty acid, and myristic acid was the only other normal saturated fatty acid present. The unsaturated fatty acids were hexadecenoic and octadecenoic acids. Both A. tumefaciens and E. coli form the cyclopropane derivatives of both mono-unsaturated fatty acids. Cyclopropane fatty acids were not found in A. agilis. However, the presence of cyclopropane fatty acids or their unsaturated derivatives depends on growth conditions and/or the age of the cells (44).

The fatty acids of the gram-positive and gram-negative bacteria differ in that the majority of the fatty acids from gram-positive bacteria are C-15 branched acids while the gram-negative bacteria possess unsaturated, saturated, and cyclopropane fatty acids(44).

(e) Proteins of the gram-negative bacterial cell envelopes.

The spectrum of amino acids derived from the hydrolysis of envelopes of gram-negative bacteria is distinctive in that it is high in acidic and neutral amino acids and low in the basic amino acids(67). A more detailed discussion of the amino acids of gram-negative bacterial envelopes will be covered in the Discussion section.

(f) Ultrastructure of gram-negative bacterial cell envelopes.

The typical envelope in thin-sections of normal gram-negative bacteria fixed in their growth medium appears as a multi-layered structure(68). On close examination, the

inner layer, i.e. the layer enclosing the cytoplasm, appears as a track-like layer which is generally considered to be the cytoplasmic membrane. Another double track-like layer can be seen as the outer layer of the envelope. A layer corresponding to the murein layer can sometimes be visualized lying between the inner and outer track-like layers(68). This layer can be seen as a single dark line in electron micrographs of thin sections.

(g) The effect of lysozyme and ethylenediamine tetraacetic acid (EDTA) on gram-negative bacterial cell envelopes.

In 1922, Fleming reported the discovery of a bacteriolytic enzyme present in nasal secretions and other animal tissues(69). The bacteriolytic effect has since been shown to be the result of an enzymatic hydrolysis of the murein layer of the cell wall(70). The term "lysozyme", coined by Fleming, has been applied to several cell wall lytic enzymes of varying specificities. However, it has been suggested by Strominger and Ghuysen(71) that the term be restricted to that enzyme activity which catalyses the hydrolysis of the β-1,4 linkage between the units of the murein component N-acetylmuramyl-N-acetylglucosamine. There are two other types of bacteriolytic enzymes which act on bacterial cell walls: endopeptidases, which split the peptide cross-links, and N-acetylmuramyl-L-alanine amidases, which cleave the junction between the polysaccharides and peptides of the murein layer(71).

For lysozyme to act efficiently on gram-negative bacteria, the divalent cations in the envelope must be removed. Repaske(72) devised a system consisting of EDTA plus lysozyme which resulted in the rapid lysis of gram-negative bacteria. In Pseudomonas aeruginosa EDTA alone will rapidly lyse the cells(73). A spheroplast can be produced following lysozyme and EDTA treatment if the external osmotic pressure of the medium is maintained. This resultant spheroplast consists of the cytoplasmic membrane plus the residual layers of the envelope after the murein layer has been removed(67).

(h) The role of divalent cations in the gram-negative bacterial cell envelope.

It is apparent from the above discussion that the divalent cation Mg^{++} plays a significant role in the maintenance of the envelope integrity of P. aeruginosa as removal of Mg^{++} by EDTA results in cell lysis(73). Furthermore, osmotically unstable rods were produced by removing Mg^{++} with EDTA and replacing the Mg^{++} with Na^{+}(74). Divalent cations have also been shown to be required in the envelope of other organisms. Calcium-deprived cells of Rhizobium trifolii developed an abnormal morphology suggestive of a weakened double-layered envelope structure(75). The normal walls of this organism were analyzed for divalent cation content, and Ca^{++} and Mg^{++} found to be present in a weight ratio of 5:1(76).

Divalent cations may have multiple functions in enve-

lopes and membranes of bacteria. A Mg^{++}-dependent re-aggregation of bacterial cytoplasmic membrane particles from Streptococcus faecalis was reported by Brown(77). Magnesium ions have also been shown to stabilize bacterial protoplasts of Xanthomonas phaseoli(78).

The role of divalent cations in the halophilic bacterial envelope may be through an interaction with lipids. The fractionation and analysis of the lipids of cell envelopes of a marine pseudomonad by Gordon and MacLeod(79) indicated that Mg^{++} (which in low concentrations will prevent lysis(38,80)) was bound to the diphosphatidylglycerol fraction. Divalent cations may interact with lipids in maintaining cell integrity of the extreme halophile Halobacterium cutirubrum, since the capacity of Mg^{++} and Ca^{++} to prevent cell envelope disintegration was markedly reduced when lipids were extracted from the cell envelope of this organism(81). It appears, therefore, that divalent cations are essential components of gram-negative bacterial cell envelopes of both halophiles and non-halophiles.

D. The envelopes of gram-negative halophilic bacteria.

The ultrastructure of marine gram-negative bacteria in thin-section appears much like that of other gram-negative bacteria: a multi-layered envelope consisting of two double, track-like layers (39,40,68,82). Attempts to visualize a murein layer in a marine bacterium have not been successful(39). The envelope of the extremely halophilic bacteria, H. halobium and H. salinarium, studied by

electron microscopy of thin-sections by Brown and Shorey consisted of only one double, track-like layer, i.e. a single membrane(83,84). However, Cho et al(85) have presented electron micrographs of thin sections of H. halobium which indicate that the envelope is multi-layered, consisting of a double, track-like cytoplasmic membrane with a single layer external to this membrane.

The results of experiments on lysis of halophilic bacteria in low ionic environments indicate that there are basic chemical and/or structural differences in the envelopes of these organisms as compared to those of most non-halophilic bacteria. Search for the nature of such differences in marine pseudomonads has not been fruitful. Muramic acid, a distinctive component of the rigid murein layer of both gram-positive and gram-negative bacteria, has been found in marine pseudomonads(40,41). Furthermore, when the phospholipids of a marine pseudomonad and of P. aeruginosa were extracted and analyzed by silicic acid column chromatography, there were no major differences between the lipids of these two organisms or other non-halophilic gram-negative bacteria(65,79).

On the other hand, analyses have shown that muramic acid is not a component of the envelope of those extremely halophilic bacteria investigated(84,86). A lipid analysis of the envelopes of the extreme halophile, H. cutirubrum, revealed that 93% of the lipids were phosphatides(87), which is similar to the composition of other gram-negative

bacterial envelopes(63). The analysis of the phosphatides revealed the presence of an unusual phosphatidylglycerol phosphate derivative containing terpenoid ether groups instead of the fatty acid ester groups commonly found(66,87, 88).

E. Objectives of this research.

The area of marine bacteriology is still in a formative stage. Relatively little is known about basic differences which may exist between bacteria from marine and terrestrial sources. A knowledge of such differences is a basic prerequisite to a detailed study of microorganisms in the ocean environment.

The research began, at the first step, with the isolation of a bacterium from the ocean. A study was then made of its ionic requirements, first for growth and subsequently for cellular integrity, to determine whether the organism was classifiable as an obligate marine bacterium. The next phase was an analysis of the behavior of the organism in low ionic environments, such as might be encountered by terrestrial organisms. This led to the formulation of an hypothesis concerning the role of cations in maintaining cellular integrity of marine bacteria.

The major objective of the research was to determine whether biochemical and/or ultrastructural differences could be found to exist between the cell envelopes of the marine bacterium and a similar bacterium isolated from a non-marine source, and whether such differences could

underlie the behavioral differences of the two organisms in low ionic environments. It was considered that these studies might provide additional criteria, to better distinguish marine from terrestrial organisms. Such knowledge could be meaningful in leading to an understanding not only of the relationship between marine bacteria and their environment, but also of the significance of ionic requirements in microbial ecology in general.

MATERIALS AND METHODS

A. Isolation and classification of bacterial strains.

I. Isolation and stock culture maintenance of marine bacterium c-Al.

A 250 ml sample of sea water was taken at high tide (6.8 feet) at Depoe Bay, Oregon. Ten ml of the sea water was incubated at 25 C to permit further bacterial growth. After 16 hr incubation, 0.2 ml was spread on artificial sea water(89) peptone agar in petri dishes. The constituents of the agar medium used throughout the isolation procedure were:

Bacto-peptone	5.0 gm,
Bacto-agar	15.0 gm,
artificial sea water	1000 ml.

The composition of the artificial sea water(89) used for these experiments was as follows: 0.402 M NaCl, 0.028 M Na_2SO_4, 0.002 M $NaHCO_3$, 0.009 M KCl, 0.01 M KBr, 0.052 M $MgCl_2$, 0.01 M $CaCl_2$, 0.15 mM $SrCl_2$, and 0.42 mM H_3BO_3.

Following overnight incubation, several colonies which had grown on the solid medium were transferred to peptone agar slants; one organism isolated by this method was designated c-Al, and is the subject of most of the research in this thesis. Later experiments have demonstrated that this isolate is unable to grow on the above medium when distilled water is substituted for artificial sea water.

A pure culture of marine organism c-Al was obtained as

follows: organisms from the slant were streaked onto a peptone agar plate and incubated 24 hr at 25 C. From the plate a single colony was picked off and restreaked on the same medium, incubated for 24 hr at 25 C, at which time a single colony transfer to a slant of the same medium was made. Stock and working cultures were maintained on the peptone agar medium until a synthetic medium was designed, after which time the synthetic medium was used. Stock and working cultures were held in the refrigerator and transferred approximately each sixty days.

II. Isolation and stock culture maintenance of terrestrial Pseudomonas 121.

Pseudomonas strain 121 was originally isolated from soil by enrichment culture for 5-hydroxytryptamine-degrading organisms by Dr. E. L. Oginsky, who provided the organism used in this study. Stock and working cultures were maintained on a medium consisting of:

Bacto-peptone	5.0 gm,
Bacto-agar	15.0 gm,
distilled water	1000 ml.

Stock and working cultures were held in the refrigerator and transferred approximately each sixty days.

III. Classification of isolate c-Al and Pseudomonas 121.

Both organisms were subjected to the classification scheme of Colwell and Liston(90), with all tests performed as described in Manual of Microbiological Methods(91). Isolate c-Al was also subjected to the classification scheme of Shewan, Hobbs, and Hodgkiss(92). The media for 121

were prepared with distilled water, whereas those for c-Al were prepared with artificial sea water(89).

B. Determination of salts and ions present in sea water required for growth of c-Al.

I. Sources and grades of salts and sugars used in this research.

(a) Merck & Co., Rahway, N.J. (A.C.S. reagent grade) - NaCl, KCL, LiCl, Na_2SO_4, $MgCl_2$, $CaCl_2$, NH_4Cl, glucose and sucrose.

(b) Mallinckrodt Chemical Works, N.Y. (A.C.S. reagent grade) - $NaHCO_3$, H_3BO_3, K_2HPO_4, Na_2HPO_4, NaH_2PO_4, $MnCl_2$, K_2SO_4.

(c) Allied Chemical, Morristown, N.J. (A.C.S. reagent grade) - KBr, $(NH_4)_2SO_4$, lactose.

(d) Sigma Chemical Co., St. Louis, Mo. - Monosodium glutamate.

(e) J. T. Baker Chemical Co., Phillipsburg, N.J. (A.C.S. reagent grade) - Sodium acetate.

(f) Matheson, Coleman & Bell, Cincinnati, Ohio (A.C.S. reagent grade) - KH_2PO_4.

(g) K & K Laboratories, Inc., Plainview, N.Y. - RbCl (99% pure).

(h) Penn Rare Metals, Inc., Revere, Pa. - CsCl(99.9% pure).

II. Selection of a carbon source in a synthetic medium for isolate c-Al.

It was necessary to devise a synthetic medium in order to determine specific and minimum sea water salt(s) re-

quired for growth. As the first step, three common carbon sources were tested separately or in combination for their ability to support aerobic growth in a medium in which NaCl, KCl, and $MgSO_4$ at sea water molarities were substituted for sea water. This substitution was based on the report that Na^+, K^+, and Mg^{++} were the only cations required by several sea water-requiring bacteria tested by MacLeod et al(93). The medium constituents tested included the following:

	Constituents		Final concentration
Group a.	Glucose		0.2%
	Sodium glutamate		0.2%
	Sodium acetate		0.2%
Group b.	NH_4Cl		0.2%
	K_2HPO_4-KH_2PO_4	(pH6.8)	0.05 M
	NaCl		0.4 M
	KCl		0.009 M
	$MgSO_4$		0.05 M

Group a components were separately sterilized by autoclaving (15 lbs for 15 min), while group b components were combined and sterilized by Millipore filtration. The growth response of c-Al was tested on medium consisting of the group b mixture plus group a components separately and in combination. Each tube containing 10 ml medium was inoculated with 0.02 ml of a 24 hr culture of isolate c-Al in sea water peptone broth. Tubes were vigorously shaken (Gyrorotary Shaker, New Brunswick Scientific Co., New Brunswick, N.J.) at 25 C for 48 hr. Optical densities at 520 mμ

were determined at 24 hr and 48 hr (Spectronic 20 spectrophometer, Bausch and Lomb).

III. Requirements of isolate c-Al for NaCl, KCl, and $MgSO_4$ in a synthetic medium.

The medium components employed for the determination of the salt requirements for growth were as follows:

	Component	Final concentration
Group a.	NaCl	0.4 M
	KCl	0.009 M
	$MgSO_4$	0.05 M
Group b.	Glucose	0.2%
	Sodium glutamate	0.2%
	NH_4Cl	0.2%
	Na_2HPO_4-NaH_2PO_4 (pH6.8)	0.003 M

Glucose and sodium glutamate were sterilized by Millipore filtration. Other components were sterilized separately by autoclaving (15 lbs for 15 min). The growth response of c-Al was tested on medium consisting of the group b mixture plus group a components separately and in combination. Total final volume in every case was 8.0 ml. Each tube was inoculated with 0.02 ml of a 24 hr culture in sea water peptone broth, incubated with vigorous shaking at 25 C for 48 hr, and growth determined visually by noting turbidity at 24 and 48 hr.

IV. The ability of related salts to substitute for NaCl in supporting growth of isolate c-Al in synthetic medium.

In order to determine whether the requirement for NaCl was specific, thereby placing isolate c-Al in the category of obligately halophilic bacteria, various related salts were substituted for NaCl in the medium below. The medium consisted of the following:

Glucose	0.2%
Sodium glutamate	0.2%
KCl	0.01 M
NH_4Cl	0.2%
$MgSO_4$	0.05 M
K_2HPO_4-KH_2PO_4 (pH7.4)	0.002 M
---	---
NaCl	0.4 M

Each of the components was autoclaved separately. The related salts (KCl, RbCl, CsCl, LiCl) were individually substituted at equimolar concentration for NaCl in the above medium or NaCl was deleted from the medium without substitution. Ten ml of medium in 50 ml flasks were inoculated with 0.02 ml of a suspension of cells which had been harvested from synthetic medium and washed twice by centrifugation and resuspension in the medium lacking glucose, buffer, and NaCl. The cultures were incubated at 25 C with vigorous shaking for 72 hr, except that media with LiCl or with no salt substituted for NaCl were incubated for 48 hr only. Growth was recorded as optical density at 520 mμ at 24, 48, and 72 hr. The above medium, with slight modifications in some cases, was used as the synthetic medium in

the experiments presented hereafter.

V. The ability of $MnCl_2$ and $CaCl_2$ to substitute for $MgCl_2$ in supporting growth of isolate c-Al in synthetic medium.

A specific requirement for $MgCl_2$ was tested by substituting related divalent cations of Mg^{++} in synthetic medium consisting of the following:

Glucose	0.2%
Sodium glutamate	0.2%
$(NH_4)_2SO_4$	0.2%
NaCl	0.4 M
KCl	0.01 M
K_2HPO_4-KH_2PO_4 (pH7.2)	0.05 M
---	---
$MgCl_2$	0.05 M
or	0.005 M
or	0.001 M

The medium was prepared and sterilized as described in the previous experiments. Manganese chloride or $CaCl_2$ was individually substituted for $MgCl_2$ at equimolar concentration in the above medium. The ability of these organisms to grow in medium lacking divalent cations was also tested. Ten ml of each medium in 50 ml flasks was inoculated with 0.02 ml of a 24 hr culture in synthetic medium. Each tube was incubated with shaking at 25 C for 48 hr. Growth was recorded as optical density at 520 mμ at 24 and 48 hr.

VI. The ability of related salts to substitute for

KCl in supporting growth of isolate c-Al in synthetic medium.

In order to determine whether KCl was required specifically, several other monovalent cations were substituted for K^+ in the following medium:

Glucose	0.2%
Sodium glutamate	0.2%
NaCl	0.4 M
$MgSO_4$	0.05 M
NH_4Cl	0.2%
Na_2HPO_4-NaH_2PO_4 (pH7.2)	0.002 M
---	---
KCl	0.01 M

The medium was prepared and sterilized as described previously. Related salts (RbCl, LiCl, CsCl, NaCl) were individually substituted at equimolar concentration for KCl in the above medium or KCl was deleted without substitution. Ten ml of each medium in 50 ml flasks were inoculated with 0.02 ml of cell suspension from a 24 hr culture grown in synthetic medium and washed once in the above medium less KCl. Each flask was then incubated with vigorous shaking at 25 C for 72 hr with optical densities (520 mμ) determined at 24, 48, and 72 hr. Because of the presence of slight turbidity at 72 hr, the contents of the flask containing RbCl in place of KCl were centrifuged and resuspended to the original volume in the above medium less KCl. Cells were again washed in the absence of KCl and 0.02 ml

of the final cell suspension was used to inoculate 10 ml of the medium containing RbCl in place of KCl. Optical densities (520 mμ) were determined at 24, 48, and 72 hr.

VII. The ability of sulfate ion to substitute for chloride ion in supporting growth of isolate c-Al in synthetic medium.

To determine whether sulfate ion could substitute for chloride ion in the growth of this isolate, all chloride ion in the medium was replaced by sulfate ion, while maintaining molarities of cations Na^+, K^+, and Mg^{++} unchanged. The following medium was employed:

Glucose		0.2%
Sodium glutamate		0.2%
$(NH_4)_2SO_4$		0.2%
K_2HPO_4-KH_2PO_4	(pH7.2)	0.05 M
$MgSO_4$		0.05 M
K_2SO_4		0.05 M
NaCl		0.4 M

The medium was sterilized by filtration as described previously. Sodium sulfate was substituted for NaCl in the above medium maintaining the Na^+ molarity unchanged at 0.4 M. Ten ml of each medium in 50 ml flasks were inoculated with 0.02 ml of 24 hr culture in synthetic medium. The cultures were incubated for 72 hr with vigorous shaking at 25 C and optical densities (520 mμ) determined at 24, 48, and 72 hr.

VIII. The ability of chloride ion to substitute for

sulfate ion in supporting growth of isolate c-Al.

The following medium was employed:

Glucose		0.2%
Sodium glutamate		0.2%
NH_4Cl		0.2%
NaCl		0.4 M
KCl		0.01 M
K_2HPO_4-KH_2PO_4	(pH7.4)	0.01 M
$MgSO_4$		0.05 M

Preparation and sterilization of the medium was as described in previous experiments. The ability of $MgCl_2$ to substitute for $MgSO_4$ in the above medium was tested, while maintaining cation concentrations unchanged. Ten ml of medium in 50 ml flasks were inoculated with 0.02 ml of 24 hr culture in synthetic medium. Cells were incubated at 25 C with vigorous shaking for 72 hr. Optical densities (520 mμ) were determined at 24, 48, and 72 hr.

IX. Growth response of c-Al to varying concentrations of NaCl in a synthetic medium.

For the determination of the growth response of c-Al to increasing levels of Na^+ the following medium was employed:

Glucose	0.2%
Sodium glutamate	0.2%
$MgSO_4$	0.05 M
KCl	0.01 M

NH_4Cl		0.2%
K_2HPO_4-KH_2PO_4	(pH7.4)	0.002 M

The above medium was prepared and sterilized as described previously. This medium was tested for its ability to support growth when supplemented with 15 different concentrations of NaCl ranging from 0 to 0.4 M. Ten ml of each medium in 18 mm x 150 mm tubes were inoculated with 0.02 ml of a suspension of 24 hr cells grown in synthetic medium and washed twice by centrifugation and resuspension in the medium without NaCl. Cultures were incubated at 25 C with vigorous shaking for 48 hr. Optical densities (520 mμ) of the culture tubes were determined at 24 and 48 hr.

C. The effect of various salts on the maintenance of cellular integrity of marine isolate c-Al.

I. The ability of various salts to maintain optical densities of cell suspensions.

The general procedure for determining the ability of salts to maintain optical densities of isolate c-Al was as follows:

(a) All manipulations were carried out at room temperature (approximately 25 C).

(b) Cells were grown in sea water peptone broth for 16 hr at 25 C with vigorous shaking.

(c) The culture was divided into equal portions in several 50 ml centrifuge tubes and harvested by centrifugation (7700 g for 10 min).

(d) Cells were washed by resuspension of the pellets

in sea water, $MgCl_2$, or NaCl solutions to the original volume by use of a vortex mixer, followed by centrifugation (7700 g for 10 min). The washing procedure was then repeated.

(e) Following the second wash, cells were resuspended in sea water to an optical density of 0.48 - 0.60 (520 mμ) in 13 x 100 mm tubes containing 4 ml of suspension.

(f) Cell suspensions were then centrifuged (2200 g for 30 min), supernates discarded and the pellets resuspended to 4 ml in the salt solution being tested.

(g) Optical densities of the test suspensions were determined over a period of several hours at 520 mμ, and in some cases, cells were stained with crystal violet and observed in the light microscope.

II. The ability of various salt solutions to maintain viability of c-Al.

The ability of 1.0 M or 0.5 M NaCl, sea water, or 0.05 M $MgCl_2$ to maintain the viability of c-Al was determined using the following procedure:

(a) Cells were grown, harvested, and washed as described in section C.I. (a-d).

(b) After the last centrifugation from wash solution, the pellets were resuspended in the appropriate salt solution to a volume equal to that of the growth medium.

(c) The number of viable cells was determined for the 16 hr culture, the second wash suspension, and the suspension in the appropriate salt solution at 0, 1, 2, and 3 hr. Viable counts were determined by the method of Miles

and Misra(94) on sea water peptone agar. All dilutions were carried out in sea water, and all plating was carried out in triplicate.

III. Studies on cell lysis of c-Al and 121 in various salts, sugars, and distilled water at 25 C.

The following standard procedure was carried out in experiments involving lysis of c-Al. Slight modifications in this procedure were made in some experiments and are noted in the Results section.

(a) Cells were grown and harvested as described in C. I. (a-c) except that 121 was grown in peptone medium with distilled water instead of sea water.

(b) The harvested pellets were resuspended to the original volume in different salt solutions containing the chlorides of divalent cation (Mg^{++} or Ca^{++}) and/or monovalent cation unbuffered at pH 6.8-7.0.

(c) Each portion of cells was washed three times by centrifugation and resuspension in the salt solution.

(d) Each thrice-washed pellet was suspended to an optical density (520 mμ) of 0.60-0.85 (13 x 100 mm tubes).

(e) Four ml replicates of each suspension were then transferred to 13 x 100 mm tubes and centrifuged at 2200 g for 30 min.

(f) Each pellet was rapidly resuspended in 4 ml of distilled water, an appropriate sugar, a solution of a monovalent cation chloride salt, or the appropriate wash

solution as control.

(g) After 30 seconds - to allow for clearance of bubbles - and periodically thereafter, depending on the experiment, optical densities (520 mμ) were determined.

(h) In some experiments the distilled water-treated cell suspensions were centrifuged at 2200 g for 30 min, cells from the pellet stained with crystal violet for observation in the light microscope, and supernates tested for the presence of leakage material by ultraviolet absorption spectra (220 mμ - 300 mμ) and/or by the presence of ninhydrin-positive material in 20 μl of the supernates spotted on Whatman No. 1 filter paper. Spots were visualized with 0.25% ninhydrin in n-butanol.

IV. Studies on cell lysis of c-Al at various temperatures.

(a) Cells were grown and harvested as described in section C.I. (a-c).

(b) Cells were washed in a solution containing 0.1 M NaCl plus 0.05 M $MgCl_2$.

(c) Each thrice-washed pellet was suspended to an optical density of 0.78 (520 mμ) in 13 x 100 mm tubes.

(d) Four ml replicates of each suspension were then transferred to 13 x 100 mm tubes and centrifuged at 2200 g for 30 min.

(e) Each pellet was rapidly resuspended in 4 ml of distilled water at a different temperature, ranging from 2 - 90 C.

(f) Optical densities (520 mμ) were determined at 30

sec.

V. Investigation for soluble lytic enzymes in the distilled water supernates of lysed cells of isolate c-Al.

(a) Cells of c-Al were grown and harvested as described in C.I. (a-c).

(b) The cells were divided into three portions and washed three times as described in C.I. (d), except that each was washed in one of the following solutions: 1.0 M NaCl, 1.0 M NaCl plus 0.05 M $MgCl_2$, or 0.05 M $MgCl_2$ only.

(c) The washed pellets were each brought to an optical density of 1.2 at 520 mμ (13 x 100 mm tubes) in the appropriate washing solution.

(d) Four ml replicates of each suspension were transferred to 13 x 100 mm tubes and centrifuged at 2200 g for 30 min, and the supernates discarded.

(e) The pellets were each resuspended in 4 ml of distilled water with a vortex mixer, immediately centrifuged at 2200 g for 30 min and the supernates retained.

(f) In the case of the distilled water supernate from cells washed in 1.0 M NaCl, several dilutions (1/2, 1/10, 1/20, 1/100) of the supernate were made in distilled water; all four diluted supernates and the three undiluted supernates were applied to Micrococcus lysodeikticus as described below.

(g) Dried cells of M. lysodeikticus (Worthington Biochemical Corp., Freehold, N.J.) were suspended in distilled water in a concentration sufficient to produce an

optical density of 0.5 at 520 mμ in 13 x 100 mm tubes. Four ml of the suspension was then pipetted into each of several 13 x 100 mm tubes, and the tubes centrifuged at 2200 g for 30 min.

(h) Each M. lysodeikticus pellet was resuspended to 4 ml in one of the above seven supernates from distilled water-suspended cells of c-Al. Four ml suspensions of M. lysodeikticus pellets in distilled water, 0.01 M NaCl, and 0.001 M NaCl served as controls. Optical densities of the suspensions were determined (520 mμ) at 30 sec and at 5 min intervals thereafter, for 1 hr.

(i) Lysozyme (General Biochemicals, Chagrin Falls, Ohio) controls at several enzyme concentrations (1 to 100 μg/ml) served to determine the minimum concentration of enzyme detectable with the M. lysodeikticus system.

VI. Optical density changes in suspensions of cell envelopes of Pseudomonas 121 and isolate c-Al in distilled water after exposure to various salt solutions.

(a) Cells were grown at 30 C in 0.5% peptone, in sea water in the case of c-Al, and in distilled water in the case of 121. Cells were harvested by continuous flow centrifugation (Ivan Sorvall, Inc., Norfolk, Conn.) after which the cells were washed three times as described previously in the appropriate wash solution: sea water for c-Al and 0.05 M $MgCl_2$ for 121. All harvesting and washing of whole cells was carried out at room temperature (approximately 25 C).

(b) Wet pellets were stored at -70 C until used.

(c) Pellets were thawed to 4 C and suspended in four volumes of the appropriate cold washing solution. All operations involved in preparation of cell envelopes were carried out in the cold (approximately 4 C).

(d) A detailed description of the standard procedure for the preparation of cell envelopes is given later in a separate section (D.). Envelopes used in the experiments described here were prepared by the standard procedure.

(e) Generally the experimental procedure employed here was like that described in section C. III. (c-g). Envelopes were thrice-washed by suspension in an appropriate solution containing, either separately or in combination, $MgCl_2$ and NaCl, followed by centrifugation at 9370 g for 15 min.

(f) Optical densities (520 mμ) of envelope suspensions after the final wash were determined, the suspensions centrifuged, the supernates discarded, and the pellets resuspended in distilled water to the original volume.

(g) Optical densities (520 mμ) were determined at 30 sec and periodically, thereafter, for 40 min.

(h) Sufficient NaCl or $MgCl_2$ was added to certain tubes after 30 min to bring the concentrations to 1.0 M and 0.05 M, respectively, after which optical densities (520 mμ) were again determined over a 30 min period.

(i) Samples of envelopes in 1.0 M NaCl, distilled water after transfer from 1.0 M NaCl, and after $MgCl_2$ was added to the system were shadow cast and observed in the

electron microscope.

VII. Cation exchange between Na^+ and Mg^{++} in the envelopes of Pseudomonas 121 and marine isolate c-Al.

(a) Cells were grown, harvested, and washed as described in section C.I. (a-d), except that 121 was grown in peptone medium in distilled water and washed in 0.05 M $MgCl_2$. Cells were stored at -70 C until used, at which time they were brought to 4 C.

(b) Cell envelopes were prepared as described later on in section D.

(c) All operations and manipulations, with the exception of the cation exchange procedure itself, were carried out in the cold (4 C).

(d) Envelopes were suspended in 0.05 M $MgCl_2$, each tube containing 1.5 ml (21 mg/1.5 ml for c-Al; 10.32 mg/1.5 ml for 121), and centrifuged at 9370 g for 15 min.

(e) The pellets were washed two times by suspension in distilled water at the original volume followed by centrifugation at 9370 g for 15 min, and the supernates discarded. One pellet of c-Al envelopes (13.0 mg) was suspended in distilled water and ashed for 4.5 hr(95).

(f) Pellets were resuspended in the appropriate concentration of NaCl or distilled water for 15 min, after which time the suspensions were centrifuged at 9370 g for 15 min.

(g) One ml of the supernate from each tube was set aside for Mg^{++} assay by the procedure of Garner(96) modi-

fied by substituting 1 N-$HClO_4$ for 10% TCA. The pellets were suspended in 2 ml of 1 N-$HClO_4$ for 15 min and centrifuged at 9370 g for 15 min.

(h) The perchloric acid supernates and the ashed samples were also assayed for Mg^{++} using a modification of the Garner(96) procedure by substituting 2 ml of 1 N-$HClO_4$ for 2 ml of 10% trichloracetic acid.

(i) The amoung of Mg^{++} present in samples was determined colorimetrically at 540 mμ by comparison with a standard curve prepared from known concentrations of Mg^{++} as $MgCl_2$.

D. Preparation of cell envelopes of isolate c-Al and Pseudomonas 121.

(a) Cells were grown, harvested, and washed as described in section C.I. (a-d) except that 121 was washed in 0.05 M $MgCl_2$ instead of sea water. Washed cells were stored at -70 C until used.

(b) Frozen pellets were thawed to 4 C and suspended in four volumes of the appropriate cold washing solution (sea water, in the case of c-Al, and 0.05 M $MgCl_2$ for 121). All operations involved in cell envelope preparation and purification were carried out in the cold (approximately 4 C).

(c) Ten ml each of cell suspension and of glass beads (0.011 - 0.12 mm) were transferred to a pre-cooled 50 ml flask designed for use with the Braun Tissue Homogenizer (Bronwill Scientific Co., Inc., Rochester, N.Y.).

(d) The flask was shaken on the homogenizer for 40 sec with continual cooling by streaming CO_2. Contents of the flask were then transferred to conical tubes, and allowed to settle for 20 min to permit beads to separate from the supernate. Supernates were collected, the beads washed with the above appropriate cold wash solution equal in volume to the supernate, and the two fractions combined.

(e) The cell envelopes were isolated and washed according to the scheme outlined in Figure 3.

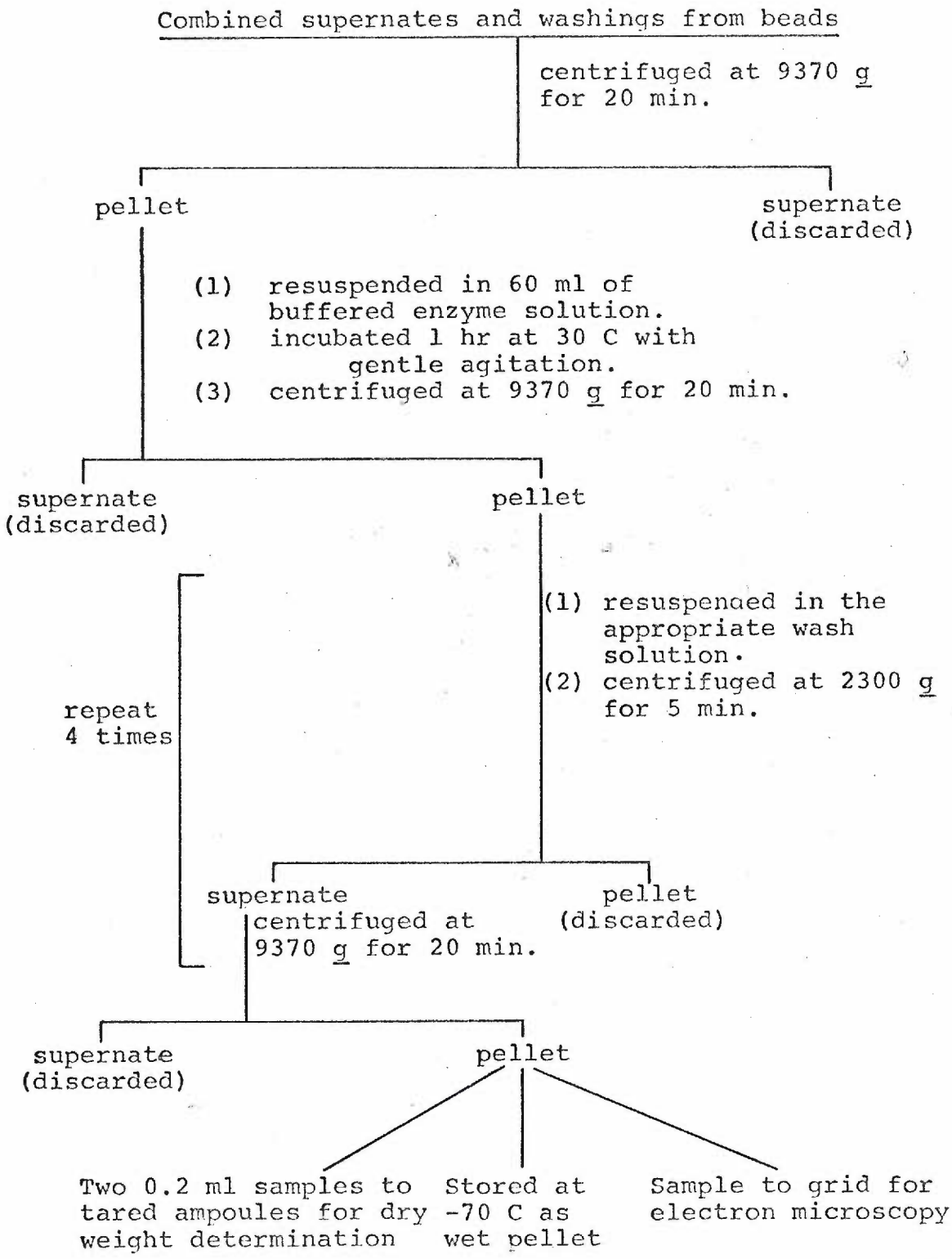
E. Electron microscopy of isolate c-Al and Pseudomonas strain 121.

I. Electron microscopy of thin sections.

For electron micrographs, cells were pre-fixed immediately following the 30 sec optical density determination in the experiment outlined in Material and Methods, section C. III. by the addition of OsO_4 in Kellenberger buffer(99) to give a final concentration of 0.1% OsO_4. Control suspensions in 1.0 M NaCl, 0.05 M $MgCl_2$, or artificial sea water were also pre-fixed by the same procedure. For subsequent fixation and embedding, the procedure of Kellenberger and Ryter (99) was followed. Vestopal W (Polysciences, Inc., Rydal, Pa.) embedded cells were sectioned on the LKB Ultrotome with glass knives. In some cases, sections were stained with lead citrate after sectioning(100). Electron micrographs were obtained with an RCA EMU-3F microscope.

II. Electron microscopy of shadowed whole cells and isolated envelopes.

Figure 3. Procedure for the isolation and washing of cell envelopes. The buffered enzyme solution contained the following: Tris(hydroxymethyl)aminomethane, 0.025 M, pH 7.8 (Sigma Chemical Co., St. Louis, Mo.); trypsin, 0.5 mg/ml (≈100,000 units/mg(97); Worthington Biochemical Corp., Freehold, N.J.); ribonuclease, 0.5 mg/ml (≈40 units/mg(98); Calbiochem, Los Angeles, Calif.); deoxyribonuclease, 0.05 mg/ml (≈100,000 units/mg(98); Sigma Chemical Co.).


Combined supernates and washings from beads
centrifuged at 9370 g for 20 min.
pellet
supernate (discarded)
(1) resuspended in 60 ml of buffered enzyme solution.
(2) incubated 1 hr at 30 C with gentle agitation.
(3) centrifuged at 9370 g for 20 min.
supernate (discarded)
pellet
(1) resuspended in the appropriate wash solution.
(2) centrifuged at 2300 g for 5 min.
repeat 4 times
supernate centrifuged at 9370 g for 20 min.
pellet (discarded)
supernate (discarded)
pellet
Two 0.2 ml samples to tared ampoules for dry weight determination
Stored at -70 C as wet pellet
Sample to grid for electron microscopy

To determine the morphology of fixed and unfixed whole cells from cultures of c-Al and 121, Parlodion (Mallinckrodt Chemical Works, St. Louis, Mo.) coated grids were placed on drops of 16 hr peptone broth cultures on dental wax for 15 min. The grids were blotted dry on filter paper. Shadowing was carried out employing a vacuum evaporator (Varion VE-10, Portland, Oregon) with carbon-platinum pellets (Ladd Research Industries, Inc., Burlington, Vermont), the shadow being cast at an angle of 12° to 16°. Envelopes were also shadow-cast following suspension in their appropriate wash solutions (section D. (b)) following the above procedure. Electron micrographs were obtained with an RCA EMU-3F microscope.

F. Biochemical analyses of cell envelopes of Pseudomonas 121 and isolate c-Al.

I. Extraction and analysis of lipopolysaccharide (lps) of Pseudomonas 121 and isolate c-Al.

The extraction and purification of lps was carried out following the procedure of Westphal and Luderitz(60) which involved the extraction of whole cells with 45% phenol at 65 C, followed by dialysis and differential ethanol precipitation to remove contaminating nucleic acid. The purity of the lps at different steps during the purification process was followed by ultraviolet absorption spectra (220-300 mμ). Infrared spectroscopy (Perkins-Elmer 221 Spectrophotometer, Perkins-Elmer Corp., Norwalk, Conn.) was employed to analyze the purified lps in accordance with the procedure of

Berst et al(64). The extraction and purification of lps from c-Al and 121 envelopes was carried out on 16 hr cells grown, harvested, and washed as in C. I. (a-d), except that 121 was grown in peptone medium without sea water and washed in 0.05 M $MgCl_2$. The extraction of c-Al was carried out in the presence of 1.0 M NaCl while the procedure for 121 employed distilled water. The purified lps was shadow-cast with platinum and examined in the electron microscope.

II. Extraction and analysis of phospholipids of Pseudomonas 121 and isolate c-Al.

For the analysis of phospholipids, the procedure was as follows:

(a) Cells were grown, harvested, and washed as described in section C. I. (a-d) and stored at -70 C until used, at which time pellets were brought to 4 C.

(b) Envelopes were prepared by the standard procedure (D. (b-e)).

(c) Envelopes (approximately 0.7 gm dry weight) of c-Al or 121 were suspended to 30 ml in sea water or 0.05 M $MgCl_2$, respectively, and extracted twice in a 20 fold volume of chloroform-methanol (2:1) by the method of Folch et al(101).

(d) The extract was filtered through chloroform-methanol washed Whatman No. 2 filter paper.

(e) Fifty ml of distilled water was added to the extract, which was then shaken vigorously and allowed to stand at room temperature until separation of the two phases was complete (approximately 2 hr).

(f) The upper phase was removed and the lower phase surface washed twice with upper phase solvent and the washings discarded. Upper phase solvent was prepared by mixing chloroform, methanol, and water in the proportions 3:48:47 by volume(101).

(g) Methanol was then added to the lower phase until a one-phase system was obtained.

(h) The extract was filtered through washed filter paper, and the filtrate taken to dryness in vacuo.

(i) The dried extract was extracted twice with distilled water, redissolved in 20 ml of chloroform-methanol (2:1), and concentrated to 5 ml in stream of N_2.

(j) Fifty ml of acetone were added to the 5 ml lipid solution with stirring to precipitate the phospholipids, which were collected by centrifugation at 7700 g for 15 min.

(k) The precipitate was redissolved in 7.5 ml of chloroform-methanol (2:1), filtered as before, and dispensed to ampoules which were flushed with N_2, sealed, and stored at -20 C for later use.

(l) The lipid extract was spotted (50 µg/spot) on Eastman thin-layer sheets of silica gel (Distillation Products Industries, Rochester, N.Y.) and developed with a solvent system containing chloroform: methanol: acetic acid: water (250:74:19:3)(102). Spots were visualized with iodine vapor and with ninhydrin - lutidine - butanol (0.3 gm ninhydrin in 5 ml lutidine brought to 100 ml with

water-saturated n-butanol) spray reagent(103) and the R_f of each spot determined.

(m) Preparative chromatograms were prepared using silica gel thin-layer sheets with 16 contiguous spots of 100 µg lipid mixture each on the origin line, and in the above solvent system. A 1 cm strip was cut from each side of the sheet, and spots visualized with iodine vapor. Areas on the remainder of the sheet corresponding in distance from origin to the various visualized spots were scraped off and eluted with a minimum volume of chloroform-methanol (2:1). Eluates were spotted on the same type of sheet and each developed in the following solvent systems:

1) Chloroform: methanol: acetic acid: water (250: 74: 19: 3) (102)

2) Isopropanol: acetic acid: water (3: 1: 1) (103)

3) Isopropanol: water

4) Butanol: acetic acid: water (63: 10: 27)

Rouser et al(104) used a solvent system with the same components as 4) above but in the ratio of 60:20:20 for the separation of beef brain lipids. To my knowledge, solvent system 3) has not been used for chromatography of lipids on silica gel thin-layers.

(n) The following phospholipid standards were chromatographed with solvent system 1) above. The source of these compounds was as follows: Applied Science Lab., Inc., State College, Pa. - phosphatidylethanolamine, phosphatidyl-

serine, phosphatidylcholine; Dr. Charles Grossman, University of Portland, Oregon - phosphatidic acid.

III. Extraction and analysis of amino sugars from envelopes of isolate c-Al and Pseudomonas 121.

(a) Cells were grown, harvested, and washed as described in section C.I. (a-d). Cell envelopes were prepared as described in section D. The hydrolysis of cell envelopes and fractionation of the amino sugars was carried out according to the procedure of Perkins and Rogers (105) with slight modifications.

(b) Envelopes (100 to 200 mg) were hydrolysed in 4 N HCl in N_2-flushed, sealed ampoules at 100 C for 5 hr.

(c) The hydrolysate was taken to dryness in vacuo, redissolved in 2.4 ml of distilled water, and concentrated to 0.5 ml under a stream of N_2.

(d) One-tenth ml of 12 N HCl was added to 0.5 ml of the hydrolysate to bring the concentration to 2 N HCl.

(e) The hydrolysate was applied to a column (5 mm diameter) consisting of a mixture of charcoal (Mallinckrodt Chemical Works, St. Louis, Mo.), 0.25 gm, and celite (Johns-Manville Co., New York), 0.25 gm, which had been previously washed 4 hr with a total of 250 ml of 2 N HCl. The amino sugars were then eluted at room temperature with 10 ml of distilled water followed by 10 ml of 5% ethanol. One ml fractions were collected (0.2 ml/min) with a Gilson model VL fraction collector (Gilson Medical Electronics, Middleton, Wisconsin), until 30 fractions were obtained.

(f) Fifty µl of each fraction was spotted on Whatman No. 2 paper which was sprayed with ninhydrin (0.25% in n-butanol). The color was developed at 80 C for 10 min.

(g) Fifty µl of each fraction containing ninhydrin-positive material was spotted on Whatman 3 MM paper and developed with n-butanol: acetic acid: water (63:10:27) in a descending direction for 17 hr with the following standards: muramic acid (50 µg) and galactosamine (20 µg), both purchased from Sigma Chemical Co., St. Louis, Mo.; glucosamine (20 µg) purchased from Calbiochem, Los Angeles, Calif. Drying of the chromatograms was carried out at room temperature in an air stream.

(h) Spots were visualized with Elson-Morgan reagents (106) as employed for paper chromatography(107). The R_{ga} (ratio of the distance of unknown from origin to the distance of glucosamine standard from origin) was calculated for each spot.

(i) Preparative chromatograms were made with Whatman 3 MM paper which had been washed three times in 1 N HCl and rinsed in distilled water until the water was free of Cl^- when tested with 0.1 M $AgNO_3$. Fractions containing material with R_{ga} similar to the muramic acid standard were spotted at 1 cm intervals along the origin. The chromatograms were developed for 17 hr in a descending direction with n-butanol: acetic acid: water (63:10:27). After drying, a 1/2" strip from the center of the paper was

sprayed as described above to detect spots.

(j) The area on the paper corresponding to muramic acid on the sprayed strip was cut out, and the material in the spot eluted in 20 ml of distilled water with stirring for 1 hr.

(k) Paper fibers were removed by centrifugation at 2200 g for 5 min, and the eluate was concentrated to dryness in vacuo.

(l) The eluate was redissolved in 2 ml of distilled water.

(m) Thirty µl of the eluate were spotted, both alone and together with 50 µg muramic acid standard, on 1/2" x 36" strips of Whatman No. 1 chromatography paper.

(n) The chromatograms were developed in an ascending direction for 24 hr in the following solvent systems:

1) Isopropanol: acetic acid: water (3:1:1)(103).

2) Isopropanol: water (4:1)(108).

3) n-butanol: pyridine: water (6:4:3)(108); the pyridine was distilled the day prior to use.

4) Phenol-saturated water.

(o) The R_f of each spot was determined.

IV. Amino acid analyses of acid hydrolysates of cell envelopes of Pseudomonas 121 and isolate c-Al.

(a) Cells were grown, harvested, and washed as described in section C. I.(a-d) and stored at -70 C until used, at which time pellets were brought to 4 C. Cell envelopes were prepared as described in section D. The hydrolysis of

the envelopes was carried out by the procedure outlined by Hill(109) with slight modifications.

(b) Envelopes (95 mg, c-Al: 150 mg, 121) were suspended in 5 ml distilled water and combined with 5 ml of concentrated HCl to give a final concentration of 6 N HCl.

(c) The 10 ml suspensions were transferred to 50 ml ampoules and flushed with N_2 for 5 min. The ampoules were sealed and maintained at 100 C for 24 hr.

(d) The hydrolysate was filtered through sintered glass and the filter twice-washed with 10 ml of distilled water.

(e) Combined hydrolysate and washings were brought to dryness in vacuo, and redissolved in a minimum of distilled water.

(f) Hydrolysates were analyzed on a Technicon Amino Acid Analyzer (Technicon Instrument Corp., Ardsley, N.Y.) using Technicon type C-2 resin with column dimensions 0.64 cm x 64 cm. Elution time was 6.5 hr. The samples were eluted with sodium citrate buffer gradient over a pH range of 2.75 - 6.10. A mixture of amino acid standards (Technicon) of known concentration was used to identify and quantitate components of the hydrolysates.

RESULTS

A. Classification of c-Al and 121 by the scheme of Liston and Colwell(90).

The purpose of testing c-Al and 121 in this classification scheme for Pseudomonadaceae was to determine the extent of the similarity of characteristics. Morphological characteristics were determined on organisms grown in peptone broth. Cultural and morphological characteristics were determined at 72 hr unless otherwise noted. All test media for c-Al contained sea water while those media for 121 were made up in distilled water. Tests of 121 in sea water media were not possible, as sea water was inhibitory to the growth of this organism. The results of these tests are presented in Table 2.

This method was originally designed for computer analysis. The organisms used in this study were not, however, analyzed by computer. The results of these tests show that c-Al and 121 have twenty-seven like and nineteen unlike characteristics. A close analysis of these data reveals that about one-half of the differences pertain to cell morphology or colony characteristics, which one might expect between any two fairly closely-related organisms. About one-quarter of the differences pertain to tests for

the hydrolysis of large molecules, such as, casein from milk, gelatin, and starch, which could be the result of the action of only two enzymes. The remaining differences, for the most part, pertain to the ability to use certain sugars. Aside from differences involving morphological and colony characteristics, the organisms differ mainly in their ability to use sugars and hydrolyze proteins. Therefore, these organisms appear to be more closely related than they appear at first glance.

Based on the results in Table 2, these organisms both are classifiable in the genus Pseudomonas.

B. Classification of isolate c-Al by the scheme of Shewan, Hobbs, and Hodgkiss(92).

Isolate c-Al was further classified by subjecting the organism to a battery of tests, some of which are included in the Liston and Colwell scheme. In addition to the results already presented, c-Al grew at 4 C and 30 C. Growth was also obtained in a medium consisting of $(NH_4)_2SO_4$, K_2HPO_4, sea water, and glucose. However, the same medium with acetate instead of glucose would not support growth.

The results of the tests in this scheme(92) indicate that isolate c-Al falls into the genus Pseudomonas Group II.

C. Requirement for salts for growth of Pseudomonas c-Al.

I. Selection of a carbon source in a synthetic medium for Pseudomonas c-Al.

The results of this experiment are presented in Table 3. As the best growth was obtained on a medium containing

Table 2

Classification of Pseudomonas strains 121 and c-A1 by the scheme of Liston and Colwell(90)

	Test	Result	
		c-A1	121
Morphological characteristics	Shape	rods, curved & straight with rounded ends	rods, straight with rounded ends
	Size (mean length & width)	1.71 μ x 0.5 μ	1.5 μ x 0.5 μ
	Gram reaction	negative	negative
	Flagellation	monopolar	lophopolar
Colony characteristics on peptone agar	Colony size	3 mm ± 0.5	2.5 mm ± 0.5
	Colony edge	entire	crenated
	Colony color	cream	cream
	Colony surface	raised	flat, raised center
	Pigmentation	none	none
Cultural characteristics in peptone broth	Comparison of characteristics at 24 & 72 hr	same	same
	Turbidity	uniform	uniform
	Sediment	slight	viscous
	Ring	no	no
	Pellicle	no	no
	Fluorescence	no	no
	Motility	yes	yes

Table 2 (continued)

		c-Al	121
Physiological characteristics	Growth at 0 C in broth and on slant	yes	yes
	Growth at 25 C in broth and on slant	yes	yes
	Growth at 37 C in broth and on slant	no	no
	Sea water required	yes	no
	Catalase test	positive	positive
	Penicillin resistant	yes	yes
	0/129 pteridin resistant	yes	yes
	Agar digested	no	no
	Gelatin liquified	yes	no
	Litmus milk reactions:		
	Peptonized	yes	no
	Acid or alkaline	decolorized	alkaline
	Surface peptonized	yes	no
	Reduced	yes	no
	NH_3 from peptone water	no	no
	Voges-Proskauer test	negative	negative
	Methyl red test	negative	negative
	Indole produced	no	no
	Growth in Koser's citrate	no	no
	Cytochrome oxidase test	positive	positive

Table 2 (continued)

Physiological characteristics	c-Al	121
Sugar utilization:		
Glucose	oxidative, no gas	negative
Maltose	oxidative, no gas	negative
Sucrose	oxidative, no gas	negative
Lactose	negative	negative
Galactose	negative	negative
Mannitol	negative	oxidative, no gas
Starch hydrolyzed	yes	no
Gluconate oxidized	no	yes
Nitrate reduced	no	yes
Nitrite reduced	no	no
Trimethylamine oxide reduced to trimethylamine	no	no
H_2S from cysteine	yes	yes

Table 3

Growth of Pseudomonas c-Al on various sources of carbon at 25 C

Carbon source (0.2%)			Optical density[1] (520 mμ)	
Glucose	Glutamate	Acetate	24 hr	48 hr
+	-	-	0.15	0.20
-	+	-	0.01	0.06
-	-	+	0.15	0.15
+	+	-	0.40	0.82
+	-	+	0.04	0.43
-	+	+	0.21	0.27
+	+	+	0.47	0.64

[1]Zero time optical density of all tubes: 0.00.

Table 4

Growth of Pseudomonas c-Al in synthetic medium with related salts substituted for NaCl (0.4 M)

Incubation time (hr)	Optical density[1] (520 mμ)					
	NaCl	KCl	RbCl	CsCl	LiCl	no salt substituted
24	0.56	0.00	0.00	0.00	0.00	0.00
48	1.50	0.00	0.01	0.00	0,00	0.00
72	1.30	0.00	0.01	0.00	-	-

[1]Zero time optical density of all tubes: 0.00.

glucose plus glutamate, the media employed in subsequent tests contained both of these carbon sources.

II. Requirements of Pseudomonas c-Al for NaCl, KCl, and $MgSO_4$ in a synthetic medium.

The results of this experiment were clear cut: no visible turbidity occurred in any of the media at 40 hr unless all three of the above salts were present.

III. The ability of related salts to substitute for NaCl in supporting growth of Pseudomonas c-Al in synthetic medium.

The results of this experiment, presented in Table 4, indicate that the NaCl requirement is specific, i.e., NaCl cannot be replaced by any of the related salts tested.

IV. The ability of $MnCl_2$ and $CaCl_2$ to substitute for $MgCl_2$ in supporting growth of Pseudomonas c-Al in a synthetic medium.

The results are presented in Table 5. Although some turbidity developed in the flask containing $CaCl_2$, the results indicate that neither of the divalent cation salts tested will effectively substitute for $MgCl_2$ in the synthetic medium employed.

V. The ability of related salts to replace KCl in supporting growth of Pseudomonas c-Al.

Four related salts were tested for their ability to replace KCl in synthetic medium. The results are presented in Table 6.

Some growth occurred in tubes containing RbCl and CsCl

Table 5

Growth of Pseudomonas c-Al in synthetic medium with $MnCl_2$ or $CaCl_2$ substituted for $MgCl_2$

Divalent cation salt	Concentration	Optical density[1] (520 mμ) 24 hr	48 hr
$MgCl_2$	1 mM	0.20	0.15
	5 mM	0.51	1.00
	50 mM[2]	0.58	1.20
$MnCl_2$	1 mM	0.00	0.01
	5 mM	0.01	0.00
	50 mM	0.00	0.01
$CaCl_2$	1 mM	0.00	0.02
	5 mM	0.10	0.12
	50 mM	0.12	0.15
$MgCl_2$ deleted; no salt substituted.	1 mM	0.00	0.00
	5 mM	0.00	0.00
	50 mM	0.00	0.00

[1] Zero time optical density of all tubes: 0.00.

[2] This concentration of $MgCl_2$ corresponds to that in the complete synthetic medium.

Table 6

Growth of Pseudomonas c-Al in synthetic medium with related salts substituted for KCl (0.01 M)

Incubation time (hr)	Optical density[1] (520 mμ)					
	KCl	RbCl	CsCl	NaCl	LiCl	no salt substituted
24	0.48	0.20	0.07	0.01	0.05	0.00
48	1.20	0.42	0.12	0.01	0.06	0.00
72	1.20	0.30	0.09	0.01	0.01	0.00

[1] Zero time optical density of all tubes: 0.00.

in place of KCl, although the growth in CsCl was extremely small. The cells from the RbCl tube were washed and used to re-inoculate a tube of fresh medium containing RbCl in place of KCl. No measurable growth occurred in this second tube at 72 hr. The small growth that occurred in the original tube was very probably due to the presence of contaminating KCl in the RbCl (99% pure) plus the residual KCl in the original inoculum. Failure to grow a second time in RbCl was probably the result of a potassium level that was too low to support growth. An alternative explanation is that the cells may have been irreversibly damaged in RbCl so that further growth was not possible.

No growth occurred in the media with NaCl or LiCl substituted for KCl. It was, therefore, concluded that none of the salts tested was able to replace KCl in the synthetic medium employed in this experiment.

VI. The ability of sulfate ion to substitute for chloride ion in supporting growth of Pseudomonas c-Al in synthetic medium.

As shown in Table 7, Cl^- cannot be replaced in this medium by $SO_4^=$. The results of the experiment can also be attributed to toxicity of $SO_4^=$. To determine whether such toxicity was a factor, the effect of the combination of these salts on bacterial growth was tested. The results of this test suggest that $SO_4^=$ at the level used may have been slightly inhibitory in the presence of Cl^-.

VII. The ability of chloride ion to substitute for

Table 7

Growth of Pseudomonas c-Al in synthetic medium with sulfate ion substituted for chloride ion

Incubation time (hr)	Optical density[1] (520 mμ)		
	Na_2SO_4 only[2]	NaCl only[2]	Na_2SO_4 plus NaCl[2]
24	0.01	0.54	0.52
48	0.01	1.20	0.99
72	0.01	1.30	1.10

[1] Zero time optical density of all tubes: 0.00.

[2] Concentration 0.4 M with respect to Na^+.

sulfate ion in supporting growth of Pseudomonas c-Al.

The ability of Cl^- to replace $SO_4^=$ in a synthetic medium was tested. The results of this experiment, presented in Table 8, indicate that growth of c-Al will occur in the absence of $SO_4^=$. However, $SO_4^=$ does enhance the growth of this organism. The culture without $SO_4^=$ decreased in optical density between 48 hr and 72 hr indicating that some lysis may have occurred after 48 hr.

VIII. Growth response of Pseudomonas c-Al to varying concentrations of NaCl in a synthetic medium.

Figure 4 shows the results of this experiment, which indicate that above 0.30 M NaCl growth was not enhanced by additional Na^+, while below 0.30 M the growth of c-Al was proportional to the concentration of NaCl. Detectable growth was observed at concentrations as low as 0.06 M NaCl. Based on these results, Pseudomonas c-Al falls into the category of slight halophilic bacterium.

The data thus far presented indicate that Pseudomonas c-Al requires for growth Na^+, K^+, Mg^{++}, and Cl^-, none of which could be replaced by the other ions tested. Although $SO_4^=$ was not required, growth was enhanced by this ion.

D. Requirement for salts to maintain structural integrity of Pseudomonas c-Al.

The effect of NaCl and $MgCl_2$ alone and in combination on maintenance of structural integrity was tested on cells which had been grown in sea water peptone broth, washed free of the medium in sea water, and resuspended in a solution of

Table 8

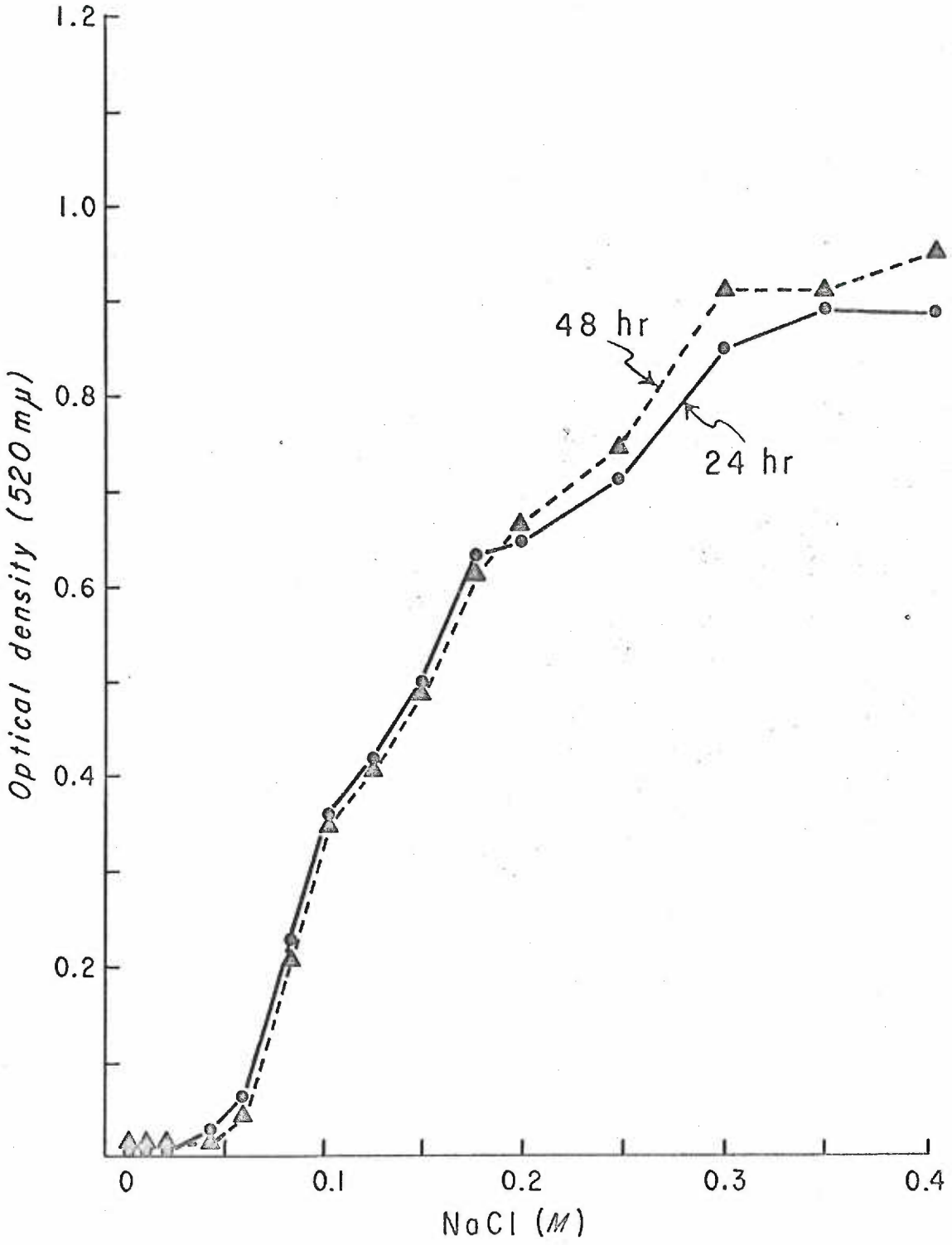
Growth of Pseudomonas c-Al in synthetic medium with chloride ion substituted for sulfate ion

Incubation time (hr)	Optical density[1] (520 mμ)	
	$MgSO_4$[2]	$MgCl_2$[2]
24	0.58	0.50
48	1.20	1.00
72	1.30	0.85

[1]Zero time optical density of tubes was: 0.00.

[2]Concentration 0.05 M with respect to Mg^{++}.

Figure 4. Growth of Pseudomonas c-Al in a synthetic medium containing varying concentrations of NaCl from 0 to 0.4 M. Growth was measured at 24 and 48 hr of incubation (25 C), as optical density at 520 mμ.


1.2
1.0
0.8
0.6
0.4
0.2
Optical density (520 mμ)
48 hr
24 hr
0
0.1
0.2
0.3
0.4
NaCl (M)

the salt(s) to be tested. Optical densities in each case were determined at the time of resuspension and periodically thereafter.

I. The ability of NaCl at various concentrations to maintain optical densities of suspensions of Pseudomonas c-Al.

The results of the experiment testing NaCl for its ability to maintain optical densities of suspensions of c-Al are presented in Figure 5. Optical densities of cell suspensions in NaCl concentrations less than 0.1 M decreased by "zero time" or 1 hr. A concentration of 0.2 M NaCl or greater was needed to maintain optical densities for 5 hr, although 0.1 M NaCl did maintain optical density for 1 hr. At NaCl concentrations which did not maintain optical density, a large part of the decrease in optical density occurred even before the "zero time" reading (recorded at approximately 30 sec). Optical density decreases were greater in 0.02 M and 0.01 M NaCl than in distilled water. The optical densities of suspensions in 1.0 M NaCl were greater than 100% throughout the 5 hr period. This could be the result of shrinkage of the cells due to loss of water when they were placed in a hypertonic solution. Others have noted a similar phenomenon with mitochondria and bacteria and have proposed the above hypothesis to account for optical density increases in hypertonic solutions(107,108).

II. The ability of $MgCl_2$ at various concentrations to maintain optical densities of suspensions of Pseudomonas c-Al.

In Figure 6 a graphic analysis of the results of this

Figure 5. The ability of NaCl to maintain optical densities of suspensions of Pseudomonas c-A1. Cells were harvested from sea water and suspended in various concentrations of NaCl (0.01 to 1.0 M), sea water, or distilled water (25 C). The percent of the initial optical density (520 mμ) was determined at "zero time", 1 hr, and 5 hr. The initial optical density was that of cells suspended in sea water at "zero time".

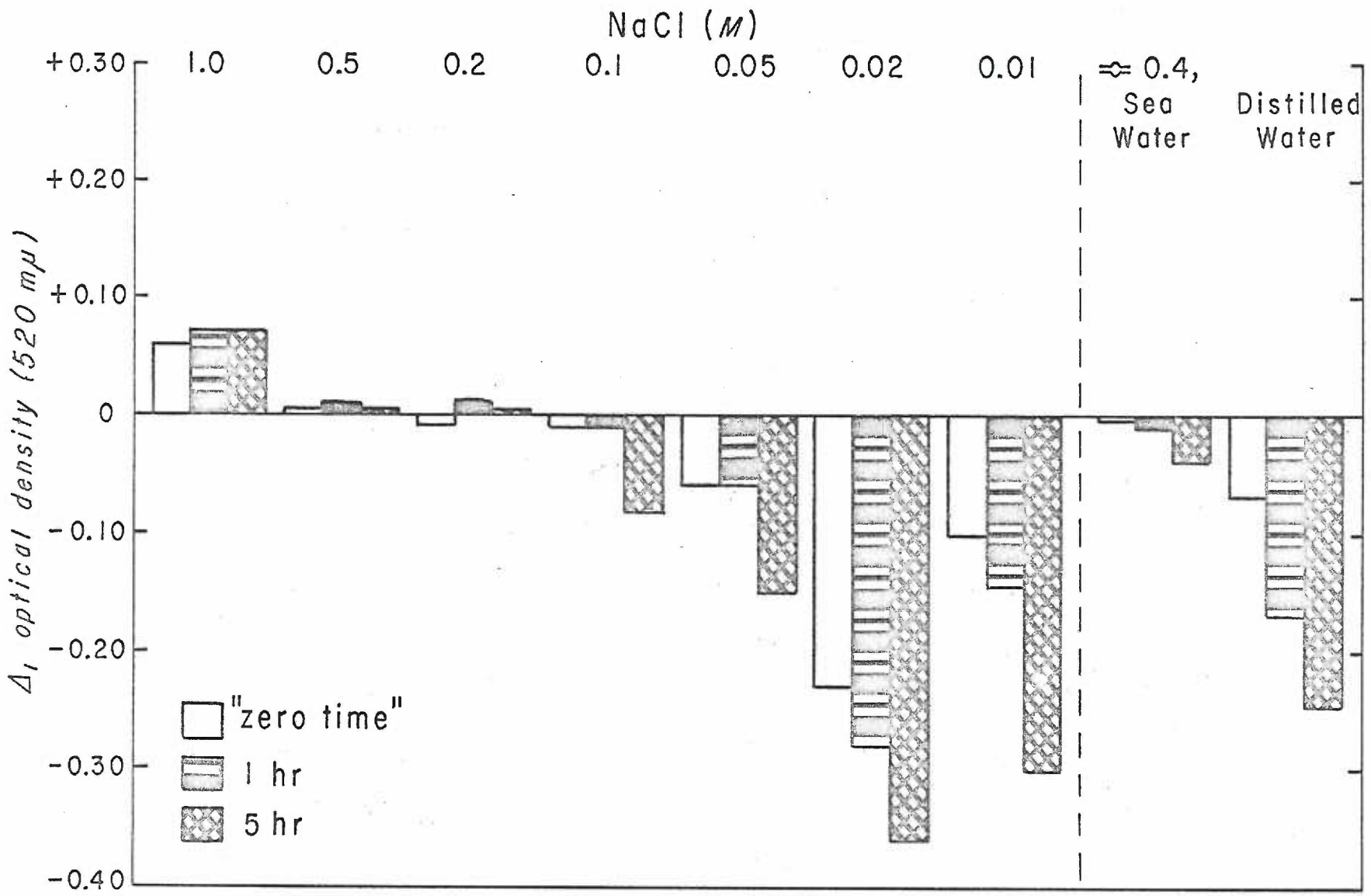
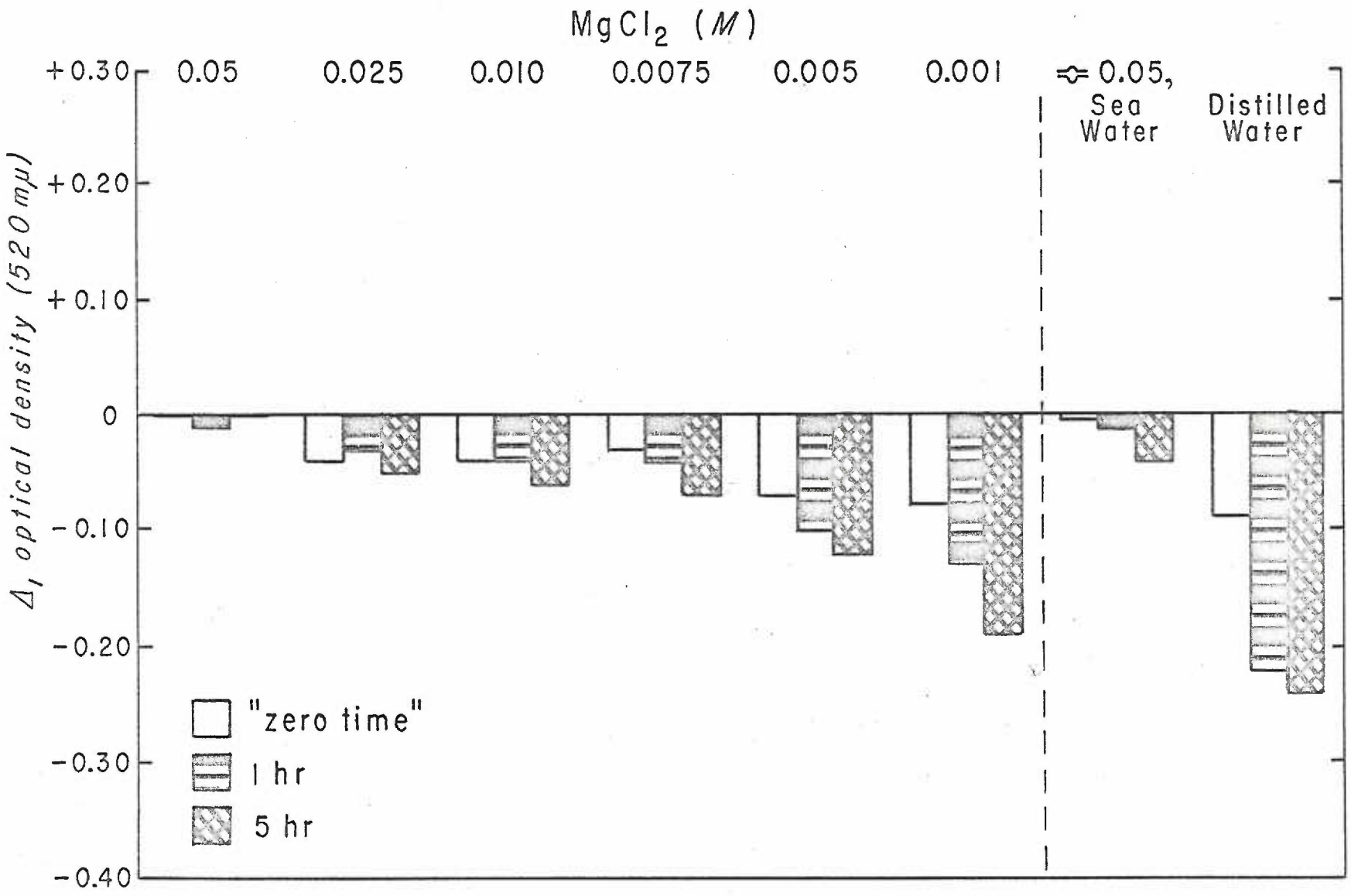

NaCl (M)
1.0
0.5
0.2
0.1
0.05
0.02
0.01
≈ 0.4, Sea Water
Distilled Water
+0.30
+0.20
+0.10
0
-0.10
-0.20
-0.30
-0.40
Δ, optical density (520 mμ)
"zero time"
1 hr
5 hr

Figure 6. The ability of $MgCl_2$ to maintain optical densities of suspensions of Pseudomonas c-A1. Cells were harvested from sea water and suspended in various concentrations of $MgCl_2$ (0.001 to 0.05 M), sea water or distilled water (25 C). The decreases in optical density were determined at "zero time", 1 hr, and 5 hr. The initial optical density was that of cells suspended in sea water at "zero time".


MgCl2 (M)
0.05
0.025
0.010
0.0075
0.005
0.001
0.05, Sea Water
Distilled Water
+0.30
+0.20
+0.10
0
-0.10
-0.20
-0.30
-0.40
Δ1 optical density (520 mμ)
"zero time"
1 hr
5 hr

experiment is presented. All concentrations of $MgCl_2$ below 0.05 M produced decreases in optical density at "zero time", 1 hr, and 5 hr; however, at concentrations between 0.0075 M and 0.05 M the optical densities were maintained within 80 to 90% of the initial optical density.

III. The ability of KCl at various concentrations to maintain optical densities and staining properties of cell suspensions of Pseudomonas c-Al following transfer from 0.05 M $MgCl_2$ or 1.0 M NaCl.

Cells of c-Al were harvested and washed in either 0.05 M $MgCl_2$ or 1.0 M NaCl as described in Materials and Methods, section C.I. Following the second wash initial optical densities were adjusted to 0.70 in $MgCl_2$ and 0.68 in 1.0 M NaCl and the suspensions centrifuged. The pellets were resuspended in KCl at the appropriate concentration. The results are given in Tables 9 and 10. The results of light microscopy studies of crystal violet-stained cells after 1 hr in KCl solutions are presented in Tables 11 and 12.

The ability of KCl to maintain optical densities of cells suspensions is strikingly different, depending on whether cells from 1.0 M NaCl or 0.05 M $MgCl_2$ are used. Following $MgCl_2$ exposure, 0.5 M and 1.0 M KCl were more effective in maintaining cells than any other molarity of KCl or distilled water. Actually, the optical density decreases in 0.1 M, 0.05 M, or 0.01 M KCl suspensions were greater than that in distilled water. This might be an indication that KCl itself plays some role in lysis.

Table 9

Optical densities of cell suspensions of Pseudomonas c-Al in solutions of KCl at various concentrations after exposure to 0.05 M $MgCl_2$

Exposure time (25 C)	Optical density (520 mμ)						
	Molarity of KCl					Controls	
	1.0	0.5	0.1	0.05	0.01	H_2O	0.05 M $MgCl_2$
Initial[1]	0.70	0.70	0.70	0.70	0.70	0.70	0.70
30 sec	0.70	0.70	0.63	0.56	0.49	0.56	0.69
10 min	0.68	0.68	0.58	0.48	0.46	----	0.70
30 min	0.64	0.64	0.50	0.43	0.40	----	0.70
60 min	0.62	0.62	0.43	0.39	0.34	0.56	0.68

[1]Initial optical density was that of the third wash suspension.

Table 10

Optical densities of cell suspensions of Pseudomonas c-Al in solutions of KCl at various concentrations after exposure to 1.0 M NaCl

Exposure time (25 C)	Optical density (520 mμ)						
	Molarity of KCl					Controls	
	1.0	0.5	0.1	0.05	0.01	H_2O	0.05 M $MgCl_2$
Initial[1]	0.68	0.68	0.68	0.68	0.68	0.68	0.68
30 sec	0.58	0.35	0.21	0.20	0.16	0.12	0.69
10 min	0.40	0.27	0.15	0.15	0.12	0.10	0.69
30 min	0.38	0.26	0.15	0.14	0.11	0.09	0.70
60 min	0.37	0.22	0.13	0.11	0.09	0.07	0.67

[1]Initial optical density was that of the third wash suspension.

Table 11

Appearance of Pseudomonas c-Al transferred from 1.0 M NaCl to various concentrations of KCl

Suspending medium (25 C)	
1.0 M KCl	Mostly amorphous debris; some darkly stained rods.
0.5, 0.1, 0.05, and 0.01 M KCl	All debris.
Distilled water	All debris.
1.0 M NaCl	All dark normal rods.

Table 12

Appearance of Pseudomonas c-Al transferred from 0.05 M $MgCl_2$ to various concentrations of KCl

Suspending medium (25 C)	Description
1.0 M KCl	Mostly debris; few darkly stained rods.
0.5, 0.1, and 0.05 M KCl	Ghosts and debris; few darkly stained rods.
0.01 M KCl	Mostly moderately stained rods with some scattered debris.
Distilled water	Moderately dark, normal rods.
0.05 M $MgCl_2$	All dark normal rods.

Cells from 1.0 M NaCl solutions underwent lysis in all KCl molarities, indicating that KCl cannot replace NaCl in maintaining cells. Furthermore, the decrease in optical density was greatest in the distilled water suspension. The ability of KCl to prevent optical density decreases after 1.0 M NaCl exposure was proportional to the concentration of KCl present.

In light microscope studies, normal cells stain darkly and the stain is retained when slides are rinsed in water. However, damaged cells do not retain the stain and appear as very lightly stained "ghosts". Debris refers to amorphous material on the slide assumed to be material released from within cells or from cell envelopes.

Such studies of cells in KCl after $MgCl_2$ exposure (Table 12) show that at no KCl molarity are cells completely protected against lysis. There were no suspensions in which stained cells were completely absent. Unexpectedly, of the cells in test suspensions, those in distilled water appeared most normal. In this suspension there were no ghosts or debris. Cells in KCl after 1.0 M NaCl were strikingly different from those described for $MgCl_2$ pre-exposed cells above. The only test suspension with rods present was 1.0 M KCl, although even this suspension consisted of mostly debris. No other KCl suspensions, as well as that in distilled water, contained evidence of whole cells or ghosts.

The findings of this experiment were the first indication that cells might be preconditioned to lysis in different ways

depending on the salt to which they were pre-exposed.

IV. The ability of LiCl and NaCl at 0.5 M to maintain optical densities of cell suspensions of Pseudomonas c-Al following transfer from 0.05 M $MgCl_2$.

The purpose of this experiment was to compare the ability of LiCl to that of NaCl in maintaining optical densities of cell suspensions. From the results presented in Table 13, it is apparent that the optical densities, and presumably the integrity, of both NaCl and LiCl suspensions were maintained equally well for 2 hr.

E. The ability of solutions of 1.0 M NaCl, 0.5 M NaCl, 0.05 M $MgCl_2$, and sea water to maintain viability of Pseudomonas c-Al.

Data from this experiment are plotted in Figure 7; it is evident that viability of c-Al was maintained relatively constant over 3 hr in all the solutions tested.

F. Studies on cell lysis of c-Al and 121 in various salts, sugars, and distilled water at 25 C.

I. Transfer of c-Al and 121 from solutions containing NaCl or $MgCl_2$ to distilled water.

In Figures 5 and 6, striking differences were apparent between the ability of NaCl and $MgCl_2$ to maintain optical densities of cell suspensions of c-Al. The suspensions of c-Al in 0.02 M and 0.01 M NaCl underwent a greater decrease in optical density than did cells in distilled water. This suggested that it was not simply the lack of sufficient NaCl to maintain cell integrity, but that NaCl in low concentra-

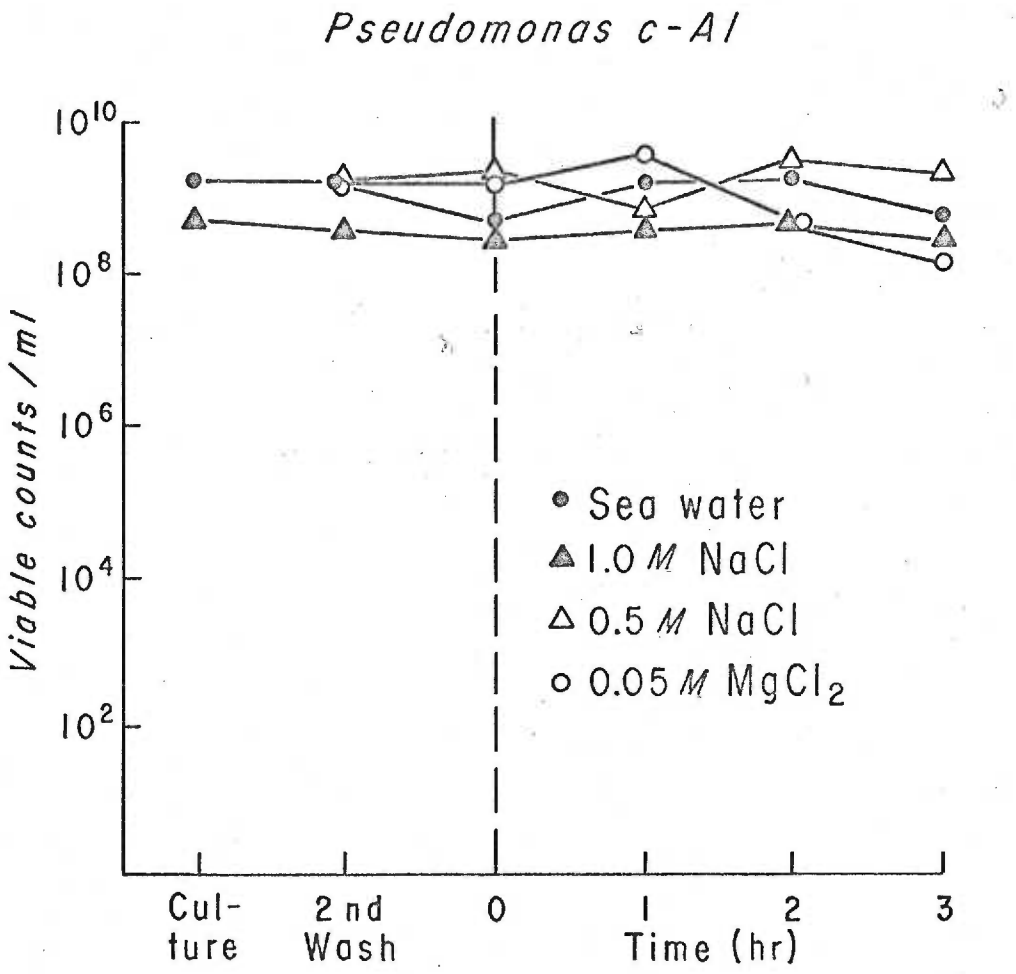
Table 13

Optical densities of Pseudomonas c-Al in 0.5 M LiCl and 0.5 M NaCl after exposure of cells to 0.05 M $MgCl_2$

Exposure time (25 C)	LiCl	NaCl
Initial[1]	0.68	0.69
30 sec	0.62	0.63
30 min	0.61	0.60
60 min	0.62	0.62
120 min	0.62	0.62

[1] Initial optical density was that of the third wash suspension.

Figure 7. Viable counts of Pseudomonas c-Al over a period of 3 hr in 1.0 M NaCl, 0.5 M NaCl, and 0.05 M $MgCl_2$ (25 C). Each point on the graph represents the mean of triplicate plate counts.

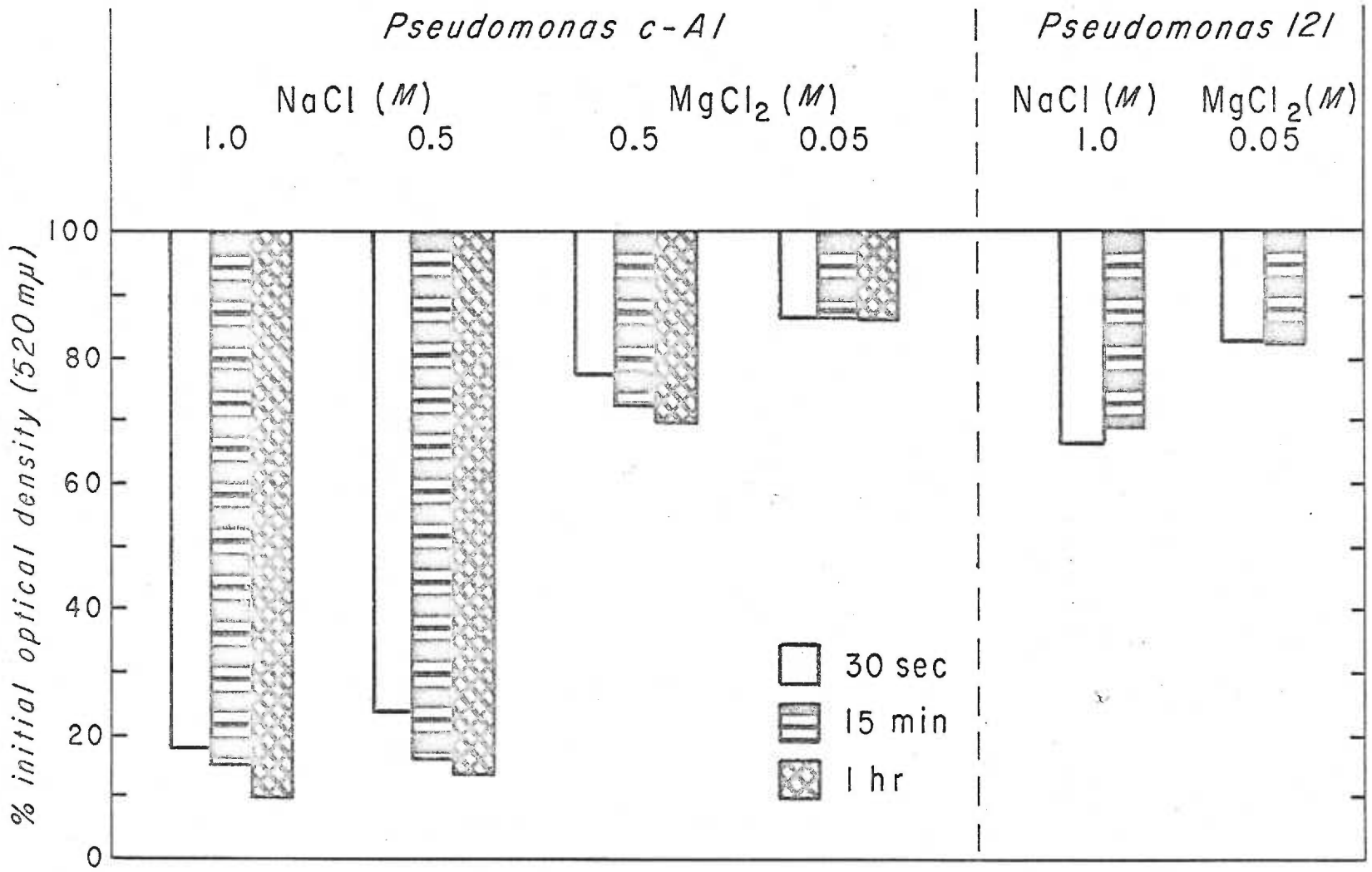

Pseudomonas c-Al
Viable counts / ml
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
Sea water
1.0 M NaCl
0.5 M NaCl
0.05 M $MgCl_2$
Cul-
ture
2 nd
Wash
0
1
2
3
Time (hr)

tions played an active role in lysis. A similar phenomenon was noted for KCl (Table 9). It was possible that Na^+ had some effect on the envelope of the cell which Mg^{++} did not, and that this Na^+-produced effect might influence the lytic pattern in distilled water, i.e. make cells more susceptible to lysis. To test this possibility, cells of c-Al were washed in different salt solutions of 1.0 M NaCl, 0.5 M NaCl, 0.5 M $MgCl_2$ or 0.05 M $MgCl_2$ as described in the Materials and Methods (C. III.). These cells were then resuspended in distilled water and optical densities of suspensions were recorded at 30 sec and periodically for one hour. The same procedure was followed for Pseudomonas 121 using 1.0 M NaCl and 0.05 M $MgCl_2$ only. The results of these experiments are presented in Figure 8. The optical density changes observed for c-Al transferred from NaCl or from $MgCl_2$ to distilled water were very different, even when equimolar concentrations of the salts were used. Equally striking was the observation that nearly all the maximum decreases in optical density had occurred by 30 sec.

Results from this experiment employing 121 were quite different from those of c-Al. The NaCl-treated 121 experienced smaller decreases in optical densities than did c-Al from NaCl solutions. In contrast, 121 transferred from $MgCl_2$ to distilled water underwent approximately the same optical density decrease in distilled water as did c-Al.

There are several possibilities that might account for the results obtained from the experiments involving c-Al:

Figure 8. The effect of distilled water (25 C) on Pseudomonas strains 121 and c-Al following exposure of the cells to 1.0 M NaCl, 0.5 M NaCl, 0.5 M $MgCl_2$, or 0.05 M $MgCl_2$. Optical densities determined at 30 sec, 15 min, and 1 hr are expressed as percent of initial optical density (520 mμ).


Pseudomonas c-A1
Pseudomonas 121
NaCl (M)
1.0
0.5
MgCl2 (M)
0.5
0.05
NaCl (M)
1.0
MgCl2 (M)
0.05
% initial optical density (520 mμ)
100
80
60
40
20
0
30 sec
15 min
1 hr

(a) exposure of c-Al to NaCl may in some way cause the cells to become structurally weakened and, therefore, more susceptible to lysis in distilled water than does exposure to $MgCl_2$; (b) the differences observed in suspensions of c-Al may have been due to osmotic factors, in which case one must then assume that NaCl gets into cells in greater amounts than does $MgCl_2$; (c) another possibility is that both (a) and (b), above are similtaneously involved during lysis.

The differences between patterns observed for c-Al and 121 could be accounted for by a more rigid envelope in 121 or an envelope in which NaCl does not affect the cells in a way that would cause them to become more susceptible to lysis in distilled water. If NaCl does not affect the lytic susceptibility of 121 by weakening structural components, then the optical density differences observed as a result of the two salts could be due to osmotic factors, i.e. to the hydrostatic pressure resulting from the entry of water into the cells upon suspension in distilled water.

The following experiments were designed to test possible mechanisms involved during lysis of Pseudomonas c-Al in low ionic environments.

II. Transfer of c-Al from 0.05 M $MgCl_2$ to various concentrations of NaCl or to distilled water.

It is believed that Mg^{++} functions as a bridge between anionic groups in membranes(110). If the role of Mg^{++} in the envelope of c-Al is that of bridging between anionic groups, such as phosphoryl head groups of phospholipids or carboxyl

groups of protein anionic side chains, then such bridges would be broken if the Mg^{++} were replaced with a monovalent cation, such as Na^{+}. Replacing Mg^{++} with monovalent cations would maintain the shielding of the negative charges preventing repulsion of the anionic groups. However, even though the integrity might be maintained in such a situation, the structure would be weakened due to a lack of bridges.

If the phosphoryl and/or carboxyl groups are bridged by Mg^{++}, then one would not expect Mg^{++} could be removed by distilled water, based on the solubility of magnesium phosphates or carbonates. An experiment was designed to determine whether Na^{+} in low concentrations was more effective than distilled water in lysing cells after their exposure to $MgCl_2$.

Several portions of cells of c-Al were washed three times in 0.05 M $MgCl_2$ with the third wash suspensions being adjusted to the desired optical density at 520 mμ. The suspensions were centrifuged, supernates discarded, and the pellets resuspended in NaCl or distilled water to the same volume as the wash suspension. Optical densities were recorded at 30 sec, and periodically thereafter for 4 hr (Figure 9), after which suspensions were centrifuged and ultraviolet absorption spectra (Figure 10) run on the supernates. The cells from the pellet were observed in the light microscope after staining with crystal violet.

Several important observations of this experiment were:

Figure 9. The effect of distilled water, 0.05 M $MgCl_2$, or various concentrations of NaCl (0.005 to 1.0 M) at 25 C on Pseudomonas c-Al following exposure of these cells to 0.05 M $MgCl_2$. Optical densities determined at 30 sec, 1 hr, and 4 hr are expressed as decreases in optical density (520 mμ).

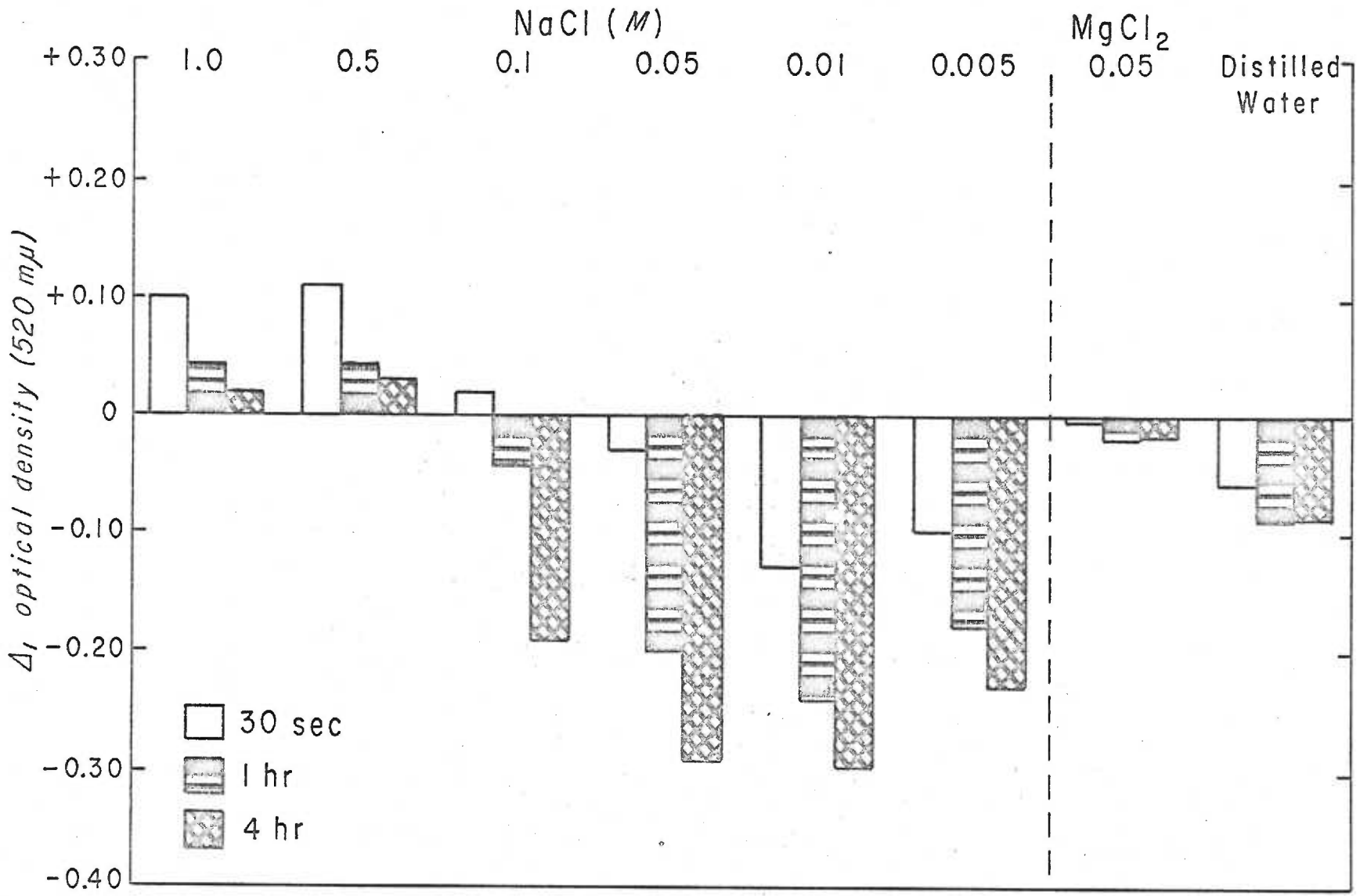
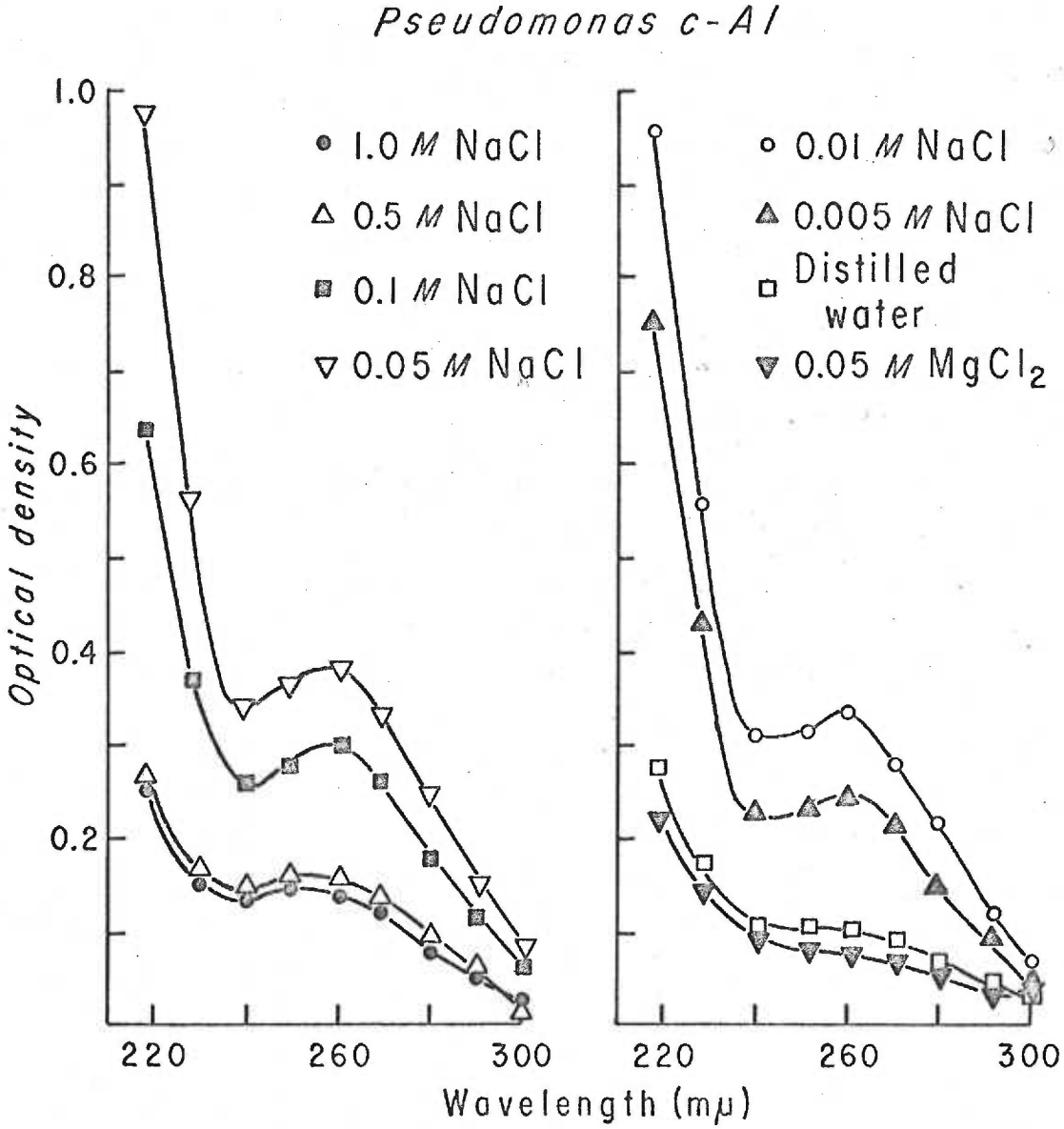

NaCl (M)
MgCl2
1.0
0.5
0.1
0.05
0.01
0.005
0.05
Distilled Water
+0.30
+0.20
+0.10
0
-0.10
-0.20
-0.30
-0.40
Δt optical density (520 mμ)
30 sec
1 hr
4 hr

Figure 10. Ultraviolet absorption spectra of supernates from Pseudomonas c-Al transferred from 0.05 M $MgCl_2$ to various concentrations of NaCl (1.0 M, 0.5 M, 0.1 M, 0.05 M, 0.01 M, 0.005 M), distilled water, and 0.05 M $MgCl_2$ and held for 4 hr at 25 C.


Pseudomonas c-Al
1.0
0.8
0.6
0.4
0.2
Optical density
1.0 M NaCl
0.5 M NaCl
0.1 M NaCl
0.05 M NaCl
0.01 M NaCl
0.005 M NaCl
Distilled water
0.05 M MgCl2
220
260
300
220
260
300
Wavelength (mμ)

(a) the optical density change during slow-stage lysis in distilled water was considerably less than observed when cells were transferred from NaCl to distilled water (see also Figure 8); (b) increases in optical density of cell suspensions in 1.0 and 0.5 M NaCl were observed - probably due to physical shrinkage of the cells - decreasing almost to normal during the time of the experiment; (c) optical density decrease was considerably greater in 0.05 and 0.01 M NaCl than in distilled water, indicating that Na^+ may play an active role in lysing cells of c-Al; (d) cells transferred from 0.05 M $MgCl_2$ to distilled water exhibit very little optical density change, indicating that Mg^{++} may remain bound in the envelope (see also Figure 8).

The ultraviolet absorption spectra (Figure 10) were as expected with absorption peaks at 260 mμ. Supernates from the suspensions that underwent the greatest decreases in optical density had the greatest absorption peaks, indicating that lysis had indeed occurred in these suspensions.

Observations of crystal violet-stained cells from these suspensions also indicated that the optical density decreases were good indications of lysis. The results of these observations presented in Table 14 led to the proposal of a working hypothesis for the role of Mg^{++} and its interaction with Na^+ in the envelopes of c-Al.

Working hypothesis: Mg^{++} acts as a bridge between anionic groups of essential structural components in the envelope. The bond formed between Mg^{++} and these anionic

Table 14

Appearance of Pseudomonas c-Al transferred from 0.05 M $MgCl_2$ to various concentrations of NaCl

Suspending medium (25 C)	Description
1.0 M NaCl	Darkly stained, normal morphology.
0.5 M NaCl	Darkly stained, normal morphology.
0.1 M NaCl	Moderately stained, normal morphology.
0.05 M NaCl	Very lightly stained ghosts.
0.01 M NaCl	Ghosts and scattered debris.
0.005 M NaCl	Ghosts and scattered debris.
Distilled water	Moderately dark cells with no ghosts.
0.05 M $MgCl_2$	Darkly stained, normal morphology.

groups is covalent in nature and therefore, resistant to attack by water. Although water does not have the force necessary to remove the Mg^{++}, some monovalent cations such as Na^{+} will displace Mg^{++}, breaking these bridges. The anionic groups, however, remain shielded, preventing mutual repulsion. Water has sufficient force to remove the Na^{+} ions, causing the anions to repel each other, as a result of which the envelope undergoes disintegration.

III. Transfer of c-Al or 121 from solutions of NaCl, $MgCl_2$ or $MgCl_2$ plus various concentrations of NaCl, to distilled water.

If the working hypothesis is valid, then exposure of cells to solutions of NaCl should condition cells of c-Al so that they would be more susceptible to lysis than cells exposed to $MgCl_2$ only. Furthermore, comparison of the sensitivity to lysis after the exposure of cells to $MgCl_2$ plus various concentrations of NaCl should indicate the amount of antagonism between Na^{+} and Mg^{++} in the envelope.

To test this possibility, cells were harvested from the growth medium, divided into several portions and each portion washed three times in one of the following solutions according to the procedure described previously in Materials and Methods C III: 0.05 $MgCl_2$ plus NaCl (0 to 1.0 M). Following the third wash, cells were suspended in the various wash solutions, the optical densities adjusted to approximately 0.74, and the suspensions centrifuged. The pellets were

resuspended in distilled water to the same volume as the wash solution. Optical densities were recorded at 30 sec to determine optical density change associated with fast-stage lysis. These results are presented in Figure 11.

Following the 30 sec optical density determination, suspensions were centrifuged, and ultraviolet absorption spectra (220-300 mμ) were carried out on the supernates. The supernates were also tested for the presence of ninhydrin-positive material as described in Materials and Methods, C. III. The ultraviolet absorption spectra and intensity of the ninhydrin reactions are presented in Figure 12.

The results of this experiment were consistent with the hypothesis: the decreases in optical density during fast-stage lysis were directly related to the concentration of NaCl to which the cells of c-Al were pre-exposed. The amount of ultraviolet absorbing material and ninhydrin-positive material present in the supernates of the lysed cells, was proportional to the optical density decrease observed in distilled water.

The same procedure was followed employing Pseudomonas 121, except that ninhydrin tests on spotted supernate samples were omitted. Cells were also transferred from 1.0 M NaCl to distilled water. These results are presented in Figures 13 and 14. Microscopic examination of these cell suspensions in distilled water indicated that the changes in optical density were not due to clumping of cells of either 121 or c-Al.

Figure 11. The effect of distilled water (25 C) on Pseudomonas c-Al following exposure of cells to various concentrations of NaCl (0 to 1.0 M) plus 0.05 M $MgCl_2$. Optical densities were determined at 30 sec, and are expressed as percent of initial optical density (520 mμ). The percent of initial optical density for cells washed in 0.05 M $MgCl_2$ only, and subsequently resuspended in distilled water, was 78%.

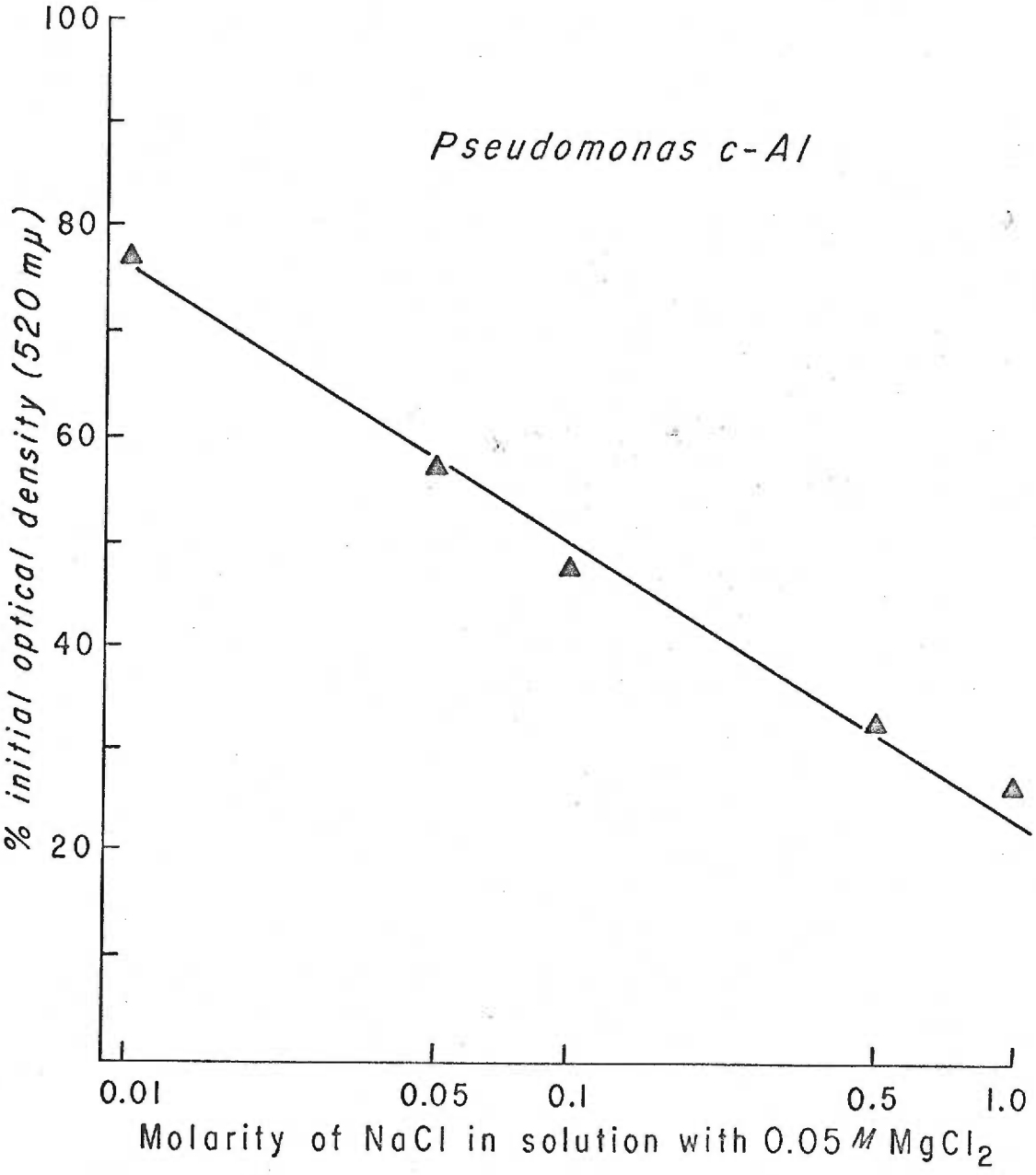

Pseudomonas c-Al
100
80
60
40
20
% initial optical density (520 mμ)
0.01
0.05
0.1
0.5
1.0
Molarity of NaCl in solution with 0.05 M MgCl2

Figure 12. Ultraviolet absorption spectra of supernates from Pseudomonas c-Al suspended in distilled water (25 C) following exposure to 0.05 M $MgCl_2$ plus various concentrations of NaCl (0 to 1.0 M). The intensities of the ninhydrin reactions are shown as (1+) to (4+).

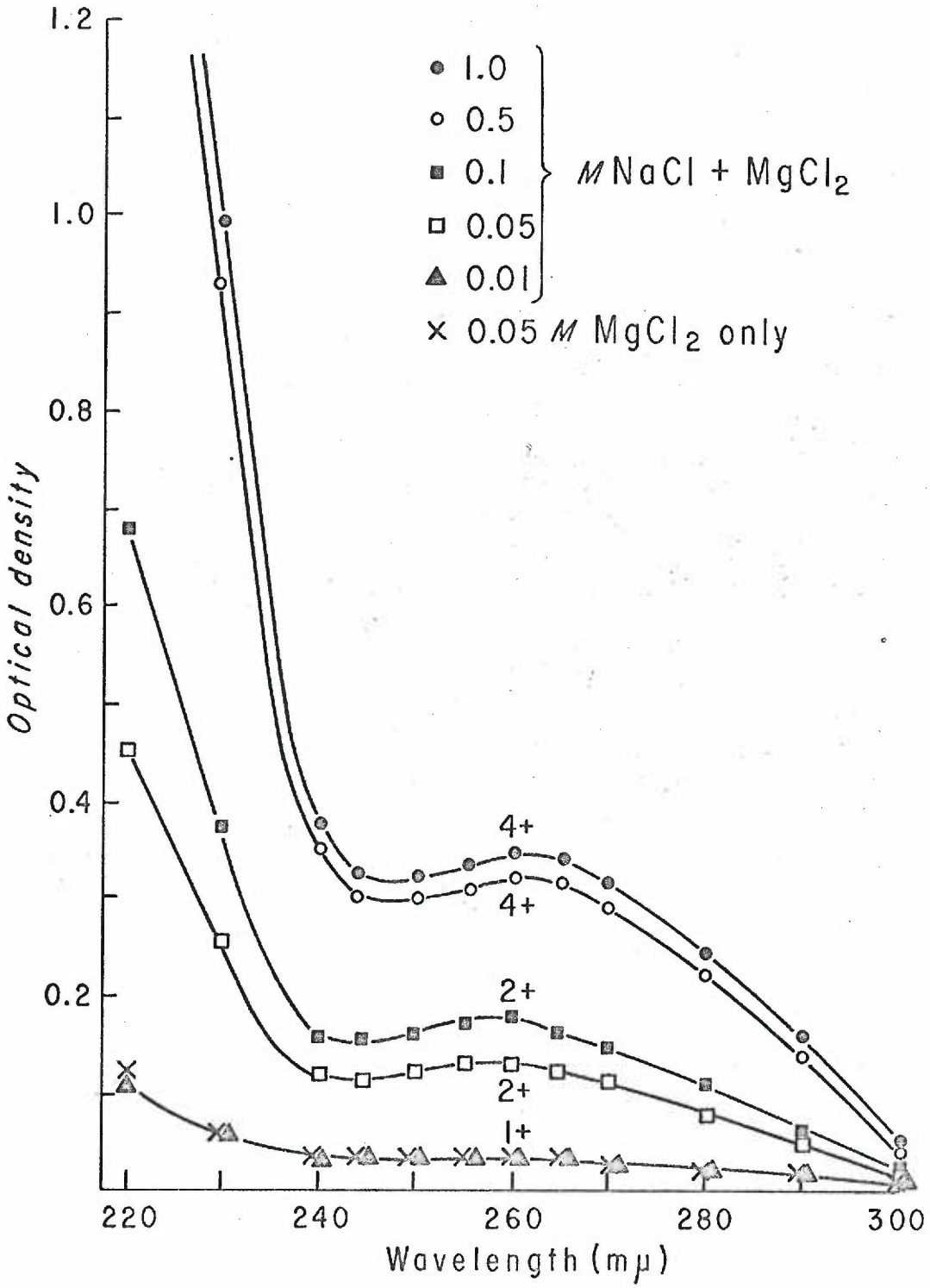

1.2
1.0
0.8
0.6
0.4
0.2
Optical density
1.0
0.5
0.1
0.05
0.01
M NaCl + MgCl2
0.05 M MgCl2 only
4+
4+
2+
2+
1+
220
240
260
280
300
Wavelength (mμ)

Figure 13. The effect of distilled water (25 C) on Pseudomonas 121 following exposure of cells to various concentrations of NaCl (0 to 1.0 M) plus 0.05 M $MgCl_2$. Optical densities determined at 30 sec are expressed as percent of initial optical density (520 mμ). The percent of initial optical density for cells washed in 0.05 M $MgCl_2$ alone and subsequently resuspended in distilled water was 82%, and from 1.0 M NaCl alone to distilled water was 66%.

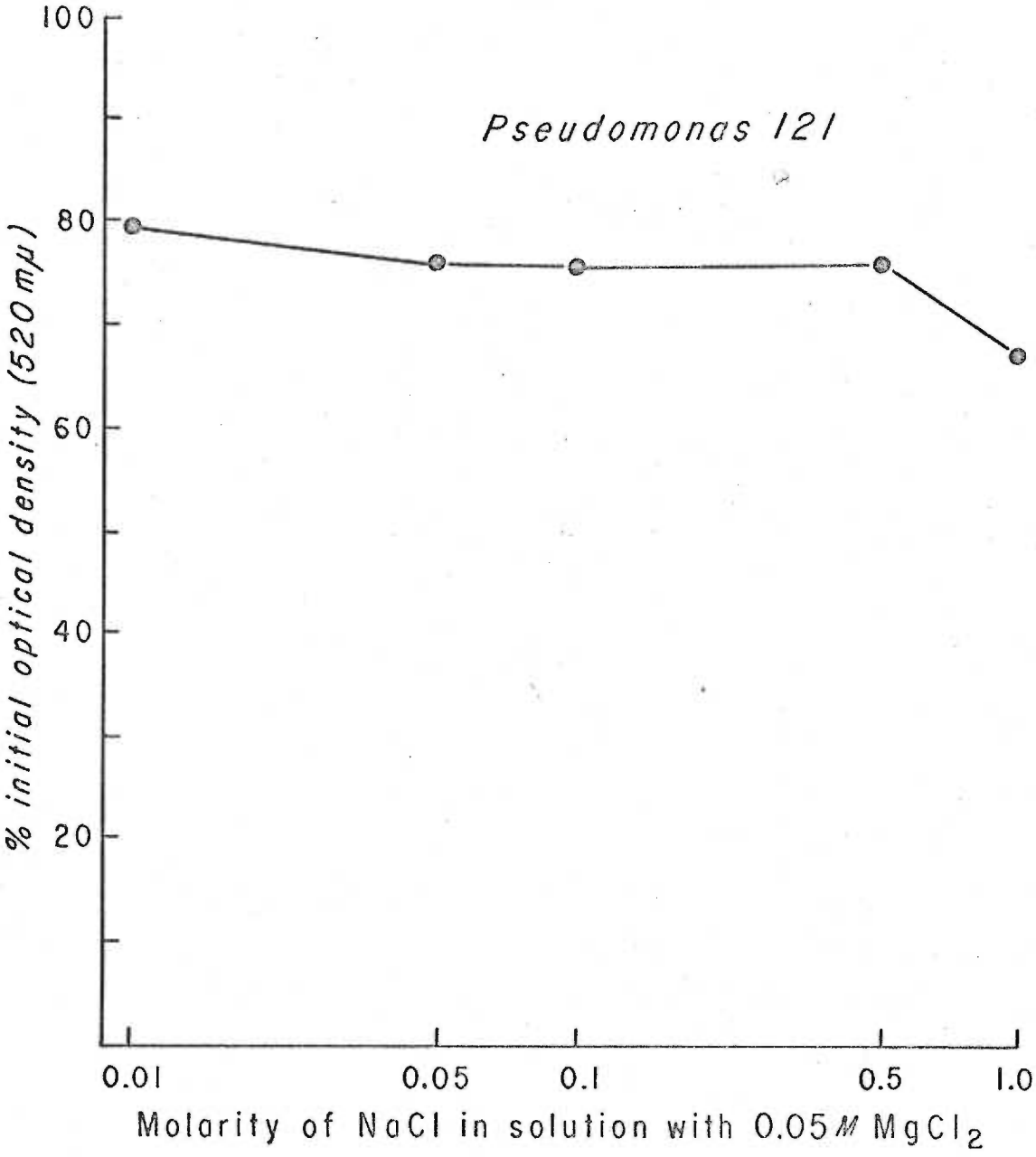
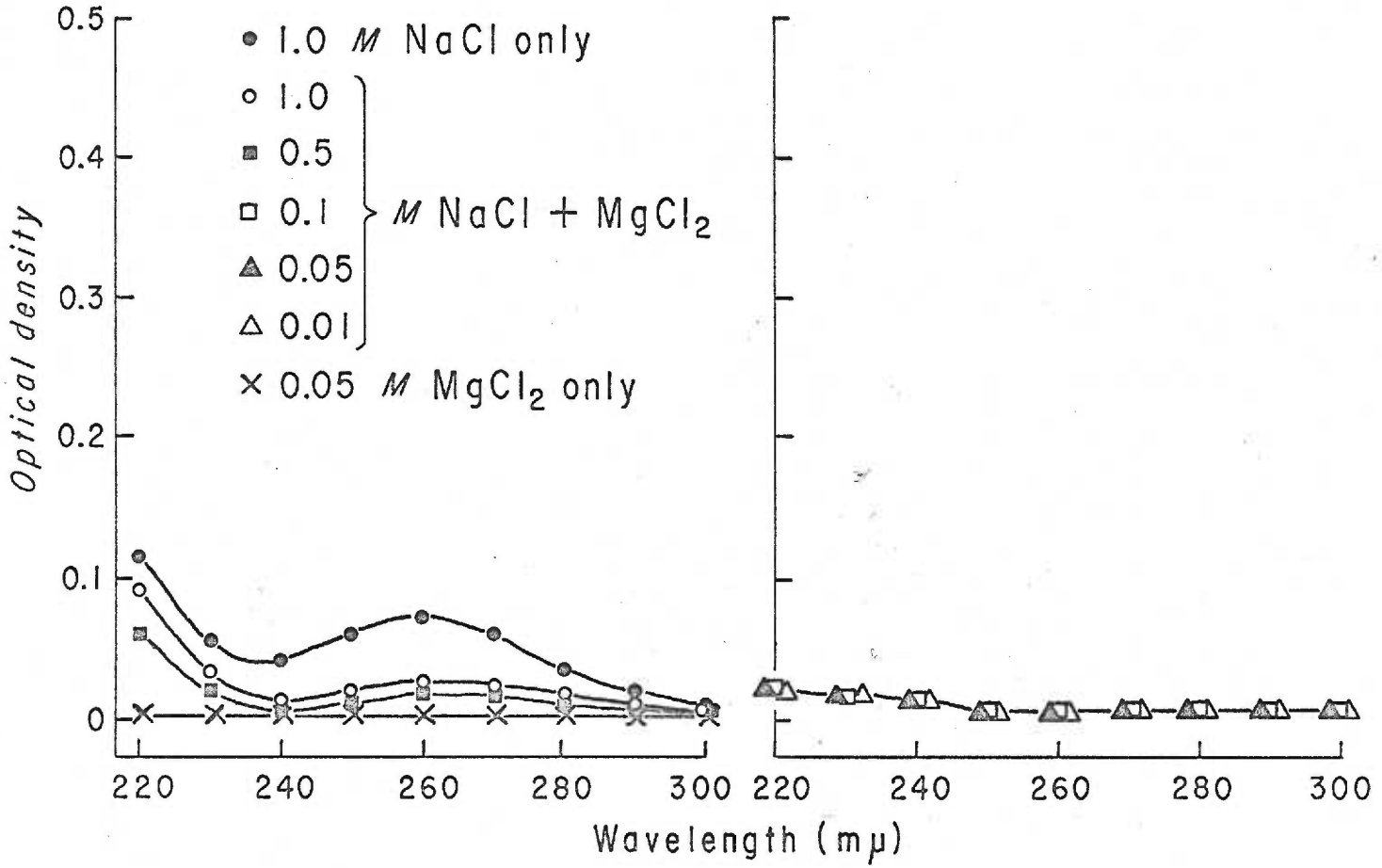

Pseudomonas 121
100
80
60
40
20
% initial optical density (520 mμ)
0.01
0.05
0.1
0.5
1.0
Molarity of NaCl in solution with 0.05 M MgCl2

Figure 14. Ultraviolet absorption spectra of supernates from Pseudomonas 121 suspended in distilled water (25 C) following exposure to 0.05 M $MgCl_2$ plus various concentrations of NaCl (0 to 1.0 M), or 1.0 M NaCl alone.


0.5
0.4
0.3
0.2
0.1
0
Optical density
1.0 M NaCl only
1.0
0.5
0.1
0.05
0.01
M NaCl + MgCl2
0.05 M MgCl2 only
220
240
260
280
300
220
240
260
280
300
Wavelength (mμ)

To determine the extent of disruption of c-Al and 121 following distilled water treatment, pellets of each were fixed, embedded, sectioned, and observed in the electron microscope. These results are presented and discussed later in a separate section devoted to electron microscopy.

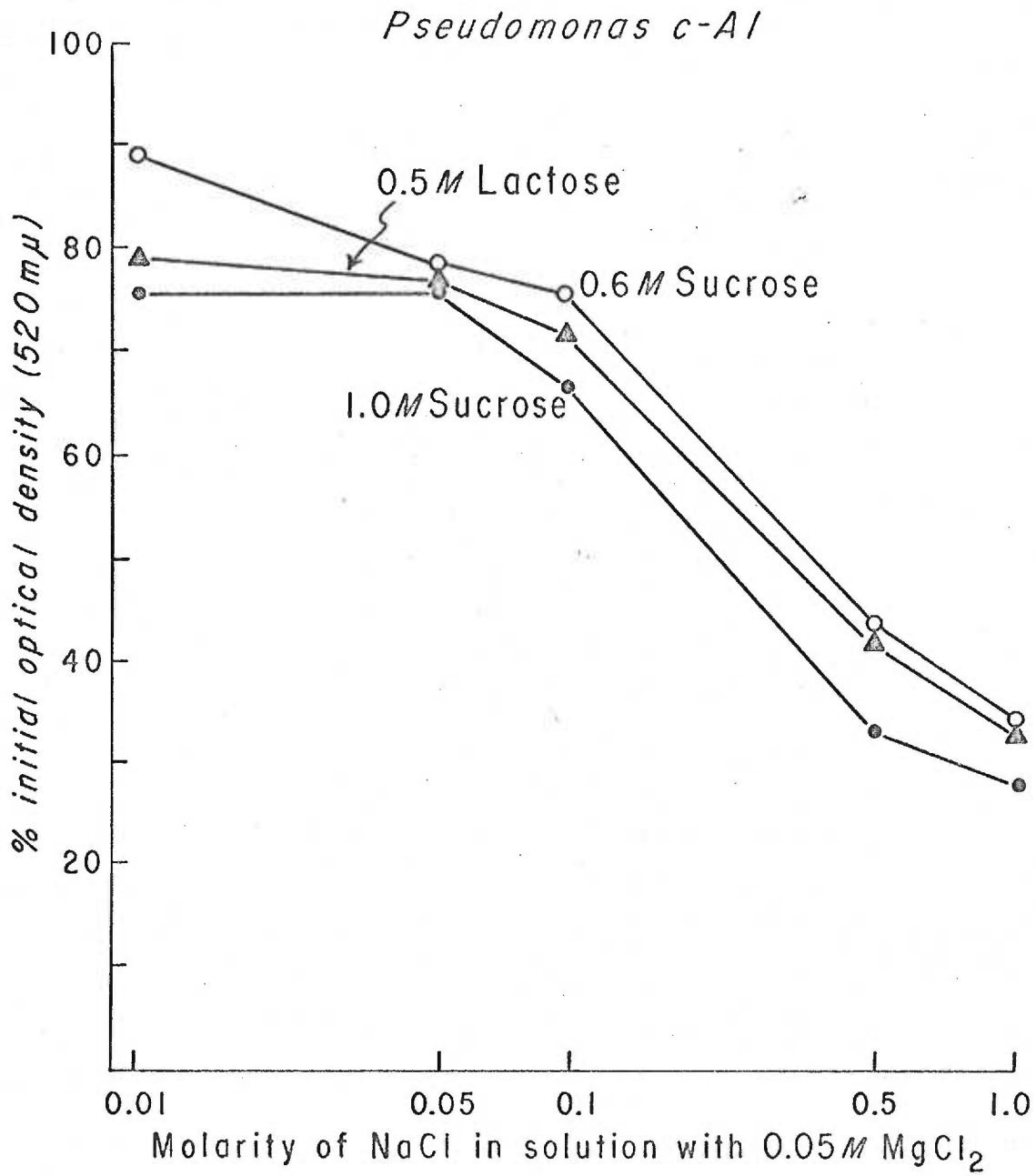
From these experiments, it appears that the terrestrial bacterium is protected against sudden osmotic changes that result in the lysis of Pseudomonas c-Al. Since the main objective of this study was the characterization of the behavior of the marine organism in low ionic environments, a further study of the terrestrial bacterium in these environments was not pursued.

IV. Transfer of c-Al to sucrose or lactose following exposure of cells to $MgCl_2$ or $MgCl_2$ plus various concentrations of NaCl.

The results of the previous experiment (F. III.) employing c-Al can be interpreted either in terms of the working hypothesis or in terms of lysis due to osmotic forces. If NaCl goes in and out of the cell freely, then one would expect the internal concentration to be similar to that of the external medium. Therefore, when cells are transferred to distilled water, the water moves into the cells causing them to burst.

To determine whether the lysis of c-Al was osmotic, the procedure for the previous experiment (F. III.) was employed but substituting 1.0 M sucrose, 0.6 M sucrose, or 0.5 M lactose for distilled water. The results are presented in Figure 15.

Figure 15. The effect of 1.0 M sucrose, 0.6 M sucrose, or 0.5 M lactose (25 C) on Pseudomonas c-Al following exposure of cells to various concentrations of NaCl (0 to 1.0 M) plus 0.05 M $MgCl_2$. Optical densities determined at 30 sec are expressed as percent of initial optical density (520 mμ). The percent of initial optical desnity for cells washed in 0.05 M $MgCl_2$ only, and subsequently resuspended in the appropriate sugar, was 80%, 88%, and 79%, respectively.


Pseudomonas c-Al
100
80
60
40
20
% initial optical density (520 mμ)
0.5 M Lactose
0.6 M Sucrose
1.0 M Sucrose
0.01
0.05
0.1
0.5
1.0
Molarity of NaCl in solution with 0.05 M MgCl2

These results are not entirely like those employing distilled water. However, it becomes immediately apparent that this lysis is not solely due to osmotic forces, although cells exposed to NaCl at concentrations of 0.1 M or less are protected considerably by the sugars.

Therefore, these results suggest that osmotic forces are probably involved in the lysis of c-Al in distilled water, but the primary factor in lysis is probably a loss of ions necessary to maintain an intact envelope.

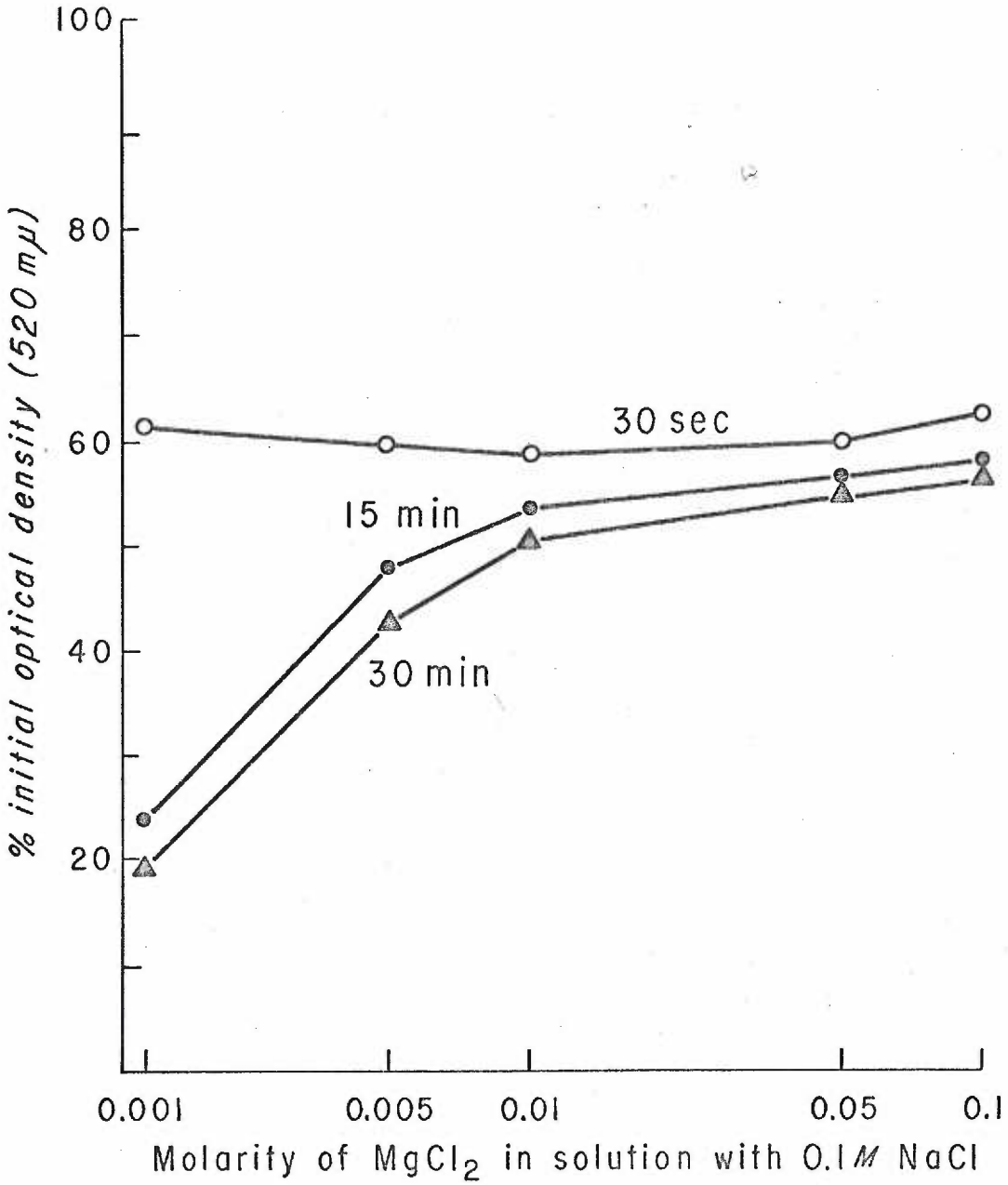
V. Transfer of Pseudomonas c-Al from NaCl plus various concentrations of $MgCl_2$ to distilled water.

The decrease in optical density, and presumably the degree of disruption to cells during lysis in distilled water, was directly related to the concentration of NaCl to which the cells were pre-exposed. In order to determine whether this was independent of $MgCl_2$ concentration, the same general procedure was used as before (Results F. III.), except that the wash solutions consisted of 0.1 M NaCl plus $MgCl_2$ at various concentrations ranging from 0.001 M to 0.1 M.

In this experiment optical densities of cell suspensions in distilled water were determined at intervals from 30 sec to 30 min. The results are plotted in Figure 16.

Whereas the decrease in optical density associated with fast-stage lysis is related to the concentration of NaCl to which cells are pre-exposed, such changes during slow-stage lysis are related to the concentration of $MgCl_2$.

Figure 16. The effect of distilled water (25 C) on Pseudomonas c-A1 following exposure of cells to various concentrations of $MgCl_2$ (0.001 to 0.1 M) plus 0.1 M NaCl. Optical densities determined at 30 sec, 15 min, and 30 min are expressed as percent of initial optical density (520 mμ).

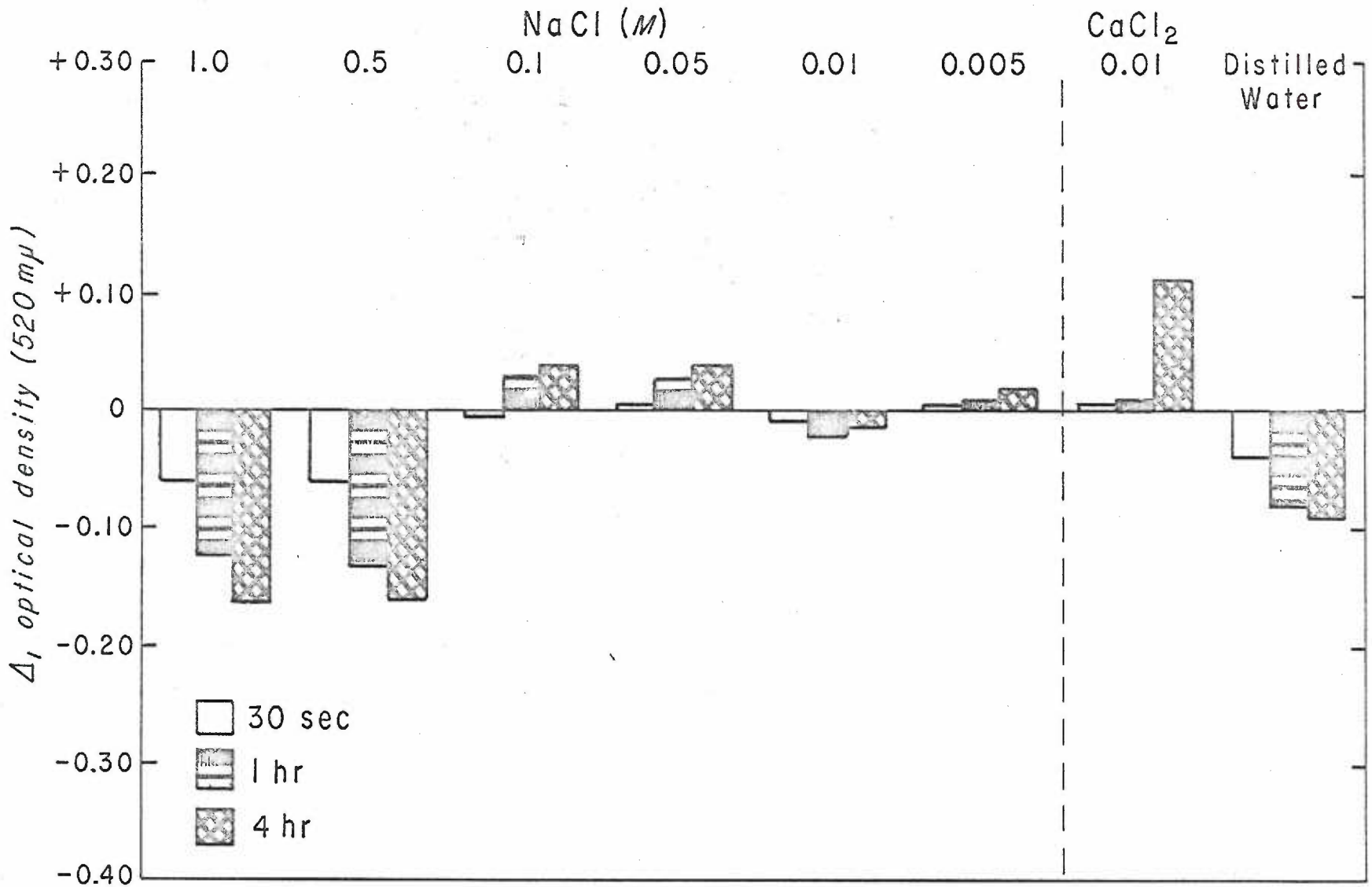

100
80
60
40
20
% initial optical density (520 mμ)
30 sec
15 min
30 min
0.001
0.005
0.01
0.05
0.1
Molarity of MgCl2 in solution with 0.1M NaCl

VI. Transfer of c-Al from 0.01 M $CaCl_2$ to various concentrations of NaCl or distilled water.

The general procedure for this experiment was that used in the experiment above (F. II.), except that 0.01 M $CaCl_2$ (sea water molarity) was substituted for 0.05 M $MgCl_2$. This experiment was carried out to determine whether Ca^{++} at sea water molarity would act in a manner similar to Mg^{++} in the previous experiment. The results of the optical density changes observed for a period of 4 hr are presented in Figure 17. Analyses of ultraviolet absorption spectra, carried out to detect any cell leakage of ultraviolet-absorbing material, indicate that all cells in NaCl or distilled water leaked material absorbing at 260 mμ. However, cells in 0.01 M $CaCl_2$ released material absorbing at 280 mμ, suggesting protein was lost from these cells. Observations of these cells in the light microscope revealed that cells from each suspending medium appeared abnormal, including cells in 0.01 M $CaCl_2$.

The results obtained with Ca^{++} presented in Figure 17 are in direct contrast to those obtained with Mg^{++} presented in Figure 9. It would seem that if Ca^{++} is bound in the envelopes, as proposed for Mg^{++}, then higher concentrations of Na^{+} are required to displace the Ca^{++} than are required to replace the Mg^{++}. The release of 280 mμ absorbing material, from envelope and/or cytoplasm, shows that Ca^{++} did not carry out the functions of Mg^{++} in this cell. As Ca^{++} at sea water molarity was, itself, not able to maintain c-Al,

Figure 17. The effect of distilled water, 0.01 M $CaCl_2$, and various concentrations of NaCl (0.005 to 1.0 M) at 25 C on Pseudomonas c-A1 following exposure to 0.01 M $CaCl_2$. Optical densities determined at 30 sec, 1 hr, and 4 hr are expressed as decreases in optical density (520 mμ).


NaCl (M)
CaCl2
1.0
0.5
0.1
0.05
0.01
0.005
0.01
Distilled Water
+0.30
+0.20
+0.10
0
-0.10
-0.20
-0.30
-0.40
Δ1 optical density (520 mμ)
30 sec
1 hr
4 hr

further studies with this salt were not pursued.

VII. Transfer of Pseudomonas c-Al from $MgCl_2$, or $MgCl_2$ plus various concentrations of LiCl, or KCl, to distilled water.

The results of experiments presented previously show that lower concentrations of KCl do not effectively maintain cells of c-Al after exposure to $MgCl_2$ (Table 9). The proposed negative charges in the envelope may be screened by K^+, but not as efficiently as by Na^+ (Figure 9). On the other hand, K^+ is probably capable of breaking the proposed Mg^{++} bridges, because transfer of cells from $MgCl_2$ to KCl solutions results in slow decreases in optical density, in the lower KCl concentration, that do not occur on transfer of cells from $MgCl_2$ to distilled water (Table 9). LiCl, at 0.5 M, also maintains the optical density of cell suspensions of c-Al (Table 13).

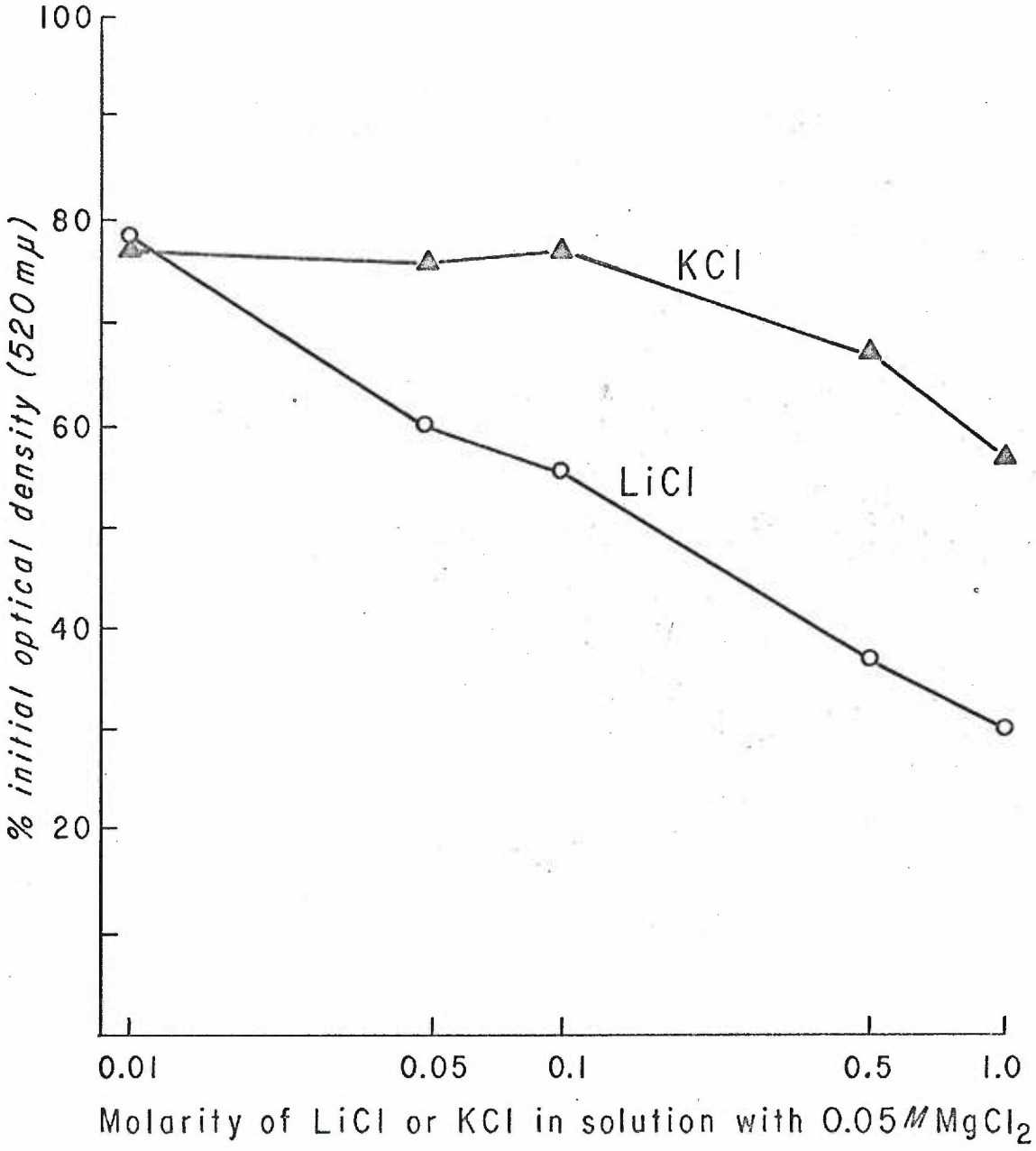
The cations Na^+ and Li^+ are more closely related in terms of ionic radii than are Na^+ and K^+. This difference in Na^+ and K^+ could account for slight differences in the ability of these ions to screen anionic groups in the envelopes. For this reason, one would expect Li^+ to act in a manner similar to Na^+ while K^+ may not. To determine whether Li^+ and K^+ would act like Na^+ in rendering cells susceptible to lysis in distilled water, LiCl and KCl were substituted for NaCl in the procedure described in Materials and Methods, section C. III., and Results, section F. III.

The results of these experiments are plotted in Figure 18. It is apparent that KCl did not render c-Al cells as susceptible to lysis as did LiCl. The results using LiCl were identical to those when NaCl was used in this system. This suggests that KCl may not compete as effectively as Li^+ or Na^+ for the proposed anionic sites occupied by Mg^{++}. Another explanation is that the Mg^{++} may be displaced, but that K^+ may not remain held at the anionic sites.

VIII. Transfer of Pseudomonas c-Al to distilled water following exposure to constant concentrations of Na^+ plus Mg^{++} with varying $SO_4^=$ concentrations.

Although the results in Figure 18 employing KCl and LiCl indicate that Cl^- is not the causative lytic factor, a further attempt was made to rule out Cl^- by substituting $SO_4^=$ in the system. Preliminary experiments not presented indicated that pre-exposure to sulfate protected cells somewhat against lysis in distilled water. However, the protection was only evident when the cation molarity was 0.1 M Na^+ or less. For this reason, an experiment was designed in which cation concentrations of suspending solutions were held constant at either 0.1 M Na^+ plus 0.05 M Mg^{++}, or 0.5 M Na^+ plus 0.05 M Mg^{++}. The $SO_4^=$ concentration in each case was varied from 0-0.1 M, and 0-0.3 M, respectively. As $SO_4^=$ concentrations were varied, Cl^- was used to maintain anions in the system.

Figure 18. The effect of distilled water (25 C) on Pseudomonas c-Al following exposure of cells to various concentrations of LiCl or KCl (0 to 1.0 M) plus 0.05 M $MgCl_2$. Optical densities determined at 30 sec are expressed as percent of initial optical density (520 mμ). The percent of initial optical density for cells washed in 0.05 M $MgCl_2$ alone, and subsequently resuspended in distilled water, was 80%.


100
80
60
40
20
% initial optical density (520 mμ)
KCl
LiCl
0.01
0.05
0.1
0.5
1.0
Molarity of LiCl or KCl in solution with 0.05 M MgCl2

The procedure employed was to wash batches of c-Al cells in solutions containing different concentrations of $SO_4^=$ as described above. Following the exposure of cells to various concentrations of $SO_4^=$, the cells were suspended in distilled water and optical densities recorded at 30 sec. The results are plotted in Figure 19.

As noted in the preliminary experiments, pre-exposure to $SO_4^=$ with Na^+ at 0.1 M protects against lysis, and the degree of protection is directly related to the concentration of $SO_4^=$ present. However, at the cation concentration of 0.5 M Na^+, $SO_4^=$ does not provide any protective advantage during lysis at any concentration tested.

IX. Transfer of Pseudomonas c-Al cells from 0.1 M NaCl plus 0.05 M $MgCl_2$ to distilled water at various temperatures from 2 C to 90 C.

If the lysis of c-Al in distilled water is the result of electrostatic interactions between cations, anionic groups in the envelope, and distilled water, then raising the temperature of the distilled water during lysis should result in a greater decrease in optical density. To demonstrate the effects of temperature on lysis, cells of c-Al were washed three times in 0.1 M NaCl plus 0.05 M $MgCl_2$ and then transferred to distilled water maintained at various temperatures. Optical densities were recorded at 30 sec. The results of this experiment are plotted in Figure 20.

These findings are consistent with the proposed relationship between increased temperature and increased lysis as

Figure 19. The effect of distilled water (25 C) on Pseudomonas c-Al following exposure of cells to 0.05 M Mg^{++} plus 0.1 M Na^{+} or 0.5 M Na^{+}, with varying concentrations of $SO_4^{=}$. The $SO_4^{=}$ concentrations were varied from 0 to 0.1 M, with respect to 0.1 M Na^{+}, and 0 to 0.3 M, with respect to 0.5 M Na^{+}, with the remainder of anion as Cl^{-}. Optical densities determined at 30 sec are expressed as percent of initial optical density (520 mμ).

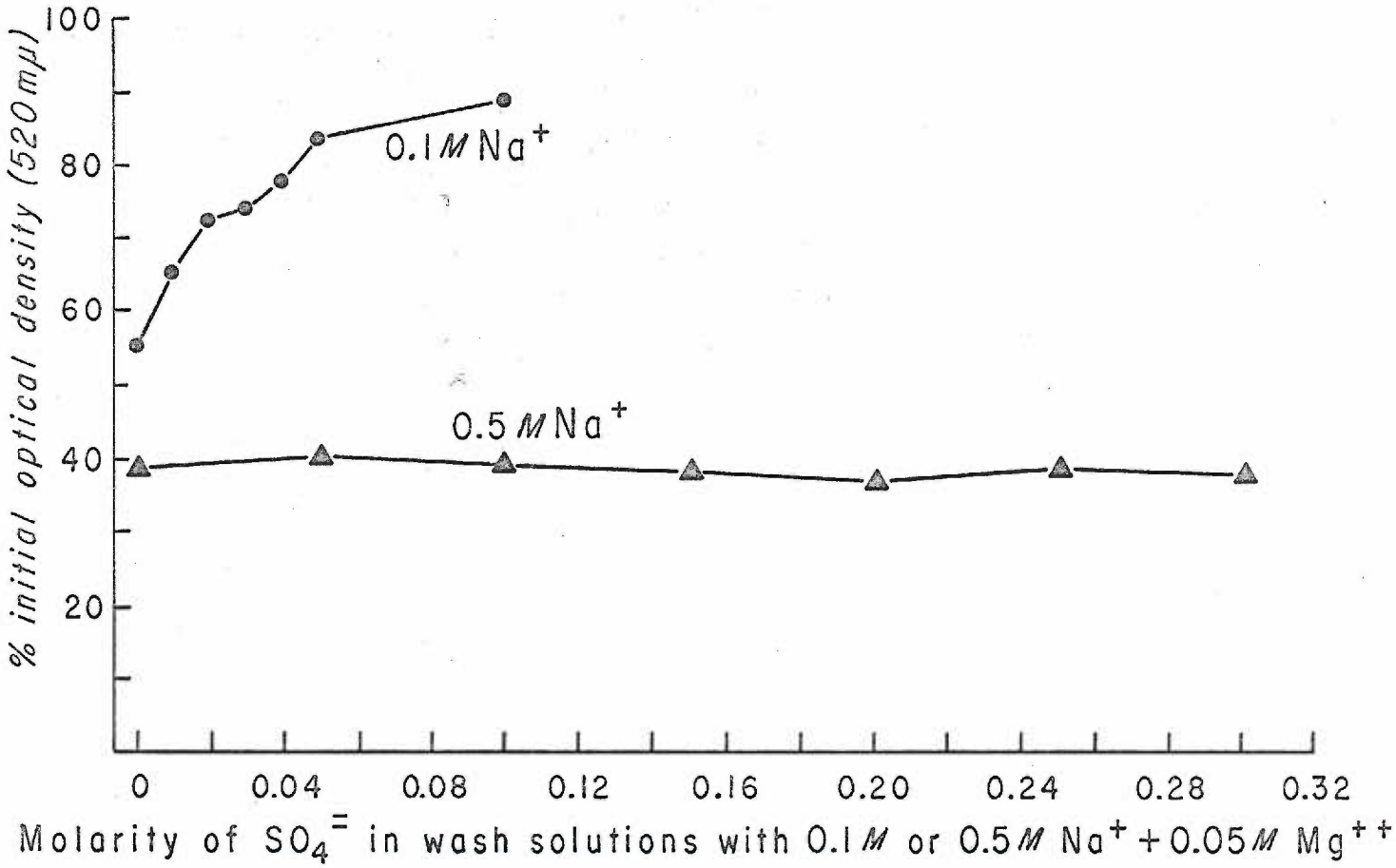
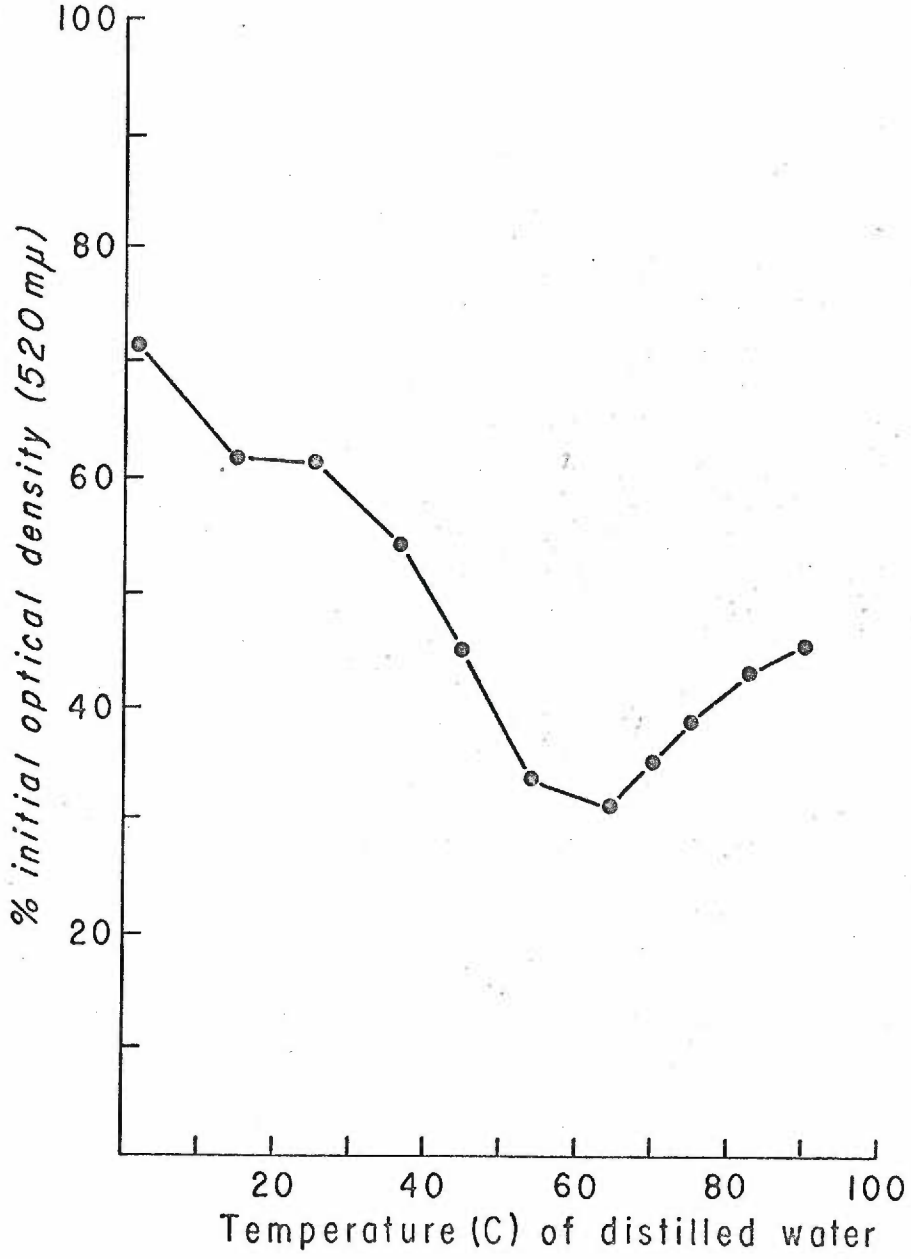

% initial optical density (520mμ)
100
80
60
40
20
0.1M Na+
0.5M Na+
0
0.04
0.08
0.12
0.16
0.20
0.24
0.28
0.32
Molarity of SO4= in wash solutions with 0.1M or 0.5M Na+ + 0.05M Mg++

Figure 20. The effect of distilled water at various temperatures on Pseudomonas c-Al following exposure of cells to 0.1 M NaCl plus 0.05 M $MgCl_2$ at 25 C. Optical densities determined at 30 sec are expressed as percent of initial optical density (520 mμ).


100
80
60
40
20
% initial optical density (520 mμ)
20
40
60
80
100
Temperature (C) of distilled water

the temperature increased up to 63 C. Above 63 C, progressively higher optical density readings were obtained, i.e. the optical density decreases were less as the temperature increased. This unexpected finding was difficult to interpret, but might have been due to denaturation of a lytic enzyme. Therefore, attempts to detect a soluble lytic enzyme were carried out.

X. Search for the presence of soluble lytic enzymes in the distilled water supernates of lysed cells of isolate c-Al.

The purpose of this experiment was to determine whether an enzyme, or enzymes, might be responsible for the lysis of c-Al in low ionic environments. It consisted of three steps: (a) cells of c-Al were transferred to distilled water from a solution of 1.0 M NaCl resulting in cell lysis; (b) cells of M. lysodeikticus used as the test organism for lytic enzymes (70), were suspended in the supernates, diluted and undiluted, of the lysed c-Al cells; and (c) M. lysodeikticus cells were suspended in solutions of known concentrations of lysozyme to determine the minimum concentration of enzyme which might be detected in this manner. Lysis of M. lysodeikticus was determined by optical density changes of cell suspensions.

To exclude the possibility that excess enzyme in the supernates of lysed c-Al may have been inhibitory, five supernate dilutions were tested on M. lysodeikticus (1, 1/2, 1/10, 1/20, 1/100). In every instance, optical densities of M.

lysodeikticus suspensions in c-Al supernates at 60 min were essentially the same as those at 30 sec, suggesting that if lytic enzymes were present in the supernates, they were present in concentrations too low to be detected. In Table 15 the results of the third part of the experiment are presented. These results indicate that the M. lysodeikticus system will detect the presence of relatively small concentrations of enzyme. At lysozyme concentrations of 100 µg/ml the optical density decrease was less than those observed at 50, 10, and 5 µg/ml. The explanation for the discrepancy is not known.

These findings do not rule out the possibility that lysis of c-Al in distilled water is due to a lytic enzyme(s). Perhaps, there are lytic enzymes which are not released into the supernate during lysis of c-Al, but rather are bound to the particulate material, and brought down during centrifugation of the lysed cells. It is also possible, although less likely, that c-Al contains lytic enzymes which are not effective on M. lysodeikticus.

Lysozyme in the presence of EDTA and Na^+ did result in the lysis of c-Al, but the rate of lysis observed was slower than that observed for Pseudomonas aeruginosa(73) under the same conditions. This suggests that the murein layer in c-Al may play a secondary role to that of cations in the maintenance of cell integrity.

XI. Transfer of envelopes of c-Al and 121 from various salt solutions to distilled water.

(a) The first experiment was designed to determine

Table 15

M. lysodeikticus in lysozyme solutions[1]

Exposure time (25 C)	Optical densities (520 mμ) µg/ml lysozyme					
	100	50	10	5	1	0
Initial[2]	0.66	0.66	0.66	0.66	0.66	0.66
30 sec	0.62	0.52	0.54	0.64	0.65	0.63
5 min	0.43	0.08	0.18	0.52	0.60	0.62
10 min	0.45	0.07	0.08	0.47	0.58	0.63
15 min	0.46	0.07	0.06	0.44	0.57	0.67
30 min	0.46	0.07	0.05	0.40	0.56	0.61
Change at 30 min	-0.20	-0.59	-0.61	-0.26	-0.10	-0.05

[1] pH 7.3 - 7.5 unbuffered.

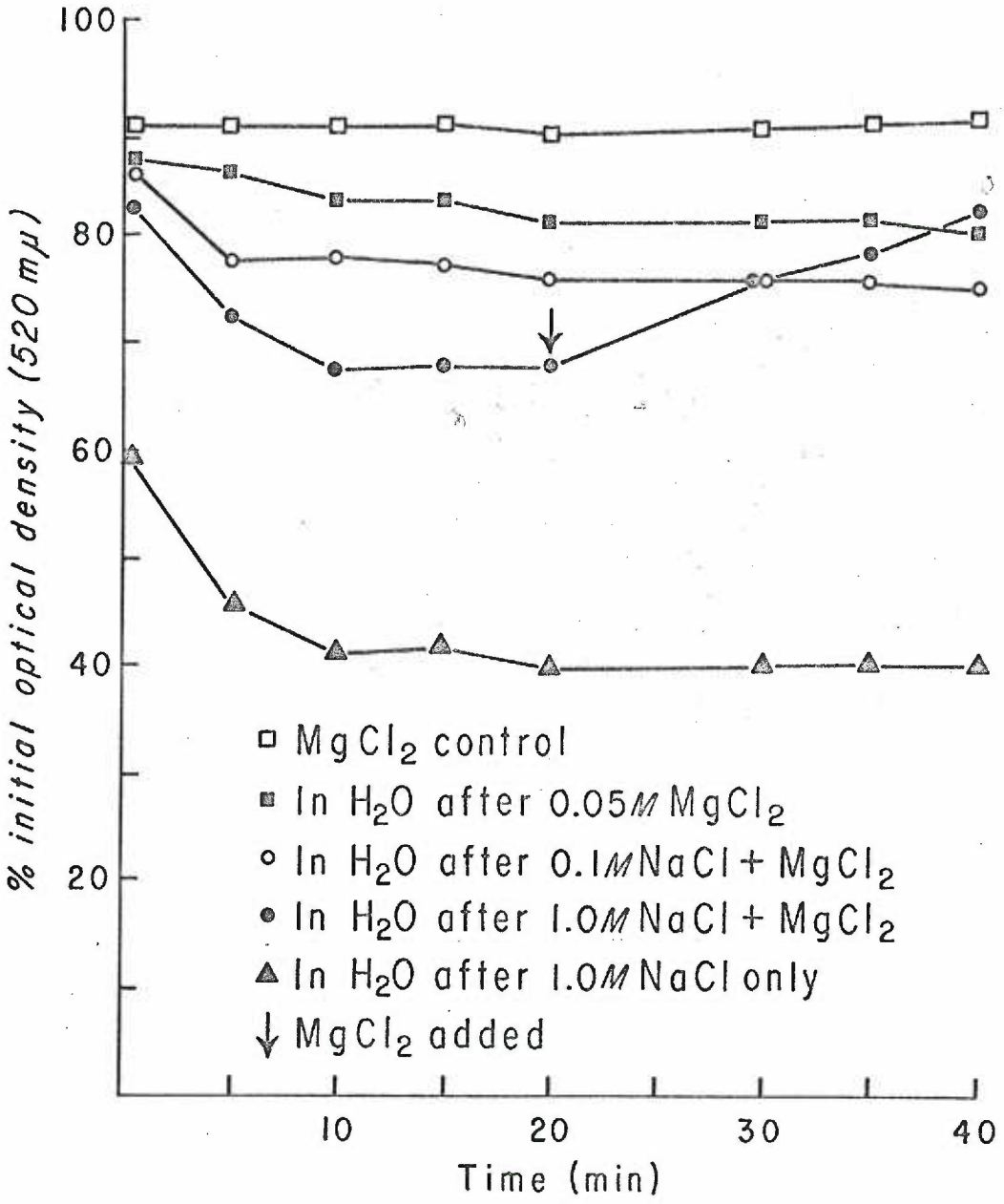
[2] Optical density in distilled water before resuspension in lysozyme solutions.

whether pre-exposure of envelopes of c-A1 to NaCl would affect the optical density changes in distilled water, as had been shown for cell suspensions. Such changes might be an indication of the extent of disintegration of the envelopes during cell lysis. Furthermore, the use of envelopes provides a means for studying the effect of electrostatic factors on lysis without the added effects of osmotic factors.

Cell envelopes were exposed to (a) 1.0 M NaCl alone, (b) 1.0 M NaCl plus 0.05 M $MgCl_2$, (c) 0.1 M NaCl plus 0.05 M $MgCl_2$, or (d) 0.05 M $MgCl_2$ alone. Envelopes were then transferred to distilled water. Optical densities were determined periodically for 40 min. At 20 min, sufficient $MgCl_2$ crystals were added to the distilled water suspension of the envelopes exposed to (b) above to bring it to 0.05 M; optical densities of this tube were recorded for another 20 min.

The results of this experiment are presented in Figure 21. As predicted by the working hypothesis, suspensions of envelopes exposed to NaCl plus $MgCl_2$ underwent greater optical density decreases when suspended in distilled water than did envelopes exposed to $MgCl_2$ alone. Furthermore, the optical density decreases were greater after exposure to the higher concentration of NaCl. When envelope suspensions pre-exposed to 1.0 M NaCl alone were suspended in distilled water an instantaneous optical density decrease of about 40% occurred; this suggested that extensive disintegration of the envelope had taken place. However, when envelopes were pre-

Figure 21. The effect of distilled water (25 C) on envelopes of Pseudomonas c-Al following exposure to 1.0 M NaCl only, 1.0 M NaCl plus 0.05 M $MgCl_2$, 0.1 M NaCl plus 0.05 M $MgCl_2$, and 0.05 M $MgCl_2$ only. Cell envelopes in 0.05 M $MgCl_2$ served as control. Optical densities were measured at intervals for 40 min, and are expressed as percent of initial optical density (520 mμ). Arrow (↓) indicates the addition of $MgCl_2$ to bring the concentration to 0.05 M.

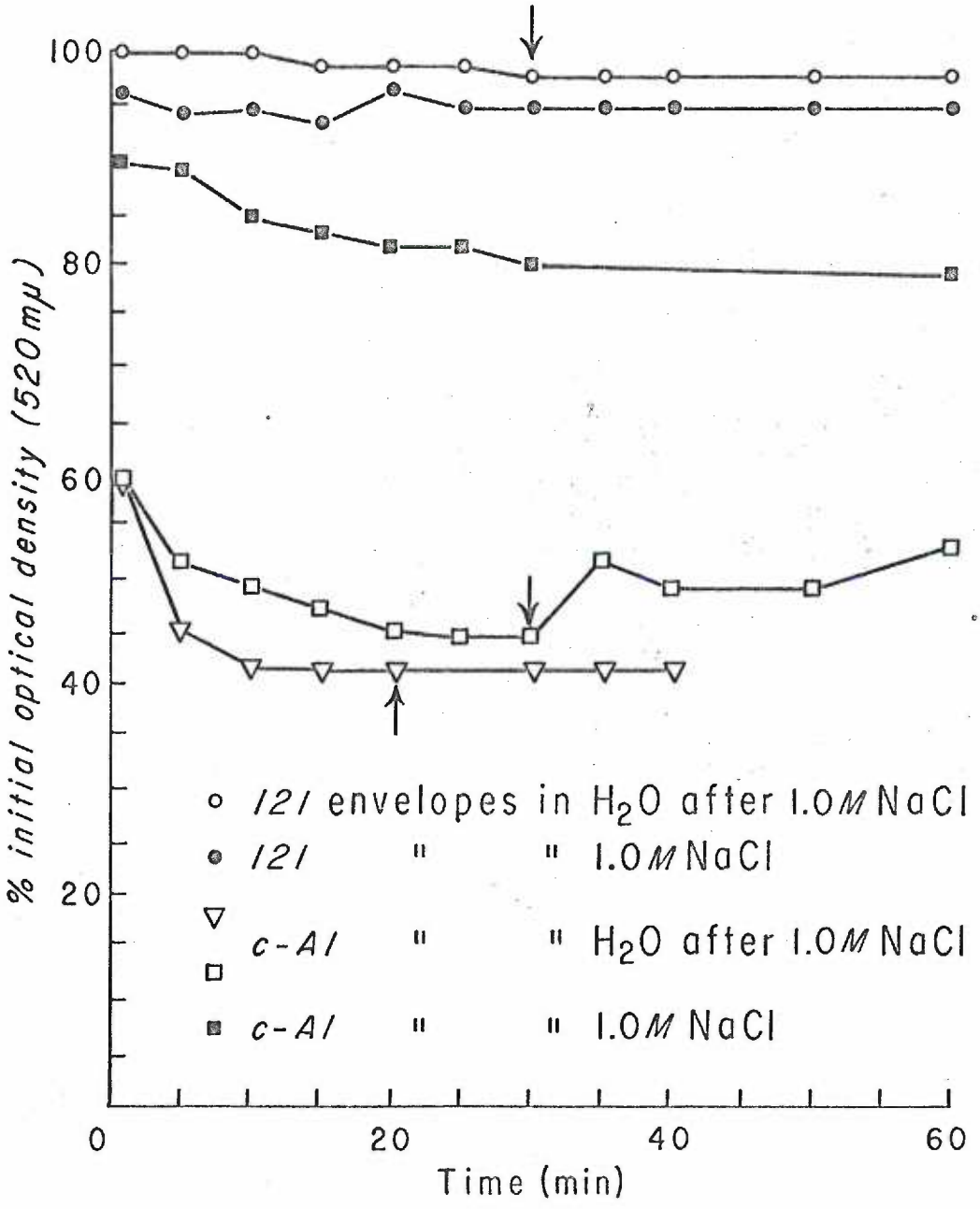

100
80
60
40
20
% initial optical density (520 mμ)
10
20
30
40
Time (min)
MgCl2 control
In H2O after 0.05M MgCl2
In H2O after 0.1M NaCl + MgCl2
In H2O after 1.0M NaCl + MgCl2
In H2O after 1.0M NaCl only
MgCl2 added

exposed to NaCl plus $MgCl_2$, the optical density was, at most, only 9% lower than that of the control envelopes in 0.05 M $MgCl_2$ (Figure 21). The gradual increase in optical density in the envelope suspension to which $MgCl_2$ was added, suggested the occurrence of reaggregation of envelope fragments produced as a result of lysis in distilled water.

As found with cell suspensions, pre-exposure to Mg^{++} appears to have protected the envelopes against disintegration in distilled water, while pre-exposure to Na^+ makes the envelopes more susceptible to disintegration. Also, the degree of susceptibility to lysis appears to be related to the concentration of Na^+ to which envelopes are pre-exposed.

(b) The second experiment was designed to determine first, whether c-Al and 121 envelopes do undergo disintegration when transferred from 1.0 M NaCl to distilled water; and second, if such disintegration had taken place, whether the addition of $MgCl_2$ or NaCl to lysed envelope suspensions would result in the reaggregation of envelope fragments. The optical density changes that occurred during this experiment are presented in Figure 22. As found with 121 cell suspensions, the 121 envelopes were not affected by pre-exposure to NaCl. However, the suspension of c-Al envelopes underwent large optical density decreases suggesting that disintegration had taken place. As previously observed (Figure 21), the addition of $MgCl_2$ after c-Al envelopes had been in distilled water for 30 min resulted in a slight

Figure 22. The effect of distilled water (25 C) on envelopes of Pseudomonas strains c-A1 and 121 following exposure of the envelopes to 1.0 M NaCl. Optical densities were determined at intervals for 60 min, and are expressed as percent of initial optical density (520 mμ). Arrows indicate the addition of $MgCl_2$ (↓), to bring the concentration of the suspending medium to 0.05 M, and NaCl (↑), to bring it to a concentration of 1.0 M.


100
80
60
40
20
% initial optical density (520 mμ)
0
20
40
60
Time (min)
121 envelopes in H2O after 1.0M NaCl
121 " " 1.0M NaCl
c-Al " " H2O after 1.0M NaCl
c-Al " " 1.0M NaCl

increase in optical density. Samples of suspensions of c-Al envelopes in (a) 1.0 M NaCl, (b) distilled water, and (c) distilled water 20 min after the addition of $MgCl_2$, were shadow-cast for electron microscopy. The appearance of the envelopes indicated that envelope disintegration had, in fact, occurred, and that the addition of $MgCl_2$ to the preparation had caused a reaggregation of fragments (Figure 23). Optical density decreases of envelope suspensions are, therefore, a valid indication of envelope disintegration. Electron micrographs of the envelopes in 1.0 M NaCl were not possible because of the abundance of salt crystals which masked the envelopes on the grid; however, normal envelopes from sea water can be seen in Figure 40. The addition of NaCl to lysed envelope suspensions did not result in an increase in optical density. This might have been predicted, since Na^+ could not act as a bridge between fragments in the manner postulated for Mg^{++}.

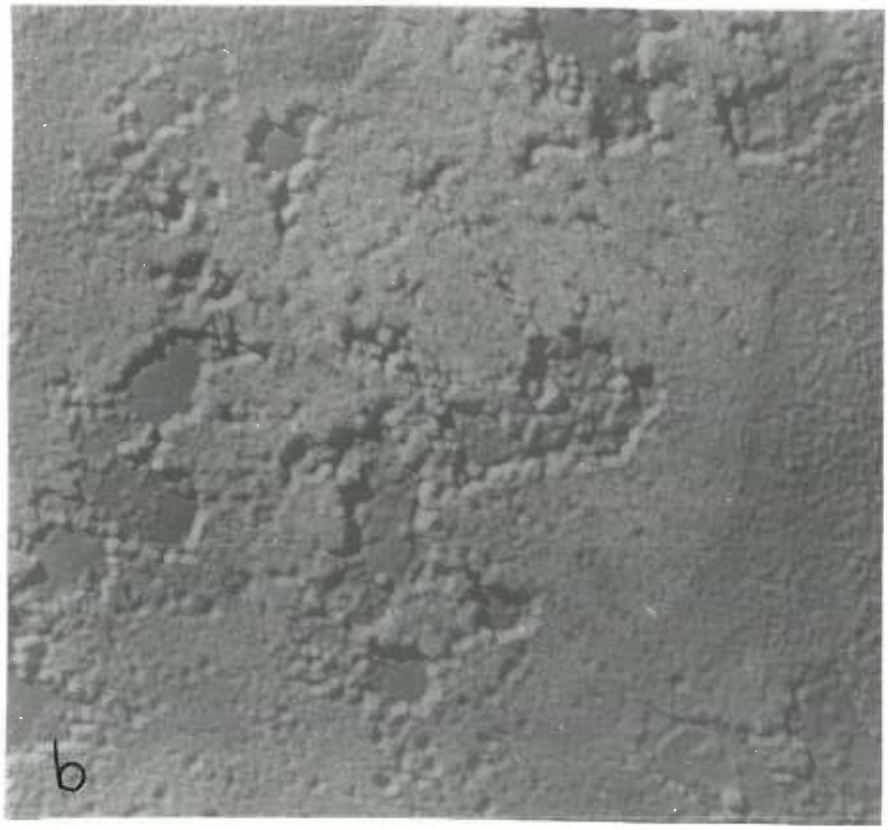
All of the results of this experiment are consistent with the hypothesis that when Na^+ replaces Mg^{++} in the envelope, Mg^{++} bridges are destroyed, resulting in the weakening of the cell envelopes. These envelopes are then susceptible to lysis in distilled water.

XII. Cation exchange between Na^+ and Mg^{++} in the envelopes of Pseudomonas 121 and Pseudomonas c-Al.

The working hypothesis, formulated early in this research, proposes that cells of c-Al are susceptible to lysis in distilled water because of a weakened envelope, the re-

Figure 23. Electron micrographs of shadowed, distilled water-lysed envelopes of Pseudomonas c-Al: (a) fragments after exposure to 1.0 M NaCl (28,000 X); (b) reaggregated forms after the addition of $MgCl_2$ (28,000 X).


a


b

sult of the destruction of divalent cation bridges. It was further proposed that these bridges are destroyed by the presence of monovalent cations which displace Mg^{++}, and that the attraction of water is not sufficient to remove Mg^{++} from these bridging sites.

All data presented thus far in this thesis have been consistent with this hypothesis, but no direct evidence for its support has been presented. An experiment was therefore carried out to determine whether Na^{+} could indeed displace Mg^{++} in the envelope of both c-Al and 121. Details of the procedure including $HClO_4$ extraction of envelopes are described in Materials and Methods C. VII. The results are presented in Tables 16 and 17.

These data indicate that Mg^{++} in the envelope of c-Al was displaced by Na^{+}, and that this displacement was proportional to the concentration of Na^{+} below 0.5 M. The low value for total Mg^{++} in the envelopes incubated in distilled water suggests that perchloric acid does not remove all of the Mg^{++}. Whether such residual Mg^{++} left in the envelopes may be removed with Na^{+}, was not determined. The displacement of Mg^{++} in the envelopes of c-Al by Na^{+} could account for the increased susceptibility to lysis of these cells in distilled water.

On the other hand, the displacement of Mg^{++} by Na^{+} in the envelopes of 121 did not render these envelopes susceptible to lysis in distilled water. The results of both these experiments indicate that cations play an extremely

Table 16

Cation exchange between Na^+ and Mg^{++} in envelope of Pseudomonas c-Al

mg Mg^{++}/gm dry envelope

NaCl M	Mg^{++} in supernate	Mg^{++} from pellet extracted with $HClO_4$	Total Mg^{++}
1.0	1.86	<0.15	<2.01*
0.75	1.86	<0.15	<2.01
0.50	1.86	<0.15	<2.01
0.30	1.65	0.30	1.95
0.10	1.34	0.60	1.94
0	<0.15	0.72	<0.87

* The Mg^{++} in the ashed envelope pellet was 2.15 mg/gm dry envelope.

Table 17

Cation exchange between Na^+ and Mg^{++} in envelopes of Pseudomonas 121

NaCl (M)	mg Mg^{++}/gm dry envelope: Mg^{++} in supernate	Mg^{++} in pellet extracted with $HClO_4$	Total Mg^{++}
1.0	2.91	0.3	3.21
0.1	1.46	1.21	2.67
0	0.25	1.99	2.24

important role in the maintenance of envelope structural integrity of c-Al, but that other mechanisms may play a more important part in the maintenance of envelope integrity of 121.

G. Electron microscopy of Pseudomonas c-Al and Pseudomonas 121.

Electron microscopic studies were used primarily in three phases of the research: (a) to characterize c-Al and 121 organisms morphologically and to compare their external surface features; (b) to observe thin sections of normal and lysed cells of both organisms; (c) to observe lysis of cell envelopes in distilled water. Part (c) has already been presented.

I. Morphology of whole cells of 121 and c-Al.

The purpose of this study was to compare the external features of these two organisms, e.g. flagellar pattern and external cell surface. Electron micrographs of unfixed cells are presented in Figures 24 through 26. An unfixed cell of Pseudomonas c-Al (Figure 24) can be seen to have a single polar flagellum, as did all flagellated cells of c-Al. An interesting feature of this cell, and other cells of this organism, is the appearance of the small round bodies at the periphery. Several of these spheres were measured and found to range in diameter from 600 to 1200 Å. The nature of these spherules is not known, although at first they were thought to be phage. However, these spherules do not appear to be attached to the exterior of the cell, but rather

Figure 24. Electron micrograph of a shadowed, unfixed cell of Pseudomonas c-Al from a 24 hr sea water peptone broth culture. Spherical bodies are visible around the periphery of the cell (41,700 X).

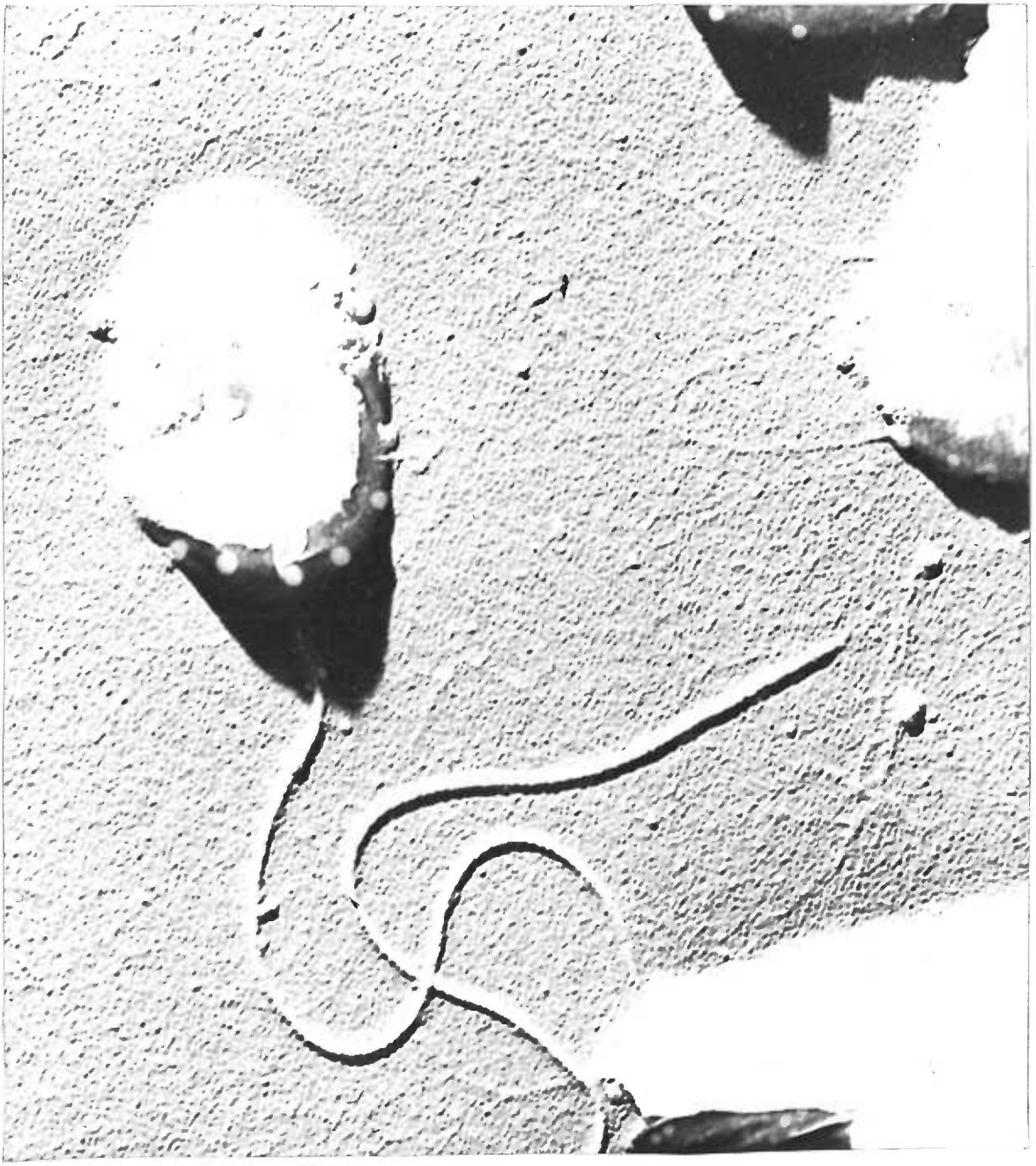


Figure 25. Electron micrograph of a shadowed, unfixed cell of Pseudomonas c-A1 from a 24 hr sea water peptone broth culture. Spherical bodies and fimbriae are visible on the periphery of the cell. The spherical bodies appear to be covered by an outer layer of the cell which has withdrawn from the bodies, probably because of desiccation (41,000 X).

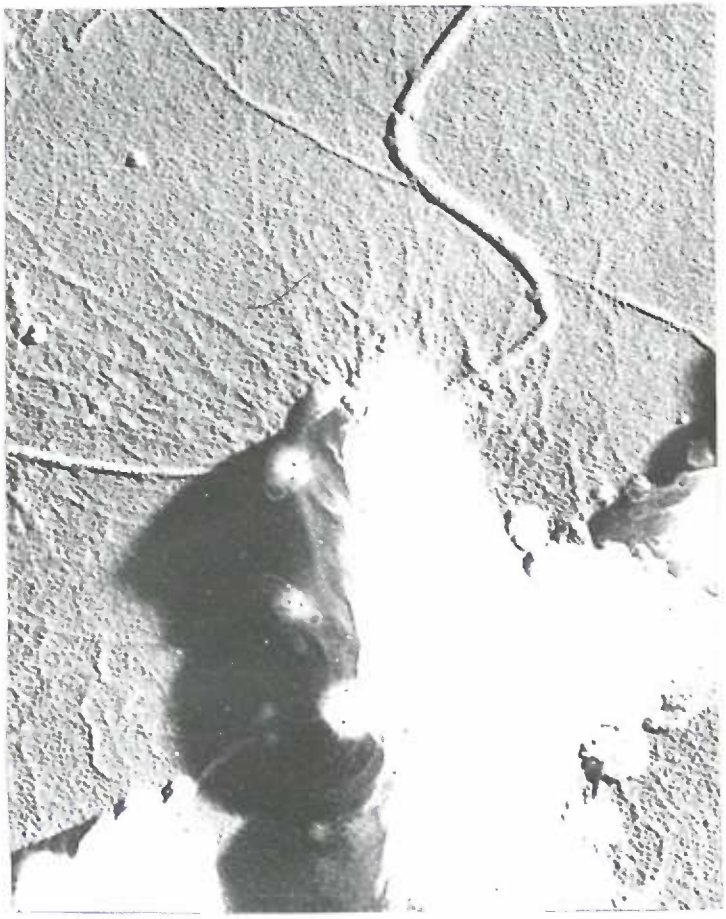
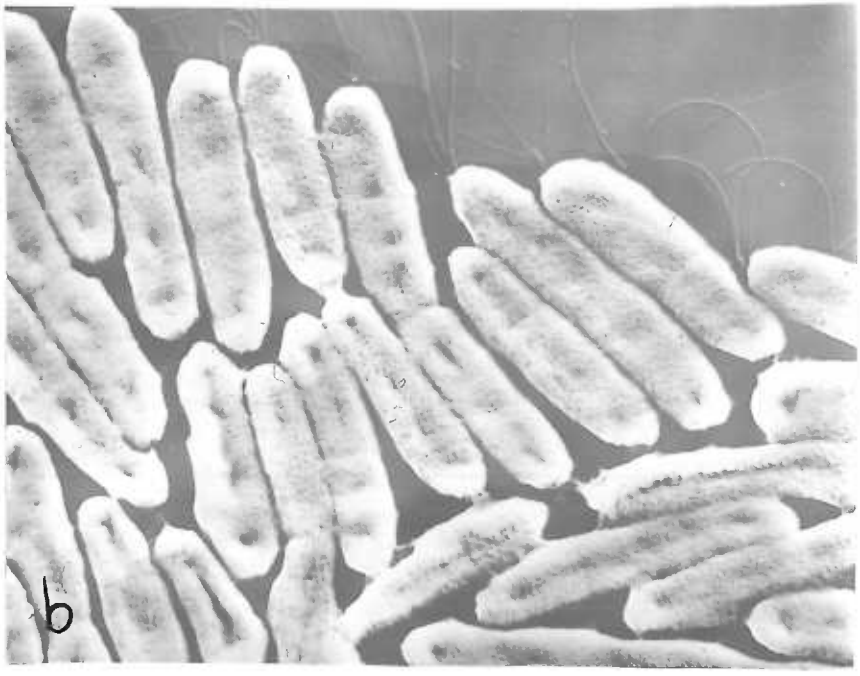


Figure 26. Electron micrographs of shadowed, unfixed cells of Pseudomonas 121 from a 24 hr culture in distilled water peptone broth. Magnifications are (a) 28,000 X and (b) 16,000 X.


a


b

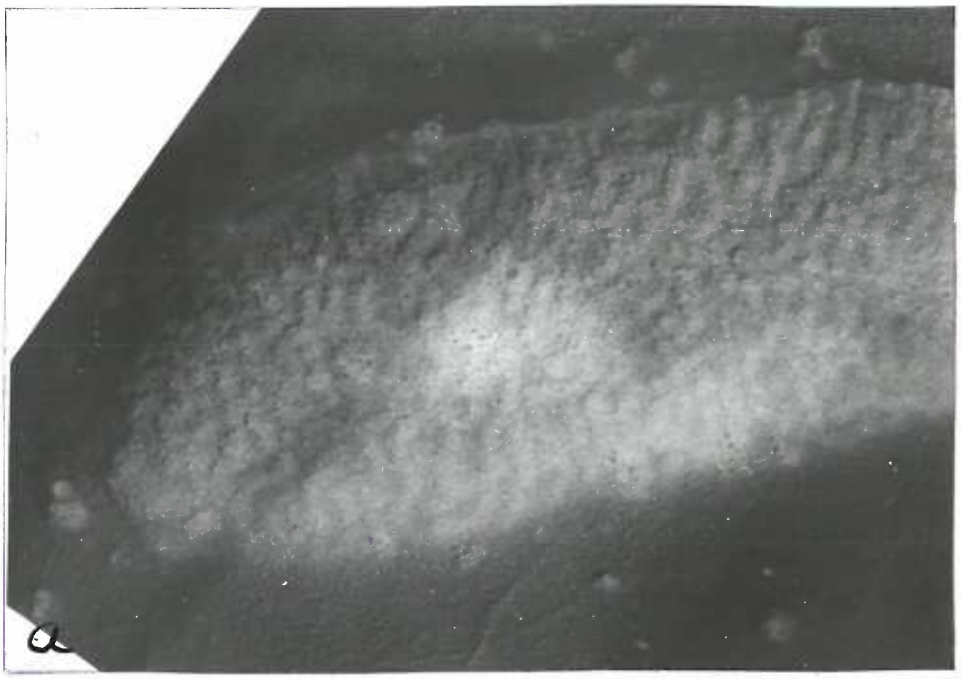
located under an external layer of the cell which can be seen (Figure 25) to have shrunken and wrinkled, apparently during desiccation of the cells. The cell in Figure 25 also has structures which appear to be fimbriae. A vast majority of the cells observed were fimbriated, not only in the polar regions but along the entire periphery of the cells.

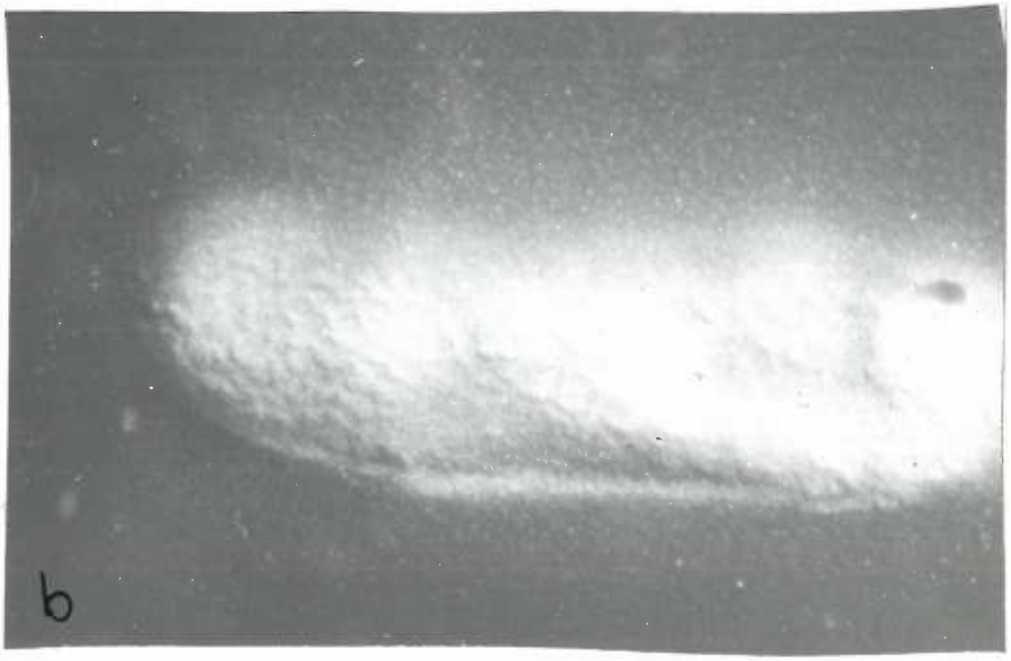
Unfixed whole cells of 121 appeared different from c-Al in shape and the flagellation pattern. The flagella were in tufts and, as in c-Al, were polar. No structures were seen that were similar to the spherules observed on c-Al. Cells of 121 fixed in sea water were not observed. To observe the effects of fixation on cell surfaces of both 121 and c-Al, cells were fixed in OsO_4, as described in Materials and Methods, E. I., in the hope that shrinking of these cells during desiccation could be prevented. The cells were then shadow-cast as before (Figure 27). The surfaces of both cells after fixation appeared similar and convoluted. There were, however, no flagella or fimbriae observed on any of the cells, nor were there any spherules surrounding c-Al.

II. Studies of normal and lysed cells of c-Al and 121.

In previous experiments, the presence of ultraviolet-absorbing material in supernates of lysed cells of c-Al was found to correlate well with decreases in optical density of lysed cell suspensions, i.e. the greater the decrease in optical density, the greater the amount of ultraviolet-absorbing material in the supernate. Furthermore, the amount

Figure 27. Electron micrographs of shadowed, fixed cells of (a) Pseudomonas 121 from a 24 hr culture in distilled water peptone broth (100,000 X) and (b) Pseudomonas c-Al from a 24 hr culture in sea water peptone broth (70,000 X).


a


b

of the optical density decrease on lysis was found to be dependent on the amount and type of salt to which the cells had been exposed prior to transfer to distilled water. To confirm the findings that optical density decreases during whole cell lysis were the result of disruption of the envelope, the following experiments were carried out.

The first step was to observe the ultrastructure of normal cells of c-Al. Previous experiments showed that optical densities and viable counts of c-Al could be maintained over several hours in sea water, 1.0 M NaCl, 0.4 M NaCl, or 0.05 M $MgCl_2$. Therefore, cells suspended in these solutions were fixed, embedded, and sectioned as described in Materials and Methods, F. I.

Thin sections of these cells are presented in Figures 28 and 29. In all cases the envelope of the cells appears as a multilayered structure. The outermost layer is membrane-like in that it appears as a double track-like structure, 75-80 Å in width. The inner layer appears also as a double track-like layer measuring approximately 75 Å. There was no layer corresponding to the murein layer seen in other gram-negative envelopes(68).

The envelopes of the cells in various salts appeared much the same with the exception of those exposed to 0.5 M NaCl. All the cells from 0.5 M NaCl appeared to have a loosely attached outer layer. The outer layers of the cells from sea water, 1.0 M NaCl or 0.05 M $MgCl_2$ showed much less separation of the layers.

Figure 28. Electron micrographs of thin-sections of Pseudomonas c-Al. Cells were fixed in (a) sea water (250,000 X), (b) sea water (114,000 X), and (c) 1.0 M NaCl (130,000 X).

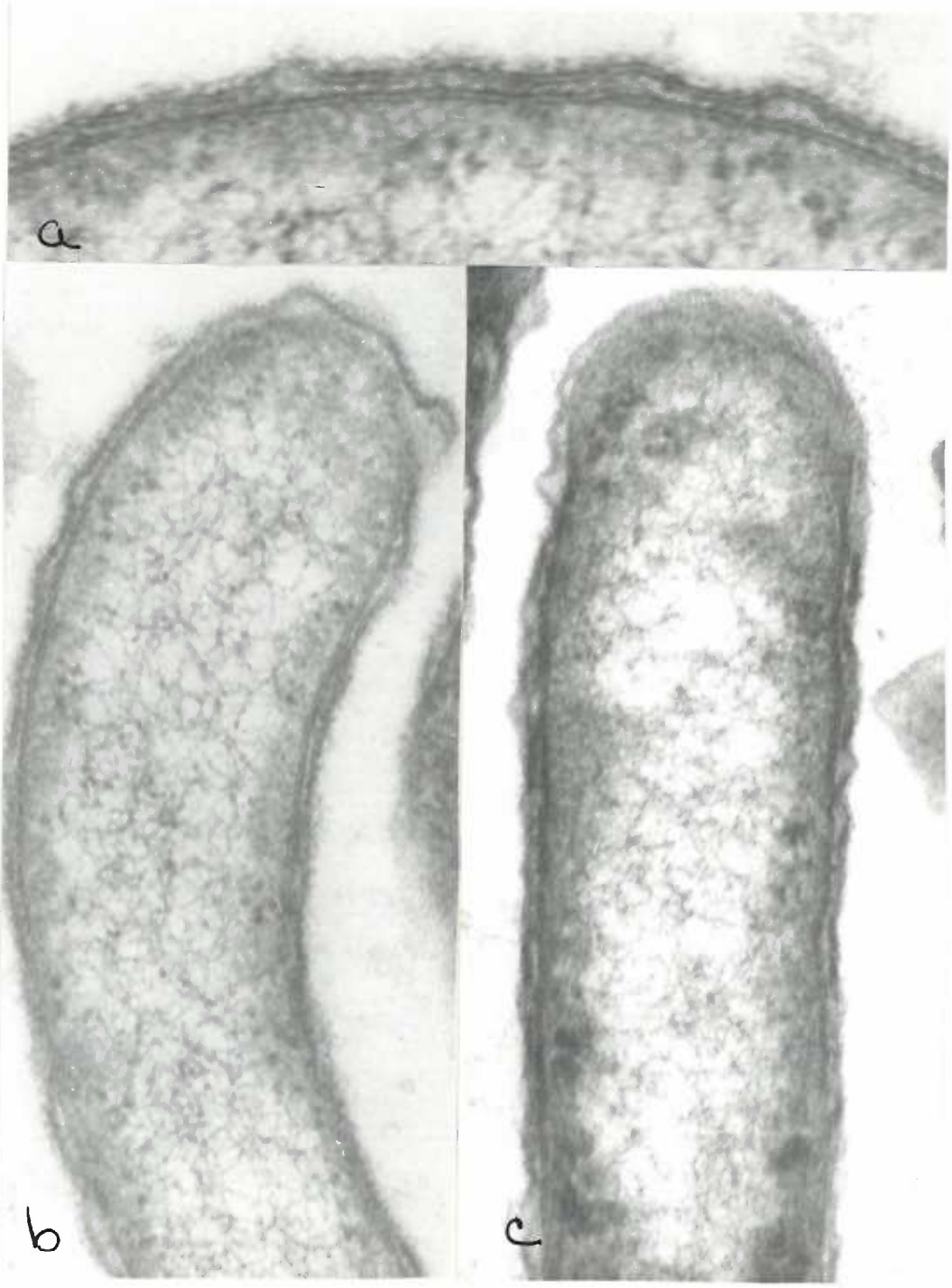

a
b
c

Figure 29. Electron micrographs of thin-sections of Pseudomonas c-Al fixed in (a) 0.5 M NaCl (150,000 X) and (b) 0.05 M $MgCl_2$ (121,000 X).


a


b

A study of the ultrastructure of lysed cells was carried out by exposing the cells to various salt solutions: (a) 1.0 M NaCl alone; (b) 0.5 M, or 0.1 M NaCl plus 0.05 M $MgCl_2$, or (c) 0.05 M $MgCl_2$ alone (as described in the Materials and Methods, C. III. and Results, F. I. and III.), followed by transfer of the cells to distilled water. After the cells had been in distilled water for approximately 30 sec, they were pre-fixed and immediately centrifuged, fixed, and embedded.

Thin sections of the lysed cells (Figures 30 through 36) indicate that the extent of disruption during fast-stage lysis is dependent on the salt to which the cells were previously exposed. Lysed cells which had been previously exposed to 1.0 M NaCl alone (Figure 30) show extensive damage, with discrete areas of rupture and extrusion of cytoplasm from the cell ("hernias"). Segments of the outer envelope layer are not usually separated or removed from the inner layer. The cell shown has maintained its rod-shape as had other lysed cells observed.

Lysed cells exposed to 0.5 M NaCl (Figure 31) prior to transfer to distilled water, look much different from those pre-exposed to 1.0 M NaCl. Many more small fragments of outer envelope layer are still attached around the periphery of the cell than from cells transferred from 1.0 M NaCl. Some of this outer layer has obviously undergone extensive damage. The cytoplasmic membrane also appears to be damaged, but to a much lesser extent than the outer layer. Hernias, similar to those in Figure 30, can also be seen along the

Figure 30. Electron micrograph of a distilled water-lysed cell of Pseudomonas c-Al after exposure to 1.0 M NaCl. Hernias are visible along the cell perphery (140,000 X).

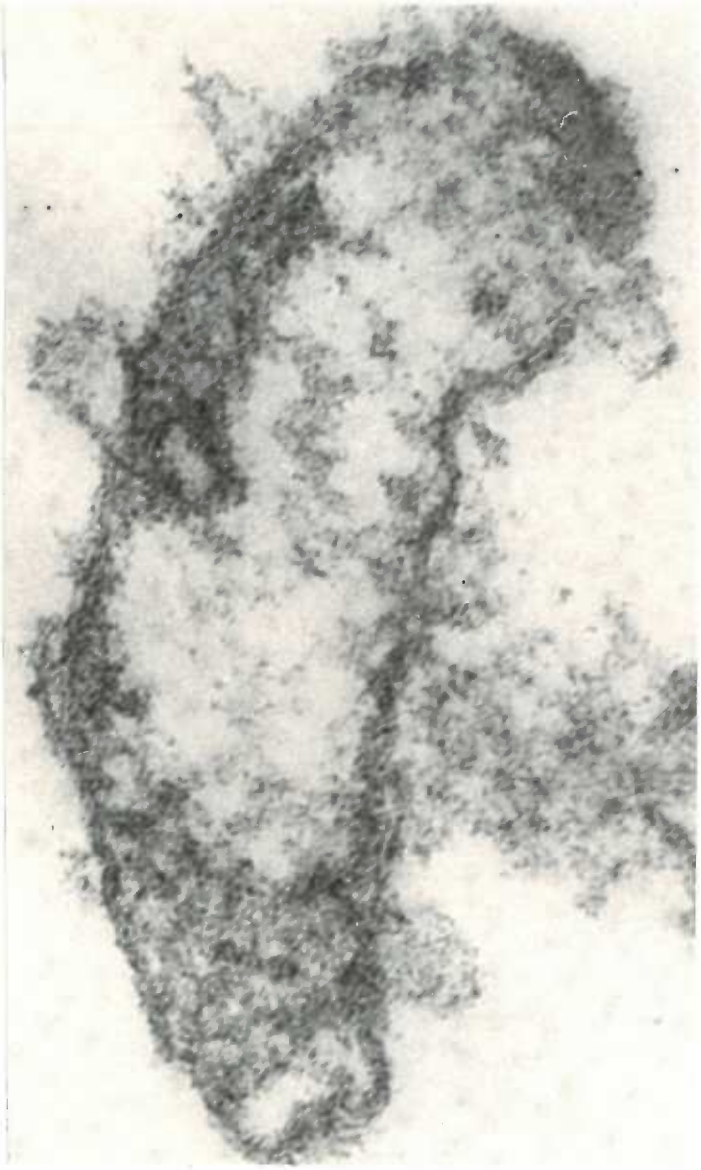


Figure 31. Electron micrograph of a distilled water-lysed cell of Pseudomonas c-Al after exposure to 0.5 M NaCl. Hernias are visible along the cell periphery (125,000 X).

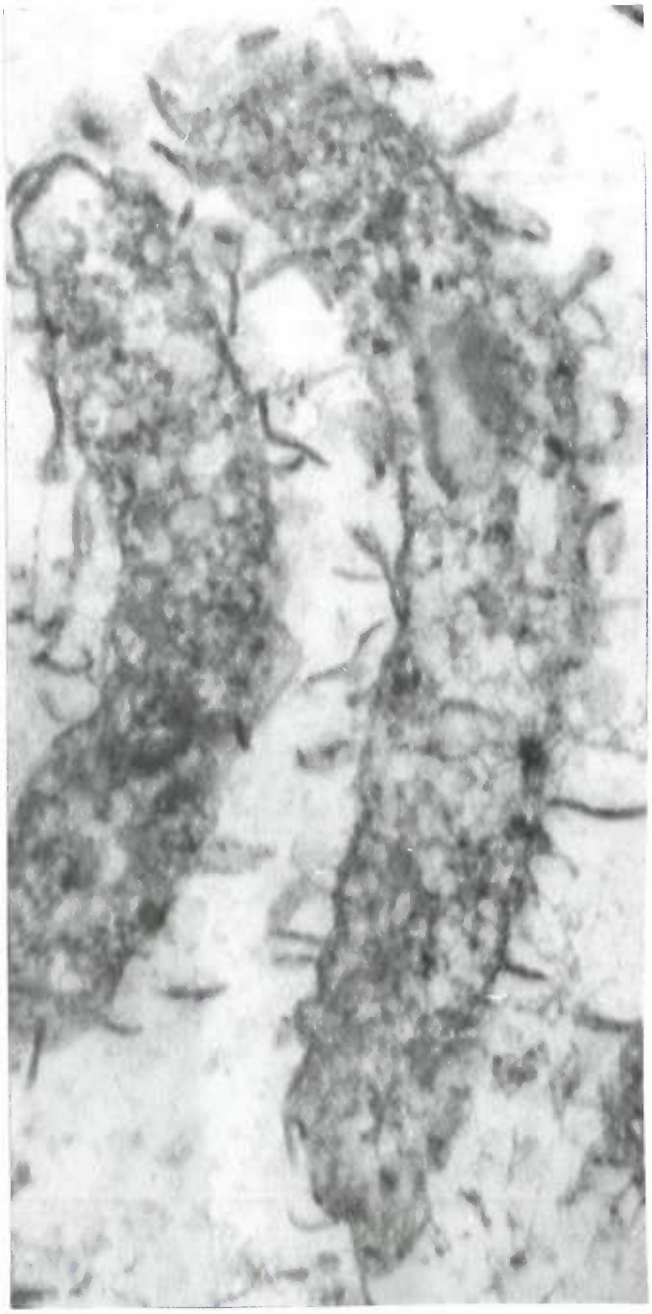


Figure 32. Electron micrographs of thin-sections of distilled water-lysed cells of Pseudomonas c-Al after exposure to (a) 1.0 M NaCl plus 0.05 M $MgCl_2$ (140,000 X) or (b) 0.5 M NaCl plus 0.05 M $MgCl_2$ (130,000 X).

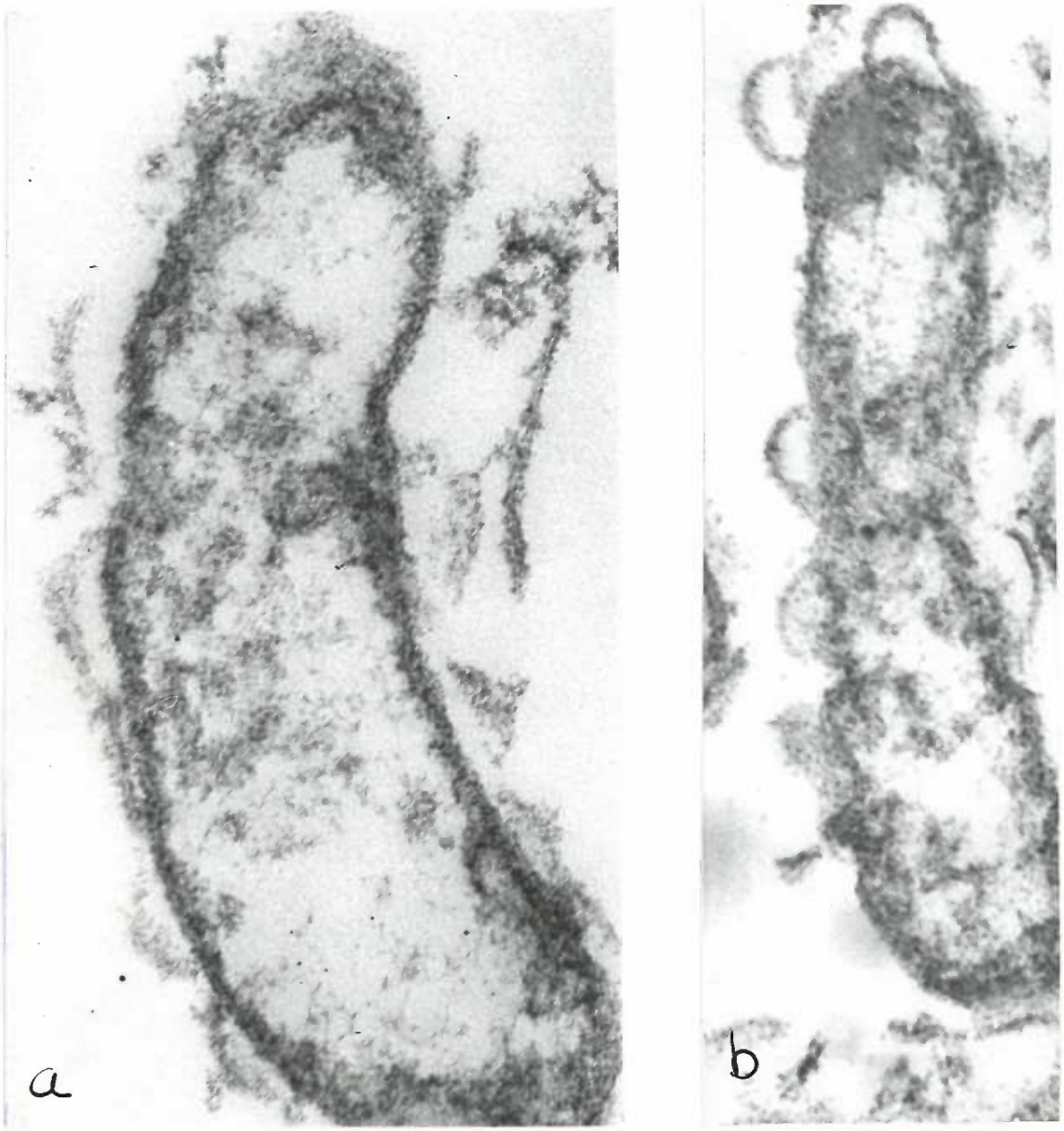

a
b

Figure 33. Electron micrographs of thin-sections of distilled water-lysed cells of Pseudomonas c-Al following exposure to (a) 0.1 M NaCl plus 0.05 M $MgCl_2$ (155,000 X) or (b) 0.05 M $MgCl_2$ alone (90,000 X).


a

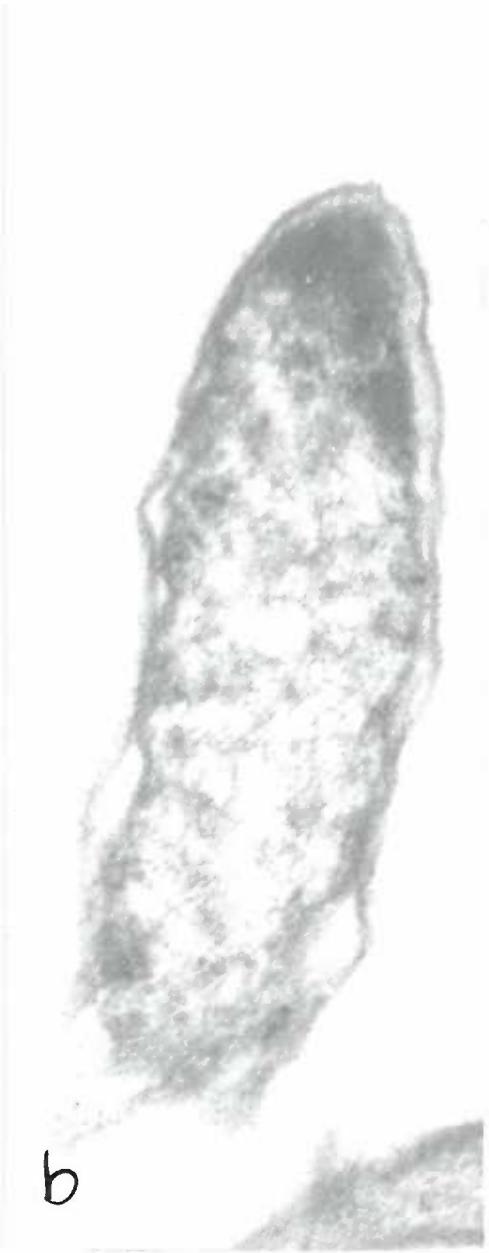

b

Figure 34. Electron micrograph of a thin-section of a distilled water-lysed cell of Pseudomonas c-Al following exposure to 0.1 M NaCl plus 0.05 M $MgCl_2$ (140,000 X).

Figure 35. Electron micrograph of a thin-section of a distilled water-lysed cell of Pseudomonas c-Al following exposure to 0.1 M NaCl plus 0.05 M $MgCl_2$ (114,000 X).

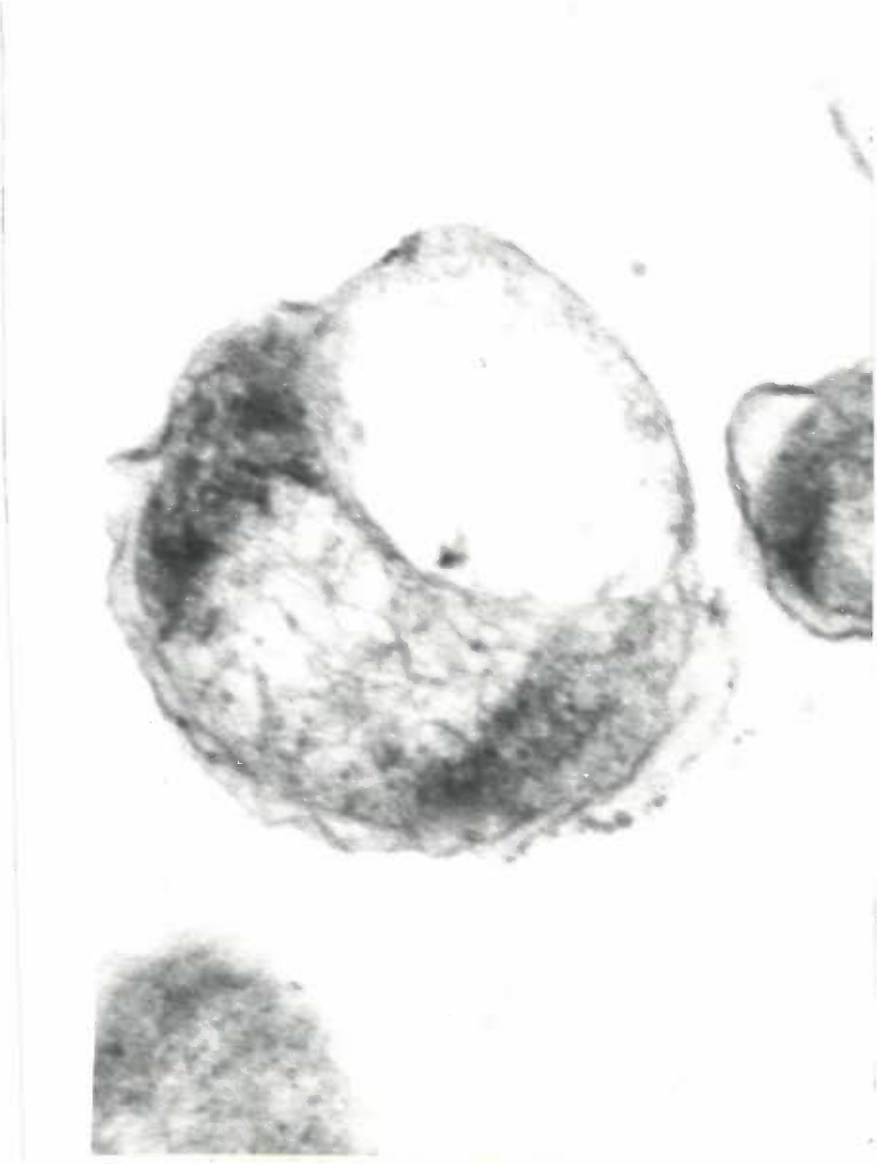


Figure 36. Electron micrographs of shadowed material from the supernates of distilled water-lysed cells of Pseudomonas c-Al pre-exposed to 1.0 M NaCl alone (9000 X).

periphery of these cells.

As described previously (Results, F. III.), suspensions of cells that had been exposed to $MgCl_2$ plus NaCl showed optical density decreases in distilled water during fast-stage lysis related to the concentration of NaCl to which they were pre-exposed. Electron micrographs presented in Figure 32 are of lysed cells which had been exposed to 1.0 M NaCl plus 0.05 M $MgCl_2$, and 0.5 M NaCl plus 0.05 M $MgCl_2$, respectively, prior to transfer to distilled water. Both cells have been disrupted extensively but to a lesser degree than cells pre-exposed to NaCl solutions alone. Large portions of the outer envelope layer appear relatively intact and cytoplasmic membrane, although disrupted to a small degree, appear intact over long segments. Hernias are conspicuously absent from these cells, and were never seen in lysed cells pre-exposed to solutions containing 0.05 M $MgCl_2$, whether or not NaCl was present.

Cells lysed in distilled water following exposure to 0.1 M NaCl plus 0.05 M $MgCl_2$ are shown in Figures 33 (a), 34, and 35. These cells have undergone a minimum of disruption during lysis. In Figures 33 (a) and 35, both the outer layer and the cytoplasmic membrane are intact and appear normal. In addition, the cytoplasm in Figure 33 (a) appears quite normal, as well. In Figure 34 the outer layer appears to have been peeled away from the cell. Many of the forms shown in Figure 35 are present in the distilled water lysed cell suspensions pre-exposed to 0.1 M NaCl plus

$MgCl_2$, but were not found when cells were pre-exposed to higher concentrations of NaCl. The cytoplasm is surrounded by the cytoplasmic membrane, and this portion of the cell maintains its rod-shape. The outer layer appears to have ballooned away from the cytoplasmic membrane on one side of the cell, so that the outer layer forms a sphere. Figure 33 (b) shows a cell which had been exposed to 0.05 M $MgCl_2$ alone, prior to its transfer to distilled water. The shape of the cell appears normal with an undisturbed envelope, but the cytoplasm appears abnormal, with aggregates and vacuolated areas. The amorphous area at the base of the cell is an artifact due to sectioning.

Cells lysed in distilled water following exposure to 1.0 M NaCl undergo extensive lysis (Figure 30). The cell in this picture was taken from a pellet sedimented by centrifugation at 2200 g for 30 min. The nature of the particulate material in the supernate was determined by centrifuging the supernate at 10,000 g for 30 min, and shadow-casting the resulting pellet (Figure 36). Large conglomerates of particles, presumably from cell envelopes, can be seen as well as flagellar fragments of various lengths. At no time was material seen resembling intact cell envelopes or whole cells.

The behavior of 121 to low ionic environments after being exposed to NaCl alone or with $MgCl_2$ was different from that of c-Al in that very little leakage of ultraviolet-absorbing material occurred, and optical density decreases

of cells suspended in distilled water were minimal (Results, section F. III.). Normal cells of this organism (Figure 37) taken from peptone broth culture and fixed, embedded, and sectioned as previously described, appear to have a multi-layered envelope. There are more layers visible (Figure 37 (b)) than in c-Al, and the layers are in very close proximity to one another, unlike those of c-Al. These cells invariably show vacuoles in the cytoplasm which are enclosed by a structure that appears as a single dark line. These vacuoles are very probably areas of poly-β-hydroxybutyrate accumulation, as they have an affinity for the lipophilic stain, Sudan Black.

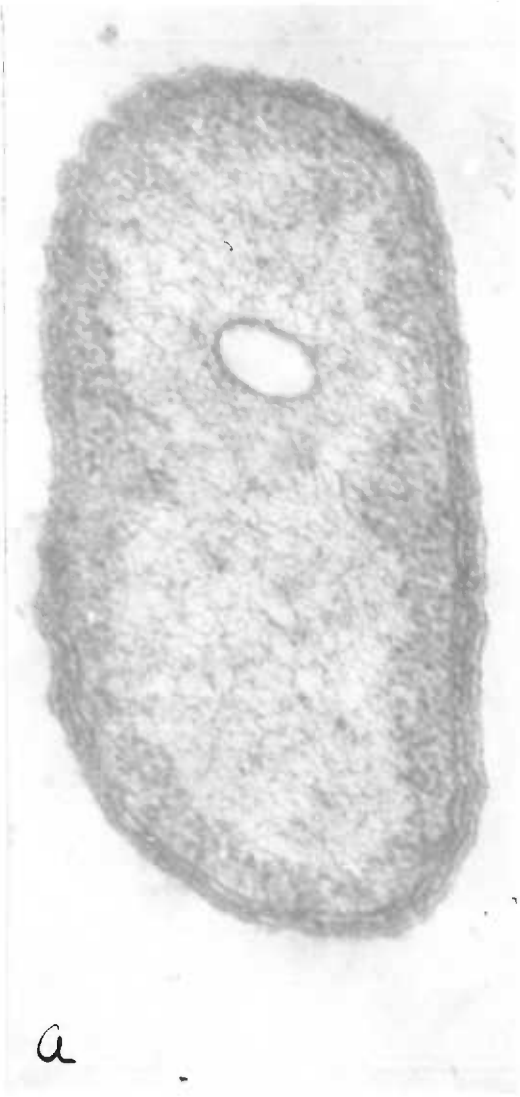
The standard procedure for lysis of c-Al was used with 121 (Results, section F. III.), and electron micrographs of these cells were obtained after transfer to distilled water. The results are presented in Figure 38. These cells appear quite normal except that the external layers of the envelope seem to have separated from the cytoplasmic membrane in some areas. This separation did not, however, result in measurable leakage of ultraviolet-absorbing materials. It was concluded that these cells have some different or additional structural component or components in their envelopes which provide a greater degree of rigidity and cohesiveness than those in the envelopes of c-Al.

III. Electron microscopy of cell envelopes of 121 and c-Al.

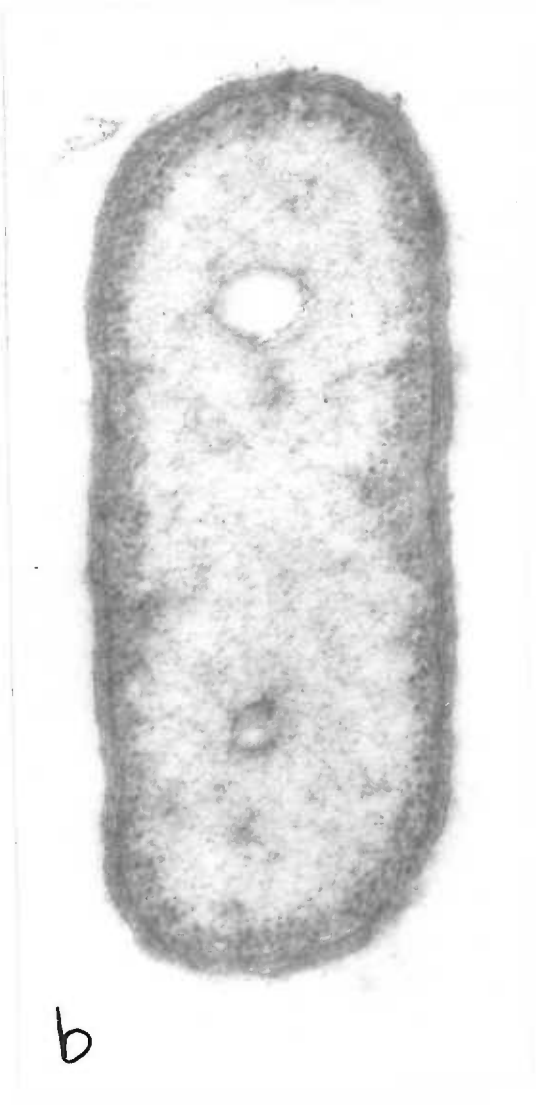
(a) Pseudomonas 121.

Electron microscopy was used to assay cell envelopes

Figure 37. Electron micrographs of thin-sections of Pseudomonas 121 fixed in distilled water peptone broth. Magnifications are (a) 140,000 X and (b) 72,000 X.

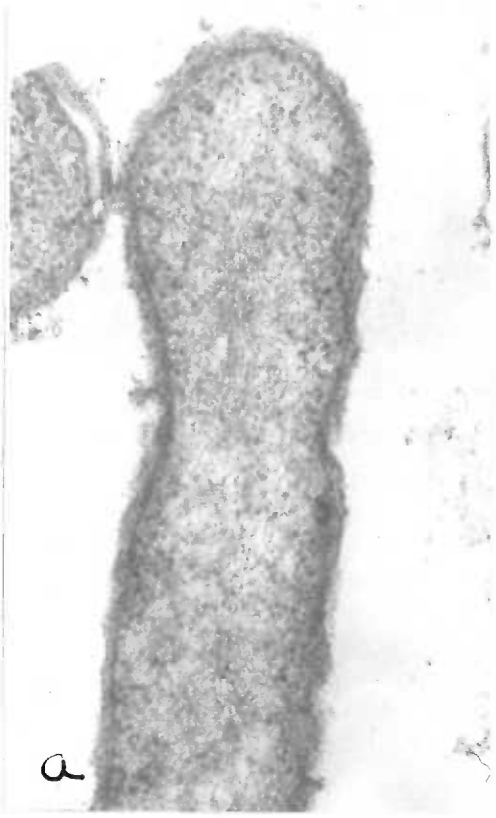


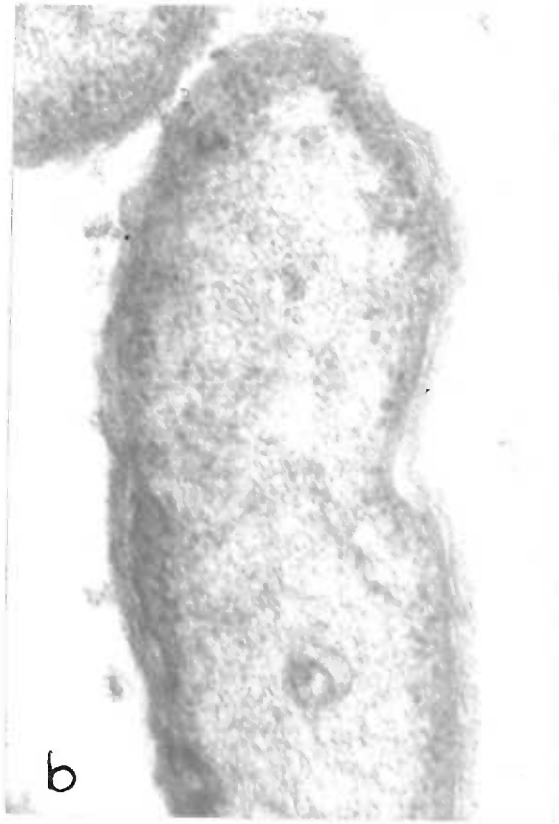
a



b

Figure 38. Electron micrographs of thin-sections of distilled water-lysed cells of Pseudomonas 121. Cells were pre-exposed to (a) 1.0 M NaCl plus 0.05 M $MgCl_2$ (57,000 X), (b) 0.5 M NaCl plus 0.05 M $MgCl_2$ (90,000 X), (c) 0.1 M NaCl plus 0.05 M $MgCl_2$ (80,000 X), and (d) 0.05 M $MgCl_2$ alone (80,000 X).


a


b


c


d

for the presence of contaminating cytoplasmic materials. Such material in large amounts could influence the biochemical studies of cell envelopes. The procedure for the preparation of envelopes of these organisms has been described in Materials and Methods, section D. The isolated envelopes were shadow-cast with platinum.

These envelopes of 121 are shown in Figure 39. Individual envelopes could not be photographed, as the envelopes of 121 were always clumped. There is evidence in this picture of some cytoplasmic debris, although much of this is probably cytoplasmic membrane fragments under the outer layers of the envelope.

(b) Pseudomonas c-Al.

The envelopes of c-Al do not show the tendency to clump, so it is possible to photograph them individually (Figure 40). The small craters in the envelopes measure between 500-700 Å. Many of the spherules, as seen in Figure 24, measure in this range. Whether the craters are produced by a loss of the spherules during envelope isolation, is not known. The large holes were very likely produced by glass beads hitting the cells during envelope isolation. There is evidence of cytoplasmic debris present, although it is slight. During the isolation procedure, the envelopes sometimes break into smaller fragments, and these can be seen in Figure 40 (b). Generally, the envelopes isolated by the described procedure are intact and relatively free of cytoplasmic debris.

H. Biochemical analyses of cell envelopes of 121 and c-Al.

Envelopes used in these studies were isolated as de-

Figure 39. Electron micrographs of shadowed envelopes of Pseudomonas 121 (25,400 X).

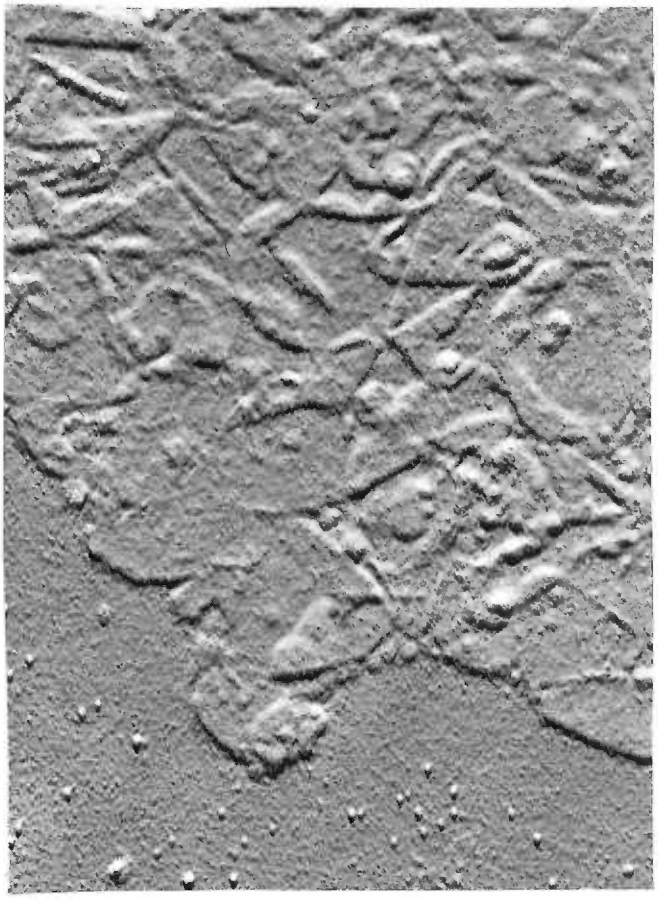
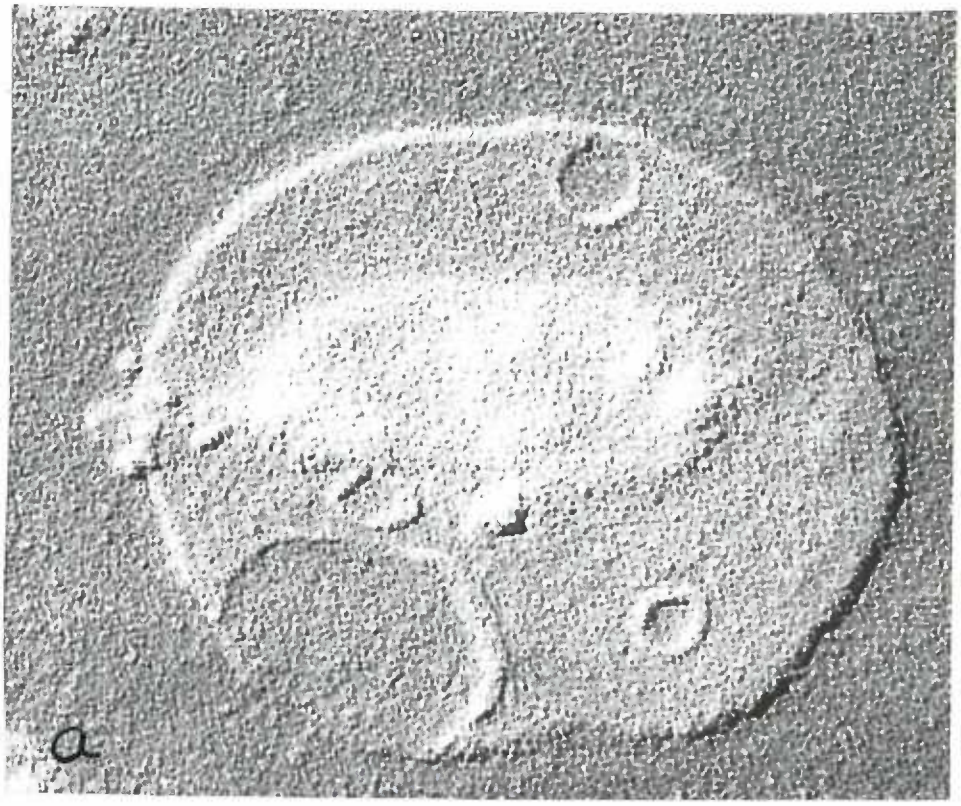
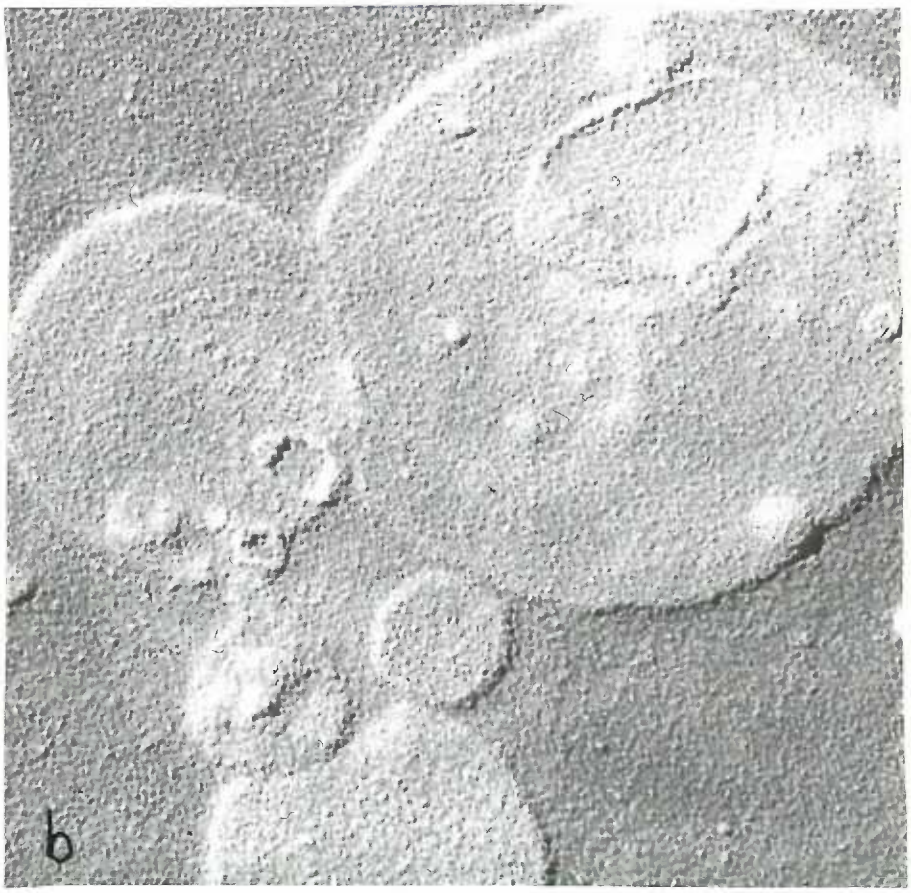


Figure 40. Electron micrographs of shadowed envelopes of Pseudomonas c-A1. Both (a) and (b) are magnified 114,000 X.


a


b

scribed in Materials and Methods, section D. Attempts to quantitate the contribution of dry envelopes to the total dry cell weight, were not entirely successful. With either organism, when the isolation procedure involved large quantities of envelopes, material was unintentionally removed with the supernates from envelope pellets. Such envelope yields were often as low as 2 to 5% (dry envelope/gm dry cells). The recovery when dealing with small quantities was much more consistent and effective, and was about 18% for 121 and 15% for c-Al. These recoveries are in agreement with 15% for E. coli determined by other workers(67).

I. Analyses of lipopolysaccharides from Pseudomonas 121 and Pseudomonas c-Al.

Lipopolysaccharide (lps) was extracted from these cells as described in Materials and Methods, F. I. The purity of the preparations was determined by assaying for ultraviolet-absorbing material in the range of 220-300 mμ. The extracted material was considered sufficiently pure when there was no longer an absorption peak at 260 mμ and/or 280 mμ.

According to Berst et al(64) there are several regions of absorbance in the infrared spectrum (500 to 4000 cm^{-1}), that are characteristic for lps.

Region 3500-2800 cm^{-1}. This region contains the bands characteristic of the groups OH, NH, and CH. A strong band in the area of 3300 cm^{-1}, in the absence of nucleic acids and proteins, may correspond to the OH and NH of sugars and amino sugars. A band at 2900

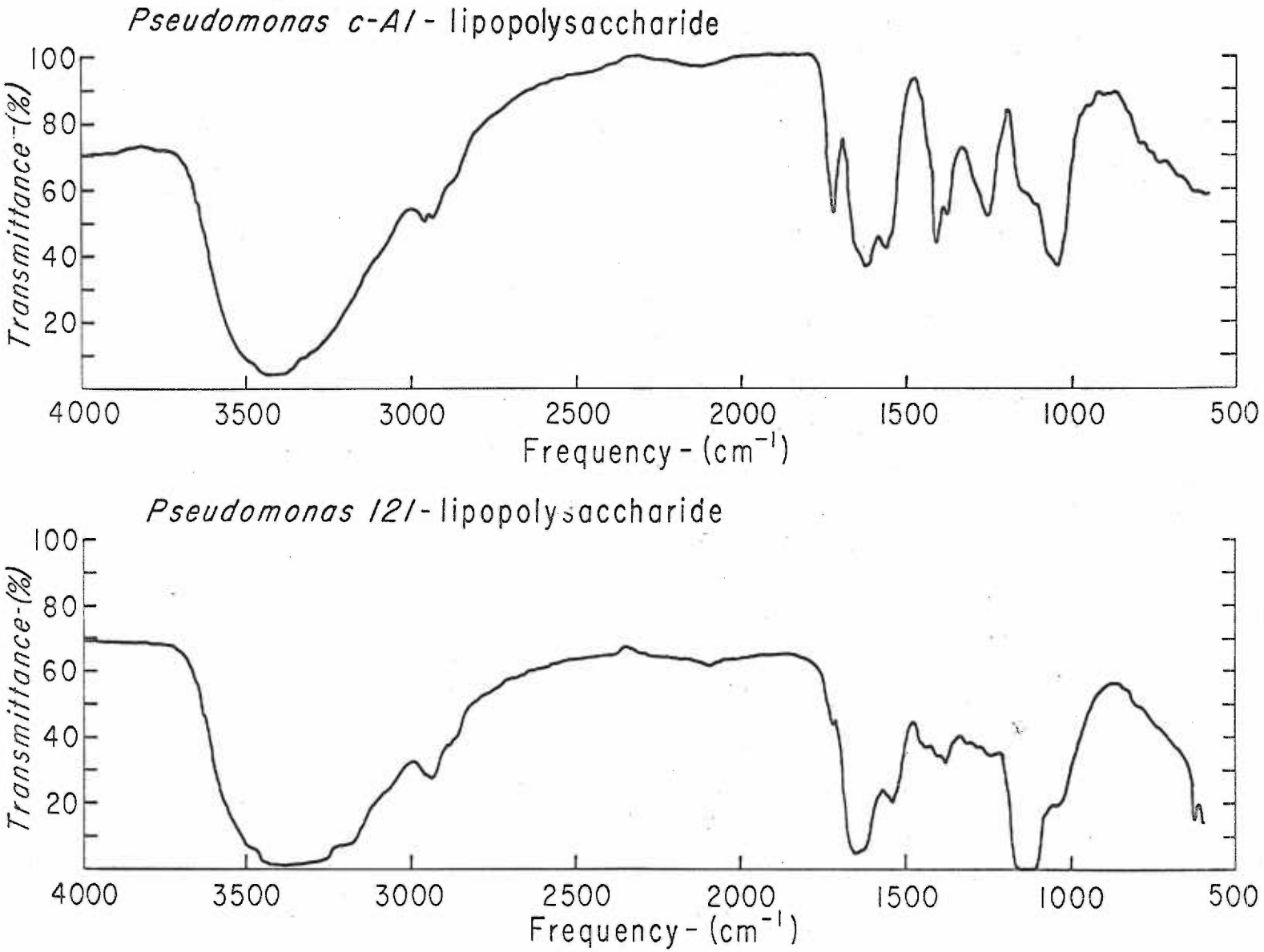
to 3000 cm^{-1} is due to the vibrations of the C-H of CH_2 and CH_3 groups which may be contributed by sugars and/or aliphatic chains.

Region 2800-1500 cm^{-1}. A band in the area of 1730 cm^{-1} corresponds to the area where the carboxyl groups of lipid esters absorb. Absorption in the area of 1650 to 1550 cm^{-1} is attributed to CO-NH group, and in the absence of proteins and nucleic acids may be from N-acetyl amino sugars.

Region 1500-1000 cm^{-1}. The COO^- group, which one finds in the uronic acids, produces a band in the area of 1410 cm^{-1}. A band at 1250 cm^{-1} can be produced by the presence of an ester, while the absorption from 1100 to 1000 cm^{-1} is characteristic of polysaccharides (64).

The results of the infrared spectra of the lps of c-Al and of 121 are presented in Figure 41. The extracted material from each organism showed a strong band of absorption in the area of 3400 cm^{-1} suggestive of the presence of OH and/or NH groups. Both extracts produced a peak at 3000 to 2900 cm^{-1}, corresponding to CH. A very strong peak at 1735 cm^{-1}, where one would expect lipid ester to absorb, was produced by the extract from c-Al while the same peak from the 121 extract was very small. The CO-NH absorption at 1650 to 1550 cm^{-1} was strong in both preparations. The 1410 cm^{-1} peak, where uronic acids absorb, was strong in the c-Al spectrum, but very weak, if present at all, in the

Figure 41. Infrared spectra of lipopolysaccharide extracted from (a) Pseudomonas c-A1 and (b) Pseudomonas 121.


Pseudomonas c-A1 - lipopolysaccharide
Transmittance - (%)
100
80
60
40
20
4000
3500
3000
2500
2000
1500
1000
500
Frequency - (cm⁻¹)
Pseudomonas 121 - lipopolysaccharide
Transmittance - (%)
100
80
60
40
20
4000
3500
3000
2500
2000
1500
1000
500
Frequency - (cm⁻¹)

121 spectrum. The band at 1235 cm^{-1} indicated the presence of an ester group in both preparations, but was particularly strong in 121. The 1100 to 1000 cm^{-1} band was strong in the c-Al spectrum but was weak in the plot from 121 material.

The spectrum from c-Al was well defined and typical of lps spectra(64). The spectrum from 121, however, was not well defined and was suggestive of a heterogeneous mixture of materials rather than a pure preparation.

The results suggest that c-Al envelopes contain lps material, as do other gram-negative, non-marine cells(64,67). The results of these studies on extracted material from 121 suggest that the lps may have been present but contaminated with other materials.

II. Extraction and analysis of phospholipids of Pseudomonas c-Al and Pseudomonas 121.

The purpose of analyzing lipids of these organisms was to determine whether gross differences might exist between them, which might be associated with their strikingly different behavior in low ionic environments.

The lipids were extracted from envelopes of c-Al or 121 with chloroform-methanol, followed by precipitation of the extract with acetone. These isolated lipids were then separated by thin-layer chromatography, each spot eluted and developed separately in four solvent systems to determine whether each spot consisted of a single lipid or a mixture.

Tracings of the thin-layer chromatograms of the lipid

mixtures from the envelopes of these organisms can be seen in Figures 42 and 43. The distribution of the spots from both organisms are very similar with the exception of an additional, very light spot (R_f 0.45) in the chromatogram of 121 lipids. Four of the spots were tentatively identified as phosphatidic acid (PA), diphosphatidylglycerol (DPA), phosphatidylethanolamine (PE), and phosphatidylserine (PS) based on their respective R_f values and those of the standards. The other spots were not identified.

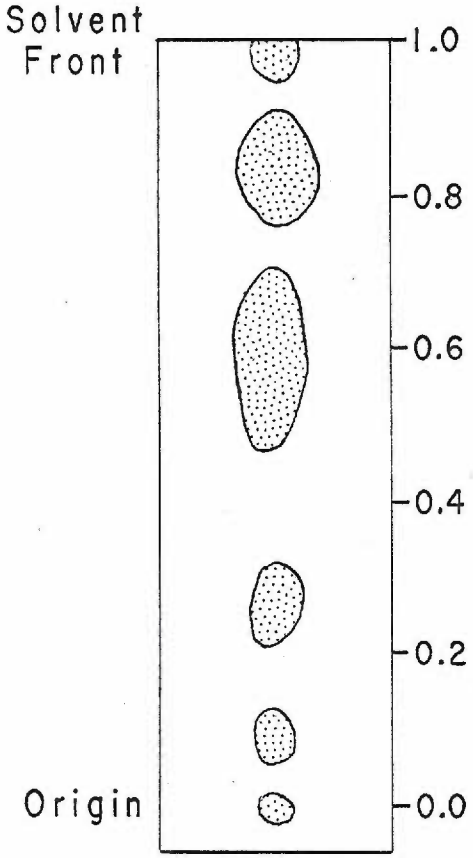
All of the spots eluted from the preparative chromatograms developed as individual spots in four different solvent systems with the exception of the spot corresponding to PE. The chromatography of this eluted spot in the N-butanol; acetic acid: water system produced one strong spot corresponding to PE and a second, extremely light spot with a slightly higher R_f value, the identity of which was not determined.

It was concluded from these studies that the phospholipid types present in these two organisms are not grossly different, unless such differences are in fatty acid types in the individual lipids. The size of the spots on the chromatograms suggests that c-Al has more PE relative to the other lipids, than does 121. The additional small spot (R_f 0.45) on the chromatogram of 121 was the only other difference noted between the organisms.

III. Extraction and analysis of amino sugars from enve-

Figure 42. Tracings of thin-layer chromatograms of envelope phospholipids of Pseudomonas c-Al. The R_f values of the standards were as follows: phosphatidic acid (PA), 0.94; diphosphatidylglycerol (DPG), 0.81; phosphatidylethanolamine (PE), 0.60; phosphatidylserine (PS), 0.28.

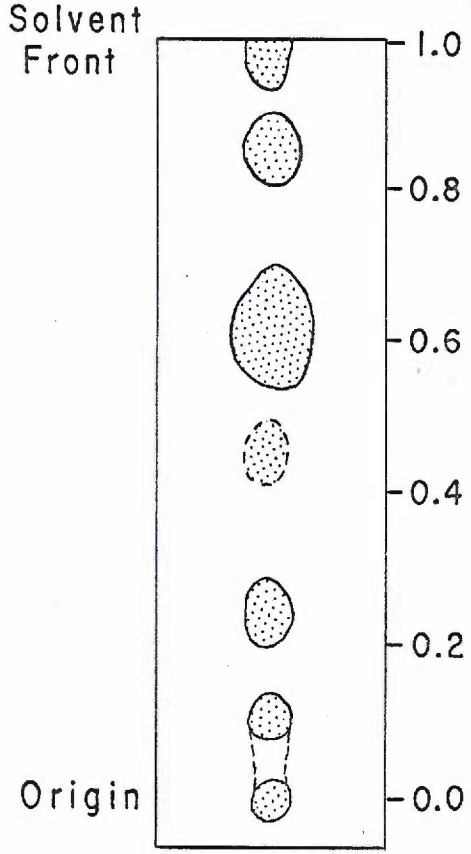
Pseudomonas c-A1



	Visualization		Possible
R_f	Ninhydrin	I_2	Identity
1.0	–	+	PA
0.8	–	+	DPG
0.6	+	+	PE
0.4			
0.2	+	+	PS
	+	+	?
0.0	+	+	?

Figure 43. Tracings of thin-layer chromatograms of envelope phospholipids of Pseudomonas 121. The R_f values of the standards were as follows: phosphatidic acid (PA), 0.94; diphosphatidylglycerol (DPG), 0.81; phosphatidylethanolamine (PE), 0.60; phosphatidylserine (PS), 0.28.

Pseudomonas - 121



	Visualization		Possible
R_f	Ninhydrin	I_2	Identity
1.0	−	+	PA
0.8	−	+	DPG
0.6	+	+	PE
0.4	+	+	?
0.2	+	+	PS
	+	+	?
0.0	+	+	?

lopes of 121 and c-Al.

The purpose of analyzing the amino sugars from the cell envelopes of these organisms was to determine whether the differences in behavior of these organisms in low ionic environments might be due to the absence of a rigid murein layer in the envelope of c-Al. One amino sugar known to be present in the murein layer of all bacteria examined is muramic acid(67). If an analysis of the envelope of c-Al were to show that muramic acid was not present, then this might be an indication that the envelope of c-Al lacked a rigid layer, and therefore, that the absence of this layer might be responsible for the fragility of this organism in low ionic environments.

The procedure for extracting the amino sugars was to hydrolyze the envelopes of both organisms in 4 N HCl and to separate the amino sugars by passing them through a charcoal-celite column. Fractions collected from the column were then chromatographed on paper in n-butanol: acetic acid: water. Individual spots were eluted and re-chromatographed separately in four different solvent systems along with muramic acid standard.

The results of these studies are presented in Figure 44 and Table 18. Figure 44 shows four Elson-Morgan positive spots from 121 and five spots from c-Al. The spots with R_{ga} values greater than 3.0 did not become visible until the third day following the application of the spray reagent. There are spots from both organisms with R_{ga} values corres-

Figure 44. Tracings of paper chromatograms of amino sugars from cell envelope hydrolysates of Pseudomonas strains 121 and c-A1. R_{ga} values were calculated by setting the distance of the glucosamine spot from the origin equal to 1. The standards were glucosamine (GA), muramic acid (MA), and galactosamine ($GalNH_2$). Fraction numbers refer to those from the charcoal-celite column.

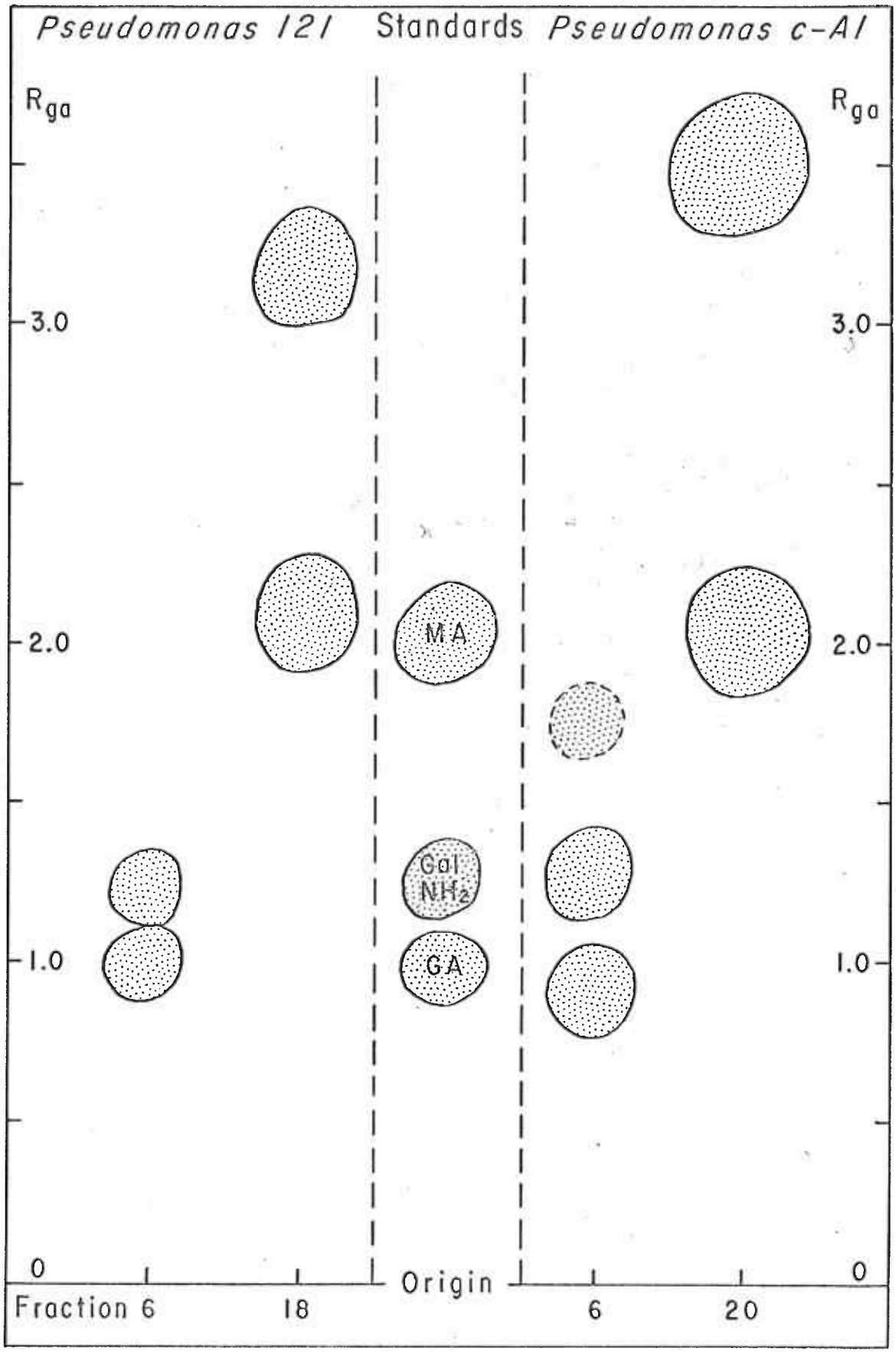

Pseudomonas 121
Standards
Pseudomonas c-Al
Rga
Rga
3.0
3.0
2.0
2.0
1.0
1.0
0
0
MA
Gol
NH2
GA
Origin
Fraction 6
18
6
20

Table 18

Re-chromatography of eluted spots with R_{ga} values similar to muramic acid

Developing solvent systems	R_f values for muramic acid standard plus unknown					
	Pseudomonas 121			Pseudomonas c-Al		
	Muramic acid	Muramic plus unknown	Unknown	Muramic acid	Muramic plus unknown	Unknown
1	0.54	0.52	0.52	0.53	0.53	0.53
2	0.37	0.36	0.36	0.34	0.34	0.31
3	0.34	0.35	0.34	0.36	0.35	0.35
4	0.57	0.58	0.56	0.58	0.60	0.59

ponding to glucosamine (GA), galactosamine ($GalNH_2$), and muramic acid (MA).

The re-chromatography of spots corresponding to muramic acid from both organisms (Table 18) indicates that muramic acid is present in both organisms. This evidence is strongly suggestive of the presence of a rigid murein layer in both organisms. In view of these findings, it appears reasonable to assume that the differences in the behavior of these two organisms in low ionic environments is not due to the absence of a murein layer in Pseudomonas c-Al. However, such differences could be due to c-Al having relatively less murein in the envelope than 121; or, the murein layer in the marine organism may have fewer peptide cross-links than in the murein layer of 121.

IV. Amino acid analyses of acid hydrolysates of cell envelopes of Pseudomonas 121 and Pseudomonas c-Al.

Cell envelope hydrolysates were analyzed to determine whether gross differences might exist in their amino acid composition. Such differences in the amino acids could indicate a basic difference in the nature of the protein in the envelopes of these organisms.

The procedure for this analysis was to hydrolyze the envelopes of each organism in 6 N HCl and to analyze the hydrolysate for its amino acid content in an amino acid analyzer. The results are presented in Table 19. These results show that the distribution of amino acids is not unique in either of the organisms when compared to those of E. coli and P. aeruginosa. However, the total protein in

Table 19

Amino acid analyses of envelopes of c-Al and 121

gm/100 gm dry envelope

Amino acid	Pseudomonas c-Al	Pseudomonas 121	E. coli[1]	Pseudomonas aeruginosa
Asp	4.0	9.75	7.1	9.3
Thr	1.87	6.93	3.8	---
Ser	1.95	4.81	3.7	5.4
Glu	3.68	7.50	6.9	5.8
Pro	1.04	2.66	1.5	---
Gly	1.63	5.66	3.5	7.1
Ala	2.20	8.53	5.6	5.1
Val	1.59	4.88	3.4	3.9
Meth Cys	0.75	1.07	0.7	---
Ile	1.63	2.49	3.7	2.8
Leu	2.69	5.57	5.3	
Tyr	1.67	3.56	3.3	4.2
Phe	2.03	4.61	3.0	7.3
Lys	1.45	4.04	4.0	1.7
His	0.53	1.30	0.9	---
Arg	1.74	4.31	3.8	1.3
Total less Cys	29.70	76.60	58.50	60.00[2]

[1] Ref. (67).

[2] Value is estimated using values from E. coli where not given for P. aeruginosa.

these two organisms is quite different. The protein content per given weight of envelope is considerably lower in the marine organism than in the terrestrial one.

The data from Table 19 were normalized for 121 and c-Al for direct comparison between the types of amino acids present in these organisms. As shown in Table 20, there appears to be little difference in the distribution of the types of amino acids from these organisms. The greatest differences lie in the neutral amino acids, of which 121 has a relatively higher amount, and the sulfur-containing amino acids, of which c-Al has the greater amount.

These results indicate that there is no gross difference in the distribution of amino acids in 121 and c-Al. Furthermore, there are no gross differences between these organisms and E. coli and P. aeruginosa in the distribution of amino acids. However, the marine organism had markedly less protein in the envelope than the other organisms. This low content of protein per envelope weight in c-Al, indicates that some other envelope component or components is present in relatively higher amounts. It is extremely unlikely that this component is murein, which would add rigidity to the envelope. It, therefore, seems reasonable to assume that the component is either lps or lipid. The amount of protein in the envelope of this organism may be significant in the response of the organism to a low ionic environment.

Table 20

Normalized analysis of amino acid content of envelopes of c-Al and 121

$$\frac{\text{Total for 121}}{\text{Total for c-Al}} = \frac{76.60}{29.70} = 2.59$$

Amino acids	gm/100 gm dry envelope c-Al (X 2.59)	121
Basic (His, Lys, Arg)	9.62	9.65
Aromatic (Tyr, Phe)	9.56	8.17
Branched chain (Val, Ile, Leu)	15.10	12.84
Sulfur containing (Met, Cys)	1.94	1.07
Neutral (Thr, Ser, Pro, Ala, Gly)	22.50	28.59
Dicarboxylic acid (Asp, Glu)	19.90	17.45

DISCUSSION

Various aspects of the relation of marine bacteria to their environment have been investigated by many workers (3,4,10). Several of these investigations have been concerned with the behavior of bacteria from the ocean in low ionic environments(4,38,39,40). The results of such investigations, although highly informative concerning the requirements for cell maintenance, have not provided direct evidence as to the specific role of ions or the interaction of such ions in the cell envelopes. The evidence which is presented in this thesis does provide insight into the role of both divalent and monovalent cations, and their interactions, in the maintenance of the cell integrity of a bacterium isolated from the ocean. This thesis also presents the results of biochemical analyses of envelopes from two bacteria, an isolate of terrestrial origin and one from the marine environment, for their content of amino sugars, phospholipids, lipopolysaccharides, and proteins. Biochemical analyses of the envelopes of other bacteria from the ocean have, to my knowledge, been confined to a lipid analysis(79), and analyses of some of the dialyzable and non-dialyzable material released from envelopes during lysis(40,41).

The body of work presented in this thesis was designed to answer certain questions basic to an understanding of

marine bacteria, and their ability to live and propagate in the ocean environment. The first of these questions concerned the ionic requirements of Pseudomonas c-Al. These aspects have been analyzed extensively by other workers(4,8, 9,26) with several organisms of varied properties. Therefore, characterization of Pseudomonas c-Al in terms of its ionic requirements was deemed a necessary phase of this research in order to compare its properties with those of other bacteria also isolated from marine environments.

The requirement of c-Al for ions can be divided into two categories, those required for growth and those required for the maintenance of cellular integrity. The object of this aspect of the work was, first, to determine those ions normally found in sea water which were required for the growth of this isolate. The findings from several experiments revealed that growth of c-Al would not occur unless the medium was supplemented with Na^+, K^+, Mg^{++}, and Cl^-. The anion, $SO_4^=$ was not required, although in low concentrations it enhanced the growth of this organism. In respect to these cations and anions, c-Al is similar to several other marine organisms investigated(4,9,93). Attempts to replace these ions required for growth with related ions were not successful. Therefore, the requirements for Na^+, K^+, Mg^{++}, and Cl^- by c-Al are specific ones. This requirement for specific ions, including Na^+, appears to be a general characteristic of those marine bacteria investigated(4). To my knowledge, the only specific roles for Na^+ in marine

bacteria thus far discovered are (a) in the transport of substrates or substrate analogs into the cell and (b) in the maintenance of intracellular solute concentrations using as a model compound the amino acid analog α-aminoisobutyric acid(9,28,111).

When the level of NaCl required for growth of c-Al was tested, maximum growth was found to occur when NaCl was present at 0.3 M; additional NaCl did not further enhance the growth of this organism. Since media devoid of NaCl would not support growth, Pseudomonas c-Al can be classified as an obligate halophilic bacterium and particularly as a "slight halophile", in agreement with findings on other marine bacteria studied for their ionic requirements(4,10).

Many of the conclusions in this thesis are based on data from optical density changes of cell suspensions. Optical density decreases were shown to be a good indicator of lysis throughout this research, with the exception of suspensions of cells in $CaCl_2$ solutions. Many other workers have also used changes in optical density as an indicator of lysis(4,10). These investigations have shown that for the most part such changes are good indications of cell lysis. However, most workers agree that optical density data should be supplemented with other confirmatory evidence, such as leakage of cytoplasmic materials into the supernate. Lysis is defined in this thesis as a disruption of the integrity of cell envelopes, evidenced as a decrease in optical density of cell suspensions, and confirmed by such leakage.

A detailed analysis of the ionic requirements for the integrity of c-Al, revealed that a combination of ions was not required for the maintenance of integrity such as was found necessary to support growth. Such analyses have revealed the same is true of all halophiles investigated(10). Tests carried out with NaCl showed that concentrations of 0.1 M or greater, maintained the optical density of suspensions of c-Al for at least one hour following transfer of the cells from sea water. Of those concentrations below 0.1 M tested, none were able to maintain optical densities. Furthermore, suspensions in NaCl at concentrations of 0.05 M and 0.01 M following transfer from sea water experienced greater decreases in optical density than did these similar cells transferred to distilled water. The significance of this finding will be discussed later. A discussion of the ability of KCl to maintain integrity of c-Al, which would be fitting at this point, will be covered following the discussion of maintenance of cell integrity by divalent cations, as the findings with KCl had a great influence on much of the subsequent work involving the action of ions in the maintenance of cell integrity.

Divalent cations have been found by several investigators to be effective in the maintenance of cell integrity of halophiles(4,10) and a necessary component in membranes from other sources(50,77). Therefore, the findings obtained with Mg^{++} in c-Al were certainly not unique or unexpected. Following exposure to sea water, the optical density of a cell sus-

pension in 0.05 M $MgCl_2$ was maintained essentially unchanged for 5 hours. Only slight decreases in optical density, ranging from 0.05 to 0.07 optical density units, had occurred by 5 hrs in cell suspensions in $MgCl_2$ concentrations of 0.05 M down to 0.0075 M; the decrease in optical density of suspensions was related to the Mg^{++} concentration, i.e. the lower the Mg^{++} concentration the greater the decrease in optical density. Contrary to the findings with NaCl, optical density decreases in distilled water were greater than those in any of the $MgCl_2$ suspensions. The significance of this observation will be discussed later.

As $CaCl_2$ is present in sea water at a molarity (0.01 M) fairly close to that of $MgCl_2$ (0.05 M), it was thought necessary to compare the ability of Ca^{++} to that of Mg^{++} in maintaining the integrity of c-Al. A comparison of tests with Ca^{++} to similar tests with Mg^{++}, revealed several unexpected findings.

The optical density of cell suspensions in $CaCl_2$ remained unchanged for a period of one hour; however, by four hours it had increased by 0.11 optical density units over that of "zero time". Ultraviolet absorption spectra of the supernates of cells in $CaCl_2$ solutions showed a peak at 280 mμ, suggestive of the presence of protein released from within the cell and/or the cell envelope. Leakage of ultraviolet-absorbing material was never detected in suspensions of cells in $MgCl_2$ solutions at sea water molarity. These findings indicate that optical density changes may not al-

ways reflect lysis in cell suspensions. Light microscopy studies of cells in $CaCl_2$ solutions, revealed that these cells did not have the normal staining properties of cells in $MgCl_2$ solutions or sea water. The stain was not retained well by these cells, indicating that the cell structure had been damaged in some way. Cells transferred from $CaCl_2$ solutions to NaCl solutions at a variety of concentrations, reacted in a manner completely opposite to cells transferred from solutions of $MgCl_2$ to NaCl solutions, the significance of which will be discussed later. These findings with Ca^{++} indicate that it is unlikely that this divalent cation at sea water concentration can replace Mg^{++} in maintaining the integrity of this organism. It is interesting to note that a variety of bacterial extracellular enzymes are activated by the presence of Ca^{++}(112). Therefore, it is conceivable that Ca^{++} reacts with, and thereby causes a release of such surface-bound proteins into the supernate of these cells, which would account for the ultraviolet absorption at 280 mμ. If this is the case, then the peak at 280 mμ may not be an indication of cell lysis.

Tests involving the reactions of c-Al in KCl solutions produced several significant findings. The first of these was that cells transferred from 1.0 M NaCl solutions to KCl solutions in a range of concentrations, reacted much differently from cells transferred from 0.05 M $MgCl_2$ solutions. Cells pre-exposed to $MgCl_2$ were maintained well in 1.0 M and 0.5 M KCl, whereas at no concentration did KCl maintain

cells following pre-exposure to 1.0 M NaCl. Furthermore, after $MgCl_2$ pre-exposure, suspensions in 0.05 M and 0.01 M KCl underwent greater decreases in optical density than similar suspensions in distilled water. Light microscopy studies confirmed these findings. These varied effects produced by the pre-exposure of cells to different salts, was an original and significant finding in this research. The results suggested that the pattern of lysis was to a great extent determined by the salt to which the cells were pre-exposed, i.e. cells were pre-conditioned to lysis.

Studies on lysis of c-Al showed that it occurred in two stages: a fast stage, occurring too rapidly to be measured spectrophotometrically, followed by a slow stage occurring slowly over several hours, most of which occurs during the first 15 minutes. This two-stage lysis is in agreement with the findings of several other workers who have investigated the lysis of halophiles(4,10).

The results of work on maintenance of cell integrity led to the proposal of a working hypothesis for the role of ions in the maintenance of cell integrity of c-Al. It was proposed that polyanionic groups are present in the envelope of c-Al, as proposed for the envelopes of other halophiles(10,32,33) and that Mg^{++} acts as a bridge between anionic groups, possibly between phosphoryl head groups of phospholipids and/or carboxyl groups of protein side chains. Bonds between Mg^{++} and these anionic groups, although ionic, are probably "covalent" in nature. This rationale was based

on the solubility properties of magnesium phosphates and carbonates. It was further proposed that water would not have sufficient attraction to remove Mg^{++} from these bridges. However, monovalent cations, e.g. Na^{+}, Li^{+}, or K^{+}, could displace Mg^{++} at the bridging sites, with the negative charges of the anion remaining screened by the monovalent cation. Sufficient screening of negative charges would prevent mutual repulsion by the negative groups and, therefore, disaggregation of envelope components. However, if the envelope is exposed to distilled water, the screening monovalent cations are removed from the anionic sites, resulting in envelope disaggregation, i.e. lysis. Several experiments were devised to test this hypothesis.

As mentioned previously, following exposure to Mg^{++} low concentrations of K^{+} (0.05 M and 0.01 M), caused greater decreases in optical density of cell suspensions of c-Al than did distilled water. Confirmatory studies with the light microscope suggested strongly that these decreases were the result of lysis. A similar experiment to that with KCl was devised with NaCl. Cells were pre-exposed to $MgCl_2$ and transferred to NaCl solutions at various concentrations. To be consistent with the proposed working hypothesis, cells transferred to low concentrations of NaCl should undergo more extensive disruption than cells in distilled water, as was the case in KCl solutions. This prediction was verified and confirmed with light microscopy studies as well as ultraviolet absorption spectra of cell

supernates. The ultraviolet absorption at 260 mμ was greatest in supernates from suspensions which experienced the greatest decreases in optical density.

These results obtained with low concentrations of Na^+ or K^+ suggest that these ions play an active role in lysing cells of c-Al, perhaps by breaking divalent cation bridges. Therefore, the very ions that in high concentration will maintain these cells, are in low concentrations active in lysing these cells. Experiments were designed to test the possible interaction of monovalent and divalent cations in the envelopes of c-Al. It was consistent with the working hypothesis that any effect of the pre-exposure of cells to monovalent cations should be seen in fast-stage lysis in distilled water. Pre-exposure of cells to monovalent cations in the presence of Mg^{++} should result in competition between these two ions for anionic sites in the envelope. Furthermore, the higher the concentration of monovalent cation in this system, the greater the antagonistic effect of this monovalent cation for Mg^{++} bridging sites. The amount of disruption during fast-stage lysis of these cells should then be proportional to the concentration of monovalent cation to which they were pre-exposed.

When this proposed antagonism between monovalent cation and Mg^{++} was tested, the results with Li^+ and Na^+ were exactly as predicted. Ultraviolet absorption at 260 mμ and ninhydrin reactions of distilled water supernates were proportional to the concentration of Na^+ to which cells were

pre-exposed. These findings were confirmed by electron micrographs of thin-sections of these cells, i.e. the cells exhibiting the greatest disruption were those which had been exposed to higher concentrations of Na^+. Thin sections of cells pre-exposed to $MgCl_2$ alone followed by transfer to distilled water appeared to have undisturbed envelopes and the cytoplasm appeared nearly normal; furthermore, these cells did not leak detectable amounts of ultraviolet-absorbing or ninhydrin-positive materials in distilled water.

Although the results employing Li^+ were identical to those of Na^+, the results with K^+ were not. Pre-exposure to K^+ did not render cells of c-Al as susceptible to lysis as did Li^+ and Na^+. Due to its larger ionic radius, K^+ may not have been able to compete as effectively with Mg^{++} for anionic sites. The relatively low polarizing power of K^+ might be insufficient to allow this ion to compete effectively for electrons of the anionic sites. Therefore, fewer Mg^{++} bridges would be broken, resulting in less disruption during lysis.

The relationship between Na^+ concentration and the degree of disruption during lysis was found to be mainly the result of electrostatic interactions with only minimum effects from osmotic forces. This became apparent when 1.0 M sucrose did not protect c-Al against lysis after cells were exposed to 1.0 M NaCl plus 0.05 M $MgCl_2$. The fact that 1.0 M KCl did not maintain cells after exposure to 1.0 M NaCl was another indication that the lysis of these cells was not solely the

result of osmotic factors.

Several workers dealing with the lytic phenomenon in halophilic bacteria have proposed that Na^+ and Li^+ are more effective that K^+ and NH^+_4 in protecting halophiles against lysis because Na^+ and Li^+ more effectively shield negative charges in the envelopes(32,35). Soo-Hoo and Brown (34) on the other hand, have proposed that Na^+ and Li^+ ions provide greater protection against lysis for Halobacterium halobium because they offer greater osmotic protection. The hydrated volumes of these ions are greater than those of K^+ or NH^+_4. It was, therefore, proposed that Na^+ and Li^+ are not able to penetrate into the cell, thereby providing osmotic protection, while K^+ and NH^+_4 readily penetrate the cell providing little or no osmotic protection(34).

The proposed relative abilities of monovalent cations to screen negative charges in the envelope(32,35) is very closely related to the proposal presented here concerning the relative ability of such cations to compete with Mg^{++} for anionic sites in the envelope. It would seem that the ions most effective in screening negative charges at anionic sites would also be more effective in the competition with Mg^{++} for these sites. The evidence presented in this thesis has shown that events taking place during lysis in distilled water are determined by the salts to which cells are pre-exposed. Cells taken from solutions of 1.0 M and 0.5 M NaCl alone and transferred to distilled water undergo extensive damage. The hernias are evidently produced by rupturing of

the envelope as a result of hydrostatic forces. It is not known whether there are specialized areas in the envelope more susceptible to hernia formation. It may be that the small craters seen in envelope preparations, or the spherules seen in preparations of unfixed c-Al cells, represent such areas particularly sensitive to stress. These hernias were never seen in lysed cells that had been pre-exposed to 0.05 M $MgCl_2$, regardless of the concentration of Na^+ present. Therefore, it would seem that Mg^{++} acts in some way to protect cells against sudden osmotic shock. Soo-Hoo and Brown(34) came to the same conclusion in studies with H. halobium. It may be that H. halobium is protected against shock by the proposed inability of water to remove Mg^{++} from the bridging sites binding together essential components of the cytoplasmic membranes or the envelope.

The inner and outer layers of c-Al envelopes appear to peel apart during lysis following exposure of cells to 0.5 M or 1.0 M NaCl plus 0.05 M $MgCl_2$. This separation of the layers suggests the breaking of bonds which hold these layers together. These layers do not separate when cells are transferred from $MgCl_2$ alone to distilled water, suggesting that Na^+ plays a role in the destruction of the bonds binding these layers together. Such separation of layers during lysis was also noted in two other marine pseudomonads(39,41,113). Lysed c-Al cells which have been pre-exposed to 0.05 M $MgCl_2$ plus 0.1 M NaCl exhibit large segments of inner and outer layers which are relatively intact. However, lysed cells

pre-exposed to higher concentrations of NaCl (0.5 M or 1.0 M) plus $MgCl_2$ not only show separation of inner and outer layers, but the layers themselves show some damage. The envelope of lysed cells pre-exposed to 1.0 M NaCl alone show extensively damaged layers in the envelope.

Observations on lysis of cell envelopes of c-Al in distilled water following exposure to 0.05 M $MgCl_2$ and/or NaCl in various concentrations were considerably different quantitatively, but not qualitatively, from similar observations on whole cells. The immediate decrease in optical density when envelopes were re-suspended in distilled water after exposure to salt solutions was much less than observed for whole cells. Even envelopes pre-exposed to 1.0 M NaCl alone exhibited only a 40% decrease before the 30 sec reading, decreasing another 15-18% over the next 15-20 minutes. Yet electron micrographs of these lysed envelopes show extensive disaggregation. Suspensions of envelopes pre-exposed to $MgCl_2$, regardless of the concentration of Na^+ present, underwent only slight decreases in optical density (up to 9%) during fast-stage lysis in distilled water. At first glance, one might conclude from these results that the role of Na^+ in the lysis of whole cells is mainly osmotic, as the pre-exposure of whole cells to Na^+ results in much greater optical density decreases in fast-stage lysis.

A short discussion is required at this point on the relationship between optical density changes in suspensions of whole cells and such changes in envelope suspensions.

When whole cells of c-Al were transferred from 1.0 M NaCl plus 0.05 M $MgCl_2$ to distilled water, the optical density decrease measured at 30 sec was always slightly more than 70% below the initial reading. The ultraviolet absorption spectra and ninhydrin tests on the supernates of these lysed cells confirm that lysis had occurred in these cell suspensions. When such lysed suspensions were centrifuged, the supernates appeared clear with negligible turbidity at 520 mμ. Electron micrographs of the material in the pellets showed that all cells were extensively damaged. Although the envelopes of these cells showed traumatic damage, the fragments of these envelopes, for the most part, were still visible around the cells, i.e. large segments of the cell envelopes were intact. One must then consider that whole cells before lysis had an optical density 70% greater than approximately the same number of lysed cell ghosts. These ghosts are much like isolated envelopes, in that the ghosts have lost at least part of their cytoplasmic material. For this reason, it is reasonable to assume that the number of envelopes required to produce a given optical density is much greater than the number of whole cells required to produce the same optical density.

Observations on lysed envelopes following 1.0 M NaCl pre-exposure showed that the extensive disaggregation of these envelopes into small fragments produced at most only a 55-58% decrease in optical density. It is therefore suggested that small decreases in optical density of envelope suspensions may be highly significant, and one should be

cautious when equating such changes to similar changes in whole cell suspensions.

The evidence presented in this thesis from studies on the role of ions is consistent with the proposed working hypothesis. Costerton et al(39) have very recently suggested that the same mechanisms may account for cell lysis in another marine pseudomonad; however, no direct evidence was presented to substantiate their proposal. Direct evidence for the working hypothesis proposed for this research has been obtained in two different experiments with cell envelopes of c-Al. First, it was shown that envelopes of c-Al exposed to 1.0 M NaCl alone underwent extensive disaggregation in distilled water. When observed in the electron microscope, this lysed-envelope material consisted of small fragments. These fragments were shown to reaggregate into large conglomerates in the presence of $MgCl_2$. This evidence presents a strong argument for the role of Mg^{++} as a bridge between envelope components in this organism. Similar reaggregation experiments with divalent cations have been carried out on membrane fragments from Streptococcus faecalis (50,77).

The second experiment providing direct support for the working hypothesis dealt with cation exchange in the envelope. It had been proposed that Mg^{++} bridges were broken by monovalent cations. If Mg^{++} could be displaced in the envelope of c-Al by monovalent cations, then it should appear in the supernate of the envelope suspension. When this hypothesis was tested, Mg^{++} was indeed found to be replaced from the en-

velopes by Na^+, and the amount of Mg^{++} displaced was proportional to the Na^+ concentration up to 0.5 M. A cation exchange experiment with envelopes of Pseudomonas 121 showed that Mg^{++} was also displaced from the envelope of this organism by Na^+. However, as 121 envelopes do not lyse in distilled water following exposure to 1.0 M NaCl, the role of Mg^{++} in the maintenance of these envelopes is thought to be a secondary one. It may be that Na^+ is not able to displace all the Mg^{++} from these envelopes, so that the amount required for maintenance of integrity remains bound in the envelopes. This possibility was not tested. Studies on cation exchange have been carried out by other workers on artificial membrane films of phospholipids in which Ca^{++} was found to be displaced by monovalent cations (114). An interesting and relevant observation was made in analyses for Mg^{++} in whole cells of Aerobacter aerogenes under various conditions(95). In this instance it was noted that "surface bound" Mg^{++}, which could not be removed by washing cells with distilled water, was readily removed by washing cells in 0.85% NaCl. This "surface bound" Mg^{++} accounted for 26% of the total cell Mg^{++}.

The discussion to this point has been concerned with the ions required for growth of c-Al, and the possible role of ions in maintaining the envelope integrity of this organism. Pseudomonas 121 has been for the most part omitted from this discussion. When 121 was tested for its response to sudden changes in salt concentration, it did not undergo

lysis. The major purpose for including Pseudomonas 121 in this study was to allow a biochemical comparison of the envelopes of the marine isolate with a closely related terrestrial bacterium. The results of electron microscopy studies on thin-sections of these organisms showed that there were major differences between these organisms in the number of envelope layers. Pseudomonas 121 appeared to have one or possibly two more visible layers than c-Al. This, in itself, implies that the envelopes of these organisms are different biochemically either qualitatively or quantitatively. Another major difference already mentioned is their responses to low ionic environments. While 121 appears quite stable in such environments, c-Al may be highly unstable, depending on the salt to which it had been previously exposed.

The final section of this discussion will deal with the results of the biochemical analyses on the envelopes of these organisms and the implications of these studies in the answers to the questions asked in the Statement of the Problem.

The purpose of this biochemical investigation was to attempt to find gross differences that might account for the ultrastructural dissimilarities observed and the variances in their responses to low osmotic environments. The most obvious answer to both these questions was that c-Al might be deficient in a rigid murein layer. As mentioned previously, the absence of such a layer could account for

the lack of protection offered by the envelope of c-Al in hypotonic environments. Furthermore, it might also account for the fewer layers in the envelope of c-Al when observed in thin-sections. However, when the envelopes of these organisms were analyzed for the presence of glucosamine and muramic acid, each organism was found to possess both sugars. The conclusion was, therefore, that Pseudomonas c-Al does indeed have a murein layer, and, as was expected, so does Pseudomonas 121. Whether there are quantitative differences between the relative amounts of murein in these two organisms or differences in the structure of their murein layers is not known. Muramic acid has been detected in the supernates from lysed cells in two marine pseudomonads(40,41). The fact that this component of the rigid layer was released on lysis led one of these investigators to suggest that the murein layer might be maintained as a polymer by the presence of cations which screen otherwise mutually repelling charges(41). This possibility was not tested in c-Al.

Another component normally found in the envelopes of gram-negative bacteria is lipopolysaccharide. One could suppose that the absence of this component might result in a less rigid envelope. The polysaccharides in lps might add rigidity, or the lipid portion through its interaction with other envelope components might also provide a more cohesive structure. Material from both 121 and c-Al was extracted by a method used for the extraction of lipopolysaccharide(60) and analyzed by infrared spectroscopy. The spectrum of c-Al

material was strongly suggestive of the presence of lipopolysaccharide. The results obtained with the extract from 121 was not as clear cut, since analysis of this spectrum indicated that this lipopolysaccharide was probably contaminated with extraneous substances, such as nucleic acids. To my knowledge, lps from marine organisms has not been analyzed by other investigators; therefore, comparison of these results with those of others is not possible. However, these spectra are very similar to those for extracted lps from other terrestrial bacteria(64).

An analysis of the phospholipids of these two organisms disclosed that there were no major differences in types of lipids found. The major portion of the lipid extract consisted of four lipids which were tentatively identified as phosphatidylethanolamine, phosphatidic acid, phosphatidylserine and diphosphatidylglycerol. The major lipid type was phosphatidylethanolamine, which appeared to be present in relatively higher amounts in c-Al than in 121. Whether the fatty acid groups in these lipids are grossly different is not known. These findings were in agreement with those of Gordon et al(79), who found that the lipids of a marine pseudomonad and P. aeruginosa were not grossly different.

The amino acid analyses of hydrolysates of the envelopes of 121 and c-Al disclosed that the distribution of amino acids from both organisms were much the same, but the total quantity of protein present in the envelopes of c-Al was less than one-half that in 121 envelopes. This finding concerning

the distribution of amino acid types is consistent with the findings of other workers(67) on E. coli and P. aeruginosa. The total protein in the hydrolysates of 121 envelopes was higher than those obtained with E. coli and P. aeruginosa(67); however, Brown and Turner estimated the protein in the envelope of E. coli to be as high as 85%(115). The protein from the envelopes of marine bacteria have not been analyzed for their relative distribution of amino acids or total quantity of protein by other workers, so comparison of these results with other marine bacteria is not possible. As the difference in the quantity of protein present in the envelopes of c-Al and 121 was the only gross biochemical difference found, it was considered to be significant, in view of the differences in the behavior of the envelopes in low ionic environments. Aside from the total quantitative difference, a close analysis of the proportions of various types of amino acids shows that there are differences in the relative amounts of sulfur-containing and neutral amino acids. Pseudomonas c-Al envelopes contain relatively twice the amount of sulfur-containing amino acids per given weight of protein than do those of 121, although the quantity of these amino acids is quite low in both envelope preparations. On the other hand, the envelopes of 121 contain proportionately 25% more neutral amino acids than do those of c-Al.

Much of this protein is no doubt required, along with other envelope components, to maintain structure. Comparison of these analyses with those of the structural protein

from Neurospora membranes isolated by Woodward(116) reveals that there are differences in the distribution of amino acids. The proportion of basic amino acids is much the same, as are those for sulfur-containing and neutral amino acids. The structural protein contains a slightly higher amount of branched-chain amino acids, while the aromatic ones are 25 to 50% higher in the envelopes. The proportion of acidic side-chain amino acids in the envelopes of the bacteria is about 20 to 40% higher than in the structural protein.

It is quite probable that the total amino acid analysis values are not representative of protein that is obligatory for maintenance of the bacterial envelope structure, i.e. structural protein. There may be many other sources of amino acids contributing to these hydrolysates. For instance, some of the amino acids are no doubt contributed by the peptides of the murein layer, while others are probably from envelope-bound enzymes that are not obligate components of the envelope structure.

The finding that the marine bacterial envelopes contained less protein than the envelopes of the terrestrial organism brings up another question. If the envelopes of c-Al have relatively less protein, then they must have a relatively higher quantity of some other component. One can speculate that the other component is lipid. As quantitative studies on envelope lipids were not carried out in this research, the question remains unanswered.

For some time the lipid matrix was thought to be the rigid structure in membranes(117). However, Fleischer et al(118) have found that lipid-extracted mitochondrial membranes appear normal when viewed in the electron microscope. This implies that the structure of the membrane is the function of the protein and not of the lipid. If this same relationship holds true in the envelopes of bacteria, then one can speculate that the envelope of the marine organism c-A1 may be different from that of 121 simply because it contains less protein. A conclusion that this is the only difference in the envelopes of these two organisms, almost certainly is an over-simplification. An organism, such as c-A1, which has its normal habitat in the salt environment of the ocean, almost certainly has a structural make-up better suited to its own natural environment than to one as might be encountered in fresh water, for instance. Therefore, it is not surprising that the envelopes of marine bacteria are not resistant to sudden osmotic changes. These organisms may not need the cohesiveness in their envelopes that terrestrial forms do. Terrestrial bacteria may encounter a variety of osmotic conditions, whereas the marine environment is relatively constant, and does not select for properties found in terrestrial bacteria.

The Statement of the Problem of this thesis posed several questions which this research was designed to answer: (a) What are the specific ions normally found in the ocean which are required by this organism for growth and for main-

tenance of cellular integrity? (b) Is this marine bacterium so basically different in its properties from its terrestrial counterparts that its survival outside the ocean environment would be tenuous? What are the primary lesions resulting from removal of the bacterium from the ocean environment? What are the causes of these lesions? (c) In what ways does this marine bacterium differ biochemically, structurally, and/or physiologically from a similar terrestrial organism? Why should such differences make this marine organism better fit for survival and propagation in the ocean environment?

This research has provided answers to several of these questions. However, the most important contributions of this research that are unique to this work are as follows: (a) that an interaction exists, specifically, an antagonism between monovalent and divalent cations in the envelope of c-Al, and that the presence of monovalent cations preconditions envelopes of c-Al so that they are more susceptible to disaggregation in distilled water; (b) that the degree of disruption during lysis is the function of the type and quantity of cation to which cells are pre-exposed; (c) that distilled water, itself, does not remove bound Mg^{++}, presumably in bridging sites, from envelopes of c-Al and 121, but that Mg^{++} is readily displaced by Na^{+}; (d) that envelopes of c-Al transferred from 1.0 M NaCl alone to distilled water undergo extensive disaggregation and that the addition of Mg^{++} results in reaggregation of these fragments;

and (e) that biochemical analyses of envelopes of 121 and c-Al indicate that envelopes of c-Al have relatively less protein than those of 121, which might account for the differences in behavior of these two envelopes in distilled water.

SUMMARY AND CONCLUSIONS

A sea water-requiring bacterium, designated strain c-Al was isolated from the ocean, subjected to a battery of classification tests, and found to be classifiable as a Pseudomonas. The ions required in the growth medium were Na^+, K^+, Mg^{++}, and Cl^-, and these could not be replaced by related ions. Pseudomonas c-Al was further classifiable as a slight halophile, based on the concentration of Na^+ required to support optimal growth. Cells transferred from sea water to NaCl or $MgCl_2$ required at least 0.1 M or 0.0075 M, respectively, for cell maintenance. Cells pre-exposed to 0.05 M $MgCl_2$ were maintained in 1.0 M and 0.5 M KCl while those pre-exposed to 1.0 M NaCl were not maintained at any KCl concentration tested. Suspensions of cells pre-exposed to 0.05 M $MgCl_2$ underwent less optical density decrease in distilled water than in low concentrations of NaCl or KCl, indicating that in low concentrations, monovalent cations might be active in lysing cells.

It was proposed that Mg^{++} formed bridges between envelope components, and that monovalent cations were antagonistic by displacing Mg^{++} from these bridging sites, thereby rendering envelopes susceptible to disaggregation in distilled water. Pre-exposure of c-Al to 0.05 M $MgCl_2$ plus varying concentrations of NaCl resulted in various

degrees of disruption of cells in distilled water; such pre-exposure had little or no effect on 121 in distilled water. This degree of disruption of c-Al was a function of NaCl concentration, as confirmed by ultraviolet absorption spectra of supernates and electron micrographs. LiCl, but not KCl, acted in a manner similar to NaCl in this experiment. Sucrose and lactose provided protection against lysis after pre-exposure to concentrations at or below 0.1 M NaCl plus 0.05 M $MgCl_2$. Transfer of c-Al from 1.0 M NaCl to distilled water produced hernias in cell envelopes, presumably the result of hydrostatic pressure. These hernias were never seen in lysed cells after pre-exposure to $MgCl_2$, regardless of the NaCl concentration. No evidence was found for lytic enzyme(s) active during lysis, although the existence of such enzymes could not be ruled out. Envelopes of c-Al pre-exposed to 1.0 M NaCl underwent extensive disaggregation in distilled water, whereas those of 121 did not. The addition of $MgCl_2$ to disaggregated c-Al envelope suspensions resulted in partial reaggregation of envelope fragments. Cation-exchange experiments disclosed that Mg^{++} was displaced from envelopes of 121 and c-Al by Na^+, and that such displacement in c-Al envelopes was proportional to Na^+ concentration up to 0.5 M.

Biochemical analyses of the envelopes of c-Al and 121 indicated that both organisms contained muramic acid, strongly suggestive of a murein layer. Infrared absorption spectra of phenol-extracted material suggested that

envelopes from both of these organisms contained lipopolysaccharide. The mixture of phospholipids from envelopes of both these organisms produced similar patterns on thin-layer chromatograms. The lipids were tentatively identified as phosphatidylethanolamine, phosphatidic acid, diphosphatidylglycerol, and phosphatidylserine. Amino acid analyses of envelope hydrolysates of 121 and c-A1 indicated no gross differences in the amino acid distribution, but envelopes of c-A1 contained less than one-half the protein of those of 121. It was suggested that the difference in the behavior of these two organisms in low ionic environments might be related to this difference in total protein in the envelopes.

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