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Part III

Response of NRC-1004 to Changes in Its Ionic Environment
or
(Role of Mono- and Divalent Ions in the Integrity of NRC-1004 Cells)

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ACKNOWLEDGMENTS

I wish to thank Dr. Donn J. Kushner who, with dedication and patience, has been the chief inspiration in the completion of this task. Beyond his responsibilities as an academic and scientific supervisor, he has known through the years how to dispense encouragement, sound advice, and sincere friendship.

It is with great appreciation that I thank Dr. A. DesMarais, past Chairman of the Department of Biology, University of Ottawa, and Drs. W. H. Cook and N. E. Gibbons, Division of Biosciences, National Research Council of Canada, who made it possible to initiate and carry out an extensive part of this research in the NRC laboratories, providing material and financial assistance.

The contributions of Drs. M. Kates and C. Nozzolillo, for helpful discussion and critical review of the manuscript; Dr. J. R. Madeley, in the construction and delicate handling of the micro-electrophoretic cell; Dr. M. Wassef and Mr. A. Mykytiuk, for their assistance in chemical analyses; Mr. G. Ben-Tchavtchavadze, for the excellent photographs; and, last but not least, Miss E. Monson, for her valuable time and experience in secretarial work, are thoughtfully acknowledged.

ABSTRACT

The marine psychrophilic gram negative bacterium NRC-1004 lyse when subjected to temperatures above 21 °C, to low ionic strengths or to pH values below 6.0. Lysis was investigated by microscopic observation, micro-electrophoresis, turbidimetry and measurement of intracellular material leakage. Under phase contrast optics the cells changed from dense rods to pale globules and finally disintegrated into amorphous aggregates.

Intact cells were homogeneous in electrophoretic mobility and behaved comparably to gram negative and gram positive bacteria. Lysis, however induced, caused an increase in electronegative mobility of cells and lysed cell populations became heterogeneous in mobility. The increase of electronegative mobility of distilled water and acid lysed cells was apparently due to adsorption of intracellular material onto the surface of lysed cells, which could be removed by washing. Temperature induced lysis caused an irreversible change in cell surface charge.

Using lipid phosphorus and hexosamine determinations as indicators of cytoplasmic membrane and cell wall, respectively, it was shown that in temperature lysed cells the envelope suffered significant degradation, and more so in the presence of salts. In distilled water lysed and mechanically broken cells, a) the cell envelope disintegrated into small particles which required centrifugation

at 100,000 x g for one hour to sediment, and b) lipid phosphorus and hexosamine were not converted to water-soluble degradation products.

Monovalent salts could not maintain the integrity of NRC-1004 cells. Divalent salts, some at very low concentration, could maintain cell structure. Effectiveness was in the order $Cu^{++} > Zn^{++} > Ni^{++} > Ca^{++} > Mn^{++} > Mg^{++}$. In the presence of 0.1 M $MgSO_4$, NaCl and, less effectively, LiCl and RbCl could maintain cell structure, but KCl, NH_4Cl, and CsCl could not. Glucose, sucrose, ethanolamine, glutamic acid, lysine, proline, methionine, arginine, cysteine, glycine, histidine and spermine were ineffective.

For growth, magnesium, sodium, and traces of calcium, iron and potassium ions were required.

RÉSUMÉ

Les recherches, dont les résultats sont presentés dans cette thèse, ont porté sur la lyse de la bactérie marine psychrophile NRC-1004. La lyse put être provoquée par la suspension des cellules dans l'eau distillée ou dans un milieu salin au pH en dessous de 6.0 ou encore en soumettant les cellules à une température au dessus de 21°C. Les observations furent faites par microscopie à contraste de phase, microélectrophorèse, turbidimétrie, et par la détermination de la perte, par les cellules, de substances intracellulaires. Durant la lyse les cellules devinrent de plus en plus pâles et passèrent de la forme de batonnets à celle de globules translucides. Ceux ci finalement se désintégrèrent et il en résultat des aggregats amorphes.

A l'électrophorèse les cellules intactes s'avérèrent homogènes du point de vue de leur mobilité et leur comportement fut comparable à celui d'autres bactéries, gram négatives comme gram positives. Quelle que fut la méthode utilisée pour provoquer la lyse, celle ci causa chaque fois une augmentation de la mobilité électronégative des cellules tandis que les populations cellulaires devinrent hétérogènes du point de vue de leur mobilité. L'augmentation de la mobilité électronégative des cellules traitées par suspension dans l'eau distillée ou soumises à un pH acide semble avoir été dûe à l'adsorption de substances intracellulaires à la surface des cellules. Ces substances purent être détachées par lavages successifs. Les

températures au dessus de 21°C eurent des effets très marqués sur la structure de l'enveloppe des cellules.

La détermination du contenu en hexosamine et en complexe lipide-phosphore, en tant qu'indicateurs des paroids et des membranes cellulaires respectivement, permirent d'apprécier les dommages causés par les températures au dessus de 21°C aux enveloppes de ces bactéries, plus particulièrement en présence de sels. La suspension en eau distillée et la rupture mécanique des bactéries NRC-1004 provoquèrent la désintégration des enveloppes en petites particules. Leur récupération exigea une sédimentation de 100,000 x g pendant une heure et on n'observa pas de dégradation chimique de l'hexosamine ni du complexe lipide-phosphore de ces structures.

Les sels monovalents ne furent pas capables de conserver l'intégrité des bactéries NRC-1004. Il fut possible de préserver leur intégrité dans des solutions de sels bivalents, même à très basses concentrations. Ils furent classés dans cet ordre décroissant de leur efficacité à préserver l'intégrité de la bactérie NRC-1004: $Cu^{++} > Zn^{++} > Ni^{++} > Ca^{++} > Mn^{++} > Mg^{++}$. En présence de 0.1 M de $MgSO_4$ il fut possible de préserver l'intégrité cellulaire de la bactérie NRC-1004 en y ajoutant du NaCl ou avec de moins bons résultats soit du LiCl soit du RbCl. Le NH_4Cl, le KCl et le CsCl ne purent pas préserver l'intégrité des cellules de NRC-1004 suspendues

dans 0.1 M $MgSO_4$. Le glucose, le sucrose, l'éthanolamine, l'acide glutamique, la lysine, la proline, la méthionine, l'arginine, la cystéine, l'histidine et la spermine s'avérèrent inefficaces.

Les ions de magnésium, de sodium et des traces des ions de calcium, de fer, et de potassium furent necessaires à la croissance de cette bactérie.

GENERAL INTRODUCTION

The microorganism which was the subject of the studies presented in this thesis is a marine psychrophilic bacterium first isolated in Norway from flounder eggs by Dr. K. Eimhjellen. This organism aroused great interest among scientists actively engaged in research on psychrophilism in yeasts and bacteria at the Division of Biosciences, National Research Council of Canada. A culture kindly provided by Dr. Eimhjellen was brought to Ottawa by Dr. P.O. Hagen and registered in the culture collection of the National Research Council of Canada under the code number NRC-1004.

First observations by Hagen, Kushner and Gibbons (80) showed that NRC-1004 was a very delicate bacterium with a maximum growth temperature of 19.5°C. Lysis, indicated by drop in turbidity and release of intracellular material, was also reported by these authors when NRC-1004 cells were suspended in cold distilled water or in sea water at 25°C.

The initial research project of the work presented here consisted of studying through various complementary approaches the effects of temperature and various ions on the integrity of NRC-1004 cells. This was done with the hope of understanding better the causes of lysis in this bacterium. Additional interest lay in the possible correlation of the data obtained in this research with those obtained with other microorganisms sharing some of the most salient

characteristics of NRC-1004. Among these were the physiological properties of marine bacteria, dependence on low temperature as a psychrophilic microorganism, and finally its lytic susceptibility in relation to the effect of various ions on the cell envelope and their role in maintaining the cell's integrity.

The introduction of this thesis has been subdivided into sections dealing with the various facets of the problems encountered during the investigation to provide a clearer presentation of the bibliographic material.

Marine Bacteria

Sea water microbiology, which Scholes and Schewan (191) somewhat picturesquely called in 1964 the "Cinderella of Marine Sciences", is coming of age, as can be seen from the increasing interest shown by numerous scientists from many nations in this rich and promising field (110, 153, 154). While in 1946 ZoBell (234) found that marine bacteria accounted for only 6.5% of bacteria described in Bergey's Manual of Determinative Bacteriology, in 1964 Scholes and Schewan (191) found that 20% of the 1550 species listed in Bergey's 1957 edition were of marine origin.

Roughly three-fourths of the earth's surface (233, 234) is covered by oceans, in which dwell, exclusive of insects, about four-fifths of all known animal species (234). Thus, it would only be just to refer to the ocean as the greatest "developing country" in our

century, rather than to describe it as "the world's largest and most efficient septic tank", as did ZoBell (234) in 1946 while arguing in favor of the hypothesis of an oceanwide microbial population. Today, no one doubts any longer the ubiquity of microbes in the ocean, a fact which was questioned by what ZoBell (234) called "land-locked biologists" who did not believe in the existence of the bacteria in the sea beyond the littoral zones influenced by the contamination from the land. While such bacteria definitely do exist, the controversial question of whether there are specific marine bacteria has still to be answered (125, 154).

Many investigators have searched for any peculiarity of marine bacteria, either in their nutritional requirements, metabolic pathways, or physical and chemical characteristics, and their work was reviewed by MacLeod in 1965 (125).

As far as the nutritional requirements are concerned, marine bacteria show a notable preference for amino acids as a source of nitrogen, carbon, and energy, as compared to microorganisms isolated from land or fresh water. Mudrak (146) in 1933 was first to notice this characteristic. While studying 10 strains of luminous marine bacteria, he observed that asparagine or aspartic acid could favorably replace peptone in the growth medium. In 1936, Bukatsch (32) showed that glutamic acid, serine, alanine, and leucine were good substitutes for peptone in the growth medium of luminous

bacteria. Three years later Ostroff and Henry (155) found that, individually, alanine, aspartic acid, and glutamic acid were able to sustain the growth of most of the 15 aerobic marine bacteria tested by them, and Doudoroff, in 1942 (48), observed that methionine was necessary for the growth of Photobacterium phosphoreum. MacLeod et al. (131) studied the nutritional requirements of 33 marine bacteria and elaborated a complex mixture of 18 amino acids which was acceptable to all as the only source of carbon, nitrogen, and energy. Those bacteria which grew only in the presence of amino acids grew well in a mixture of glutamic acid, alanine, and aspartic acid. This mixture was also satisfactorily used by Burkholder and Bornside (34) to grow marine bacteria which decomposed marsh grass.

Vitamins, in particular biotin and niacin (33, 131), are also important growth factors for marine bacteria. Worthy of mention here is the experiment performed by Burkholder (33) with two vitamin-deficient microorganisms. These he suspended together in a growth medium where the two different vitamins required by these organisms were absent. Nevertheless both microorganisms grew well in this medium, thus showing that they could overcome their respective deficiencies, each providing to the other the vitamin it was not capable of synthesizing.

MacLeod concluded (125) that, apart from a characteristic preference for amino acids, there is nothing that could be considered

unique about the organic nutritional requirements of these micro-organisms.

Bacteria in the Marine Environment

The failure of the early attempts to grow marine bacteria in media which did not contain sea water indicated that some ions present in sea water are indispensable for the normal growth of marine bacteria.

Johnson and Harvey (98) studied a group of luminous bacteria and observed that luminescence and respiration decreased when sea water was diluted more than two-fold, and stopped completely when sea water was reduced to 10% of the suspending solution. In 1926 Lipman (121) plated sea water samples on sea water media increasingly diluted with distilled water and observed that, for the samples taken one mile offshore, the greatest number of bacteria were found on the less dilute sea water media and only a few colonies on the most dilute plates. On the other hand, the samples collected near the wharf still showed an impressive amount of bacteria in the most dilute media. ZoBell (235) collected bacteria from various sources, i.e. mouth microflora, various marine muds, sewage, tap water, and raw sea water. He inoculated all samples in growing media made up of decreasing concentration of sea water and he observed that the growth-promoting properties of these media were proportional to the concentration of sea water for marine bacteria, while the reverse was true for

bacteria from other origin.

ZoBell and his collaborators (237, 238) went on further to check Korinek's suggestion (108) that marine bacteria can be differentiated on the basis of their salt tolerance. While Korinek (108) did not observe any change in the difference in salt tolerance between sea water and fresh water bacteria grown in his laboratory over a period of one year, ZoBell and Michener (237) and ZoBell and Upham (238) claim to have observed that sea water bacteria developed the ability to grow in fresh water media after cultivation on sea water media for periods of 2 to 12 years in their laboratory. ZoBell (235), however, agrees with Korinek (108) that sea water is the best medium for primary isolation of bacteria of marine origin.

Richter in 1928 (169) was first to notice that Na^+ is a necessary element in the growth medium of marine bacteria. Further studies by other investigators (32, 45, 146, 217) confirmed this early observation. While K^+ was shown to be required by some luminous marine bacteria (32), it could not replace Na^+ (45, 130, 162, 217). MacLeod and Onofrey (128-130) studied in detail the inorganic requirements of different types of marine bacteria. They remarked that, in addition to Na^+ and K^+, marine bacteria required also Mg^{++}, $PO_4^{\equiv}$ and $SO_4^{\equiv}$ as well as iron. The optimum concentration of NaCl for growth of marine bacteria in MacLeod and Onofrey's experiments (130) was found to be about one-half of the Na^+ concentration in sea water, and

below 0.2-0.3 M NaCl the rate and extent of growth of marine bacteria was proportional to the concentration of NaCl in the growth medium. Only K^+ and sucrose showed a slight sparing action on the amount of Na^+ necessary for growth.

Tyler et al. (217) and Pratt (162) confirmed these observations. In 1963, Pratt (162) compared the growth of bacteria from the Atlantic Ocean and a small fresh water pond on media containing varying amounts of Na^+ and sucrose. Bacteria of marine origin grew best on the medium containing 0.35 M NaCl and did not grow at all at NaCl concentrations of 0.025 M or below. Pratt observed about one-half maximal growth when 0.32 M KCl or 0.5 M sucrose was added to the medium containing 0.025 M NaCl.

Another interesting aspect of the salt requirements of marine bacteria is the relatively high amount of Mg^{++} necessary for growth of these bacteria as compared to non-marine bacteria, and a marked interaction between Mg^{++} and Ca^{++} ions (125, 129). MacLeod and Onofrey (129) also showed the importance of chloride and bromide salts of Na. Of the six marine bacteria they tested, three showed an absolute requirement for these halides; for the other three, a marked increase in the growth rate could be observed in the presence of Na chloride or Na bromide. In these experiments chloride and bromide could be used interchangeably on a mole for mole basis.

While non-marine bacteria may show a degree of salt

tolerance as great as, and sometimes even greater than, marine bacteria, MacLeod (125) draws the conclusion that marine bacteria, in the present state of knowledge, can only be differentiated from non-marine specimens by their unique ability to survive and grow in sea water. Kriss (110) proposed three main criteria to distinguish marine bacteria:

1) their ability to reproduce in the sea and not just in isolated samples of water where conditions might be very different from the natural ones;
2) the growth and multiplication of the organism in fairly large amounts at a distance from shore which would assure that there is no contamination from land; and
3) the frequency of collection of single cells in the open sea.

These are evidently very demanding standards and only one new class, named Krasilnikoviae by Kriss (110), does indeed fill these requirements. However, neither of the two species described by Kriss, K. capsulata and K. incapsulata, was ever grown in the laboratory, and Sorokin (201) has questioned the validity of the description of these organisms as well as the technique of collection by Kriss. Sorokin claims that they are nothing more than colloblasts of ctenophore tentacles.

Biochemical Activities of Marine Bacteria

The biochemical activities of bacteria in a marine environment have been the subject of many investigations. Much attention has been paid to the role played by bacteria in the sulfur

cycle (232). Baas Becking and Wood (11) studied the relation between sulfur bacteria and the other bacteria which coexist with them, an interplay which has been called metabiosis (12). Rubenchick (176) had already observed the complementary action between cellulolytic organisms and sulfur bacteria, the former providing products of the degradation of cellulose as the source of electrons for anaerobic reduction of sulfates by the latter. The sulfur cycle is in part chemical and part biological. Sulfates are reduced to sulfydryl or sulfides by plants, heterotrophic microorganisms (110) and chemo-autotrophic anaerobic bacteria (233). The green and purple sulfur bacteria catalyse the endothermic photosynthetic oxidation of sulfides while the thiobacilli catalyse the aerobic exothermic oxidation of sulfur to sulfate (233).

Many marine microorganisms fix nitrogen; however, little information on this process is yet available (233). Nitrification is brought about by bacteria which oxidize ammonia to nitrite or nitrate (38, 202). This process has been observed mainly in sediments (90, 192). Denitrification by bacteria appears to take place readily in the sea but most organisms reduce nitrate only to nitrite (24). Heterotrophic bacteria make great use of organic nitrogen and we have mentioned previously the preference for amino acids of marine bacteria. Ammonia can be used by autotrophs and many heterotrophs to synthesize their own cell substance (233).

Phosphorus is found in phospholipids, nucleoprotein and

tricalcium phosphate of animal bones. ZoBell (234) states that tricalcium phosphate, either in the form of bones or crystals, provides an excellent surface for the attachment of marine bacteria and suggests three possible ways by which bacteria might dissolve it: a) by generation of acids; b) by the decomposition of organic matter associated with the calcium phosphate of bones, mechanically liberating calcium phosphate; and c) by uptake of insoluble tricalcium phosphate into cell phosphoproteins and incorporation into phospholipids. Wood's (231, 233) work has provided evidence that at least reactions (a) and (c) do occur.

Marine bacteria also decompose organic matter. Liston and Colwell (122) observed that bacterial flora varied greatly with different hosts and that there was also considerable geographic variation mainly in relation to the availability of nutrients.

Baas Becking et al. (11) have found, in estuarine water, bacteria capable of oxidizing ferrous into ferric iron, while Hutton and ZoBell (89) found sea water autotrophic bacteria oxidizing methane and hydrogen.

These few examples show that marine bacteria are extremely active in their environment. Much work still has to be done to assess the degree to which marine bacteria do affect and modify their environment, and how this can in turn be used profitably by man.

The Psychrophiles

The study of the ability of marine bacteria to grow at relatively low temperatures is of great importance in view of the fact that, with the exception of surface waters where the temperature can vary according to the latitude and the seasons, the sea temperature is around 5°C (234). The very early marine microbiologists had already noticed that marine bacteria were extremely sensitive to thermal changes and to any rise in temperature. ZoBell and Conn (236) showed that the number of cultures on agar plates was directly proportional to the temperature of plating. Bedford (20) showed that 40 of the 71 cultures of marine bacteria with which he was working were killed at a temperature of 37°C, while the optimum temperature for all of his cultures ranged between 20 to 25°C, and all grew well at 0°C. ZoBell and Conn (236) obtained the highest number of cultures from marine samples at temperatures between 18 and 22°C, and for that reason did not consider these bacteria to be psychrophiles.

The whole question of the definition of psychrophiles is highly controversial. In bacteriological textbooks (239-241), psychrophiles are variously defined as bacteria which grow best at temperatures below 30, 25, or 20°C. Schmidt-Nielsen (189) first used the word "psychrophile" to qualify bacteria able to grow at 0°C. Since then, it has been used rather loosely every time some species was found to be able to grow near freezing temperature. In his review on psychrophilic bacteria,

Witter (230) states that the most numerous definitions of psychrophiles can be found among applied microbiologists, and within this group those interested in milk, soil, and food products in general seem to have come up with almost as many definitions as there are investigators. He further states four ways by which psychrophiles could be classified, i.e. the optimum growth temperature of the microorganism studied, its ability to grow at low temperature, and criteria independent of temperature and used by some investigators, such as the belief that psychrophiles do not survive pasteurization and are predominantly gram negative rods. However, it must also be said that food microbiologists have been the most concerned about those organisms which affect the preservation of food by the cold. For that reason, and according to the behaviour of the species they were dealing with, they in turn chose the adjective which they felt was best suited to that particular organism. Thus Eddy (59) enumerates a few variants on the same theme, i.e. "Glaciale Bakterien", psychrotolerant, eurythermic, and thermophobic. He supports Mossel's (144) suggested use of the term psychrotrophic for bacteria able to grow at 5 °C and below, whatever might be their optimum temperature. Ingraham and Stokes (93) list two more terms: cryophiles and rhigophiles. Rubentschik (177) suggested the term psychrocartericus (cold conquering). Horowitz-Wlassowa and Grinberg (87) proposed unsuccessfully the use of "psychrobe" for the true psychrophilic bacteria and the retention of psychrophile for the other cold tolerant microorganisms.

Physiological Aspects of Psychrophilism in Microorganisms

Optimum growth temperature has been one of the main criteria used in defining psychrophiles. However, depending on whether one considers the optimal growth temperature that which gives the fastest rate of multiplication or the highest cell yield, one reaches different conclusions. The latter temperature gives the highest cell yield and is generally much lower than the former. Sometimes the crop of bacteria can be several times as great at 5°C as the population of a culture from the same bacteria grown at 20°C or above (76, 218). Sinclair and Stokes (196) have shown that this difference is at least in part due to the fact that there is more oxygen dissolved in growth media mechanically shaken at low temperature. This would explain the greater number of aerobic microorganisms growing in continuously shaking broth than in stationary cultures.

Many so-called psychrophilic organisms have shown a surprisingly high maximum growth temperature. For this reason, Hucker (88) proposed that strict psychrophiles are those which cannot grow at 32°C and facultative psychrophiles those that do grow at that temperature and above.

Witter (230) claimed that, since the maximum growth temperature of organisms capable of low temperature growth are so variable, the inclusion of a limiting maximum growth temperature in the definition of a psychrophile would serve no useful purpose. In order to

exclude mesophiles, Stokes (209) proposed to define psychrophiles as microorganisms which grow rapidly enough at 0°C to become macroscopically visible in about a week. He also supported the suggestion of Baxter and Gibbons (17) to subdivide psychrophiles into strict or obligate and facultative whether they grow most rapidly below 20°C or at and above 20°C. Rose (174) proposed, on the other hand, to set the maximum growth temperature of obligate psychrophiles at 25°C and of facultative psychrophiles at 30°C.

Many attempts have been made to uncover the mechanisms which permit psychrophiles to grow at low temperature. These mechanisms are probably quite complex, with an interplay of many factors like enzymatic activities at the extracellular and intracellular levels as well as the nature of the cell wall and the protoplasmic membrane.

Brown (25) observed that the end products of glucose oxidation are the same for a mesophilic and for a psychrophilic strain of marine Pseudomonas. Both strains, however, showed a marked difference with respect to the temperature coefficient of glucose and gluconate oxidation. When cells of the mesophilic strain were grown at a lower temperature, their behaviour was closer to that of the psychrophilic strain as the temperature coefficient of glucose oxidation decreased in resting cells. However, this coefficient remained always lower for the psychrophilic strain than for the mesophilic strain

whenever they were measured at the same temperature.

Ingraham (91) compared the growth of two strains of Pseudomonas isolated from chickens left to spoil at 3°C and a psychrophilic P. fluorescens with strains of mesophilic E. coli and P. aeruginosa. He observed that when the natural logarithm of growth rate is plotted against the reciprocal of the absolute temperature there is a striking difference in the results for both groups. The values of the temperature coefficient (μ of the Arrhenius equation) for the psychrophilic strains varied between 8,700 and 9,400 calories, while that of the mesophile was 14,000 calories, and he suggested that this difference in temperature coefficient might be due to the fact that psychrophiles and mesophiles have corresponding enzymes with different activation energies. In collaboration with Bailey, Ingraham (92) sought evidence for this hypothesis by examining the effect of temperature on the activities of glucose-6-phosphate, isocitrate-, and malate dehydrogenase in cell-free extracts of psychrophilic and mesophilic Pseudomonas. The effects of temperature on rate of reaction catalysed by these enzymes were the same for preparations from psychrophiles and mesophiles, and, in general, the activation energies were very similar, if not identical, for the enzymes examined. The temperature coefficient differences they observed between mesophiles and psychrophiles paralleled those reported (91) for the temperature characteristic of growth. They concluded that the temperature response differences between psychrophiles and

mesophiles for growth and catabolism is probably a result of some aspect of cellular organization rather than of enzymatic differences.

It is interesting to note that Baxter and Gibbons (17) had already suggested that the basis of the difference in response to low temperature may be accounted for by cell organization. Comparing a strict psychrophilic and a mesophilic strain of the yeast Candida, they found that in the psychrophile the uptake of glucosamine is only slightly temperature dependent between 0°C and 30°C, while in the mesophile virtually no uptake occurred at 0°C and at 20°C the rate was usually less than a third that at 30°C. The oxidation of glucose is completely inhibited in the mesophile at 0°C, whereas in the psychrophile it proceeds at an appreciable rate. Rose and Evison (175) compared the sugar transport in psychrophilic and mesophilic related organisms (Arthrobacter, Candida, and Corynebacterium). They observed that while the psychrophiles were able to grow and to transport sugar into the organism at 0°C, the mesophiles could not grow at such a temperature, due possibly to the inactivation of the mechanism for transporting sugars at temperatures below 10°C. However, a mesophilic strain of Corynebacterium xerosis was still capable of accumulating intracellular sugar below the minimum temperature of growth of that organism.

In similar experiments, Sinclair and Stokes (197) observed that the rate of fermentation of a psychrophilic strain of Candida is much less sensitive to a decrease in temperature than that of a mesophilic

strain. The fermentation rate for the psychrophile decreases 37% as the temperature is lowered from 25 to 10°C, compared to a reduction of 72% for the mesophile. On the other hand, the fermentative activity of the psychrophilic yeast was practically lost after exposing the cell suspension for 20 min at 35°C, while 71% of the oxidative activity was still present. Exposure of the mesophilic yeast for 3 hr to 35°C did not impair either activity.

Morita and Burton (142) and Burton and Morita (35) studied the activity of malic dehydrogenase in Vibrio marinus, a psychrophilic marine bacterium with an optimum growth temperature of 24.5°C and a maximum of 30°C. They observed that there were probably three malic dehydrogenases present and that the inactivation of the enzyme complex took place very rapidly at 30°C. In whole cells the enzyme appeared to be working very much below its maximum capacity. When cells were exposed to temperatures above the maximum for growth, the activity was notably increased, but this led to the denaturation of the intracellular enzyme. The enzyme obtained from heat- (30°C) and Triton X100-lysed cells showed a rate of activity 20 times greater than the rate of malic dehydrogenase activity in intact cells. Another interesting observation was that the lysed cell enzyme lost almost twice as much activity with each 10°C drop in temperature as did the intact cell enzyme. These facts strongly support the hypothesis of a cellular control of enzymatic activity.

Cell permeability could be considered a regulatory factor for this enzyme complex. Heating cells affects their permeability, favoring simple diffusion over active transport of material into the cell. This would account for the increased enzyme activity. The loss of active transport might account for the relatively greater loss of activity on cooling.

Hagen and Rose (81, 82) studied the effects of temperature on a psychrophilic species of Cryptococcus. This microorganism could grow at 16°C but failed to grow at 30°C. When the medium was supplemented with α-ketoglutarate, citrate, or DL-isocitrate, cells grown previously at 16°C for 2 hr were capable of growing at 30°C. The ability to synthesize α-ketoglutarate at 16°C but not at 30°C was suggested to explain a marked decline in the size of the amino acid pool and in particular the rapid utilization of glutamic acid pool at 30°C. These authors considered that the temperature sensitivity of this microorganism was due to thermolabile enzymes involved in the synthesis of α-ketoglutaric acid.

In conclusion, heat sensitivity of enzymes seems to be one, if not the principal, of the known factors limiting the maximum temperature for growth.

In a recent paper Stanley and Morita (205) described the striking effects of the salinity of the growth medium on the maximum growth temperature of two psychrophilic strains of Vibrio marinus and

two bacteria isolated from the Antarctic. They reported that at a salinity of 0.7% (lowest salinity permitting growth), the maximum growth temperature of Vibrio marinus was 10°C lower than at a salinity of 3.5%. This last value is the salinity of normal sea water. The two other bacteria, one an unidentified gram positive coccus and the other an unidentified gram negative bacillus, showed a less marked difference (approximately 6°C). They also tested the effectiveness of various cations in restoring the normal maximum growth temperature of Vibrio marinus MPI (maximum growth temperature 21.5°C in normal sea water) and placed them in the following order: $Na^+ > Li^+ > Mg^{++} > K^+ > Rb^+ > NH_4^+$. These salts were all tested in the presence of 0.1 g per liter of NaCl as this salt was shown to be essential for growth.

Sodium chloride, bromide, iodide, nitrate, and phosphate have the same effect on these cells, but sodium sulfate was inhibitory. Finally the lowest concentration of NaCl at which growth took place in a chemically defined medium containing also $MgSO_4$, K_2HPO_4, and polypeptone was 0.15 M. In this medium the maximum growth temperature recorded for Vibrio marinus MPI was 11.5°C.

More data has yet to be obtained from psychrophilic organisms, more particularly from obligate psychrophilic bacteria, before it will become possible to know with certainty which mechanisms are involved in such intriguing phenomena as psychrophilism. Concurrently this knowledge would help in understanding the extreme cold

sensitivity of certain mesophilic organisms like the cold sensitive mutant of E. coli isolated by O'Donovan and Ingraham (151) which fail to grow below 20°C due to hyperfeedback inhibition of histidine synthesis.

Ionic Requirements of Marine Microorganisms to Maintain Cellular Integrity

According to the recent review of MacLeod (125) on marine bacteria, the lysis of such organisms, when suspended in diluted sea water, was first noticed by Harvey (84) in 1915. The marine luminous bacteria Harvey had isolated eventually lost their luminescence and lysed due to the decrease in osmotic pressure caused by diluting the sea water suspension with distilled water. They maintained their ability to produce light when suspended in 1.0 M sucrose. Hill (86) and Johnson and Harvey (98) further confirmed these observations. However, in 1955 Pratt and Riley (163) observed that while sodium and magnesium ions protected the cells of a marine obligate halophile gram negative bacterium against lysis, glucose, glycerol, and ethanol did not, and solutions of potassium chloride and ammonium chloride accelerated and intensified lysis. MacLeod and Onofrey (130), while studying the salt requirements for growth of marine bacteria, observed that marine bacteria required 0.2 M to 0.3 M NaCl for optimum rate and extent of growth, and that sucrose and potassium exerted only a slight sparing action on the sodium requirements. They concluded from these observations that

osmotic pressure was not the sole or even primary function of Na^+ in the growth of the cells.

Tomlinson and MacLeod (216) found that cells of many marine bacterial species lysed quickly when washed in distilled water but that magnesium or manganese prevented lysis. MacLeod and Matula (126) investigated further the effect of various ions on the lysis of marine bacteria in distilled water and remarked that Ca^{++} and Ni^{++} were more effective than Mg^{++} in preventing cell lysis, and Mn^{++} and Co^{++} were equally as active as Mg^{++}.

In a later work, the same investigators (127) studied the lysis in distilled water of five marine bacteria. They found that while the lytic susceptibilities varied among some of the organisms, sodium and lithium were more effective stabilizing agents than potassium or ammonium. In general, divalent cations (Ca^{++}, Ni^{++}, Mg^{++}, Co^{++}, and Mn^{++}) were more effective than the monovalent ones in preventing lysis, and this property appeared to be similar to their capacity to form chelate complexes. All but one of the organisms studied maintained their integrity better at low concentrations of Na_2SO_4 than in equivalent concentrations of NaCl. In one organism (B10) this effect could be detected after 2 hr of incubation, while in the case of some others 18 hr of incubation were required. One organism (B9) showed the opposite effect, being less stable in solutions of Na_2SO_4 than in NaCl. Glucose and sucrose prevented lysis to a lesser degree than NaCl, and ethanol

and glycerol were ineffective.

Similar studies were carried out on halophilic bacteria and the results of these investigations were reviewed by Brown (27) and are summarized below. Christian and Ingram (40) studied the lysis of Vibrio costicolus by osmotic shock. They observed that lithium chloride and magnesium chloride gave similar protection to that of sodium chloride while potassium chloride and ammonium chloride afforded very little protection. Univalent anions, bromide, nitrate, thiocyanate, acetate, and sulfate behaved similarly to chloride with either sodium or potassium as cation.

Abram and Gibbons (1), studying the turbidity of suspensions and the morphology of Halobacterium cutirubrum, a red pigmented rod which grows in NaCl concentrations ranging from 3 to 5 M, observed increased turbidity as the salt concentration of the suspending medium decreased until 2 M; below this concentration the optical density decreased abruptly. When the salt concentration is reduced gradually, halophilic bacteria change from rods to spheres. In a subsequent paper, Abram and Gibbons (2) observed the effects of the chlorides of various monovalent cations on the turbidity as well as the morphology of H. cutirubrum cell suspensions. Turbidities of the cell suspensions decreased gradually with decreasing concentration of all salts but sodium chloride. In potassium, rubidium, and cesium chlorides, changes in morphology similar to the one observed in sodium chloride occurred

with a decrease in the salt concentration. Sodium was the cation most effective in stabilizing H. cutirubrum followed in descending order by lithium, rubidium, potassium, ammonium, and cesium. Mohr and Larsen (141) studied the effects of various cations on the morphology of H. salinarium and found that sodium was the most effective one followed by potassium, magnesium, calcium, manganese, strontium, zinc, lithium, ammonium, and cadmium.

Buckmire and MacLeod (31) studied the mechanism of lysis of a marine pseudomonad and concluded that NaCl did not prevent lysis of the cells through osmotic action. This conclusion stemmed from their observation that the intracellular concentration of Na^+ was always the same as the concentration of Na^+ in the suspending medium. The same was true for the respective concentrations of Cl^-. They concluded with the following statement: "Since so far as NaCl was concerned, no gradient was maintained between the inside and outside of the cell, NaCl could not prevent lysis of the cells through osmotic action." While Brown (26) suggested that cations acted on the conformation of the membrane proteins, Buckmire and MacLeod (31) rather favor the hypothesis by which the mucopeptide layer of the cell membrane is made up of units held together by cross linkages between polyanions of adjacent units. "When the negative charges on the polyanions are screened by the cations of a salt, the units would then be close enough to form a continuous layer."

On the other hand, Kushner (113), studying the lysis and dissolution in salts of envelopes of H. cutirubrum, observed that sodium chloride is much more effective than potassium and ammonium chloride in preserving the integrity of intact cells but only slightly more effective in preserving the integrity of mechanically prepared envelopes. Magnesium chloride is much more effective in preserving the integrity of envelopes of formalin treated cells than intact cells.

Recent work by Kushner and Onishi (116) showed that envelopes of H. cutirubrum from which protein had been removed did not require salt for stability. When envelopes were suspended in low salt concentration, their protein became more susceptible to trypsin, chymotrypsin, and other proteolytic enzymes. The requirement for divalent cations, in particular magnesium, was increased, as well as the one for sodium, but not the one for potassium. They concluded that the requirement for high salt concentration by extreme halophiles is due to mutual repulsion between negatively charged groups of protein. The phosphate groups on the lipids would support the envelope structure as sites of binding for divalent cations, especially magnesium. Later work by McClare (138) indicated that in the envelope of Halobacterium halobium the lipids are bound to at least two types of protein by different kinds of bonds: polar and apolar.

(Fig. 1)

McClare stated further that in vivo the lipids of Halobacterium halobium are bound hydrophobically to one group of

FIGURE 1

McClare's hypothesis

Top: Magnesium ions form chelates that hold together lipid and protein.

Bottom: Proposed membrane model for Halobacterium halobium showing a single layer of lipid binding together two types of proteins which were isolated from envelopes of this organism.

Illustrations are taken from C.W.F. McClare, Bonding between proteins and lipids in the envelopes of Halobacterium halobium, Nature 216: 766-770 (1967).

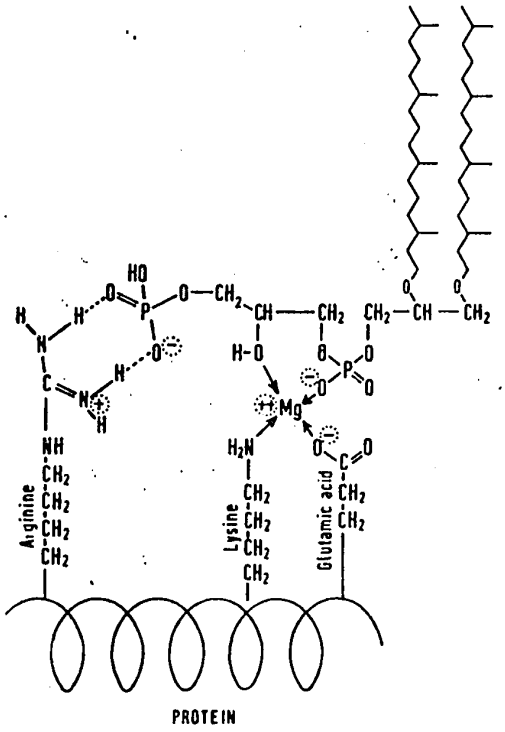


Fig. 5. Possible binding between lipid and protein.

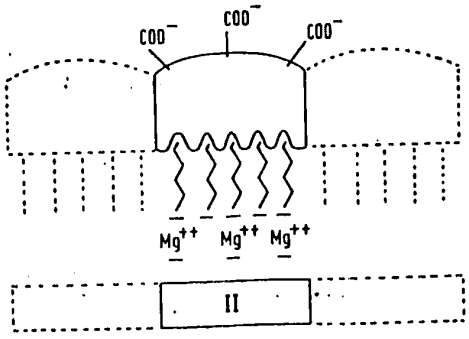


Fig. 6. Possible membrane model.

proteins and by polar group to another and that these lipids seem to be part of the same structure. As he showed the presence of magnesium in the envelope, he suggested that it forms a chelate between groups on the protein and the lipid diphosphate head groups.

Another interesting aspect of this work was the hypothesis that the membrane of Halobacterium halobium is constituted of a single layer of two types of protein bound by a single layer of lipids instead of the double layer of lipids of the "unit membrane".

The Lytic Phenomenon

Lysis of bacterial cells is a striking phenomenon that has been defined as the destruction of the cell (210). While the lysis of an individual cell can be observed occasionally under the microscope, more commonly a whole population may undergo lysis as in an old culture.

The term "lysis" is used here to mean the breakage into small units and/or the dissolution into the environment of the cell's envelope accompanied by the release of the cytoplasmic constituents.

The difficulty in studying bacteriolysis resides mainly in the fact that there is no precise measure yet available of the amount of destruction suffered by a bacterial population. Three criteria are used to measure lysis:

1) measuring the drop in turbidity of a suspension;
2) measuring the amounts of intracellular substance appearing in the suspending solution; and

3) observing under the microscope samples from a bacterial suspension.

In the first instance intact cells evenly distributed in a physiological solution give a higher turbidity value than a lysed suspension, due chiefly to the fact that bacteria do contain material in more concentrated solution than the environment and hence have a higher refractive index than the solution in which they are suspended. The higher diffraction index can easily be observed by phase contrast microscopy (27).

When bacterial cells break up, the turbidity and the amount of scattered light of the suspension decrease, the contents of the cells diffuse into the solution, and this forms the basis of the second approach to the assessment of lysis. The light scattering of bacteria in suspension has also been used as an indication of lysis and population changes. While it was generally accepted that the amount of light laterally scattered by bacteria is an osmotic response to the environment and due to cell shrinkage in a hypertonic solution, only very recently did Bateman (16) investigate the exactitude of this hypothesis. The gram negative bacterium Serratia marcescens showed that the amounts of light scattered at 90° increased as the microorganisms were suspended in ever increasing concentrations of NaCl. Bateman claims that the initial increase in the scattering of monochromatic light followed by a small decrease, which was generally interpreted as an indication of osmotic cell shrinkage with a subsequent decrease in light scattering caused by

the entry of the osmotically active solute into the cell, is not necessarily true but may apparently result from the use of concentrated cell suspensions in which multiple scattering is important. In this paper Bateman did not provide microscopic observations of the bacterial cells on which he performed his experiments.

The measure of the amounts of intracellular material released into the solution is a valuable indication of the destruction of the permeability barrier of the cell. This so-called permeability barrier is in fact a physiological entity by which cells are able to choose which of the substances available in the solution may enter the cell and which metabolites might leave it. For this reason it is difficult to interpret as lysis the appearance of intracellular material in the suspending solution. Such an occurrence might not necessarily be due to the destruction of the physical integrity of the cell but rather to the loss of the discriminating capacity of the cell's envelope. For instance, Abram and Gibbons (2) have shown that cells of Halobacterium cutirubrum, which normally lyse when suspended in distilled water, do not lyse in distilled water after being fixed overnight in formalin. Kushner (113) observed that such formalin treated cells do release intracellular inorganic phosphate when they are suspended in distilled water.

Salton and Horne (186, 187) have studied the ultraviolet absorption spectra of cell wall suspensions and cytoplasmic supernatant fraction after mechanically breaking cells of Streptococcus faecalis and

centrifugation. They observed that the cytoplasmic fraction has a sharp peak at 260 mμ corresponding to that of the nucleic acids whereas the wall gives a smooth scattering curve. The presence of 260 mμ absorbing material has been widely used for many bacterial species to measure the escape of intracellular material into the suspending solution (80, 120, 127, 143, 211, 212).

Moss and Speck (143) showed that the loss of 260 mμ absorbing material does not accompany either lysis or death of E. coli suspended in phosphate buffer. From all these considerations it appears that a valid appreciation of lysis has to come from the comparison of all these individual indicators.

Bacteriolysis can be divided into two types according to the agent causing it. Lysis in effect can be induced by biological agents such as enzymes or viruses or by mechanical, physical, or chemical agents.

Stolp and Starr (210) have recently reviewed the different known biological agents that cause bacteriolysis. Among these agents the oldest known is probably the enzyme lysozyme whose lytic properties were first described by Fleming in 1922 (66). Lysozyme is particularly active against gram positive organisms from which it can remove the whole cell wall, but in some of these organisms and in gram negative bacteria it is necessary to use "sensitizing" agents such as trypsin, quaternary ammonium compounds, thyoglycolic acid, polymyxin, or

ethylenediaminetetraacetic acid (EDTA) (167). All these agents are supposed to act on the lipid sheath covering the cell wall, thus permitting lysozyme to attack the wall (214). Repaske's work (95) suggests that EDTA chelates the heavy metals bound to the cell wall and interferes with the lysozyme substrate complex. Thacore and Willet (214) and Sato et al. (188) induced lysis in Mycobacterium tuberculosis with lysozyme and glycine. Jeynes (97) and Diena et al. (46, 47) induced the formation of Salmonella typhi spheroplasts with glycine. Glycine is supposed to interfere with the synthesis of the cell wall mucopeptide component responsible for cell rigidity (46, 136).

Bacteriophages are known to produce two types of lysozyme, one acting from inside the cell after cellular infection, the other from without (208, 210). Bacteria also produce antibiotic substances, called bacteriocins, capable of lysing bacteria. The action of these substances has been reviewed by Ivanovics (95). There are various types of bacteriocins and some can cause bacterial death without inducing lysis. Generally the biosynthesis of bacteriocins has a lethal consequence for the organism producing them and their activity is quite specific either to the same species or to closely related ones (210). The action of the numerous antibiotics on bacterial cells is of prime interest for therapeutics, and bacteriolysis due to such substances has been observed. For instance, penicillin is known to interfere with cell wall synthesis and growing cells are particularly sensitive. While in some

cases this can lead to spheroplast formation (117, 118), Bartman and Bliss (15) have observed that penicillin can induce lysis without previous formation of spheroplasts.

Two polypeptides, Gramicidin and Tyrocidin, from Bacillus brevis, lyse streptococci and pneumococci by destroying the cytoplasmic membrane (210). Various Myxobacteria induce bacteriolysis. Gram positive bacteria are sensitive to an antibiotic produced by Myxobacteria but gram negative bacteria are resistant to the lytic action of this antibiotic. Nevertheless many of these same gram negative bacteria lyse rapidly in the presence of various Myxobacteria and the cause of this lysis has not yet been explained (105, 149).

Cochrane (41) has reviewed the lysis of bacteria by enzymes from Actinomycetes, and Stolp and Starr (210) reviewed their own work on the lysis caused by the bacterial parasite Bdellovibrio bacteriovorus which is itself a bacterium. In this case the lytic process does not seem to be directly related to the action of an exogenous enzyme complex. They believe the parasitic bacterium drills a hole in the host cell and then injects an unknown substance that induces autolysis in the host.

Autolysis has been defined as lysis occurring spontaneously without the aid of an exogenous agent (210, 228). Autolysis is a complex process which basically stems from the cell's own dynamic equilibrium. The cell's envelope is presumably constantly renewed by

the action of various enzymes. One group of enzymes is synthesizing new envelope while another group is hydrolyzing the same material. Consequently one can expect that under certain conditions or factors such a highly sensitive system would be thrown out of balance with resulting lysis (210).

Furthermore, lysing bacteria may release autolytic enzymes which can induce the lysis of the rest of the population (140). There is still very little information available on the autolytic enzymes released by lysing bacteria. In Bacillus cereus, Kronish et al. (111) have observed that during autolytic cell wall destruction and formation of protoplasts, a peptide and a mucopeptide are released at different rates from the cell wall, probably as the result of the combined action of two different enzymes. Later work by Singer and Church (198) showed that in this bacterial species there exists an enzyme capable of breaking the ether bond of muramic acid which releases dialyzable lactate peptides. Pelzer et al. (158) have identified in E. coli several cell wall degrading enzymes.

While it could be expected that the imbalance of the synthesizing-hydrolyzing system of the cell wall can produce lysis of starving cells, the control system which prevents lysis in such a situation has not yet been elucidated (227). Hadjipetrou and Stouthamer (79) have, however, reported that cultures of Bacillus subtilis lyse rapidly after glucose depletion.

In conclusion, the lytic phenomenon is the irreversible result of the breakdown of the bacterial envelope. Thus, lysis can be understood only in the light of the knowledge of the structural and physiological characteristics of the envelope itself.

The Bacterial Envelope

The "skin" of the bacterial cell is constituted of concentric layers which can be classified in the following way (181):

a) The outermost layer called ionic layer made up by the net charge resulting from the various charged substances of the surface components.

b) Underneath the ionic layer is sometimes found a capsule which is not an essential structural element of the bacterial cell. The presence of a capsule around a bacterial cell is dependent upon the bacterial species and environmental factors (54, 55). The absence of the capsule in otherwise encapsulated organisms can be due chiefly to two factors: mutations (54, 55), and the activity of enzymes as, for instance, hyaluronidase which attacks the hyaluronic acid capsule of streptococci (18, 100).

Bacteria grown in conditions that prevent capsule formation or cells whose capsule was removed by enzymatic degradation nevertheless retain their morphological integrity and maintain their viability (5, 10, 181).

It is generally possible to differentiate chemically the capsule from the cell wall material (179, 180, 203).

c) Bacterial cells are at times surrounded by a layer of adsorbed materials such as slimes and gums which are products of cellular metabolism (181). These substances may be removed by repeated washings of the cells but some components may be strongly adsorbed. An example of this occurrence is the deoxyribonucleic acid layer found by Smithies and Gibbons (199) on the surface of halophilic organisms grown in sodium chloride-deficient media that produced cell wall instability (213).

d) Salton (181) also mentions another type of surface layer, different from capsules, slimes, and gums, constituted by substances causing the cells to adhere to each other, as in Lampropedia hyalina and Sarcina ventriculi. Canale-Parola et al. (37) have shown that in S. ventriculi cellulose is the intercalating material. Chain-forming bacteria, however, seem to be united by cell substances (a polysaccharide or a glycosamino-peptide) normally present in the cell wall (123).

e) The following layer has received different denominations from various authors, and Salton (181) has made a commendable attempt to clarify the situation so created. He lists the following terms, "envelope", "hull", "shell", "coats", "membrane", and "wall", which in his opinion reflect the same concept, namely a covering, framework, continuous sheet or wrapper around the bacterial cell.

In our work, the term "envelope" is used to describe

the whole of the shell in which is contained the cytoplasm. The complexity of the envelope increases from a single surface membrane such as Van Iterson and Ruys (220) have described in pleuropneumonia-like organisms (Mycoplasma), to the very complex structure observed in Lampropedia hyalina, Micrococcus radiodurans, and certain tetrad-forming cocci (147). The bistructural constitution of the envelope has been shown in gram positive as well as in gram negative bacteria. Two different layers have been observed. The outer layer is generally called "cell wall" and the inner one "cytoplasmic membrane" (181).

Electron microscopic observations of preparations of gram negative bacterial envelopes have shown that generally both the cell wall and the cytoplasmic membrane are multilayered but that gram positive cell walls are single layered. Furthermore, in gram negative cell walls the layers are lipoprotein, lipopolysaccharide and mucopeptide, while in gram positive walls only the mucopeptide layer is present (31, 104, 147). The cell wall is thought of as the structure that confers its rigidity and possibly its shape to the cell, and Brown et al. (28), studying the autolysis of a marine Pseudomonad NCMB 845, concluded that in this organism the inner layer of the cell wall is the more rigid. Buckmire and MacLeod (31) stated that in the marine Pseudomonad B-16, the mucopeptide ("rigid") layer lies between the cell wall and the cytoplasmic membrane of this organism and is probably involved in holding them together. Murray et al. (148) did an electron microscopic survey of the cell wall of various gram negative organisms (Escherichia coli,

Spirillum serpens, Vitreoscilla sp. and Simonsiella sp.) and found that in E. coli the inner layer of the cell wall is readily lost when spheroplasts are formed by penicillin treatment or by treatment with lysozyme-EDTA; in the other species lysozyme also removed this mucopeptide structure from isolated cell walls. This layer was also found to be lysozyme-sensitive in the other organisms studied.

Generally the most characteristic substance found in the cell wall of most bacteria so far studied, with the exception of Halobacterium cutirubrum (115), is a "backbone" formed by a mucopeptide complex which, in the words of Murray (147), "acts as it were a tough fabric in the midst of complex layers." The mucopeptide has also been referred to as glucosaminopeptide, glycopeptide, peptidopolysaccharide, Techoin and Murein. Salton (181) discourages the use of the term "mucopeptide" given by Mandelstam and Rogers in 1959 (134) to this complex of amino acids and amino sugars for being "chemically vague and physico-chemically misleading," and advocates the generalization of the term glycosaminopeptide. To this Rogers and Perkins, in their recent review (172), and Fig. 2 answered that none of the terms presently in use gives any true idea of the chemical structure of these polymers and that a definitive chemical name can be recommended only when this structure will be exactly known. It should also be mentioned that Weidel and Pelzer (227), supported by Martin (137), proposed the name Murein to

FIGURE 2

Structure of mucopeptide

proposed by Rogers and Perkins (172)

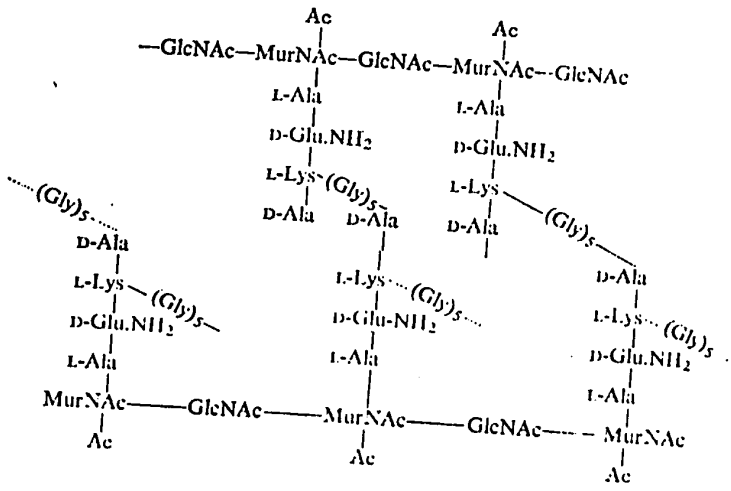
glcNAc: N-acetyl glucosamine

MurNAc: N-acetyl muramic acid

Glu: Glutamic acid

$(Gly)_5$: pentaglycine bridges which run from the NH_2 group of lysine in one peptide chain to the terminal -COOH of the D-alanine in a neighbouring chain. The dotted lines indicate pentaglycine bridges running out of the plane of the paper.

In: Cell Walls and Membranes, p. 250. Spon Pub. (1968).


Ac
Ac
—GlcNAc—MurNAc—GlcNAc—MurNAc—GlcNAc
L-Ala
L-Ala
D-Glu.NH2
D-Glu.NH2
L-Lys
(Gly)5
D-Ala
D-Ala
D-Ala
(Gly)5
D-Ala
L-Lys
(Gly)5
L-Lys
(Gly)5
D-Glu.NH2
D-Glu.NH2
L-Lys
(Gly)5
D-Glu.NH2
L-Ala
L-Ala
L-Ala
MurNAc—GlcNAc—MurNAc—GlcNAc—MurNAc
Ac
Ac
Ac

this heteropolymer substance found in the cell wall of all microorganisms with the probable exception of the obligatory halophilic bacteria (115).

Electron microscopic studies done by Weidel et al. (226) on E. coli and by Martin (137) on Proteus mirabilis (both gram negative organisms), showed that a protein was attached to the murein. When this protein was dissolved by proteolytic enzymes it liberated what Weidel and Pelzer (227) called the "murein sacculus". This chemical structure is constituted by alternating units of muramic acid (3-0-carboxyethyl-D-glucosamine) and N-acetylglucosamine [6-0-(N-acetylglucosaminyl)-N-acetylmuramic acid] (147, 204). The peptide part is made up by a number of amino acids such as D-alanine, D-glutamic acid, lysine, diaminopimelic acid, glycine, aspartic acid, serine, and threonine (147, 171, 181, 204).

In general, muramic acid has been found only in microbial cell-wall glycosaminopeptides and the nucleotide precursors of the wall (181). However, neither muramic acid nor diaminopimelic acid were detected in the pleuropneumonia-like organism Mycoplasma mycoides (159) or in Halobacterium cutirubrum (115), H. halobium and H. salinarium (29).

Other components which have been found mostly in cell walls of gram positive organisms are the teichoic acids which are phosphate polymers containing either glycerol or ribitol residues and ester-linked D-alanine (8). It is now believed that teichoic acids are

found neither in the cell wall nor in the cell membrane but rather sandwiched between these two structures (172). As they do not seem to play a role in the rigidity of the cell wall, Archibald et al. (7) suggested that they may contribute to the passage of ions across the cell surface. From a more practical point of view, teichoic acids have shown great value in the determination of serological properties (immunological specificity) of the cells from which they come and more particularly in staphylococci (172).

The Cytoplasmic Membrane

Underneath the cell wall is found another sheath containing the cytoplasm which is therefore called the cytoplasmic membrane.

In his pioneering work Weibull (223, 224) observed that when cells of Bacillus megaterium KM were suspended in 0.1 to 2.0 M sucrose and 7.5% w/v of polyethylene glycol the cell wall was completely digested by lysozyme. Each rod released two or three spherical "protoplasts" which lysed when suspended in distilled water leaving empty membranes or ghosts. These membranes are generally considered to represent the cytoplasmic membrane of bacterial cells (133, 181, 223, 224).

The same technique was successfully used to obtain cytoplasmic membranes of other bacterial species such as Micrococcus lysodeikticus (132), Sarcina lutea (30, 132), S. flava (42), Bacillus subtilis (229), and Streptococcus faecalis (194).

Gram negative organisms like Rhodospirillum rubrum

and Escherichia coli (164) were also subjected to the action of lysozyme alone or lysozyme combined with EDTA (67). As a result of this treatment the rod-shaped organisms increased in size and acquired a spherical shape. The same effect was produced when E. coli was grown in a medium containing penicillin or in a medium lacking diaminopimelic acid (132). The final structure was considered as "protoplasts" similar to those observed in gram positive organisms, but Salton (181) deduced from his observations on penicillin treated Vibrio metschnikovi and Salmonella gallinarum that the spherical shape of these gram negative bacteria was probably due to only partial degradation of the cell wall accompanied by a loss in rigidity and a consequent modification in shape. In gram negative bacteria these abnormal forms are called "spheroplasts" to distinguish them from the protoplasts of gram positive organisms. When spheroplasts of Vibrio metschnikovi are induced by growth in the presence of penicillin, this organism takes the aspect of a poached egg, i.e. an outer envelope of much greater diameter than the centrally located true protoplast (181). Van Iterson (219) demonstrated very elegantly how the cytoplasmic membrane of Bacillus subtilis receded from the cell wall when this bacterium was plasmolysed.

When it became possible to study thin sections of bacteria under the electron microscope it was discovered that many bacteria possess intracellular membranes, called "mesosomes" by FitzJames (65). In cross sections of Micrococcus lysodeikticus the mesosomes appear as

a lamellar type of structure resulting from the coiled up membranes, and in Bacillus sp. they appear as vesicles surrounded by the invaginated plasma membrane (182). Ryter and Landman (178) have shown that this second type of mesosome is released when the cell wall breaks down during protoplast formation. Salton (182) suggests that the only difference between a mesosome membrane and a plasma membrane may be in its anatomical location in fixed sections of whole bacteria. Furthermore, with the present methods of membrane preparation it is not yet possible to separate into different fractions the mesosome and the cytoplasmic membrane of bacteria (182, 184).

The yields of membrane material quantitatively isolated from 24-hr-old cultures of some gram positive bacteria have been calculated by Salton and Freer (185) to vary between 10.6% for Bacillus licheniformis and 25% for Micrococcus lysodeikticus and Sarcina lutea. Bacterial cell membranes are constituted chiefly of lipids and protein which accounts for 55-75% and 20-30% respectively of the weight of the membrane (182) and in this respect are similar to other cell membranes.

The "unit membrane" theory is presently used as the basic approach to the investigation on the nature of the bacterial cytoplasmic membrane. According to Danielli and Davson's (43) theory implemented by Robertson (170), the unit structure of the biological membrane called "unit membrane" consists of a bimolecular leaflet of phospholipids held together by Van der Waals forces and covered by

a layer of protein. The phospholipids are oriented perpendicularly to the protein layer in such a way that the polar side of the phospholipid is facing the protein. The overall thickness of this structure is about 75Å.

The evidence in support of the "unit membrane" theory is of three types: chemical, electron microscopic, and X-ray diffraction data. X-ray diffraction data have been gathered from the myelin sheath of peripheral nerve cells (63, 109). Myelin is a multilayered structure covering the axon of peripheral nerves and it appears to be an internal proliferation of the Schwann cell which surrounds the axons of all nerves.

The first X-ray diffraction observations of Schmidt and his collaborators (190) seemed to indicate the presence of a radially oriented repeating unit with spacing of about 180-185Å in mammalian peripheral nerves. These data were interpreted in terms of repeating units containing two bimolecular leaflets of phospholipids each 60-70Å thick interspersed with a protein layer of approximately 25Å (109). Finean (62) proposed a model where the smallest repeating unit consisted of one bimolecular leaflet 50-55Å thick with a protein layer 30Å thick.

Fernandez et al. (61) compared X-ray diffraction data with observations from electron micrographs of nerve cells at various stages of preparation. From this work it appears that the major differences in the observations from both techniques are due to the

process of fixation of the nerve cell, chiefly to the effect of OsO_4. When the nerve cells were fixed with $KMnO_4$, which seems to stabilize the structure without "reorganizing" the constituents as OsO_4 does, there is a good agreement between X-ray diffraction data and electron micrograph observations. The plasma membrane of the Schwann cell, which represents half of the myelin sheath, fixed in $KMnO_4$, appears on micrographs as a 75Å thick structure, constituted by two dense zones about 25Å thick separated by a less opaque region of similar thickness (109). The myelin sheath is the only biological membrane on which comparative studies have been carried out with the electron microscope and by X-ray diffraction; all other cases deal with simple phospholipid models such as lecithin and cephalin (19, 63). The interaction of various proteins with phospholipid monolayers has been given a great amount of interest by numerous investigators during the past 12 years. Finean in 1966 (63) and Level and Ito in 1967 (168) reviewed the information gathered from investigations on the possible types of interaction between lipids and proteins in model systems. While the models of bimolecular leaflets show electrical properties similar to those measured in cell membranes as well as a striking electron micrographic similarity to what is observed in the cell membrane, one has to bear in mind the fact that models are usually prepared from relatively simple compounds and do not contain the proteolipids, lipopolysaccharides and glycolipids known to be present in the cell membrane (109).

From the chemical point of view, Gortner and Grendel (74) in 1925 calculated the ratio of surface area occupied by lipids extracted from erythrocytes to the surface of these cells to be about 2.0. From the data collected in recent publications, Korn (109) calculated that the ratio of surface area of phospholipids and cholesterol extracted from human erythrocyte ghosts to the surface area of these cells is about 1.40. Korn states that this value might approximate the required value of 2.0 but that this approximation is not close enough to be considered a satisfying argument in favor of the validity of the "unit membrane" theory.

The lipids extracted from different biological membranes vary in their chemical composition. For instance, sphingomyelin has been isolated from erythrocyte ghosts (221) and myelin (150) but has not been found in membranes of microorganisms. Phosphatidyl ethanolamine has been found in myelin (150), erythrocyte ghosts (221), and various bacterial membranes (99, 152). Moreover it is the only phospholipid found in Azotobacter agilis. On the other hand, cholesterol, which has been found in membranes of plants and animals, has not as yet been observed to be present in the membrane of bacterial cells (183).

The chemical constituents of the cytoplasmic membrane isolated from various bacteria studied to date differ only slightly in their content. Nevertheless there are, within close limits, some variations

which have attracted the attention of investigators. These variations, particularly in the percentage of lipid and protein content, seem to depend on the bacterial species, the particular strain used, the age of the bacterial culture, and the chemical and physical methods of isolation and analysis used by the investigators (183). The difficulty lies mostly in the "purity" of the membrane preparation. For instance, two membrane preparations of Sarcina lutea made in different laboratories gave the following values as percentages of the membrane's dry weight: proteins, 57 and 39.8%; lipids, 27 and 28.9%; RNA, 5.4 and 1.2%. Similar variations were observed in the analysis of five membrane preparations of three different strains of Bacillus megaterium in four different laboratories (102, 183). Proteins varied between 58 and 85%, lipids between 6 and 27%, hexoses between 0.2 and 4.8%, and RNA between 1.2 and 15% of the membrane's dry weight.

Two other interesting facts have equally been noted. Firstly, one type of phospholipid generally accounts for 50% or more of the total membrane phospholipid content, and in the second place membranes do show a high degree of individuality as to the type of phospholipid. Salton (183) cites as an example the strain KM of Bacillus megaterium which contains phosphatidyl ethanolamine as the dominant phospholipid, whereas in the strain M of the same species this lipid appears to be absent.

Another very interesting aspect of the bacterial

phospholipids is the fact that gram negative organisms contain almost exclusively saturated and monounsaturated C_{16} and C_{18} fatty acids (39, 85) while membranes of gram positive bacteria have been found to contain mainly branched chain C_{15} and C_{17} fatty acids and in the case of Linolea longa and Micrococcus lysodeikticus no unsaturated fatty acids are present (85). Other substances which have been reported to be found in cytoplasmic membranes of gram positive microorganisms are pigments such as carotenoid pigments in Micrococcus lysodeikticus and cytochrome pigments in Bacillus megaterium (102).

These examples show the complexity of the problem and considerable work lies ahead before the elaborate structure forming the first layer around the cell will be finally deciphered.

The biosynthesis of the membrane as well as the physiological aspect of membrane structure as the site of extensive enzymatic activities (103) are probably the most important aspects of the question and might provide in the future the key to the problem of the true constitution of the cytoplasmic membrane.

AIMS OF THE PRESENT RESEARCH

The investigations presented in this thesis were intended to provide information on the chemical and morphological changes caused by temperature and distilled water induced lysis in the marine psychrophilic bacterium NRC-1004. Previous investigations carried out on NRC-1004 which led to the research whose results are presented here are summarized below.

Lysis of NRC-1004 cells suspended in sea water at room temperature was observed by Hagen et al. (80). These authors also showed that NRC-1004 has a temperature range of 0-19°C. The shortest lag phase was recorded at 15.4°C. Growth rate was not significantly influenced by temperatures within the cardinal points nor were the final turbidities. No growth was observed at 19.1°C or above. By viable counts of cells in stationary growth phase and suspended in sea water-tryptone medium the authors observed that no viable cells could be detected after 2 hr incubation at 25°C or 30 min at 30°C. About 90% of the cells were dead after 15 min at 25°C but lysis as indicated by a decrease in turbidity and the leakage of 260 mμ absorbing material from the cells was detected only after 30 min at 25°C.

A number of metabolic inhibitors, which included NaF, KCN, NaN_3, sodium arsenate, mercuric chloride, and p-chloromercuric benzoate, did not prevent lysis. Lysis was not affected by methanol,

ethanol or n-propanol but butanol increased the rate of lysis by 20%. Glucose or spermine did not prevent lysis nor affect the rate of lysis.

Small amounts of hexosamine (21 to 26% of the cell's total hexosamine) were released by lysed cells at 25°C and 27°C. They noted a decrease in lipid phosphorus at 25°C and 35°C. At 35°C nearly complete disappearance of lipid phosphorus resulted after 90 min. In mechanically prepared envelopes lipid phosphorus was broken down at a slower rate than in cells.

Hagen et al. (80) proposed that temperature induced lysis is probably caused by the breakdown of the cytoplasmic membrane due to the action of a membrane-bound phosphatidase of the phospholipids. It was decided to observe the changes that could occur in the electrophoretic behaviour of heat and distilled water treated cells on the assumption that any chemical change taking place on the cell envelope would produce a variation in the net electrical charge of the envelope and hence affect the electrophoretic behaviour of these cells.

The lysis of cells suspended in distilled water has been generally attributed to osmotic pressure. It was believed that there was a possibility that some ions might play a significant role in the integrity of the cell. A systematic study of the effectiveness of mono- and divalent ions in preventing lysis of NRC-1004 cells in distilled water at temperatures below 19°C was undertaken.

The effects of temperature (25 and 38°C) and distilled

water treatment on envelopes of NRC-1004 cells were to be investigated by chemical methods.

Morphological changes of NRC-1004 cells subjected to temperatures above 20°C and distilled water treatments were to be observed under phase contrast microscopy.

The data obtained from the different approaches mentioned above were thought to

1) clarify the role of ions in maintaining cellular integrity of the marine psychrophilic bacterium NRC-1004,
2) determine to what extent the lysis of temperature (25 and 38°C) and distilled water treated NRC-1004 cells could be related to the breakdown of the cell envelope constituents.

Part I

Microelectrophoretic Studies on the Lysis of NRC-1004

THEORY

When an electrical current passes through a colloidal suspension in an aqueous solution, the particles of the colloid flow at a constant velocity either to the positive or negative pole of the field. The velocity per unit strength of the applied electrical dc field is called the "electrophoretic mobility" and is a characteristic property of the colloidal system that is being studied (157). Electrophoretic mobility shows the existence of a charge on the surface of the particles. This electrical charge may be due to two factors:

1) the ionization of specific chemical groups such as a -COOH or $-NH_2$ present on the surface of the particle,
2) the adsorption of charged ions from the surrounding medium onto the surface of the particle.

Consequently the movement of the particles varies with the pH and the ionic strength of the medium and the nature of the electrolytes. When these variables are maintained constant, it is possible to determine the composition of the colloidal surface of simple organic particles.

To facilitate the task of interpretation of electrophoretic mobility, Overbeek, Wiersema and their collaborators proposed the following physical assumptions, applicable to microorganisms in suspension, and which are quoted from their most recent review on this topic (157):

1. Only a single particle is considered, which implies that the interaction between colloid particles is neglected.
2. It is assumed that the colloidal solution follows Ohm's law; in other words, all terms non-linear in the dc field are neglected.
3. The Brownian motion of the colloid particle is neglected.
4. The colloid particle (plus the adjacent layer of liquid that moves with it) is treated as a rigid sphere.
5. The dielectric constant is supposed to be the same everywhere in the sphere.
6. The electric conductivity of the sphere is supposed to be zero.
7. The viscosity coefficient of the liquid surrounding the sphere is assumed to be independent of position.
8. The dielectric constant is independent of position.
9. The small electrolyte ions are point charges that interact with the particle through Coulomb forces only.
10. The charge of the sphere is distributed uniformly on its surface.
11. Only one type of positive and one type of negative ions are considered to be present in the solution.

Abramson et al. (3) have shown that it is possible to differentiate ionogenic compounds such as proteins from non-ionogenic substances such as hydrocarbons. This was done by calculating the differences in the mobility/pH values at constant ionic strength of the ions in the suspending solution and the mobility/ionic strength plots at a constant pH.

The identification of the different surface components of bacteria by electrophoresis has been pioneered by Dyar and Ordal (57) and Dyar (56). These authors used the anionic detergents dodecyl, tetradecyl, and hexadecyl sulfonic acids as charged indicators. Their theory was that the hydrocarbon end of these molecules would be specifically adsorbed to surface lipid and that the negatively charged end would increase the net negative charge on the particle which, in this case, was Micrococcus (Staphylococcus) aureus. In the presence of the anionic detergents these workers observed an increase in the negative mobility (reflecting the negative charge) of the bacteria and of droplets of pure hydrocarbons and lipids. No change in mobility was observed when proteins such as egg albumin and bacterial nucleoproteins, polysaccharides such as cellulose and bacterial dextran, and pyrex glass particles were tested (23).

Valuable information on the ionizable groups found in the surface structure of cells can be obtained from the variation of mobility of the cell with the pH. Phosphate or sulfate groups, which are strongly

acidic, are considered to be responsible for the maintenance of a high charge at a pH as low as 3.0. The fall in mobility below pH 5.0 is likely to be due to the loss of charge by weaker groups such as carboxyl groups. Katchalski and Gillis (101) suggest that this gradual decrease in mobility in the latter-mentioned case is caused by an interaction effect when the ionizing capacity of a particular -COOH charged group is modified by near neighbours such as polyelectrolytes (proteins).

The electrophoretic mobility of bacteria suspended in salt solutions has been shown to depend on the physiological condition of the cells and upon the treatment these receive after harvesting and before mobility is measured (23). Conflicting observations were reported for various microorganisms whose electrophoretic mobility was measured at different times during their growth curve. For instance, the electrophoretic mobility of Escherichia coli was 20% lower in the lag and logarithmic growth phases than in the stationary phase (145). Variations in electrophoretic mobility with the age of culture were also observed in Brucella and Salmonella typhosa (207). However, no variation of electrophoretic mobility during the growth cycle could be detected with Aerobacter aerogenes, nor did the nature of the growth medium affect the electrophoretic mobility of this organism (13, 14). The electrophoretic mobility of Escherichia coli was also shown to be independent of the type and concentration of the growth medium (145, 215). However, Dyar (56) reported higher mobilities for Micrococcus aureus when this microorganism

was cultivated in glucose medium than when cultivated in ordinary broth. The presence or absence of capsular material or flagella may also affect the electrophoretic mobility of bacteria (23). For this reason the treatment to which the organism is subjected before electrophoresis becomes of paramount importance. It is therefore imperative, so as to procure reliable and reproducible results from electrophoresis, that the cells should always be grown in the same medium, harvested at the same age, and washed in the same solution after harvesting.

MATERIALS AND METHODS

Apparatus

The apparatus used to determine electrophoretic mobilities was built according to the directions of Douglas (49, 50, 53). It consists of a "micro-cell" shown in Figs. 3 and 4. There are two main parts measuring 3 in. x 1-1/2 in. The bottom section, A, is made out of:

1) the base, constituted by a microscope slide 1 mm thick covered by
2) a cover-slide 0.2 mm thick from which a channel of 6 cm in length and 1 cm in width was cut out,
3) a second cover-slide identical to the former but without the channel. Two holes 1 cm in diameter and 5 cm apart were pierced through it.
4) A microscope slide 1 mm thick with the corresponding holes to those drilled in cover-slide 3. In the middle of this top microscope slide a hole of 2 cm in diameter was pierced to permit the observation of particles in the channel using phase contrast optics.

FIGURE 3

The microelectrophoretic cell—exploded view. 1-4, section A; 5-11, section B.

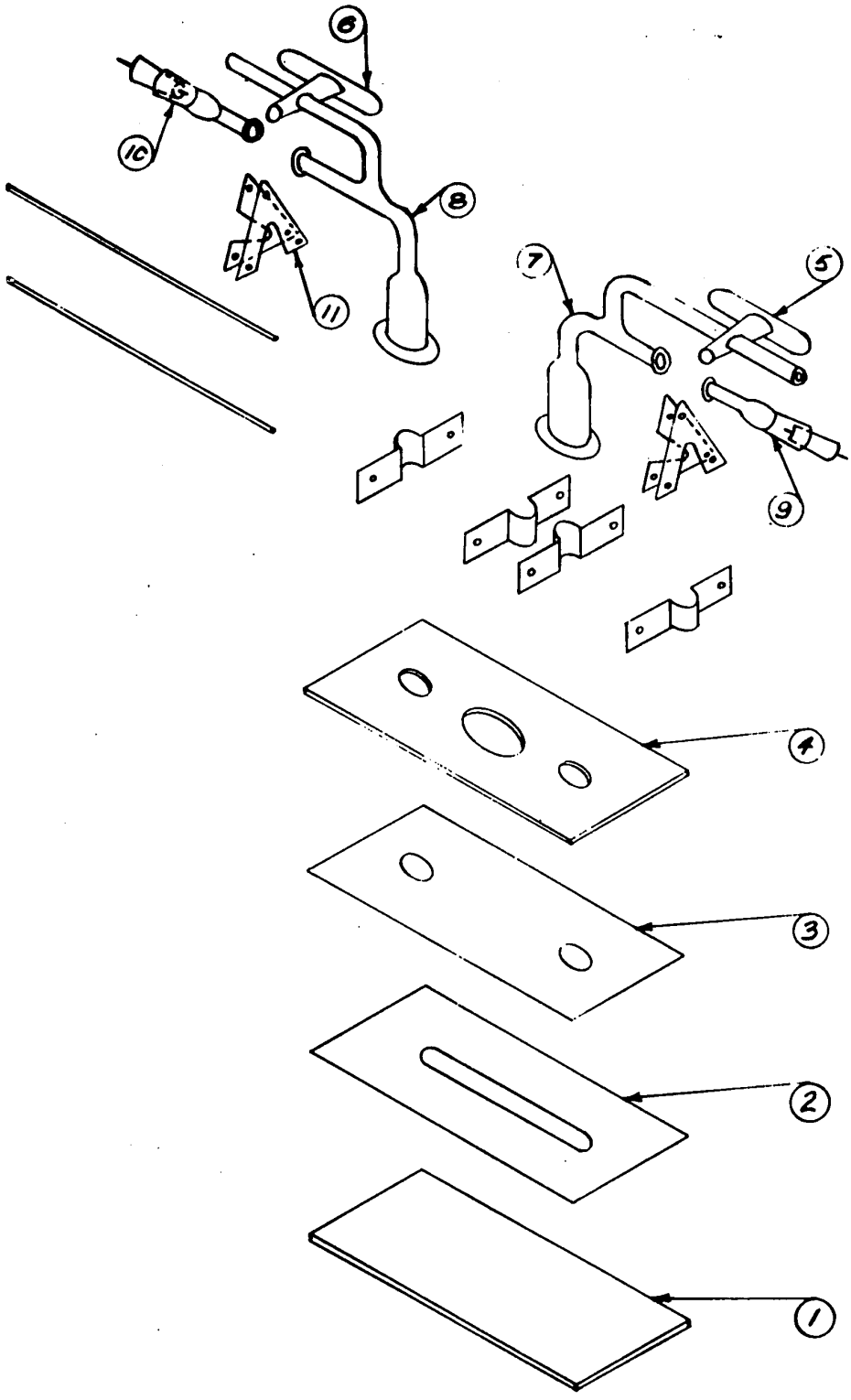
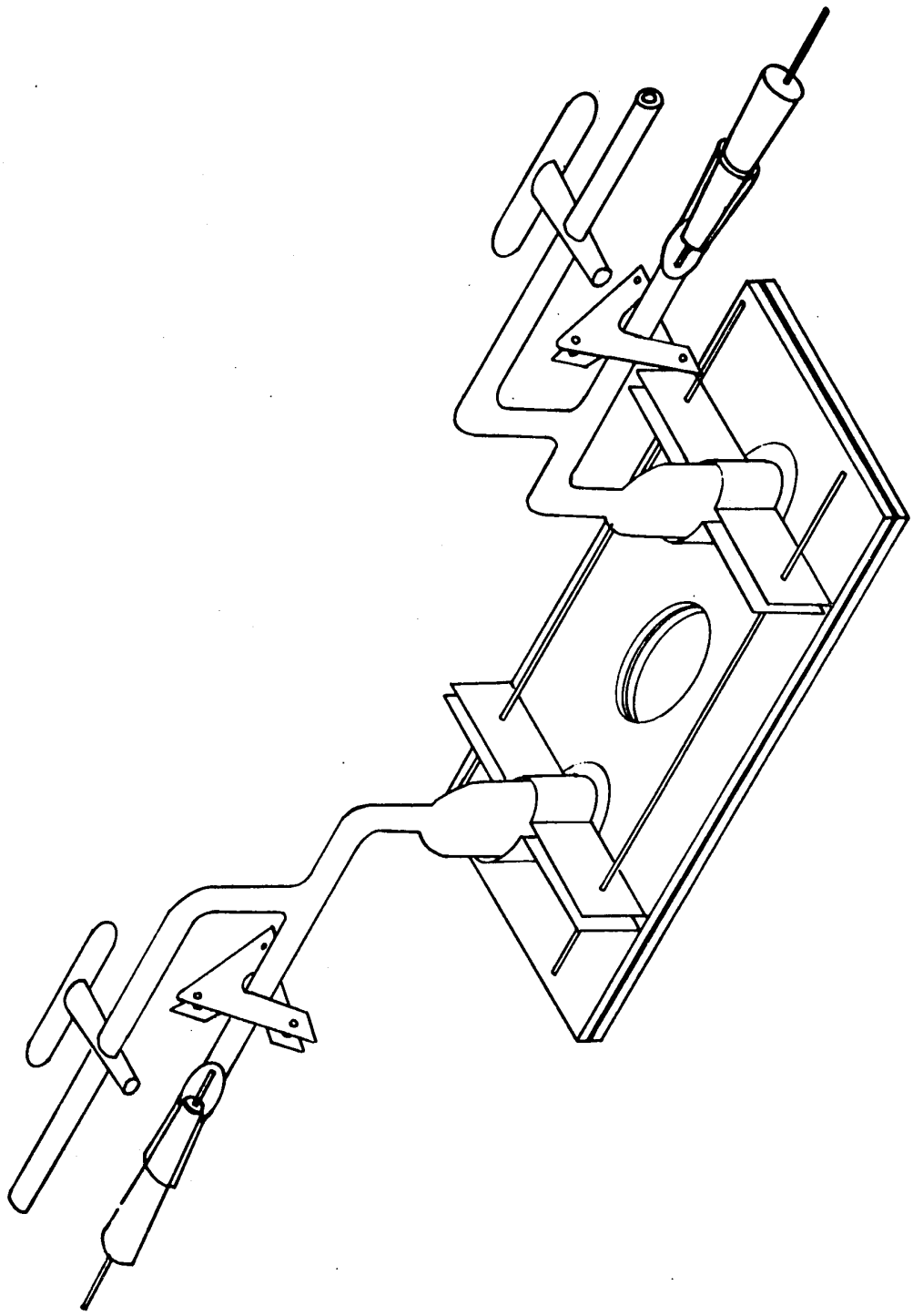

6
10
8
11
7
5
1
9
4
3
2
1

FIGURE 4

The microelectrophoretic cell.



The various holes were made with a hollow brass drill and fine carborundum paste. The central channel of cover-slide 2 was made by joining with a writing diamond two holes previously drilled 5 cm apart in the slide. The channel was removed by gentle tapping of the glass.

The various slides of this section were cemented together using "Araldite D syrup" with 7% by weight of "Hardener 951" and solidified for 3 hr at 60°C.

The top section, B, was constructed from 4 mm bore Pyrex glass tubing with the inlet tap 5 and the outlet tap 6 for filling and emptying the micro-cell. The corresponding arms 7 and 8 ended with ground glass flanges for the attachment of the electrode vessels 9 and 10. Both parts of section B were cemented to section A using Araldite stick adhesive. The bracing device shown on top of Fig. 3 was used to keep both parts of section B securely in place during the cementing procedure which lasted 3 hr at 140°C. After cooling, the bracing device was carefully removed. The electrode vessels 9 and 10 were closed by means of rubber plugs of the appropriate size through which passed the copper electrodes immersed in 1.0 $\underline{M}$ $CuSO_4$. The opposite ends were closed by means of rolled filter paper plugs previously soaked in a solution of 0.5 $\underline{M}$ NaCl and 0.1 $\underline{M}$ $MgSO_4$. The electrode vessels were attached to the arms 7 and 8 by means of metal clamps 11 and rubber washers as protection against excessive pressure. The joints were sealed with Silicone high vacuum grease.

Electrical Circuit

The voltage supply, from a main outlet, was applied through a transformer to a rectifier tube, the direct current (110 v) was smoothed through a resistor-capacitor network and finally stabilized by a voltage regulator tube. The potential difference across the micro-cell was adjusted by a Variac control and the current measured on a Leeds and Northrup potentiometer having 0-150 μA, 0-1.5 mA, and 0-15 mA scales. Also incorporated into the circuit was a double pole, triple throw switch which enabled the current to be reversed and had a central neutral position.

Measurement of Electrophoretic Mobilities

The electrophoretic mobility of the particles was observed in a constant temperature room at 12 °C with a Zeiss microscope using phase contrast optics at a magnification of 640X. The microscope was tilted to a horizontal position so that the micro-cell on the microscope stage was in fact in a vertical position. This was done to avoid particles under observation going out of focus due to the effect of gravitational sedimentation.

The channel depth was measured by focusing first on the organisms adhering to the top cover-slide and then on those adhering to the bottom slide. The distance was obtained from the reading on the Vernier scale of the micrometric knob. This procedure was repeated 15 times to assure a standard deviation no greater than 1% for the mean of the channel's depth.

The current was adjusted so that particles in either stationary level took 10-15 sec to travel 60 μ. This distance was measured on the scale of an ocular micrometer.

Abramson et al. (4), studying the micro-electrophoretic behaviour of gelatin-coated particles of asbestos and of particles of collodion, observed that on application of a potential difference two phenomena take place:

1) the movement of the colloidal particles in suspension towards one of the poles. This is electrophoresis.
2) the movement of the fluid along the glass walls of the micro-cell which become charged and the convergent return flow in the center of the channel. This is called electro-osmosis.

Due to this cause the mobility of the particles varies according to their location in the channel. Proper electrophoretic mobility of particles can therefore only be measured at the levels where electro-osmotic flow at the edges of the micro-cell, and the return flow close to the center of the micro-cell, cancel each other out. These particular levels are called stationary levels and have been calculated by various investigators (107, 112, 200) to be at 27% of the distance from either the top or the bottom of the micro-cell.

For any single determination the microscope was focused on one of the stationary levels and a single particle was timed, the current reversed and a particle timed in the other direction. This was

repeated 4 times at each level and the entire procedure then repeated giving a total of 32 readings. Any slight drift in the absence of an applied field was allowed for by averaging the reciprocals of the times in each set of four. Symmetry of flow in the cell was confirmed by agreement between values averaged in this way for the two stationary levels. The electrophoretic mobilities correspond to the mean of these averages. Frequency distribution histograms for the mobilities of intact and lysed cells at pH 7.0 were drawn from 120 individual timings in each case (Fig. 7).

The conductivity measurement of the suspension was made at 25°C using a Mullard type cell and a Radiometer conductivity meter type CDM2. Thus all mobility measurements relate to 25°C (161).

Buffer System

The buffer system used in the electrophoretic studies was the sodium veronal + acetate + HCl system (final buffer concentration 0.014 M) proposed by Michaelis (139) and used by Douglas (50). This covered a wide pH range (3.0-9.0) and the final pH was not appreciably affected by the addition of 0.5 M NaCl and 0.1 M $MgSO_4$ necessary to maintain the cells. Below pH 3.0 the pH of the salts solution was adjusted by the addition of HCl. The final pH of all buffers was checked using a Beckman pH meter and glass electrode.

Organism

The organism used in the present studies was isolated

from flounder eggs by Dr. K. Eimhjellen of the Technical University of Norway (Trondheim). It is registered as NRC-1004 in the culture collection of the National Research Council of Canada (Ottawa).

At 12°C under phase contrast microscopy at a magnification of 1200X, these bacteria may be seen as short rods with rounded ends, some slightly curved. The majority appear singly but some form short chains of two or, more rarely, three individuals. They produce a red pigment which seems to be closely related if not identical to prodigiosin (103). They are gram negative. Electron micrograph preparations made by McDonald and Chambers at the National Research Council of Canada showed only polar flagellation (242). These observations were corroborated by those of Jean Yves D'Aoust at the Department of Biology, University of Ottawa (242). Eimhjellen and Felsen (60) in Norway reported earlier that some young cultures can be peritrichous.

On the basis of the fatty acid composition of the cells, Kates and Hagen (103) suggest that this microbe belongs to the genus Serratia rather than to the red pigmented marine pseudomonads (119). However, the classification of this bacterium is still an unresolved problem in view of the peculiarities it manifests compared to the mesophile Serratia marcescens grown at 10°C. Palmitic and hexadecenoic acids were the major fatty acids identified in both microorganisms. Phosphatidyl ethanolamine was the major phosphatide in both microorganisms but phosphatidyl serine and an unidentified ninhydrin positive phosphatide, probably phosphatidyl o-amino acyl

glycerol, present in Serratia marcescens were not found in NRC-1004. There was a high proportion of octadecenoic acid in Serratia marcescens which was virtually absent in NRC-1004. Furthermore, NRC-1004 contained tetradecenoic or pentadecenoic acid which was not found in Serratia marcescens. Another important difference between both microorganisms noted by Kates and Hagen was that NRC-1004 lacked the ability to synthesize cyclopropane acids and that only traces of these were produced when the mesophilic Serratia marcescens was grown at 10°C. Further work is necessary to find a proper taxonomic place for NRC-1004.

Growth Characteristics

Previous investigations (80) have shown that NRC-1004 grows optimally at approximately 15°C, a temperature at which it has also the shortest lag phase. Below and above 15°C the length of the lag phase increases proportionally to the raising or lowering of the temperature. Below 20°C in growing cultures, the final turbidity is not affected to any significant degree by the temperature but is rather a factor of the amount of nutrient material present in the medium (80).

Effects of Temperature

Above 20°C this microorganism dies. Hagen et al. (80) showed by viable counts that 90% of the cells were dead after 15 min exposure to 25°C while the turbidity began to decrease only 15 min later. The loss of colony-forming ability therefore precedes lysis. The process of lysis, i.e. decrease in turbidity and release of 260 mμ absorbing

material in the suspension, cannot be stopped by transferring the cultures back to 5 °C.

Growth Medium

NRC-1004 has been grown previously in sea water enriched with 0.2% (w/v) of Bacto tryptone (about 0.7 mg protein/ml; 80). Turbidity of the growth medium at 660 mμ did not exceed 0.600 after 72 hr of incubation. In our work the sea water growth medium was enriched with 0.4% (w/v) of Bacto tryptone to obtain more sizeable cell yields per culture. The average turbidity for the same time of incubation was between 0.800 and 0.950 (1.0-1.3 mg of protein/ml). Stock cultures were maintained in sea water-tryptone medium (0.4% w/v), pH 6.8-7.0, to which 1.5% of Bacto agar was added. They were kept at 0-2 °C and transferred every three weeks.

To obtain cellular material for our experiments the following procedure was adopted. A 250 ml flask containing 100 ml of sea water-tryptone medium, pH 6.8-7.0, at 5 °C, was inoculated with a loopful from the stock culture. After 2 days incubation at 5 °C on a rotary shaker (100 rpm), 2 ml of this culture was inoculated into 1000 ml of sea water-tryptone medium in a 4 litre baffled bottom flask and shaken for 72 hr at 5 °C.

Preparation of Cell Suspensions

The cells were harvested by centrifugation in cold Nalgene cups, in a Sorvall refrigerated centrifuge, at 6000 x g for

10 min at 0°C. The cells were then resuspended in 500 cc of cold sea water with an inverted 10 ml pipette, taking care to avoid blowing respiratory CO_2 into the suspension. The introduction of CO_2 was thought to cause a decrease in pH and be responsible for the lysis of the cells. The suspension was centrifuged again and the cells washed twice more in the same fashion, using a buffered solution consisting of 0.5 M NaCl, 0.1 M $MgSO_4$, and 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.0 (Sigma brand), referred to as psychrophile suspension medium (PSM). The purpose of these washings was to remove the greatest possible amounts of growth medium and extracellular metabolites from the cell suspension.

RESULTS

Effect of pH on Mobility

Measurements of the effect of pH on mobility over a wide pH range were complicated by the fact that cells lysed at pH 5.0 or below. This type of lysis was investigated at a later stage and observations on it are reported in detail in Part II, page 84. If mobility measurements were made no more than 15 min after suspension at each pH value, the lower (solid) mobility curve shown in Fig. 5 was obtained. Above pH 6.0 the mobility of (intact) cells was independent of pH, and the mobility changed little until the pH was reduced to 4.0 (Fig. 5). Below pH 3.0 the electrophoretic movement became too slow to measure, and only the sign of the charge could be determined. The iso-electric point lay

FIGURE 5

Top: Effect of pH on turbidity and release of intracellular substances.

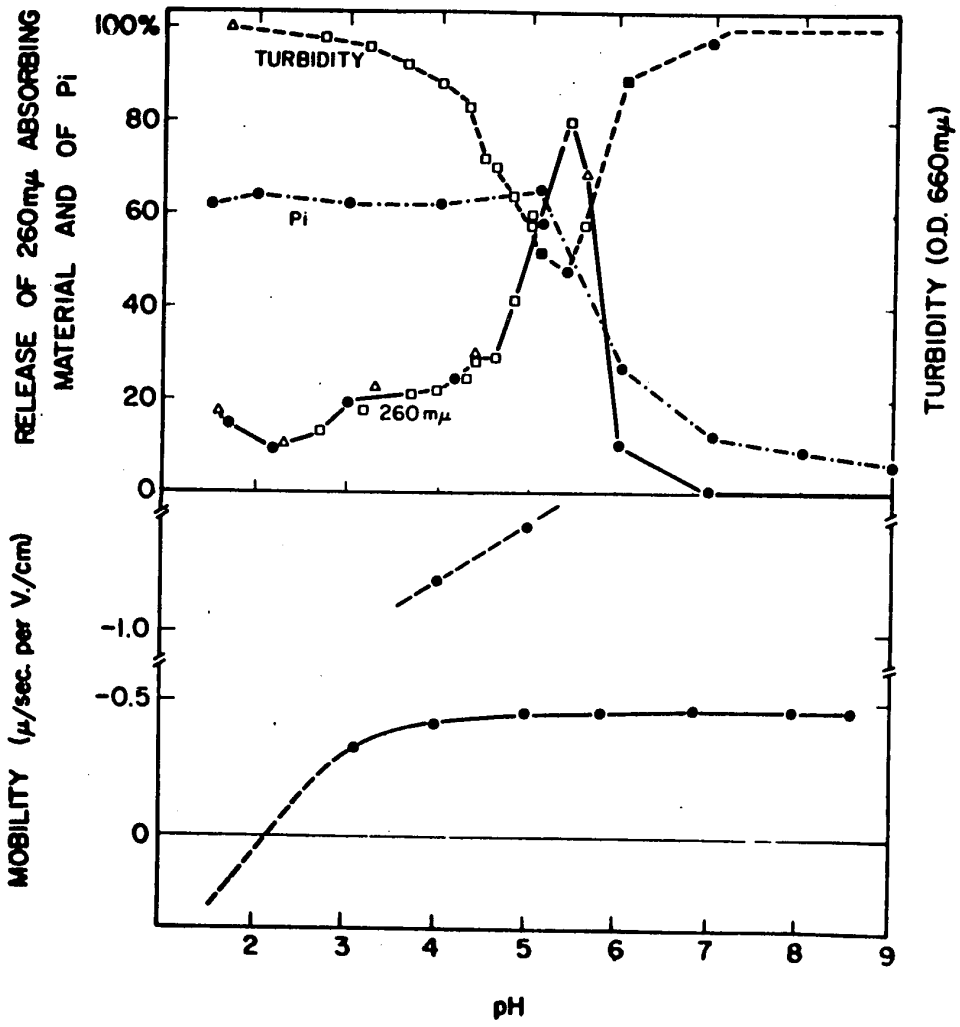
----- Turbidity. 100% = Turbidity of cells suspended in sea water.

-·-·- Release of inorganic phosphate (Pi).

——— Release of 260 mμ absorbing material.

This part of Fig. 5 is discussed in detail, page 87, and is placed here to provide information on the leakage of intracellular material from cells at pH 5.5 and below.

Bottom: Effect of pH on the electrophoretic mobility of intact cell suspensions. Upper curve shows "fast" cells measured after 20 min. The scale on the ordinate is the same above and below the break.


RELEASE OF 260mμ ABSORBING MATERIAL AND OF Pi
100%
80
60
40
20
0
TURBIDITY
Pi
260 mμ
TURBIDITY (O.D. 660mμ)
MOBILITY (μ/sec. per V./cm)
-1.0
-0.5
0
2
3
4
5
6
7
8
9
pH

between pH 2.0 and 2.5.

Cells did not lyse at pH 6.0 or higher, and their electrophoretic behaviour did not change as long as the suspensions were kept cold, but lysis at pH 4.0 or 5.0 led to progressive changes in electrophoretic behaviour. On exposure to each pH the cells became heterogeneous and could be divided into two distinct populations: a "fast" population and a "slow" population (Fig. 5), whose mobility was close to that of intact cells at pH 6.0 or 7.0. "Fast" forms appeared after about 20 min and their number increased until, after 3 hr, over 90% of the total population was "fast". When these cells were washed repeatedly in cold Michaelis buffer/salts, pH 7.0, and then reexamined electrophoretically, they had all become "slow" again. These results suggested that intracellular material had been adsorbed on the surfaces of lysed cells, and this possibility was further investigated. Cells were lysed by suspending them in Michaelis buffer/salts solution, pH 5.0. The pH values of portions of the suspension were adjusted to 4.0 or 7.0, or left at 5.0, and the lysed cells removed by centrifugation. Addition of supernatants at pH 5.0 or 7.0 to intact cells did not change the electrophoretic mobility observed at these pH values after 1 hr (Table I). At pH 5.0, all the cells became "fast". Thus, there was no adsorption, or effect of adsorption, of intracellular material onto intact cells or cells lysed at pH 4.0, though there may have been some effective adsorption onto cells lysed at pH 5.0.

TABLE I

Mobilities of intact and lysed cells:

the effect of washing and lysed cell supernatants on these mobilities

Exp.	Methods of lysis	Intact cells		Treatment after lysis: None		Lysed cells washed		Supernatant + lysed cells		Supernatant + intact cells	
		Mobility at pH									
		5.0	7.0	5.0	7.0	5.0	7.0	5.0	7.0	5.0	7.0
1	pH 5.0	-1.40[a]	-	-1.38[a]	-0.88[b]	-	-	-	-	-	-
		-0.43[a]	-0.44	-0.44[a]	-0.42[b]	-0.45	-0.42	-0.42	-0.43	-1.09	-0.42
2	Temperature	-	-0.45	-1.11	-1.03	-0.72	-0.71	-	-0.74	-	-0.45
3	Distilled water	-	-0.44[c]	-1.04	-1.08	-0.45	-0.43	-0.44	-0.43	-	-

[a]Mobilities recorded for "fast" and "slow" populations at pH 5.0.

[b]The mobility recorded after suspension at pH 7.0 for 5 min reverted to the control value after approximately 20 min.

[c]Mobilities of distilled water lysed cells suspended in distilled water were measured during a period of 100 hours and found to remain constant.

If cells were suspended for 2 hr at more acid pH values (3.0 and 2.0) and then washed at pH 7.0, their mobility at this pH was the same as that of intact cells.

Effect of Temperature-Induced Lysis on Mobility

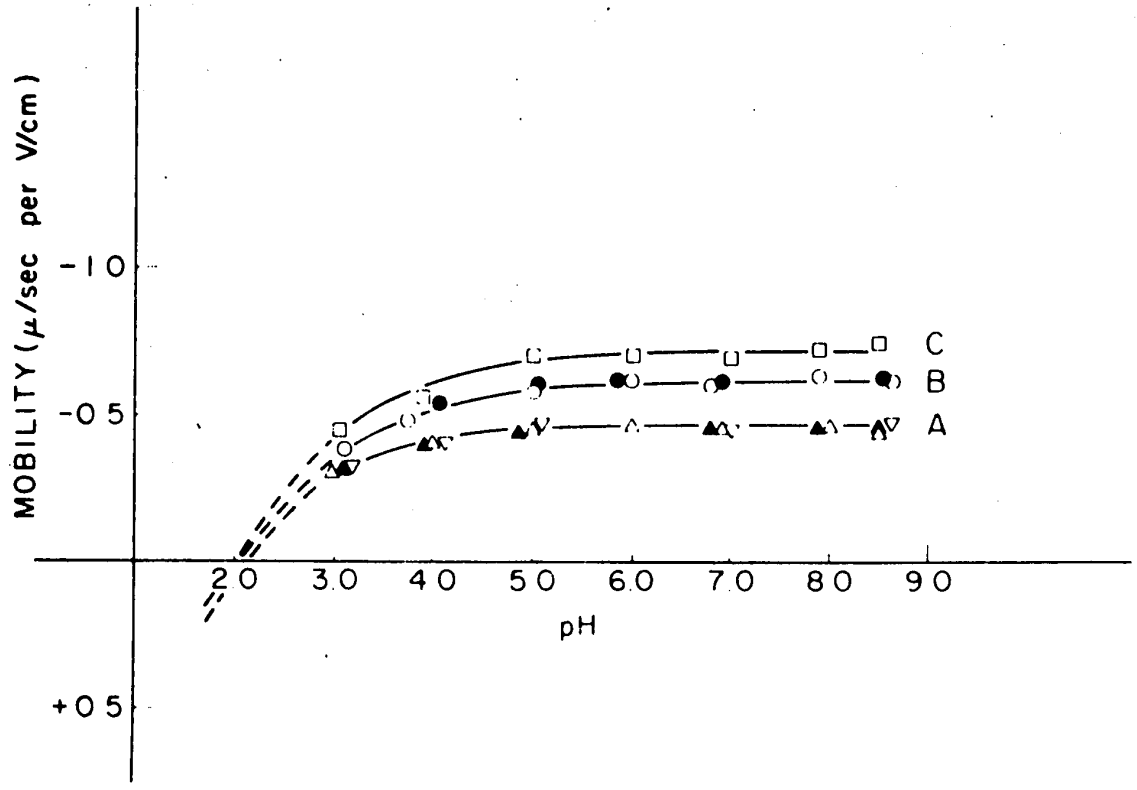
In contrast to their homogeneity at 5°C, cells at 25°C in Michaelis buffer/salts, pH 7.0, became electrophoretically heterogeneous after 30 min. After 2 hr the "fast" forms predominated but some "slow" cells remained. It was necessary to expose the cells to 25°C for at least 3 hr at a concentration no higher than 10^9 cells/ml before all became "fast". The mobility of suspensions exposed to 25°C for 1 hr was reduced to the control values (-0.45 μ/sec per volt/cm) at pH 7.0 by washing the cells three times in a cold Michaelis buffer/salts mixture; however, after more prolonged exposure, washing three times or more only reduced the mobility to between -0.60 and -0.72 μ/sec per volt/cm. No adsorption of intracellular material from temperature-lysed cells onto intact cells or washed lysed cells could be detected electrophoretically (Table I).

The increased electrophoretic mobility of cells lysed at 25°C appeared over the whole pH range studied (Fig. 6), and an even greater effect was observed after lysis at 35°C (Fig. 6), although neither temperature caused a change in the isoelectric point. The mean mobilities of cells lysed at 25°C and then washed varied between -0.60 and -0.72 μ/sec per volt/cm but were always higher than control values

FIGURE 6

Effect of temperature and water treatment on mobility of washed cells.

Curve A		Intact cells (no symbols).
	△	Water-lysed cells at 5 °C.
	▲	Water-lysed cells at 5 °C followed by 25 °C.
	▽	Exposed to distilled water at 25 °C.
Curve B	O	Temperature-lysed cells at 25 °C.
	●	Temperature-lysed cells at 25 °C followed by water, 5 °C.
Curve C	□	Temperature-lysed cells at 35 °C.


MOBILITY (μ/sec per V/cm)
-1 0
-0 5
+0 5
2 0
3.0
4 0
5 0
6.0
7.0
8 0
9 0
pH
C
B
A

(Fig. 6). In view of this variation, the difference between cells lysed at 25 °C and 35 °C shown in Fig. 6 may not be significant.

Effect of Lysis in Distilled Water on Mobility

When cells were lysed by suspension in distilled water for 2 hr at 5 °C, direct electrophoretic measurements in Michaelis buffer/salts at pH values of 5.0 and 7.0 showed that all cells were "fast". The mobilities recorded in Table I are similar to those for cells lysed at 25 °C. Subsequent washing of the suspensions in Michaelis buffer/salts, pH 7.0, reduced the mobilities at all pH values to those of intact cells (Fig. 6). Thus, water lysis did not cause a permanent measurable change in the external cell surface.

Combined Effects of Temperature and Suspension in Water on Mobility

Fig. 6 shows the mobility/pH curves for the resulting cell structures after:

1) exposure to 25 °C in Michaelis buffer/salts, pH 7.0, for 3 hr followed by suspension in distilled water at 5 °C for 2 hr;
2) suspension in distilled water at 5 °C (2 hr) followed by treatment at 25 °C for 3 hr in Michaelis buffer/salts, pH 7.0; and
3) exposure to 25 °C in distilled water for 3 hr.

All cells were washed three times in Michaelis buffer/salts, pH 7.0, at 5 °C between and after treatments.

When temperature treatment preceded suspension in distilled water the resulting mobility/pH curve was displaced upwards

from the curve for intact cells, and was similar in both form and absolute mobility values to the curve for "temperature lysed cells" (Fig. 6). After all other treatments, the resulting mobility/pH curves approximated closely that for intact cells.

Electrophoretic Homogeneity of Intact and Treated Cell Samples

The electrophoretic homogeneity of cell populations must be known to assess the effect of any treatment on mobility. Fig. 7 records the frequency distribution histograms at pH 7.0 of intact and treated cells. To allow for the small drift velocity sometimes observed at a stationary level in the absence of an applied electric field, half the difference of the mean mobilities in the two directions was halved and added to the individual mobilities of the smaller mean and subtracted from the greater. A similar correction was made for the upper and lower stationary levels. The mobilities recorded in the histograms are corrected in this way. The normal (Gaussian) distribution curves, calculated from the standard deviations, are also shown.

DISCUSSION

To ascertain the validity of any comparison of the data obtained, the homogeneity in electrophoretic behaviour of intact and treated cells must be considered. The histograms were always unimodal (Fig. 7) and corresponded well with the normal distribution curves of the same standard deviation. For intact cells and cells lysed in water the

FIGURE 7

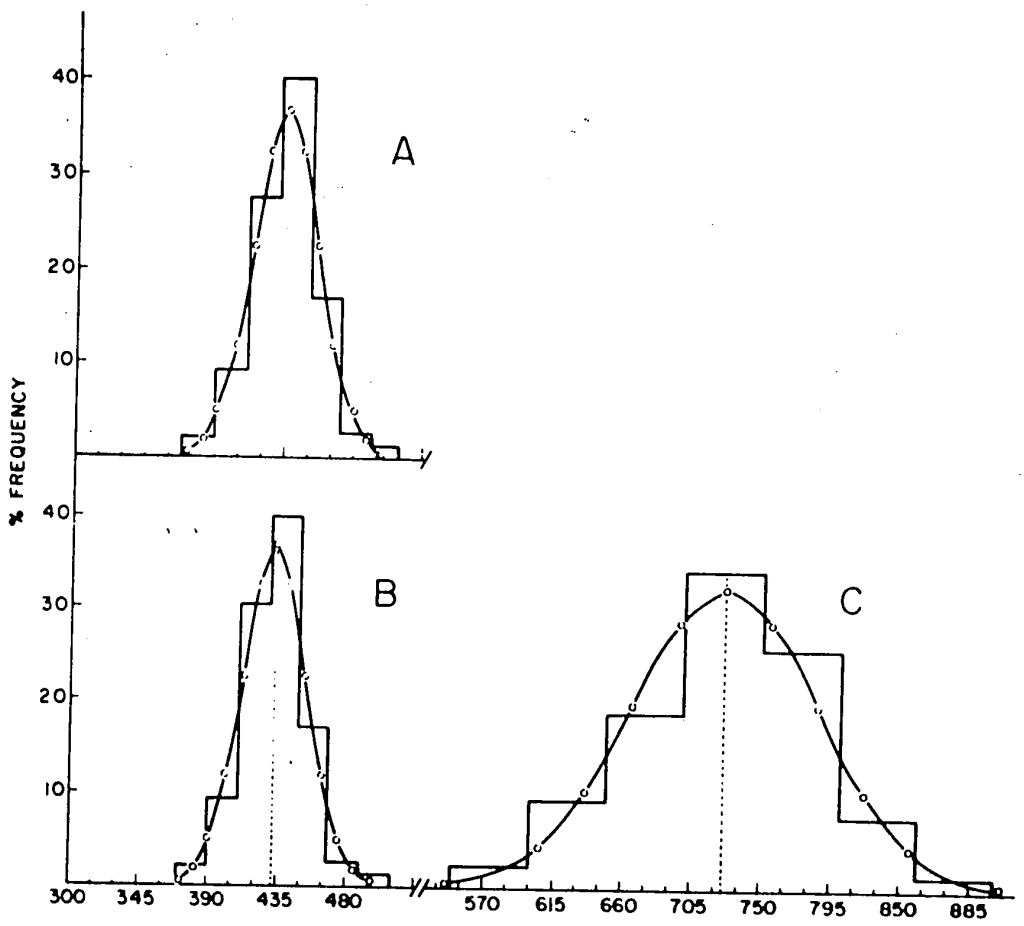
Frequency distribution histograms for intact and lysed cells.

Abscissae represent mobilities in (μ/s per V/cm) x 10^{-3}.

A Intact cells, standard deviation 0.03.

B Water-lysed cells (ghosts), standard deviation 0.03.

C Temperature-lysed cells (ghosts), standard deviation 0.06.


% FREQUENCY
40
30
20
10
A
B
C
300
345
390
435
480
570
615
660
705
750
795
850
885

histograms prepared from four separate experiments at pH 7.0 were surprisingly reproducible, with a coefficient of variation between 6 and 7%. A difference of about 10% between two mobility values (averaged from 32 timings in each case) can be considered significant, so that the mobilities for intact and water-lysed cells must be considered the same within experimental error.

Since the mobilities of temperature-lysed cells at pH 7.0 varied between -0.6 and -0.72 μ/sec per volt/cm for different experiments, the histogram was prepared from one set of measurements only, where 128 particles were timed. After temperature-induced lysis the resulting structures were less homogeneous (Fig. 6) with a coefficient of variation of 9% but the mean mobility and, indeed, the entire range of mobilities were significantly higher than those of both intact and water-lysed cells.

The nature of the chemical groups that determine the charge on cell surfaces cannot be deduced from the mobility/pH curves at high ionic strengths in the presence of $MgSO_4$, since ion adsorption could mask any charged groups present. Nor is it possible to determine whether the observed mobility arises from built-in charged groups or by non-specific ion adsorption. However, in solutions of the same composition, ionic strength, and pH, identical surfaces should behave in the same way with respect to the accumulation of counter-ions at the surface and their distribution within the double layer. The

electrophoretic mobility therefore would be the same and any mobility differences could reflect surface differences. If the mobility of two preparations were the same, however, one could not necessarily conclude that the surfaces were identical, but only that they had the same net charge. It is on this basis that the electrophoretic data will be discussed.

The form of the mobility/pH curve for intact cells is similar to that obtained for other bacterial cells, both gram positive and gram negative, over the same pH range (51, 72, 96), even though the high ionic strength of the suspending medium necessary to maintain cell structure makes the absolute mobility values recorded here low. In relatively concentrated solutions the double layer is thinner and the potential drop with distance from the interface is steeper (156) causing slower electrophoresis.

The initial electrophoretic effects of lysis, however induced, were the same: the cell populations became heterogeneous; that is, with time, an increasing proportion of cells showed increased negative mobility. When lysis was complete, as measured by the loss of turbidity of suspensions, all lysed cells had high negative mobilities. After repeated washings the populations became homogeneous with the original, slower mobility values. The effect of washing lysed cells suggested that the initial alteration in their electrophoretic behaviour was due to adsorption of intracellular material. However, adding

supernatants from distilled water lysates to intact or lysed and washed cells, in Michaelis buffer/salts, did not alter their mobilities (Table I). Possibly the intracellular material causing increased mobility had already been adsorbed onto the lysed cells from which the supernatant was prepared. Another possible, though less likely, explanation is that intracellular material is adsorbed only on the cell from which it originally leaks.

After the cells were washed, the mobility of those lysed in distilled water or in salts at pH 5.0 was the same as in untreated, intact cells. Thus, these kinds of lysis do not permanently alter the charge in the external cell surface. The increased permeability, which leads to the loss of intracellular substances, is probably due to changes within or at the cytoplasmic membrane.

Osmotic surges probably play an important part in lysis in distilled water. However, these cells, like other marine bacteria, require specific ions as well as an adequate osmotic pressure for stability. Cells lysed in distilled water formed extremely stable suspensions maintaining a constant optical density and electrophoretic mobility for several days (Table I). The lysed cells were stable at 25 °C or above, whereas the turbidity of cell envelopes prepared by Mickle disintegration fell at this temperature (80). If an enzyme is responsible for cellular breakdown at 25 °C, it must be inactivated at low ionic strength. Exposure of cells to 25 °C in distilled water also leaves the surface unchanged electrophoretically.

In contrast to water lysis and pH lysis, complete temperature-induced lysis led to significantly higher mobilities in the pH range 3.0-9.0, that is, to a change in charge of the cell surface. The breakdown of phospholipids occurring during temperature-induced lysis observed by Hagen et al. (80) leads to the suggestion that probably a phosphatidase might be involved in cellular breakdown.

Part II

Effect of pH on Cellular Integrity of NRC-1004

INTRODUCTION

The strange behaviour shown by NRC-1004 cells at low pH raised serious doubts concerning the validity of electrophoretic studies under these conditions. The fact that the electrophoretically "fast" cells became "slow" after washing led to the belief that changes in the electrophoretic behaviour, not being of a permanent nature, were caused rather by adsorption of some substances onto the cell surface than by a drastic change in the chemical and physical constitution of the cell envelope. It was assumed that the substances adsorbed on the surface were of intracellular origin and consequently experiments were designed to provide further information on the characteristics of lysis of NRC-1004 cells at acidic pH. These experiments and their results are described below.

MATERIALS AND METHODS

Lysis as a Function of pH

In conjunction with microscopic observation under phase contrast, two methods were used to measure lysis of NRC-1004 cells suspended in salt solutions at various pH values.

A. Turbidimetry

Cells grown for 72 hr (O.D. at 660 mμ: 0.800) in

sea water-tryptone medium (0.4% w/v) were washed twice in 0.5 M NaCl, 0.1 M $MgSO_4$, 0.01 M Tris buffer and resuspended in the same salt solution without buffer at a concentration which, diluted 100-fold, gave in sea water an optical density of 0.250 at 660 mμ. Fall of turbidity was measured in 18 mm calibrated test tubes with a Coleman Junior spectrophotometer against blanks of the same salt/buffer solutions. Cell suspensions were incubated at 0-2°C for 120 min. The turbidity of cell suspensions in sea water corresponded to 100%. Cell suspensions were thoroughly mixed for 3 sec before each reading with a Vortex junior mixer.

After 120 min all cell suspensions were centrifuged at 2°C for 10 min at 6000 x g. Cell-free supernatants were used to measure the release of 260 mμ absorbing material and of inorganic phosphate.

B. Release of intracellular material

1. Material absorbing at 260 mμ

The release of intracellular 260 mμ absorbing material in supernatants was measured using a 1 cm cuvette and a Beckman DU spectrophotometer (182). At 260 mμ the values obtained from supernatants of cells lysed in distilled water varied between 0.950 and 1.050 and were used in each experiment as indicating 100% release of intracellular 260 mμ absorbing material.

2. Increase of inorganic phosphate in supernatant

Four ml of supernatant were transferred into a glass

centrifuge tube containing 1 ml of 10% trichloroacetic acid to precipitate proteins. This solution was centrifuged for 10 min at 6000 x g in the cold. The same method was used to prepare the corresponding blanks. Inorganic phosphate was measured by the method of Fiske and SubbaRow (64). Total phosphate of NRC-1004 cell suspensions in PSM giving an O. D. of 0.250 at 660 mμ averaged 20 μg (100% phosphate).

Buffer Systems

Three different buffers were used:

1) succinic acid-sodium tetraborate (pH 3.0-5.8),

2) Michaelis buffer (sodium veronal + acetate + HCl) (pH 3.0-9.0),

3) Tris [tris(hydroxymethyl)aminomethane] (pH 5.0-7.5).

For pH values below 3.0 the pH of the salt solutions was adjusted by the addition of HCl.

Salt solutions consisted of 0.5 M NaCl + 0.1 M $MgSO_4$. Final concentration of buffers were 0.014 M for Michaelis buffer and 0.010 M for the two other buffers.

In one experiment a mixed buffer consisting of Michaelis buffer (0.014 M) and Tris buffer (0.010 M) was used. The final pH of all buffers and cell suspensions in salt/buffer solutions was checked using a Beckman pH meter and glass electrodes.

RESULTS

Microscopic Examination of Cell Suspensions in Salt Solutions at Various pH

Cells were observed by phase contrast microscopy. A drop of the cell suspension was transferred to a cold microscope slide

covered with a cold coverslip and observed for a period not exceeding 3 min. The observation period was limited to 3 min because gradual warming of the slide caused loss of cell motility followed by morphological changes (see Fig. 20) and eventual lysis. When cells were suspended in suitable salt solutions and where lysis was caused by pH or temperature, loss of motility always preceded lysis. A gradual decrease in the speed of free moving cells in the microscopic field could be noticed after 4-5 min of observation. No motile cells could be detected after 8-10 min.

Motility was observed only at pH values between 6.5 and 8.0. Under microscopic examination cells suspended in salt solutions at pH values below 6.5 showed changes in shape characteristic of lysing cells. Bacteria lost their rod-like appearance to become round, globular, and swollen. They became less dense and some dark spots could be seen at the periphery. The envelope became less and less distinct till it became impossible to distinguish a definite structure and finally only very small particles of various sizes could be seen.

Effect of pH on the Turbidity of Cell Suspensions and on the Measurement of 260 mμ Absorbing Material Released by Lysed Cells

Cells suspended in salt/buffer solutions within the pH range 6.0-9.0 showed very little change in turbidity over a 2-hr period (Fig. 8). At pH lower than 6.0 the turbidity fell sharply. The lowest optical densities were recorded at pH 5.3, where they were 45-50% of

FIGURE 8

Effect of pH on turbidity and release of intracellular substances.

----- Turbidity.

-·-·- Release of inorganic phosphate.

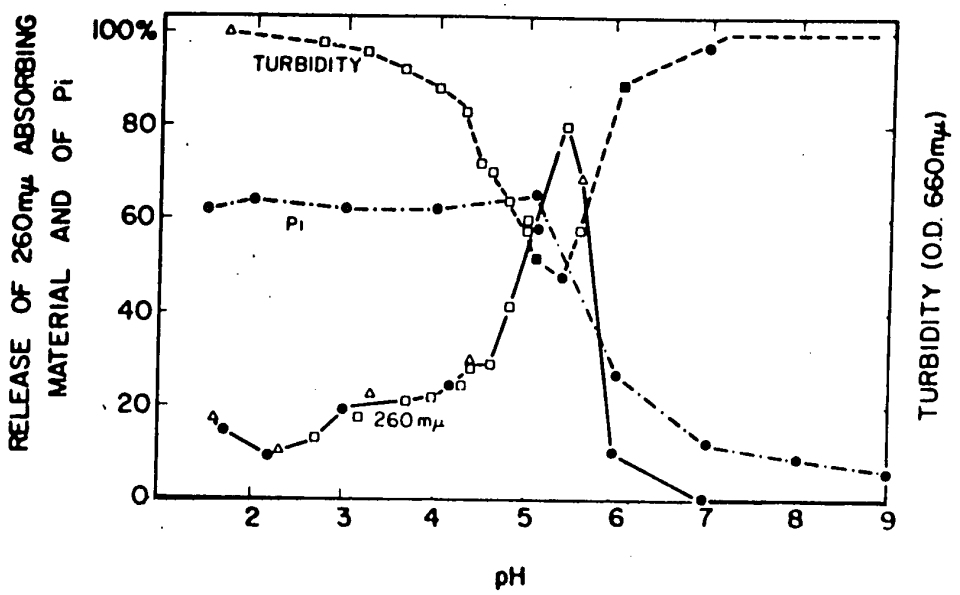
——— Release of ultraviolet-absorbing material.

□ Succinic-sodium tetraborate buffer.

■ Tris buffer.

● Michaelis buffer (0.01 M buffer, 0.1 M $MgSO_4$, 0.5 M NaCl).

△ Michaelis buffer + 0.01 M Tris buffer.


RELEASE OF 260mμ ABSORBING MATERIAL AND OF Pi
TURBIDITY (O.D. 660mμ)
100%
80
60
40
20
0
TURBIDITY
Pi
260 mμ
2
3
4
5
6
7
8
9
pH

the turbidity of control cell suspensions in sea water, pH 7.0. Below pH 5.3 the O.D. values increased as the pH was further decreased reaching the turbidity of control suspensions at about pH 3.5.

Concurrently with the fall in turbidity there was an extensive release of 260 mμ absorbing material into the supernatants at pH's below 6.0. The greatest release occurred at pH 5.3, where it was 80% of the amount released by cells suspended in distilled water. Below pH 5.3 the amount of 260 mμ absorbing material detected in the supernatants decreased and below pH 4.0 it represented only between 10 and 20% of the amount released by cells lysed in distilled water (Fig. 8). As the observations made by phase contrast microscopy showed that, in fact, there was complete cellular lysis at all pH values below 6.0, it was necessary to establish the causes of the discrepancies that seemed to exist between microscopic observations and turbidimetric as well as release of 260 mμ absorbing material measurements.

The acidification of the salt solutions was thought to cause coagulation of cellular proteins. This would account for the high turbidities at low pH while cells actually lysed. The increase in 260 mμ absorbing material released was probably due to the precipitation of this material at low pH. Two series of experiments (A and B below) were undertaken to clarify this point.

A. Effect of acidification of supernatants on the measurement of 260 mμ absorbing material released by cells suspended in salt solutions at various pH

Cells were suspended for 2 hr at 2°C in salt/buffer solutions at various pH and labelled "control PSM". Inoculum consisted of 0.1 ml of washed cells resuspended in 9.9 ml of salt solution. O.D. at 660 mμ in sea water was 0.250. After 2 hr the suspensions were centrifuged and in each case the supernatants divided into two aliquots. Aliquot I consisted of 1.0 ml of supernatant. Aliquot II consisted of the remainder of the supernatant to which 0.1 ml of 15 N HCl was added to bring the pH down to 1.0 (acidified control, PSM HCl). The released 260 mμ absorbing material was measured in 1 cc cuvettes with a Beckman DU spectrophotometer. The results described in Table II and in Fig. 9 show quite clearly that when the pH of the supernatant at pH 5.0 was lowered to 1.0 there was an extensive decrease in the measurable amount of 260 mμ absorbing material. While in supernatants at pH 5.0 the 260 mμ absorbing material measured represented 72% of the amount measured in the supernatants of distilled water lysed cells, this value was reduced to only 28% when the supernatants at pH 5.0 were acidified to pH 1.0 with HCl. When a supernatant at pH 5.0 was acidified to 1.0 with HCl there was a 60% decrease in the measurable amount of 260 mμ absorbing material. As the pH of the supernatants decreased the difference between measurable 260 mμ absorbing material in the original low pH supernatants

TABLE II

Effect of acidification of supernatants on the measurement of 260 mμ absorbing material released by cells suspended in salt solutions at various pH

pH	Per cent turbidity	Per cent release of 260 mμ absorbing material measured in supernatants	
		Before acidification	After acidification with HCl
1.5	115	22	17
2.0	88	12	15
3.0	90	19	15
4.0	85	46	25
5.0	51	72	28
6.0	92	3	3
7.0	100	2	2
8.0	100	1	1
9.0	100	3	3
Sea water	100	5	-
Distilled water	10	100	-

FIGURE 9

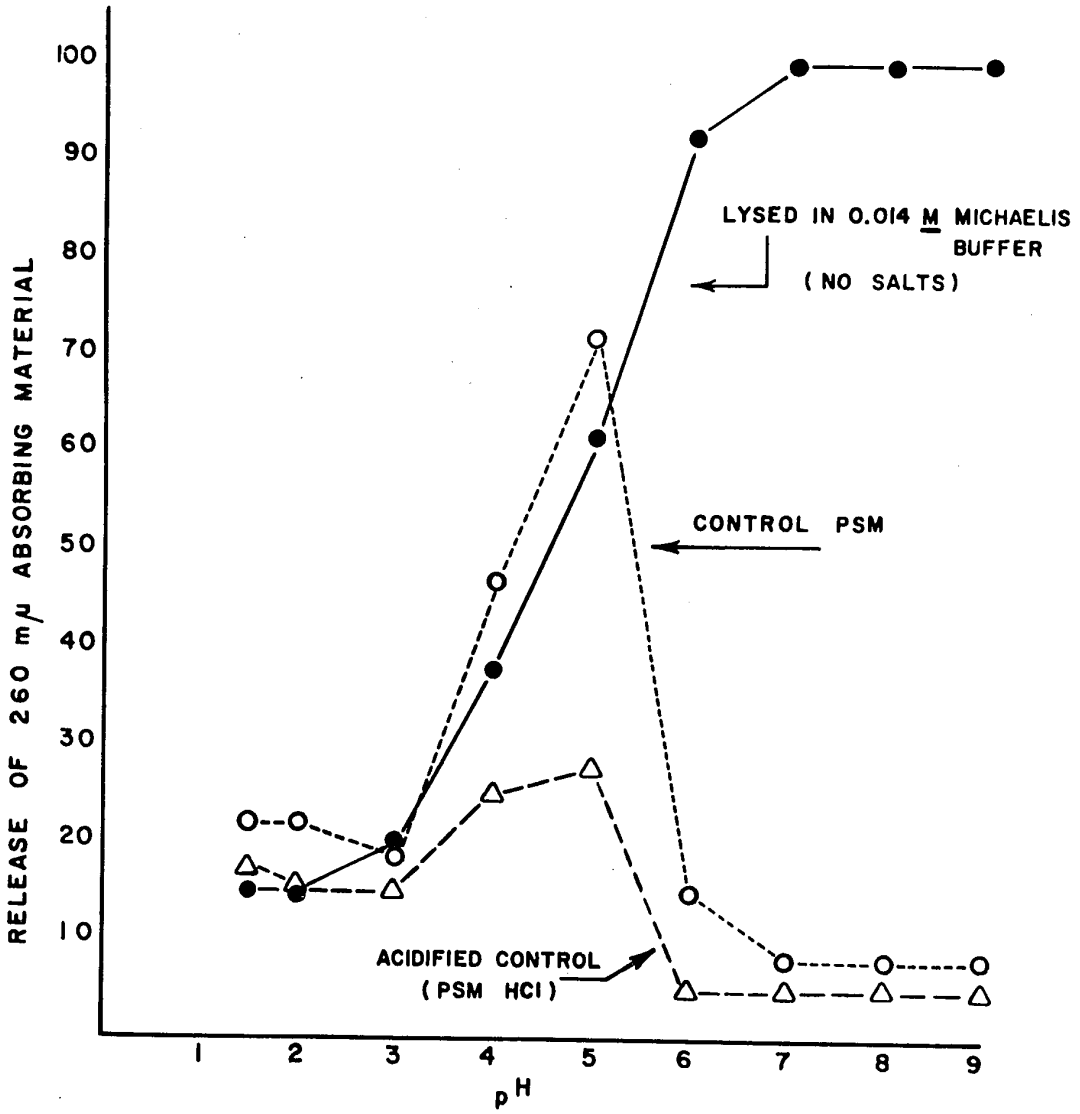
Effect of pH on the measurement of released 260 mμ absorbing material.

100% = 260 mμ absorbing material released in supernatants of cells suspended in distilled water.

○ 260 mμ absorbing material in supernatants of cells suspended in PSM.

△ Same as above but HCl was added to supernatants.

● 260 mμ absorbing material in supernatants of cells suspended in Michaelis buffer (0.014 M) alone.



EFFECT OF pH ON THE MEASUREMENT OF RELEASED 260 mμ ABSORBING MATERIAL

and in their respective HCl acidified samples was less marked. Turbidity, which dropped at pH 5.0 to half the O.D. of the cell suspensions in sea water, gradually increased as the pH decreased. However, under microscopic observation, all cells suspended in solutions with pH below 6.0 had lysed.

B. Effect of acidification on the measurement of 260 mμ absorbing material released in supernatants of cells lysed in water

In these tests the released 260 mμ absorbing material was measured in supernatants of cells suspended in distilled water to which 0.015 M Michaelis buffer was added. Below pH 3.0, HCl was used to acidify the water. Cells were suspended in the fashion described previously and the 260 mμ absorbing material was measured after 2 hr incubation at 2°C in the supernatant of the centrifuged lysed cell suspensions. Results are shown in Table III. All cells lysed, with the exception of those suspended in sea water. The turbidities of the suspensions lysed in water at pH 5.0 and below are close to that of suspensions in sea water. On the other hand, the measured release of 260 mμ absorbing material in the supernatants of suspensions whose pH ranged between 6.0 and 1.5 showed a gradual decrease. Above pH 7.0 the amount of 260 mμ absorbing material released in the supernatants was the same as in unbuffered distilled water.

These results confirm those already obtained in test A, and point out the shortcomings of the measurement at acid pH of lysis by

TABLE III

Effect of acidification on the measurement of 260 mμ absorbing material released in supernatants of cells lysed in water

pH	Per cent turbidity	Per cent release of 260 mμ absorbing material
1.5	84	15
2.0	94	15
3.0	100	20
4.0	86	38
5.0	97	62
6.0	7	93
7.0	13	100
8.0	20	100
9.0	20	100
Unbuffered distilled water	9	100
Sea water	100	8

turbidimetry and by the release of 260 mμ absorbing material. An alternate method for the measurement of lysis of cell suspensions at pH below 7.0 had to be used. This method consisted of measuring the release of inorganic phosphate in the supernatants, a procedure which has been satisfactorily used by Kushner and Bayley with Halobacterium cutirubrum (114).

The general procedure was the same as in the two previously described tests. Cell suspensions in salt solutions at various pH values were incubated for 120 min at 0-2°C. After centrifugation at 6000 x g for 10 min with cold, proteins in supernatant were precipitated with 10% trichloroacetic acid and centrifuged again at 6000 x g for 10 min at 0°C. Inorganic phosphate present in the supernatants was determined by the method of Fiske and SubbaRow (64).

Measurement of Inorganic Phosphate Released in Supernatants by Cell Suspensions at Various pH

Table IV shows the averages, for three different experiments, of inorganic phosphate released in supernatants by cell suspensions at various pH values. These results are compared with those of turbidimetry and the measurement of the release of 260 mμ absorbing material (Fig. 8). There is an increasing amount of inorganic phosphate released with a maximum at pH 5.0 amounting to 65% of the amount released by cells lysed in distilled water. Decreasing the pH below 5.0 did not cause any further release of inorganic phosphate nor any

TABLE IV

Measurement of inorganic phosphate released in supernatants by cell suspensions at various pH

pH	Per cent inorganic phosphate released
1.5	62
2.0	64
3.0	62
4.0	62
5.0	65
6.0	27
7.0	12
8.0	9
9.0	6
Distilled water 120 min	100*
Distilled water 10 min	69

*50 μg P = 100%.

significant variation in the measurement. There is, however, a difference in the amount of inorganic phosphate in the supernatant of distilled water lysed cells when it is measured 10 min or 120 min after inoculation (Table IV) and 100% release of inorganic phosphate represented the amount released by cells suspended in distilled water for 120 min.

DISCUSSION

Lysis at different pH values has been measured as the loss of turbidity at 660 mμ, and as leakage of cellular constituents (UV-absorbing material and inorganic phosphate) into the surrounding medium. The greatest fall in turbidity occurs between pH 4.0 and pH 6.0. Similarly, the maximal release of UV-absorbing material occurs at about pH 5.5 (Fig. 8). The release of inorganic phosphate gives a different and probably more realistic picture of the effect of pH on cell breakdown. At all pH values below 6.0, most of the inorganic phosphate is released into the external medium. Kushner and Bayley (114), working with the extreme halophile Halobacterium cutirubrum, used similar methods to indicate cellular leakage at low pH and found discrepancies in the release of inorganic phosphate and UV-absorbing substances, although these gave closely comparable results when leakage was caused by low salt concentrations (22). It was concluded (114) that precipitation of about 80% of the UV-absorbing material under acid conditions made the release of such material useless as an index of cell leakage at low

pH values.

Lysis of the psychrophile at pH 5.0, followed by acidification of the supernatants to pH 1.0, caused the optical density at 260 mμ to drop to less than half the value recorded at pH 5.0 (Fig. 8). Therefore, with this organism also, measurement of loss of inorganic phosphate appears to be a more reliable index of cell leakage at acid pH.

Precipitation of macromolecules probably also caused the increased turbidity of suspensions as the pH was lowered from 5.3 (Fig. 8), and such precipitation obviously limits the reliability of this convenient method of measuring lysis.

The release of inorganic phosphate from cells in salts at various pH values after 2 hr was expressed as a percentage of the release in distilled water after the same time (Fig. 8). The inorganic phosphate released in water after 2 hr was 30% greater than after 10 min. This increased release may have been due to the action of hydrolytic enzymes which are active in distilled water at 5°C, but are inactive at low pH values. It is probably more accurate to relate the inorganic phosphate released at different pH values to that in water after 10 min incubation. By doing this, over 90% release could be obtained below pH 6.0.

Part III

Response of NRC-1004 to Changes in Its Ionic Environment

or

(Role of Mono- and Divalent Ions in the Integrity of NRC-1004 Cells)

INTRODUCTION

A difficulty encountered at the start of the studies on electrophoretic behaviour of NRC-1004 was to produce a salt solution which would a) prevent the escape of intracellular substances from these bacterial cells, b) assure that the cell suspensions had the same turbidity as in sea water, c) maintain the morphological characteristics of the cells, and d) have an ionic strength low enough to prevent the adsorption of ions on the cell surface whose presence would mask the charged groups forming part of that surface.

A systematic study of the effect of various mono- and divalent ions commonly found in sea water was undertaken to clarify the importance of these ions on the cell's integrity and to what extent the effects caused by these ions could be explained by osmotic pressure.

Investigations were also undertaken on the chemical changes that occurred when NRC-1004 cells were heat treated (25 and 38°C) or suspended in distilled water. Comparative studies were made

of the effects of temperature and distilled water treatments on whole cells and mechanically prepared cell envelopes.

MATERIALS AND METHODS

Microorganism

NRC-1004 was grown in the condition stated in the Materials and Methods section of Part I. Cell suspensions in sea water and PSM were prepared in the same fashion as described in Part II.

Solution for Washing and Suspension of NRC-1004 Cells

The salt solution adopted consisted of a mixture of 0.5 M NaCl, 0.1 M $MgSO_4$, and 0.014 M Michaelis buffer, pH 7.0. A similar solution was used to wash the freshly harvested cells replacing Michaelis buffer by 0.01 M Tris buffer. This last solution is referred to as Psychrophile Suspending Medium or PSM.

Mechanical Preparation of Envelopes

All PSM washed cells recovered from a 1000 ml medium of 72 hr of growth were resuspended in a total volume of 30 ml of cold PSM. This suspension was divided into two cold glass side-baffled flasks containing 5 gm each of Ballotini beads. Both flasks were stoppered, placed in the respective containers of a Mickle disintegrator and shaken for 20 min in a 5 °C room. The contents of both flasks were then transferred into two cold Nalgene centrifuge tubes and centrifuged for 10 min at 2000 x g to remove the beads. Unbroken cells were

separated from the supernatant by centrifugation at 6000 x g for 10 min. Envelopes were recovered from the supernatant by centrifugation at 15,000 x g for 20 min, resuspended in cold PSM and a sample was observed by phase contrast microscopy to check if whole cells were present. If they were, the suspension was centrifuged once more at 6000 x g for 10 min. The supernatant was washed three times in cold PSM at 15,000 x g for 20 min.

Preparation of Water Lysed Cells

Water lysed cells were prepared by suspending a heavy concentration of PSM washed cells in 500 ml of cold glass-distilled water for 2 hr. The water lysed cells were recovered by centrifuging the suspension for 20 min at 15,000 x g and subsequently washed three times in cold PSM at 15,000 x g for 20 min.

Chemical Methods

Inorganic phosphate was measured after precipitation of proteins with 10% trichloroacetic acid by the method of Fiske and SubbaRow (64). Lipids were extracted by the method of Bligh and Dyer (21) and lipid-P was analyzed by Allen's (6) method. Chromatography of lipids on silicic-acid impregnated paper were made by the method of Marinetti et al. (135) using diisobutyl ketone as a solvent. Hexosamine was measured by the method of Rondle and Morgan (173). Hexoses were determined with anthrone reagent (173).

Measurement of Lysis in Whole Cell Suspensions

A. Turbidimetry

Washed cells were resuspended in cold PSM in a concentration which gave an optical density of 0.250 at 660 mμ when diluted 100-fold in either sea water or PSM. Turbidity was measured in calibrated 18 mm diameter test tubes with a Coleman Junior spectrophotometer at 660 mμ. The procedure followed consisted in preparing 25 ml of the salt solutions in which the cells were to be suspended. A 10 ml volumetric flask was filled to about half volume with the cold solution, 0.1 ml of cell suspension in PSM was added and the volume completed to 10 ml with the salt solution. The flask was stoppered, shaken vigorously for a few seconds, and the solution transferred immediately into a cold calibrated test tube. The cell mass was thoroughly distributed through the solution by placing the test tube for 3 sec on a Vortex junior mixer before each turbidimetric measurement against a blank of the same salt solution.

The same procedure was carried out to test the effects of suspension in distilled water and in PSM or sea water at 25 °C and 38°C. Turbidity in PSM and/or sea water is taken as 100%.

B. Release of intracellular material

The release of intracellular material was measured by the method described in the corresponding section of Materials and Methods of Part II (page 85).

Methods for the Measurements of Chemical Changes Due to Temperature Lysis

An inoculum of 1.0 ml of a sea water suspension of whole cells, distilled water lysed cells or mechanically prepared envelopes, was suspended in 100 ml of sea water at 0°C in a cold Nalgene centrifuge tube and mixed for 30 sec. Four 10 ml aliquots of this suspension were transferred into calibrated test tubes, two of which were placed in a test tube rack inside a water bath filled with crushed ice, and the other two similarly placed in a water bath kept at a temperature of 25°C. Two more aliquots of 10 ml were used to measure the total lipid phosphate or hexosamine present at the start of the experiment at 0°C. The values obtained from these samples were used as the 100% values to which those obtained from the other samples were compared.

To account for any chemical change that could take place in the samples used as controls at 0°C during the 2 hr of incubation, four 10 ml aliquots were transferred into cold glass centrifuge tubes and centrifuged for 10 min at 6000 x g at 0°C. The cell pellets were resuspended in 10 ml of distilled water at 0°C. Two were used as control time 0 and two were placed into the test tube rack in the crushed ice bath for 2 hr.

Separate experiments were done in duplicate for the determinations of glucose, hexosamine, and lipid-P.

RESULTS

Lysis in Distilled Water

In cold (3°C) distilled water cells lysed rapidly. The O.D. of suspensions fell to about 50% of that in cold sea water or PSM within a few seconds, and by 10 min had fallen to a minimum value of 10-15% of this value. The rate of lysis in distilled water was not appreciably increased by raising the temperature to 40°C (Fig. 10). During a 10 min period the optical density of suspensions in cold sea water or PSM did not fall.

Effect of Monovalent Salts on Lysis

Monovalent salts alone could not maintain cell integrity. The same amount of lysis as in distilled water was observed when cells were suspended in 0.5 M LiCl, CsCl, RbCl, KCl, and NH_4Cl. Cells lysed completely in up to 1.5 M NaCl (Table V).

In the presence of 0.1 M Mg^{++}, however, certain monovalent cations were able to maintain cell integrity. Fig. 11 shows that 0.5 M NaCl in the presence of this concentration of $MgSO_4$ was as effective as sea water (or PSM), both in maintaining turbidity of suspensions and preventing leakage of intracellular substances. If 0.5 M NaCl was present, $MgCl_2$ could substitute for $MgSO_4$. LiCl was less effective than NaCl, since 0.9 M LiCl produced the same turbidity as 0.5 M NaCl, and even at this LiCl concentration some intracellular compounds were released. RbCl was less effective than

FIGURE 10

Time course of lysis in distilled water.

● 0°C.

O 40°C.

Curves represent average values from three experiments.

Zero time readings represent cells diluted 100 times in PSM and the curves show the effect of 100-fold dilution in distilled water.

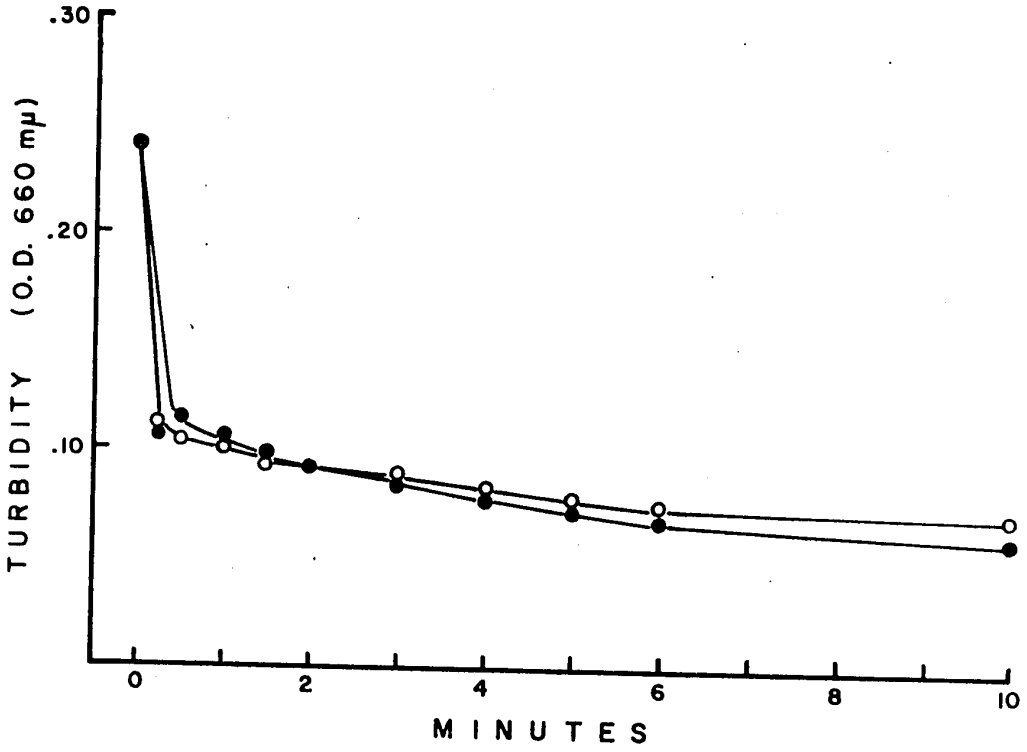

.30
.20
.10
TURBIDITY (O.D. 660 mμ)
0
2
4
6
8
10
MINUTES

TABLE V

Effect of monovalent salts on lysis of NRC-1004 cells

Solution	Per cent turbidity	Per cent release of 260 mμ absorbing material
PSM	100	8
Sea water	100	10
0.5 M LiCl	11	100
0.5 M CsCl	10	100
0.5 M RbCl	10	100
0.5 M KCl	11	100
0.5 M NH_4Cl	10	100
0.2 M NaCl	11	100
0.5 M NaCl	11	100
0.7 M NaCl	10	100
1.0 M NaCl	11	100
1.5 M NaCl	11	100
0.5 M LiCl + 0.5 M NaCl	11	100
Distilled water	11	100

FIGURE 11

Effect of NaCl, LiCl, and Tris buffer on cell integrity in the presence of 0.1 M $MgSO_4$.

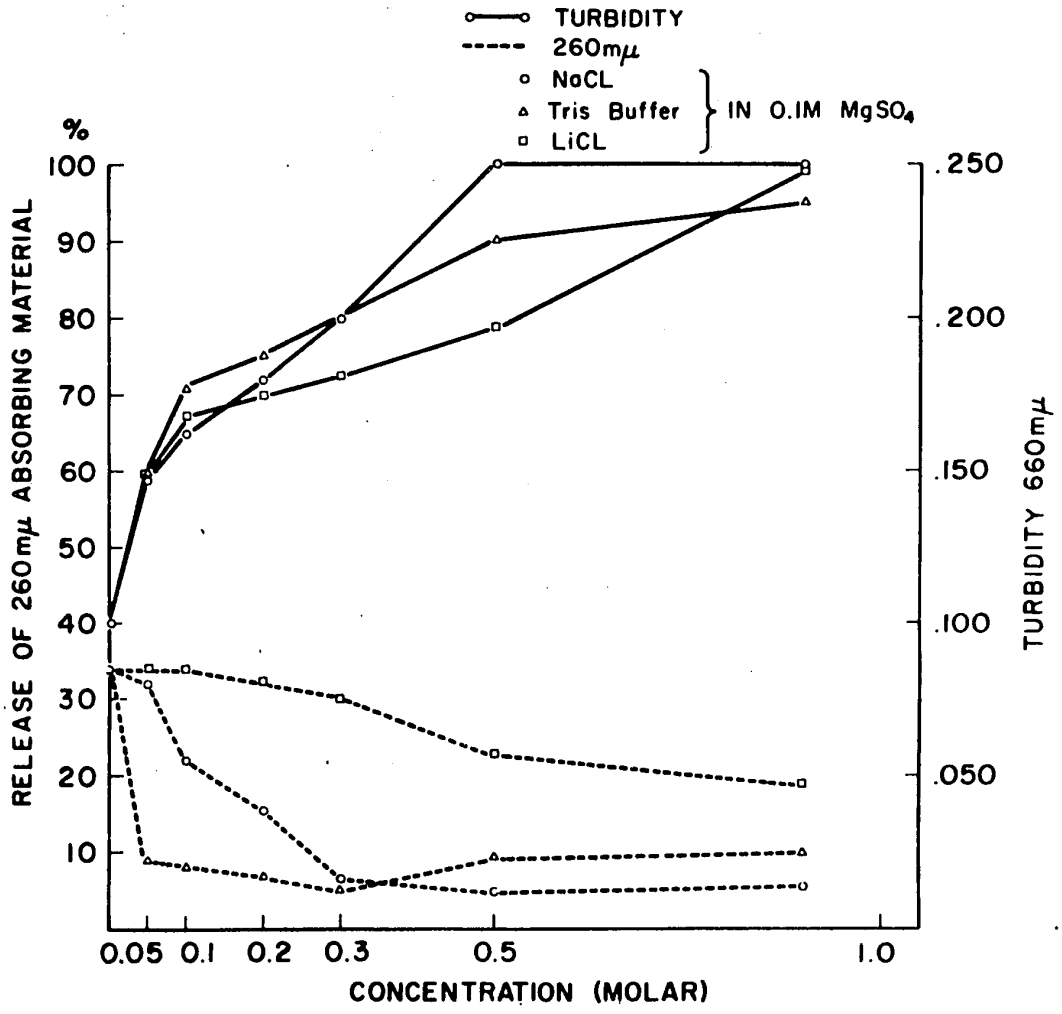
O NaCl.

□ LiCl.

△ Tris buffer.

——— Turbidity.

----- Release of ultraviolet-absorbing material.


TURBIDITY
260mμ
NaCL
Tris Buffer
LiCL
IN 0.IM MgSO4
%
100
90
80
70
60
50
40
30
20
10
RELEASE OF 260mμ ABSORBING MATERIAL
.250
.200
.150
.100
.050
TURBIDITY 660mμ
0.05
0.1
0.2
0.3
0.5
1.0
CONCENTRATION (MOLAR)

LiCl, KCl had almost no effect, and CsCl was lytic, concentrations of this salt higher than 0.3 M lowering the turbidity and causing an almost complete release of UV-absorbing substances (Fig. 12). NH_4Cl was also strongly lytic; concentrations as low as 0.05 M lowered turbidity and caused 100% release of intracellular substances in the presence of 0.1 M $MgSO_4$.

These results show that Na^+ was the essential ion in NaCl. Other sodium salts were also effective in maintaining cell structures (as measured by turbidity) in the presence of 0.10 M $MgSO_4$ (Table VI). Sodium nitrate, nitrite, sulfate, molybdate, and succinate maintained turbidity of suspensions as well as or better than NaCl. Sodium bromide, iodide, acetate, and tartrate were more effective than NaCl in the concentration range 0.1-0.2 M but less effective at a concentration of 0.5 M. Turbidity fell as the sodium tartrate concentration was increased. In the presence of 0.1-0.5 M sodium phosphate, cells lysed.

Divalent Salts

In concentrations higher than 0.2 M, $MgCl_2$ maintained turbidity of cell suspensions and prevented leakage almost as well as PSM or sea water; $MgSO_4$ was as effective in preventing leakage, but less so in maintaining turbidity (Fig. 13).

Very low concentrations of certain divalent metal ions (Cu^{++}, Zn^{++}, and Ni^{++}) and somewhat higher concentrations of Mn^{++} and Ca^{++} prevented lysis (Fig. 14). Cupric ions were roughly 1000 times

FIGURE 12

Effect of RbCl, KCl, and CsCl on cell integrity in the presence of 0.1 M $MgSO_4$.

O RbCl.

△ KCl.

□ CsCl.

——— Turbidity.

----- Release of ultraviolet-absorbing substances.

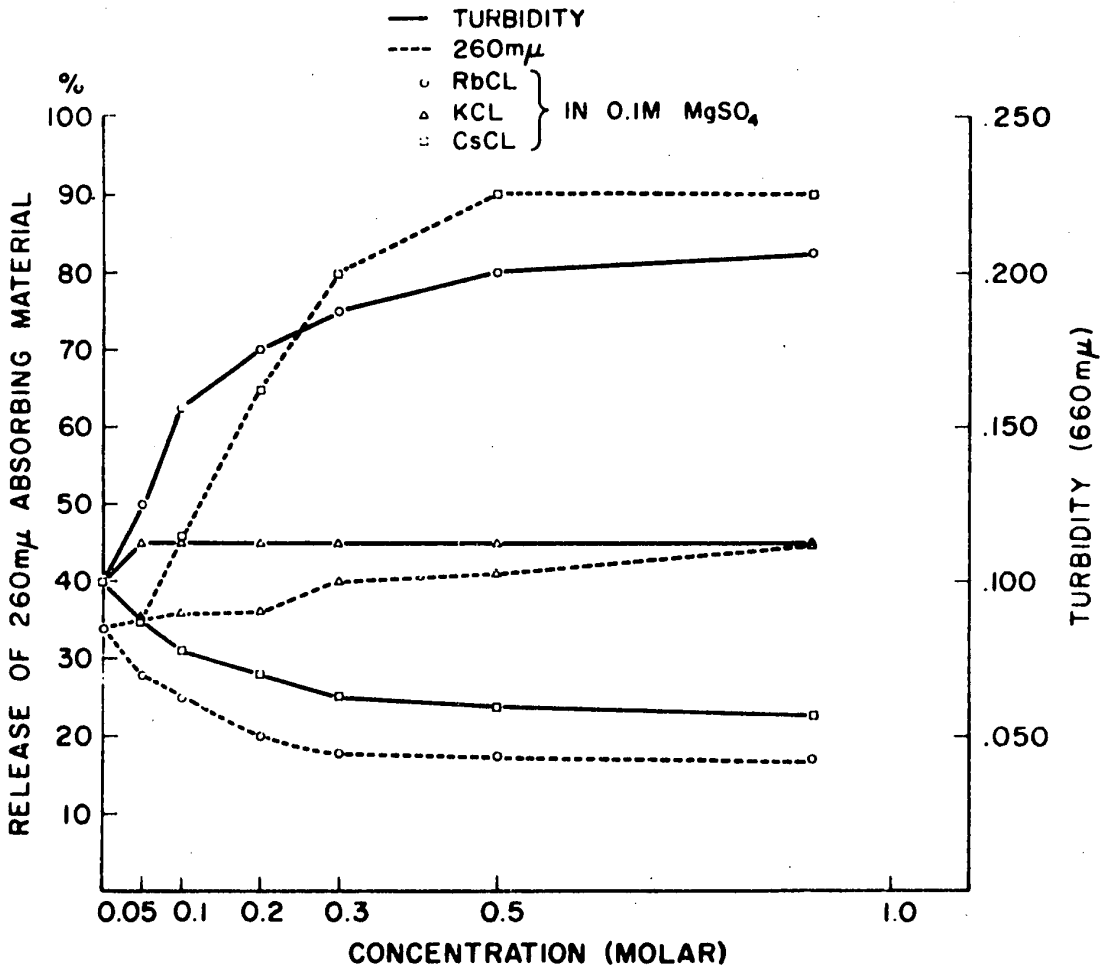

TURBIDITY
260mμ
RbCL
KCL
CsCL
IN 0.1M MgSO4
%
100
90
80
70
60
50
40
30
20
10
RELEASE OF 260mμ ABSORBING MATERIAL
.250
.200
.150
.100
.050
TURBIDITY (660mμ)
0.05
0.1
0.2
0.3
0.5
1.0
CONCENTRATION (MOLAR)

TABLE VI

Effect of sodium salts on turbidity of cell suspensions in the presence of 0.1 M $MgSO_4$

Anion	Per cent turbidity				
	0.0 M	0.1 M	0.2 M	0.3 M	0.5 M
Chloride	32	55	62	70	100
Bromide	32	45	79	87	68
Iodide	32	84	84	68	87
Nitrate	32	68	90	95	105
Nitrite	32	74	80	94	121
Sulfate	32	95	63	90	131
Phosphate	32	11[a]	11[a]	11[a]	11[a]
Molybdate	32	53	100	95	126
Acetate	32	63	84	84	84
Succinate	32	55	97	100	95
Tartrate	32	84	84	68	53

All solutions were adjusted to pH 7.0, using NaOH or the appropriate acid to avoid introducing other anions.

[a]On microscopic examination only cell fragments could be seen.

FIGURE 13

Effect of $MgSO_4$ and $MgCl_2$ on cell integrity.

● $MgSO_4$.

O $MgCl_2$.

——— Turbidity.

----- Release of ultraviolet-absorbing material.

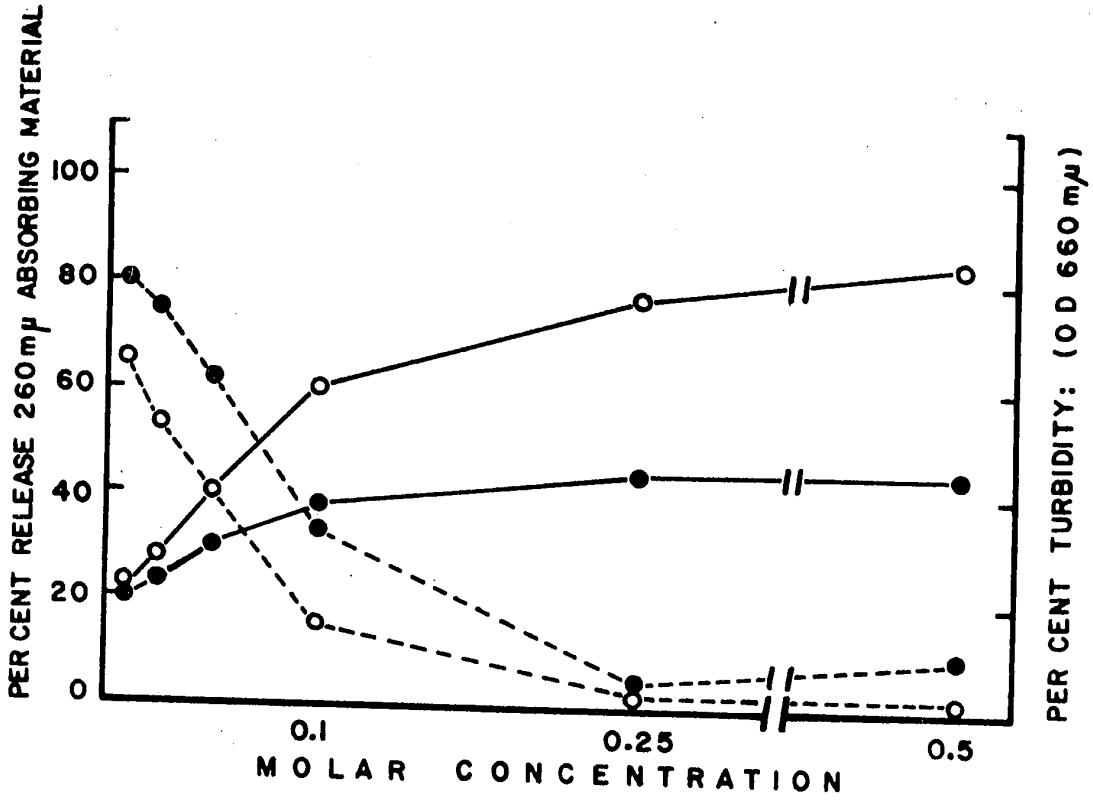

PER CENT RELEASE 260mμ ABSORBING MATERIAL
100
80
60
40
20
0
0.1
0.25
0.5
MOLAR CONCENTRATION
PER CENT TURBIDITY: (O D 660 mμ)

FIGURE 14

Effect of divalent cations on cell integrity.

● $CaCl_2$.

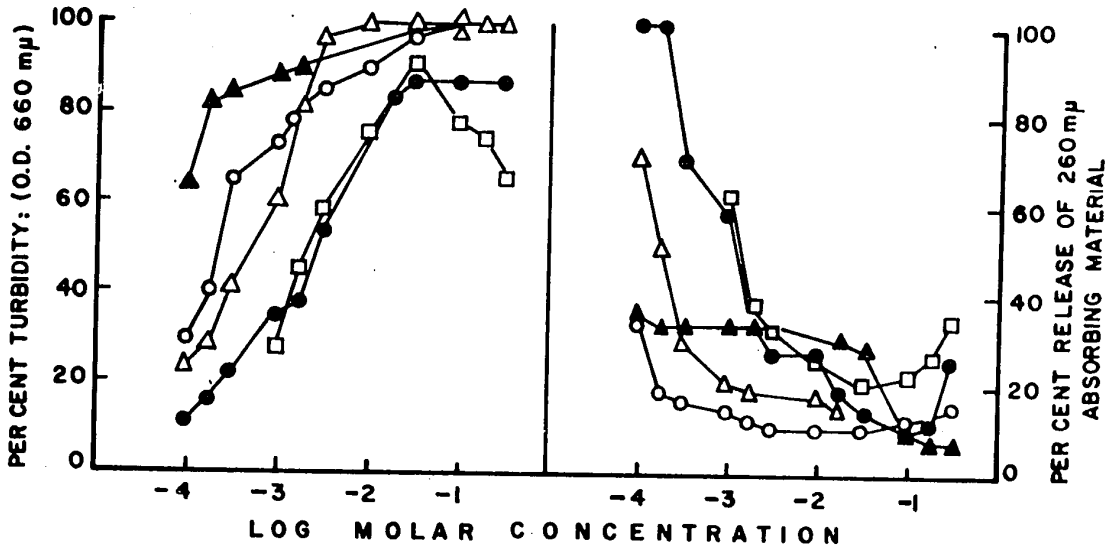
○ $ZnCl_2$.

▲ $CuSO_4$.

△ $NiCl_2$.

□ $MnCl_2$.

Concentrations are expressed as $\log_{10}$.


PER CENT TURBIDITY: (O.D. 660 mμ)
100
80
60
40
20
0
-4
-3
-2
-1
LOG MOLAR CONCENTRATION
PER CENT RELEASE OF 260 mμ ABSORBING MATERIAL

as effective as Mg^{++}; Zn^{++}, 200 times; Ni^{++}, 100 times; and Ca^{++} and Mn^{++} only 2 times as effective. As discussed below, some of these ions could also preserve cell shape.

The ability of very low concentrations of heavy metal ions to prevent lysis is probably the cause of a curious effect noted in some experiments in which Tris was used to buffer the salt solutions.

Effect of Tris Buffer on Lysis

The turbidities of cells suspended in various concentrations of $MgCl_2$ (Fig. 15) were higher in the presence of Tris buffer (0.01 M) than in its absence, while at the same time the leakage of 260 mμ absorbing material decreased notably at $MgCl_2$ concentrations below 0.25 M. Similarly, the presence of Tris buffer prevented leakage of 260 mμ absorbing material from NRC-1004 cells suspended in various concentrations of $MgSO_4$. However, the turbidities of cell suspensions in $MgSO_4$, which at all tested concentrations of $MgSO_4$ were lower than the turbidity of cell suspensions in PSM, did not increase in the presence of Tris buffer (Fig. 16).

Tris buffer had a striking effect on the turbidities of cell suspensions in $CaCl_2$ (Fig. 17). In the presence of 0.01 M Tris buffer and 0.125 M $CaCl_2$ turbidities increased to reach 90% of the turbidity of cells suspended in PSM, and the release of 260 mμ absorbing material was virtually the same as controls. With increase in $CaCl_2$ beyond 0.250 M concentration, the turbidities fell and 260 mμ absorbing

FIGURE 15

Effect of Tris buffer on cells suspended in various concentrations of $MgCl_2$.

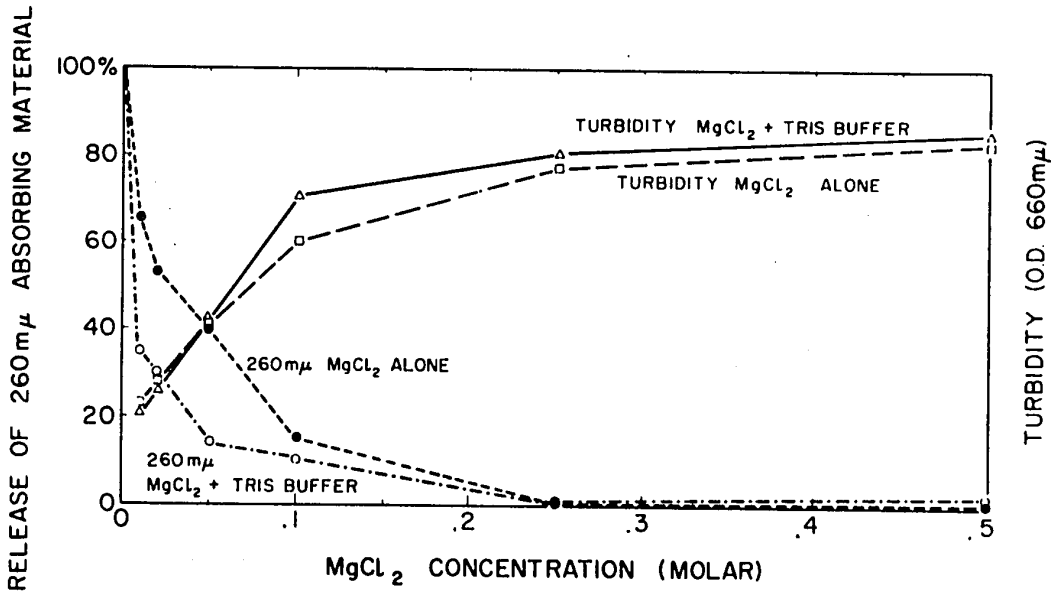

RELEASE OF 260mμ ABSORBING MATERIAL
100%
80
60
40
20
0
0
.1
.2
.3
.4
.5
MgCl2 CONCENTRATION (MOLAR)
TURBIDITY (O.D. 660mμ)
TURBIDITY MgCl2 + TRIS BUFFER
TURBIDITY MgCl2 ALONE
260mμ MgCl2 ALONE
260mμ
MgCl2 + TRIS BUFFER

FIGURE 16

Effect of Tris buffer on cells suspended in various concentrations of $MgSO_4$.

——— Turbidity.

----- Release of ultraviolet-absorbing material.

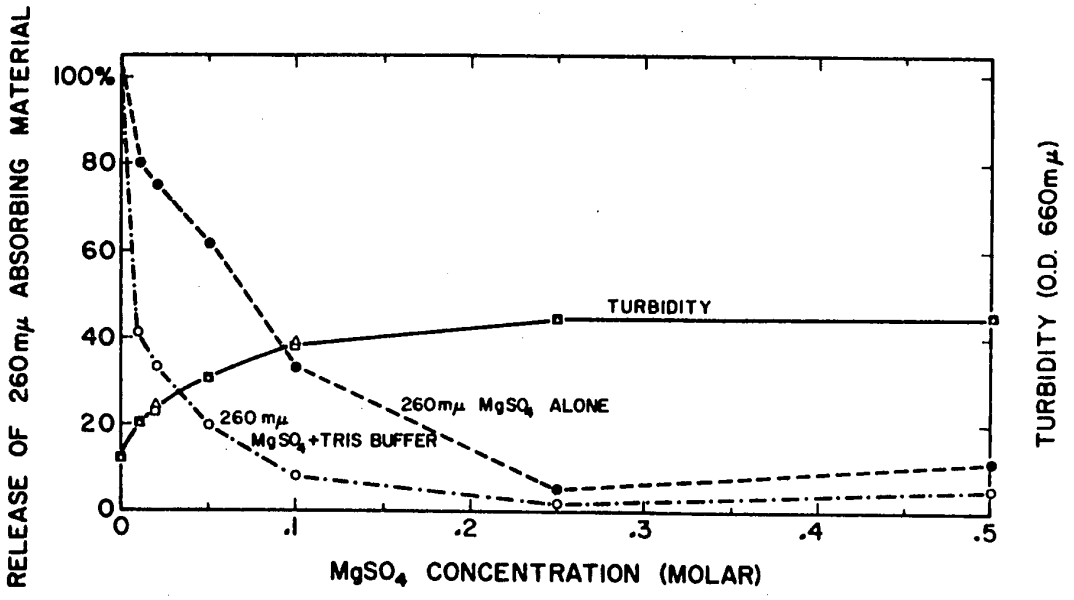
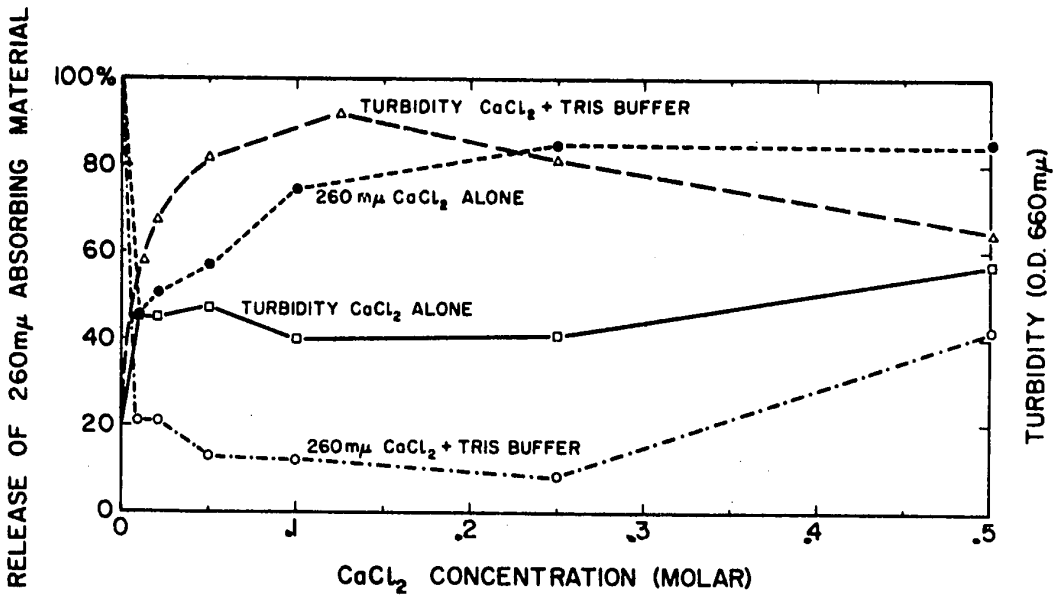

RELEASE OF 260mμ ABSORBING MATERIAL
100%
80
60
40
20
0
0
.1
.2
.3
.4
.5
TURBIDITY
260mμ MgSO4 ALONE
260 mμ
MgSO4+TRIS BUFFER
MgSO4 CONCENTRATION (MOLAR)
TURBIDITY (O.D. 660mμ)

FIGURE 17

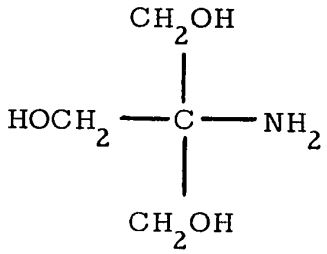
Effect of Tris buffer on cells suspended in various concentrations of $CaCl_2$.


RELEASE OF 260mμ ABSORBING MATERIAL
100%
80
60
40
20
0
TURBIDITY CaCl₂ + TRIS BUFFER
260 mμ CaCl₂ ALONE
TURBIDITY CaCl₂ ALONE
260mμ CaCl₂ + TRIS BUFFER
0
.1
.2
.3
.4
.5
CaCl₂ CONCENTRATION (MOLAR)
TURBIDITY (O.D. 660mμ)

material increased proportionally in the supernatants. The sparing effect of Tris buffer could be detected at a concentration as low as 0.001 M Tris buffer in 0.1 M $CaCl_2$ cell suspensions (Fig. 18).

NRC-1004 cell suspensions in LiCl concentrations between 0.025 M and 0.5 M lysed to the same extent as when suspended in distilled water. When 0.01 M Tris buffer was added to the LiCl solutions there was an increase in the turbidities of the cell suspensions accompanied by a decrease in the leakage of 260 mμ absorbing material into the supernatants (Fig. 19). Finally, while cells suspended in concentrations up to 1.0 M of NaCl lysed to the same extent as those suspended in distilled water, the presence of 0.01 M Tris buffer significantly reduced the fall of turbidity and the release of 260 mμ absorbing material when the cells were suspended in 0.5 M NaCl, as shown in Table VII.

The possibility that the beneficial activity of Tris buffer might be due to the amino group was investigated by testing various amino acids (Table VIII). No effect was shown by ethanolamine or by



Tris buffer
tris(hydroxymethyl)aminomethane

any one of the amino acids tested (glutamic acid, lysine, proline,

FIGURE 18

Effect of various concentrations of Tris buffer on cells suspended in 0.1 M $CaCl_2$.

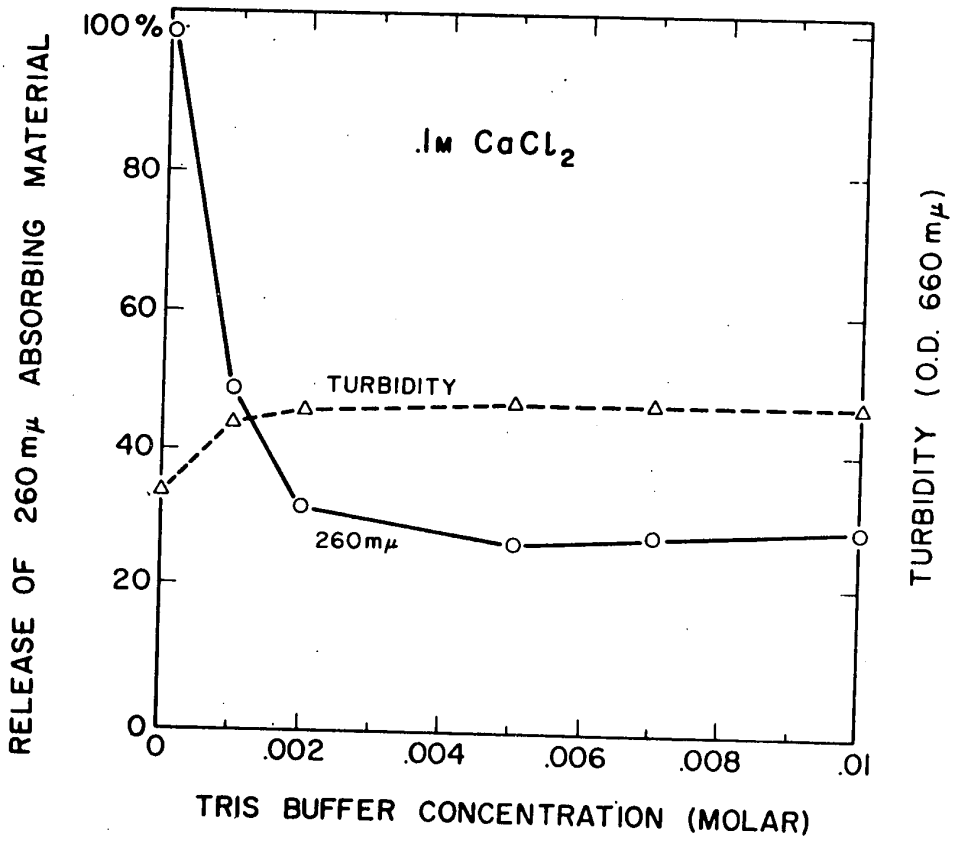

100%
80
60
40
20
0
.1M CaCl2
TURBIDITY
260mμ
RELEASE OF 260 mμ ABSORBING MATERIAL
TURBIDITY (O.D. 660 mμ)
0
.002
.004
.006
.008
.01
TRIS BUFFER CONCENTRATION (MOLAR)

FIGURE 19

Effect of Tris buffer (0.01 M) on the turbidity and 260 mμ absorbing material release of cells suspended in various concentrations of LiCl.

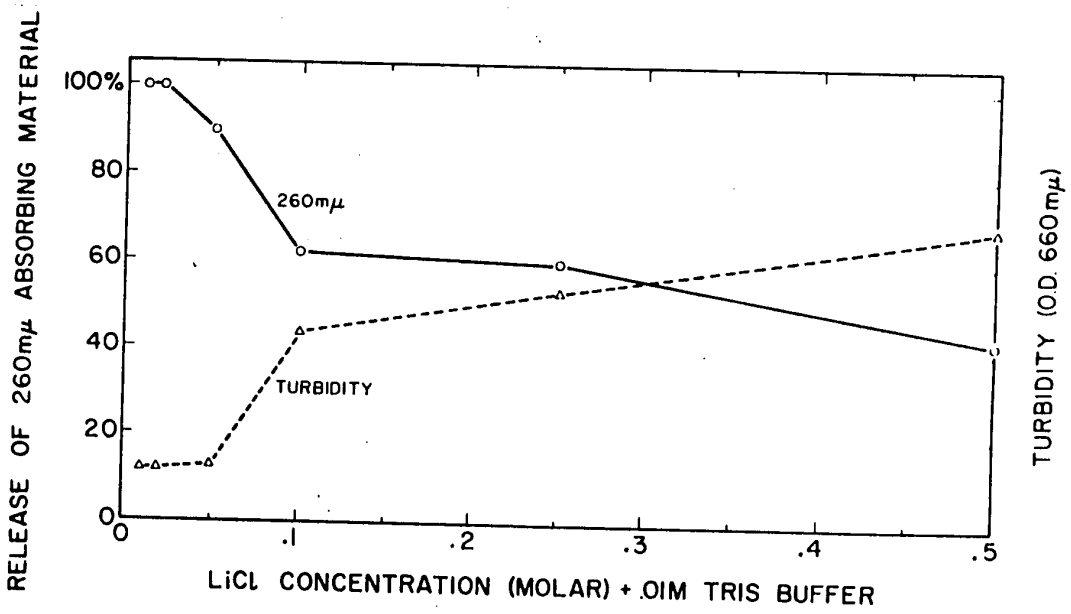

RELEASE OF 260mμ ABSORBING MATERIAL
100%
80
60
40
20
0
260mμ
TURBIDITY
0
.1
.2
.3
.4
.5
LiCl CONCENTRATION (MOLAR) + .01M TRIS BUFFER
TURBIDITY (O.D. 660mμ)

TABLE VII

The effect of Tris buffer and ashed Tris on lysis

Suspending medium	Per cent turbidity (O.D. 660 mμ)	Per cent release of 260 mμ absorbing material
H_2O	11	100
0.5 M NaCl	12	100
0.01 M Tris	11	100
0.5 M NaCl + 0.01 M Tris	71	31
0.5 M NaCl + 0.01 M ashed Tris	55	50
0.5 M NaCl + 0.01 M Tris + EDTA, 10^{-4} M	61	79
10^{-2} M	64	80
10^{-1} M	61	100

TABLE VIII

Effect of ethanolamine, Tris buffer, and various amino acids on the turbidity and release of 260 mμ absorbing material of cells in the presence of 0.5 M NaCl

Suspending medium	Molar concentration	Per cent turbidity	Per cent release of 260 mμ absorbing material
H_2O		10	100
Tris	0.1	65	38
	0.01	50	35
Ethanolamine	0.1	12	100
	0.01	11	
Glutamic acid	0.1	13	100
	0.01	11	100
Lysine	0.1	10	100
	0.01	10	100
Proline	0.1	11	100
	0.01	10	100
Methionine	0.1	10	100
	0.01	9	100
Arginine	0.1	11	100
	0.01	10	100
Cysteine	0.1	12	100
	0.01	10	100
Glycine	0.1	10	100
	0.01	10	100
Histidine	0.1	10	100
	0.01	10	100
PSM		100	8

methionine, arginine, cysteine, glycine, and histidine) at concentrations of 0.01 M and 0.1 M. The possibility that the presence of trace amounts of impurities in the commercially available product might be responsible for the beneficial activity of Tris buffer was then checked.

Evidence for the second possibility was provided by the utilization of ashed Tris solution instead of the normal buffer solution. The following procedure was followed: 1.26% of Sigma Tris buffer was ashed in the presence of nitric acid (reagent grade) for 24 hr at 600°C and resuspended in 10 ml of glass-distilled water. The final concentration of Tris in all suspensions was 0.01 M. Results in Table VII show that solutions of ashed Tris had almost the same effect as the buffer solution normally used.

Following this, spectographic analysis of the commercial sample used showed that it contained traces of several heavy metals. From the content of ashed Tris buffer it was calculated that 0.01 M Tris contained at least:

2.8×10^{-6} M B

1.2×10^{-5} M Mg

5×10^{-6} M Si

5×10^{-7} M Fe

1×10^{-6} M Al

4×10^{-6} M Ca

2×10^{-7} M Cu

The effects of Tris buffer could be attributed to the heavy metal ions that contaminate it. In support of this, it was found that adding EDTA removed the protective action of Tris and increased lysis of cells suspended in Tris plus 0.5 M NaCl (Table VIII).

Postgate and Hunter (160) found earlier that certain commercial samples of Tris buffer contained substance(s) toxic to bacterial growth, probably copper, whose effect could be neutralized by EDTA.

Effect of Sugars

From the previous experiments it seems that osmotic pressure plays only a secondary role in maintaining the integrity of NRC-1004 cells. For further clarification of the role of osmotic pressure, sugars were tested instead of salts. As we can see in Table IX, D-glucose in concentrations up to 3.5 M was unable to prevent lysis. Sucrose at an osmoequivalent concentration to 0.5 M NaCl, in the presence of 0.1 M $MgSO_4$, had a rather detrimental effect on NRC-1004 cells. Cells suspended in 0.1 M $MgSO_4$ alone showed a higher turbidity and a lower amount of 260 mμ absorbing material release than when suspended in 0.1 M $MgSO_4$ + 1.0 M sucrose.

Effect of Spermine

The polyamine, spermine, helps preserve marine bacteria and protoplasts of other bacteria against lysis in hypotonic media (77). Adding from 10^{-6} to 10^{-1} spermine to cells in 0.5 M NaCl did not

TABLE IX

Effect of D-glucose and sucrose

on NRC-1004 cell suspensions

Suspending medium	Molar concentration	Per cent turbidity	Per cent release of 260 mμ absorbing material
PSM	-	100	8
H_2O	-	10	100
D-glucose	0.01	42	100
"	0.05	44	100
"	0.1	43	100
"	0.2	45	100
"	0.3	52	100
"	0.5	55	100
"	1.0	65	100
"	1.5	69	100
"	2.0	70	100
"	2.5	70	100
"	3.0	40	100
"	3.5	40	100
$MgSO_4$	0.1	72	19
$MgSO_4$ + NaCl	0.1 0.5	100	6
$MgSO_4$ + sucrose	0.1 1.0	40	28

prevent lysis, as measured either by the fall of turbidity or by the release of UV-absorbing substances. It was previously shown that spermine did not prevent heat-induced lysis in these bacteria (80) (see Table X).

Microscopic Studies of Changes in Different Ionic Environments

When cells were placed in distilled water the normal rod shape (Fig. 20a) quickly became swollen and pale, as observed under phase contrast (Fig. 20b). After 10 min the cells broke down, forming more or less amorphous aggregates (Fig. 20c). Dark intracellular bodies appeared in the swollen cells and many had two or three dots located on the periphery. Such dots could also be seen in the cell debris (Fig. 20c). In PSM at 25°C lysing cells slowly became swollen, then their size decreased below that of intact cells (Fig. 20d, e, f).

Cells suspended in all concentrations of divalent salts that prevented lysis had normal or near-normal shapes. Thus, in 0.005 M $ZnCl_2$ or $CuSO_4$, the size and shape of cells were the same as in sea water or PSM. In 0.005 M $NiCl_2$ and $CaCl_2$, cells became rounder than normal but remained phase-dense, while in this concentration of $MnCl_2$ cells were globular and pale. These microscopic observations correspond to findings made by measurements of turbidity and release of intracellular substances (Fig. 14). Together, these observations permit classification of cations in order of decreasing ability to prevent lysis as $Cu^{++} > Zn^{++} > Ni^{++} > Ca^{++} > Mn^{++} > Mg^{++}$.

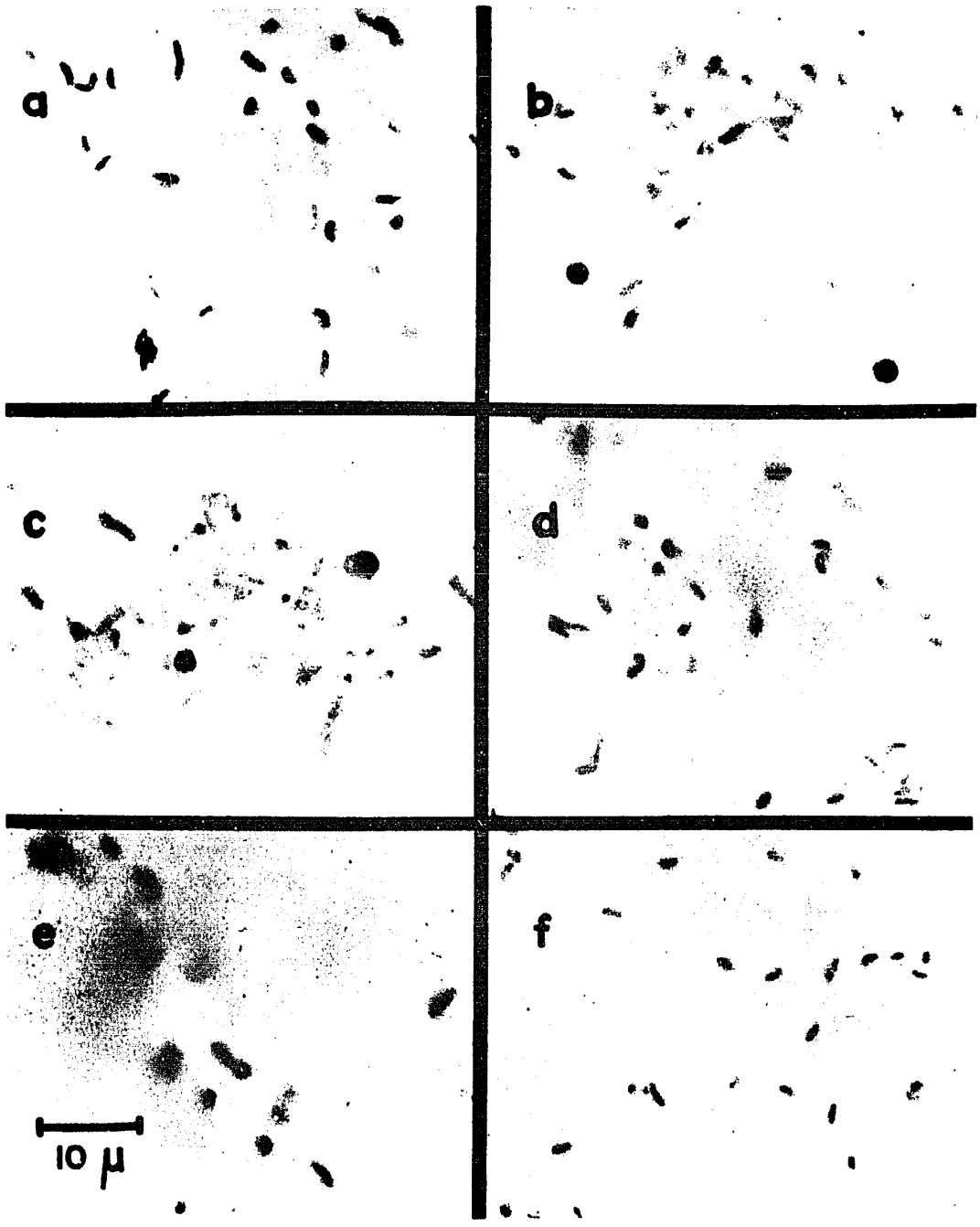
TABLE X

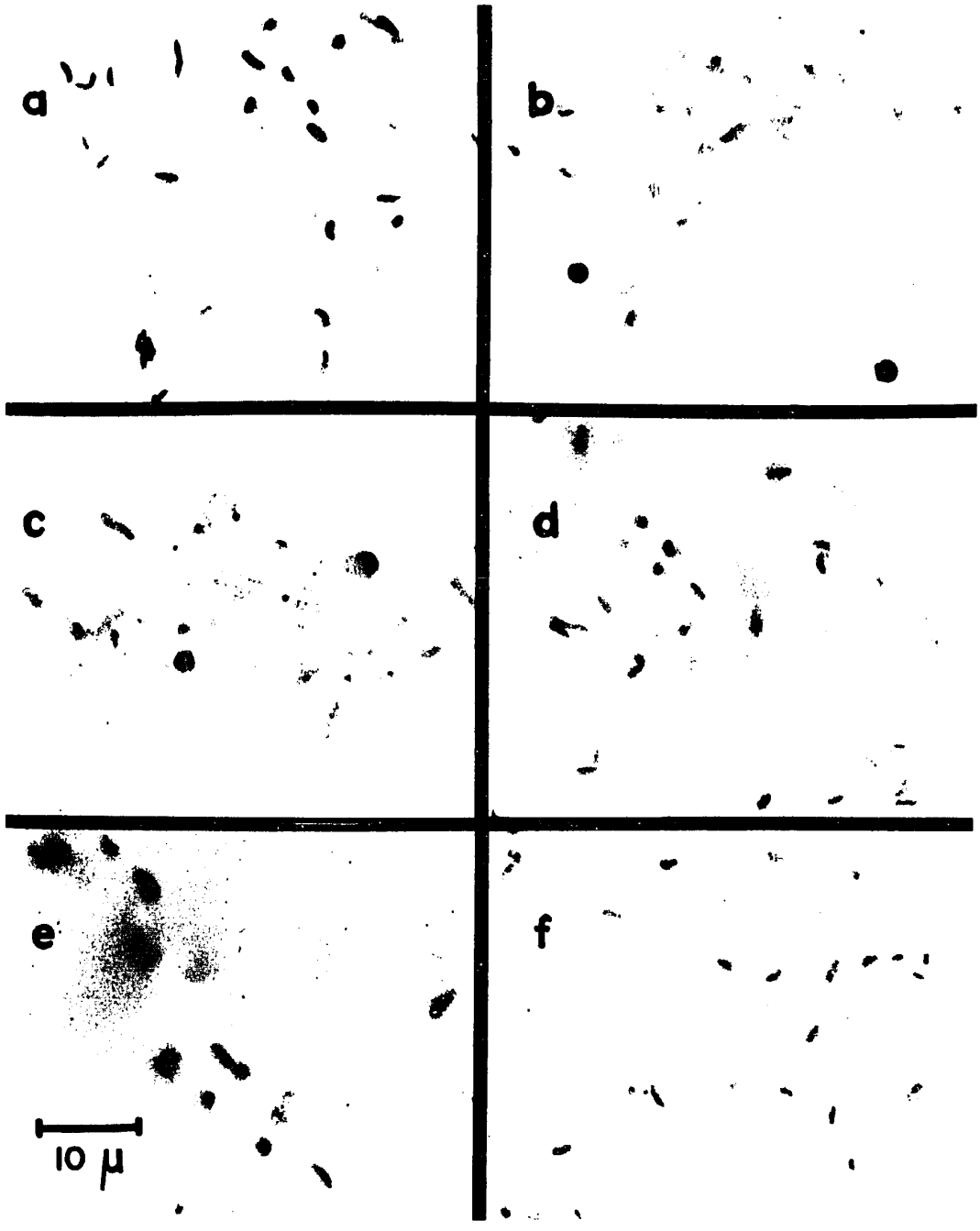
Effects of various concentrations of spermine on the turbidity and 260 mμ absorbing material release of NRC-1004 in the presence of 0.5 M NaCl

Molar concentration of spermine	Per cent turbidity	Per cent release of 260 mμ absorbing material
10^{-1}	23	50
10^{-2}	13	62
10^{-3}	7	71
10^{-4}	14	80
10^{-5}	14	85
10^{-6}	13	82
H_2O	11	100
PSM	100	9

FIGURE 20

Morphological effects of exposure to distilled water at 0°C and to salts (PSM) at 25°C. a) Normal cells (PSM, 0°C); b) Distilled water, 0°C, 3 min; c) Distilled water, 0°C, 10 min; d) PSM, 25°C, 20 min; e) PSM, 25°C, 75 min; f) PSM, 25°C, 120 min.


a
b
c
d
e
f
10 μ


a
b
c
d
e
f
10 μ

Effects of Salts on Bacterial Growth

In order to evaluate further the physiological effects of the ionic environment, the abilities of the various salts studied here to support growth were examined. In these experiments, 0.4% tryptone, whose ionic content is unknown, was used as a nutrient source. Table XI shows results after 96 hr incubation at 5°C. At that time turbidities were maximal, O.D. at 660 mμ of sea water-tryptone media were 0.800. Nevertheless, with the exception of the sea water-tryptone media, all cultures were kept shaking for a total of 15 days. Turbidities of the magnesium-sodium-CaFeK-tryptone media declined gradually after 96 hr. No growth was observed either turbidimetrically or microscopically in any one of the other media at the end of this experiment. The need for small amounts of Ca, Fe and K for the growth of NRC-1004 had been previously observed by Dr. I.J. McDonald and A.K. Chambers of the Division of Biosciences, National Research Council of Canada. The importance of magnesium for the growth of gram positive and gram negative bacteria has been studied by Webb (222), who showed that in the complete absence of magnesium bacteria did not grow. Gram positive bacteria (Cl. welchii, Micrococcus T38, B. subtilis, L. plantarum, and Strep. faecalis) had a much greater requirement for magnesium than gram negative bacteria (Chromobact. violaceum, Bact. cloacae, Bact. lactis aerogenes, Ps. aeruginosa, and Ps. prunicola). In a deficiency or excess of magnesium, rod-shaped bacteria became filamentous.

TABLE XI

Effects of various salts on growth of NRC-1004

Divalent ion (0.1 M)	Monovalent ion (0.5 M)	CaFeK	Per cent growth at 96 hr at 5 °C
Sea water	-	-	100
$MgCl_2$	-	+	0
$MgCl_2$	NaCl	+	53
$MgSO_4$	-	+	0
$MgSO_4$	NaCl	+	67
$MnCl_2$	NaCl	+	3
$ZnCl_2$	NaCl	+	5
$CuSO_4$	NaCl	+	0
$CaCl_2$	NaCl	+	6
$MgCl_2$	KCl	+	0
$MgCl_2$	NH_4Cl	+	0
$MgCl_2$	LiCl	+	0
$MgSO_4$	NaCl	-	0

All media contained 0.4% Bactotryptone (Difco).

CaFeK = CaCl, 0.00002 M; $FeNH_4(SO_4)_2$, 0.0004% (w/v); KCl, 0.005 M. O.D. at 660 mμ of sea water-tryptone media at 96 hr, 0.800.

Of all salts added, only 0.5 M NaCl plus 0.1 M $MgCl_2$ or $MgSO_4$ were able to support growth, and this only if small amounts of other salts (0.005 M KCl, 0.00002 M $CaCl_2$, and 0.0004% (w/v) $Fe(NH_4)(SO_4)_2$ were present. Other salts tested included 0.1 M $MgCl_2$, 0.1 M $MgSO_4$, 0.1 M $MgCl_2$ plus one of the following: 0.5 M KCl, 0.5 M LiCl, 0.5 M NH_4Cl, and 0.25 M NaCl + 0.25 M KCl. In addition, 0.1 M $ZnCl_2$, $MnCl_2$, $CuSO_4$, and $CaCl_2$ were tested in the presence of 0.5 M NaCl. Tryptone and the traces of other ions described above were added to each combination. None was able to support growth of the red psychrophile (Table XI).

Release and Destruction of Structural Components during Lysis and Mechanical Cell Disruption

It has been shown that during lysis in sea water at 25 °C or higher some hexosamine was released and the lipid-soluble phosphorus fraction decreased (80). Some of the changes in the lipid phosphorus and hexosamine fractions accompanying lysis of these cells in distilled water were studied (as possible indices of cytoplasmic membrane and cell wall material respectively (106, 181)) and compared with those accompanying lysis in salts or in sea water at 25 °C or higher. In the first experiments, lysed cells were removed by centrifuging for 10 min at 8000 x g and lipid phosphorus and hexosamine were determined in both the cell pellet and the supernatant. These experiments (Tables XII and XIII) showed that there was a fundamental difference between temperature-induced and

TABLE XII

Effect of lysis on lipid phosphorus distribution

		Per cent lipid phosphorus in					
		Pellet		Supernatant		Total	
Treatment	Exp.	1	2	1	2	1	2
Sea water 0°C, 2 hr		79	68	11	18	90	86
25°C, 2 hr		36	27	12	9	48	36
Distilled water 0°C, 2 hr		33	36	67	64	100	100

Turbidity (O.C. 660 mμ) of cell suspensions: Exp. 1, 0.350; Exp. 2, 0.750. 100% lipid phosphorus (μg/ml): Exp. 1, 4; Exp. 2, 12.

After incubation as shown, suspensions were centrifuged at 8000 x g for 10 min and lipid phosphorus determined in sedimented and supernatant fractions.

TABLE XIII

Effect of lysis on hexosamine distribution

		Per cent hexosamine in					
		Pellet		Supernatant		Total	
Treatment	Exp.	1	2	1	2	1	2
Sea water 0°C, 2 hr		87	90	13	10	100	100
25°C, 2 hr		54	59	32	28	85	87
Distilled water 0°C, 2 hr		19	15	81	85	100	100

Turbidity (O.D. 660 mμ): Exp. 1 and 2, 0.750. 100% hexosamine, 7 μg/ml in both experiments. Procedure as in Table XII.

distilled-water lysis, a difference already inferred from the electrophoretic experiments. In temperature-induced lysis, more than half the lipid phosphorus could not be recovered in the pellet and supernatant fractions, which confirms the observations made by Hagen et al. (80). These authors had found by chromatographic examination that after prolonged incubation at 25°C, the two main phospholipids of NRC-1004, phosphatidyl ethanolamine and phosphatidyl glycerol, disappear*. This was thought to result from the action of a cytoplasmic membrane-bound phosphatidase. In distilled water, about half the lipid phosphorus was released from the cell, but was recovered from the supernatant. The amounts of lipid phosphorus remaining cell bound after 2 hr in sea water at 25°C and after 2 hr in distilled water at 0°C were approximately the same. Separate experiments showed that the effects of lysis in salts (PSM) and in sea water at 25°C were similar.

In sea water at 25°C about 15% of the total hexosamine was destroyed in 2 hr and about 30% was released from the cell (Table XIII). By contrast, in distilled water at 0°C no hexosamine was destroyed, but more than 80% was released from the cell in 2 hr.

When cells were mechanically broken in cold salts or sea water, their lipid phosphorus and hexosamine were solubilized to the same extent as when they were suspended in distilled water. In each case, about half the cell's lipid phosphorus was contained in

*Kushner, D.J., Personal communication, showed that at 25°C the disappearance of lipid phosphorus is accompanied by a proportional increase in water-soluble organic-P and inorganic-P in the supernatants.

particles that could be sedimented at 100,000 x g for 1 hr, but not at 15,000 x g for 30 min (Table XIV). A similar pattern of hexosamine sedimentation was also found in each preparation (Table XV).

The lipid composition of the fractions of water lysed cells that sedimented or that remained in the supernatant after 30 min at 15,000 x g were the same, chromatographically, and did not differ from the composition of intact cells previously described by Kates and Hagen (103). From the R_f values and staining behaviour, these likely consisted mostly of phosphatidyl ethanolamine, with minor amounts of phosphatidyl glycerol and polyglycerol phosphatides, and possibly neutral lipids containing free fatty acids.

The changes occurring when mechanically prepared envelopes and cells lysed in cold distilled water were suspended in sea water or distilled water at different temperatures were also studied. Both preparations lost little turbidity when suspended in distilled water at 0°C, 25°C, or 38°C, even for as long as 6 hr. In sea water, turbidity fell at the higher temperatures and lipid phosphorus was destroyed. There was a greater destruction of lipid phosphorus in both preparations in sea water than in distilled water, but hexosamine was not destroyed. In water lysed cells suspended in water at 25°C or 38°C the lipid phosphorus content fell but turbidity did not (Table XVI). These observations suggest that salts contribute to but are not essential for the activity of the cytoplasmic membrane bound phosphatidase responsible for the breakdown of the lipid phosphates, whereas other enzymes that require salt for activity may be responsible for the fall in turbidity.

TABLE XIV

Sedimentation of lipid phosphorus compounds

from water lysed and mechanically broken cells

Centrifugal force and time	Per cent lipid phosphorus in supernatant			
	Distilled water lysed cells		Mechanically broken cells	
Exp.	1	2	1	2
None	100	100	-	-
2,000 x g, 10 min	-	-	100	100
15,000 x g, 30 min	54	54	68	56
30,000 x g, 30 min	47	47	40	43
100,000 x g, 60 min	11	13	11	11

Lysis in distilled water took place at 0°C for 2 hr. The suspensions of water lysed cells contained 3 μg/ml lipid phosphorus (Exp. 1) and 3.5 μg/ml lipid phosphorus (Exp. 2). After breaking cells mechanically in PSM, intact cells were centrifuged down at 2,000 x g for 10 min and the lipid phosphorus content of the supernatant (4 μg/ml in Exp. 1; 7 μg/ml in Exp. 2) taken as 100%.

TABLE XV

Sedimentation of hexosamine

from water lysed and mechanically broken cells

Centrifugal force and time	Exp.	Per cent hexosamine in supernatant				
		Distilled water lysed cells		Mechanically broken cells		
		1	2	3	4	5
None		100	100	-	-	-
2,000 x g, 10 min		-	-	100	100	100
15,000 x g, 30 min		56	60	51	36	82
30,000 x g, 30 min		45	50	-	-	76
100,000 x g, 60 min		19	25	-	-	20

Method as in Table XIV. 100% hexosamine in distilled water lysed cells was 9 μg/ml and in mechanically broken cells 62 and 52 μg/ml, Exp. 1 and 2; 62 μg/ml, Exp. 3 and 5; 14 μg/ml, Exp. 4.

In Exp. 3 cells were broken poorly by shaking, and were then treated sonically (Braunson Sonifier) for three 5-sec cycles.

TABLE XVI

Effect of temperature and salts on the turbidity and lipid phosphorus content of mechanically broken and water lysed cells

Material	Suspending solution	Treatment	Per cent turbidity	Per cent lipid phosphorus
Water lysed cells	Sea water	0°C, 0 hr	100	100
		0°C, 6 hr	95	77
		25°C, 6 hr	62	57
		38°C, 6 hr	57	55
	Distilled water	0°C, 0 hr	100	100
		0°C, 6 hr	99	89
		25°C, 6 hr	100	65
		38°C, 6 hr	95	66
Mechanically prepared envelopes	Sea water	0°C, 0 hr	100	100
		0°C, 6 hr	91	71
		25°C, 6 hr	76	52
		38°C, 6 hr	65	53
	Distilled water	0°C, 0 hr	100	100
		0°C, 6 hr	96	88
		25°C, 6 hr	87	84
		38°C, 6 hr	92	81

Water lysed cells were prepared by 2 hr exposure to distilled water at 0°C, and washed twice in distilled water before being resuspended in distilled water or sea water. 100% turbidity = O.D. 0.7-0.9 for water lysed cells, 0.3-1.1 for envelopes; 100% lipid phosphorus = 11-16 μg/ml for water lysed cells, 3-13 μg/ml for envelopes. Most figures represent the mean of at least two experiments.

Effect of EDTA on Water Lysed Cells

The experiments just cited show that suspensions of cells lysed in distilled water and washed retained their turbidity in water for several hours. However, if 10^{-2} M or higher EDTA was added, turbidity fell rapidly (Fig. 21); this suggests that residual ions bound to water lysed cells are important in maintaining the structure of the remaining envelopes.

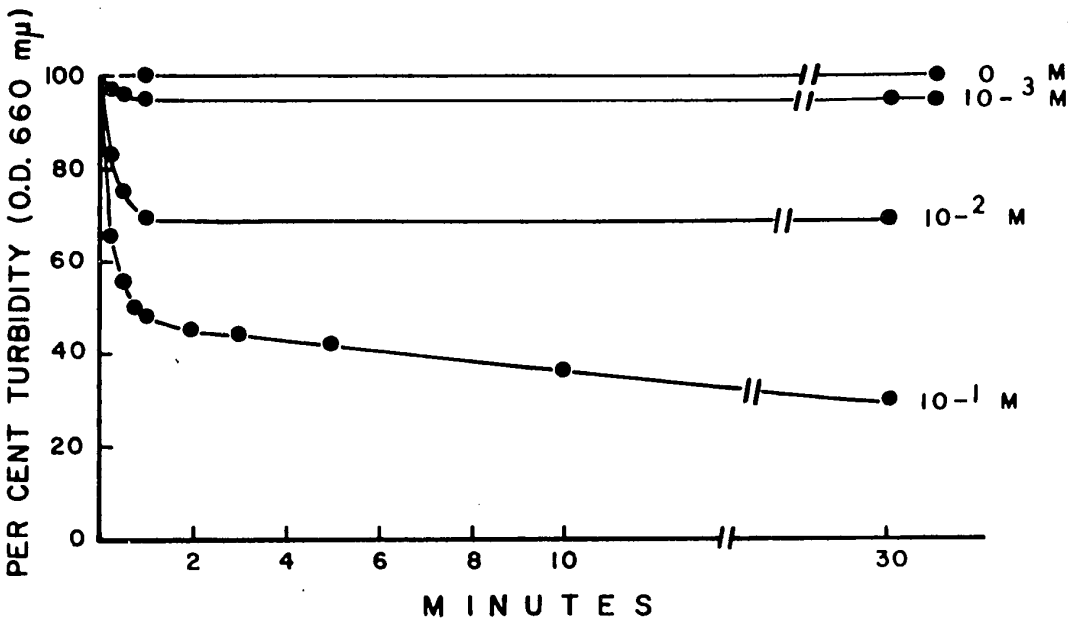
DISCUSSION

Several marine bacteria require salts for stability and exhibit some specificity in their response to ions. In general, NaCl and LiCl are more effective than KCl or NH_4Cl, and divalent cations are more effective than monovalent cations (125-127). MacLeod and Matula found that five marine bacteria, which differed considerably in their susceptibility to lysis when the salt concentration of the suspending medium was lowered, responded specifically to mono- and divalent cations, and could also be stabilized by sucrose and glucose, though not by glycerol or ethanol (127). Such studies demonstrate the role of certain ions in maintaining cell stability as well as in affecting transport and other metabolic processes. The effect of solutes cannot be ascribed solely to their osmotic action, and this is also true for moderately and extremely halophilic bacteria (22, 125).

NRC-1004 is very exacting in its environmental requirements. It lyses if the temperature rises above 20°C or if the pH falls

FIGURE 21

Effect of EDTA on turbidity of distilled water lysed cells. Cells were exposed to distilled water at 0°C for 2 hr, then washed and resuspended in the presence or absence of EDTA. 100% turbidity = that of PSM or sea water. The figures by each curve show the EDTA concentration used.


PER CENT TURBIDITY (O.D. 660 mμ)
100
80
60
40
20
0
2
4
6
8
10
30
MINUTES
0 M
10-3 M
10-2 M
10-1 M

to 5.0 or below. It also has relatively rigid ionic requirements for stability. Thus, monovalent salts alone, even 1.5 M NaCl, the most effective monovalent salt, cannot preserve cell structure. A divalent cation is essential. A mixture of 0.1 M $MgSO_4$ or $MgCl_2$ and 0.5 M NaCl can prevent lysis and support growth if traces of other ions are added. In the presence of Mg^{++}, LiCl can also support structure, though not as well as NaCl, but KCl and other monovalent salts do not support structure, and CsCl and NH_4Cl are lytic; sucrose and glucose are also ineffective. Divalent cations alone can also preserve cell integrity; Cu^{++} and Zn^{++} are able to do so in very low concentrations indeed.

The ability of most divalent cations to maintain cell structure is directly related to their ability to form complexes with organic ligands, which follows the order $Mg^{++} < Mn^{++} < Ni^{++} < Cu^{++} < Zn^{++}$ (94). Ca^{++} is more effective than Mg^{++} or Mn^{++}, though it forms complexes less well. A lipid complex with Mg^{++} has been extracted from the extreme halophile, Halobacterium cutirubrum (165), and there is other indirect evidence that Mg-lipid complexes are important in preserving membrane structure of this organism (116). The divalent cations Ca^{++}, Mg^{++}, and Zn^{++} are components of the cell wall of Pseudomonas aeruginosa and play a part in maintaining its structure (9,58). Recently, Ghosh and Carroll (71) have demonstrated that Mg^{++} must be present to maintain the chemical and physical integrity of the

membrane of Listeria monocytogenes. Butler et al. (36) have shown that cell membranes of Micrococcus lysodeikticus, disaggregated into sub-units by treatment with detergent (sodium lauryl sulfate), could be reaggregated into membranous structures in the presence of magnesium. However, the nature of the complexes formed by the other divalent ions studied here is still unknown. One striking effect of Zn^{++} and Cu^{++} is that these ions, in concentrations much too low to have any significant osmotic effect, are able to prevent the distortion that occurs when cells are transferred from sea water to distilled water. This may well indicate that distension in distilled water is not primarily due to osmotic pressure but rather to the changed ionic environment, an interpretation supported by the fact that lowering the pH of a suspension of these bacteria in salts also leads to distension.

The importance of ions in maintaining envelope structure is also indicated by the fact that EDTA can break down the residual structures remaining after distilled water lysis. Several other workers (75, 166) have used EDTA to show the importance of ions in maintaining the structure of bacterial cells and envelopes. Repaske (167) states that EDTA promotes lysis of gram negative bacteria by forming chelates with the heavy metals that presumably help in maintaining the envelope together. Thus EDTA would weaken the envelope and make it more sensitive to the action of lysozyme.

Earlier studies of the chemical changes taking place in

cells during temperature induced lysis were extended to include those accompanying lysis in distilled water. Temperature induced lysis is accompanied by a breakdown, presumably enzymic, of phospholipids, among them phosphatidyl ethanolamine, and a release from the cell of up to 1/3 the total hexosamine with some accompanying hexosamine destruction (80). In distilled water at 0°C, however, more than half the lipid phosphorus and about 80% of the hexosamine are released from the cell but are not destroyed. Chromatographically, the solubilized and sedimented lipids have the same composition. The chemical nature of the corresponding hexosamine fractions is still unknown. Evidence is also presented that the destruction of lipid phosphorus at higher temperatures is increased by the presence of salt and that other lytic events, manifested as a fall of envelope turbidity, require salt (103).

It is instructive to compare the results presented here with those obtained by Buckmire and MacLeod, using their marine pseudomonad (31). These workers prepared envelopes in 0.5 M NaCl (a concentration in which cells were stable) and found that in 0.01 M NaCl these envelopes lost turbidity and released non-dialyzable material containing hexosamine and diaminopimelic acid. In contrast, envelope suspensions of the red psychrophile NRC-1004, prepared in PSM, did not lose turbidity on dilution with water. It is not known if they lost hexosamine, lipid phosphorus, or other material in water; however, it is unlikely, first because such a loss would probably be

accompanied by a fall of turbidity, and second because breaking cells mechanically in PSM led to as great a loss of these compounds as did lysis in water.

Lipid phosphorus is considered an index of membrane material in gram positive bacteria (106, 115). In Streptococcus faecalis, for example, cytoplasmic membranes, containing virtually all of the cell's lipid phosphorus, sedimented in 30 min at 25,000 x g (106, 194). Much of the lipid phosphorus of the red psychrophile NRC-1004, however, is bound to particles that do not sediment under these conditions. It is unlikely that these particles exist as such in the cytoplasm and more probable that they are derived from a membrane system so fragile that it breaks into small particles under even such a comparatively mild treatment of the cell as mechanical disruption.

SUMMARY AND CONCLUSION

It has been shown that NRC-1004 is a microorganism of great sensitivity to temperature, pH, and ionic strength changes in its environment. This has been made evident from its readiness to undergo lysis at temperatures generally considered moderate (20°C or above). It also lysed when the pH of the suspending solution was below 6.0. Finally, it required the presence of divalent ions in its environment in order to maintain its cellular integrity.

When NRC-1004 cells were lysed either by temperatures above 20°C, pH below 6.0, or suspension in distilled water, under phase contrast microscopy these bacteria changed from rods to pale globules with gradually fading contrast to their environment. Finally only small particles, apparently remains of the cell envelope, were visible. Optically it was not possible to distinguish between the particles resulting from distilled water or temperature lysed cells and envelopes prepared by mechanical disruption of the cells.

More information on lysis of NRC-1004 cells was provided by the observation of the cells' behaviour in an electrical field at various pH values. Microelectrophoretically the behaviour of NRC-1004 intact cells was comparable to the behaviour of other gram negative and gram positive bacteria. All lysed cell populations were electrophoretically heterogeneous with "fast" and "slow" cells present. After repeated washing it was possible to distinguish between distilled water, acid

lysed, and temperature lysed cells. The mobilities of distilled water and pH lysed cells returned to the values obtained for intact cells and cell populations became homogeneous. The increase in negative mobility of distilled water and acidic pH lysed cells was considered to be caused by the adsorption on the surface of the cells of intracellular material which was removed by subsequent washing.

Temperature lysed cells retained their high negative mobility after several washings, an indication of the irreversible change in the charge of the external surface of the cells. Temperature treatment did not produce any change in the electrophoretic behaviour of the distilled water lysed cells. A change in electrophoretic behaviour such as the higher negative mobility of temperature lysed cells compared to the mobility of intact cells was interpreted as evidence for a change in the chemical structure of NRC-1004 cell envelope.

Electrophoresis only provides information on the net electrical charge on the surface of a particle. Two particles with the same net surface charge, hence showing the same microelectrophoretic behaviour, may not necessarily have identical surfaces. The high ionic strength of the suspending solution required to maintain the integrity of NRC-1004 cells prevented identification of the nature of the chemical groups responsible for the charge on the cell surface. Another limitation of the microelectrophoretic technique due to the presence of ions in the suspending solution was the possibility that the cell mobility

could be caused by the adsorption of ions onto the cell surface. Adsorbed ions may also mask built-in charged groups present on the cell surface.

The importance of hexosamine and lipid phosphorus as constituents of the bacterial cell wall and cytoplasmic membrane, respectively, have been reviewed. The fundamental difference between temperature induced lysis and distilled water lysis inferred from the distinct microelectrophoretic behaviour of temperature and distilled water lysed cells was confirmed by determination of hexosamine and lipid phosphorus in centrifuged lysed cell pellets and supernatants. Hagen et al. (80) had reported phosphatidase activity in temperature treated NRC-1004 cells and mechanically prepared envelopes. In the present work these observations were confirmed and the action of enzymes in the degradation of the cell wall and the cytoplasmic membrane of temperature and distilled water lysed NRC-1004 cells was investigated.

Extensive destruction of the cytoplasmic membrane and, to a lesser extent, of the cell wall of NRC-1004 cells took place during temperature lysis. About half of the lipid phosphorus and 15% of the hexosamine was destroyed by temperature treatment in whole cells. Lipid phosphorus was also destroyed when distilled water lysed cells and mechanically prepared envelopes were exposed to 25 and 38°C. Enzymatic activity was enhanced by the presence of salts as 33% of the lipid phosphorus was destroyed in distilled water and 45% in sea water at 25 and 38°C.

Suspension of NRC-1004 cells in distilled water and mechanical disruption of cells caused disaggregation of cell wall and cytoplasmic membrane into very small particles. This was shown by sedimentation. Centrifugation at 100,000 x g for 60 min was required to sediment about 80% of the hexosamine and 90% of the lipid phosphorus from distilled water lysed cells and mechanically prepared envelopes. Less than half of both envelope constituents was sedimented at 30,000 x g for 30 min in distilled water lysed cells and mechanically prepared envelopes.

In 1963 Kolb et al. (106) proposed the use of lipid phosphorus as an index of the cytoplasmic membrane in gram positive bacteria. In gram negative bacteria there is evidence that the phospholipids are closely associated with the membrane (102, 182). Characteristically the lipid phosphorus particles from NRC-1004 preparations have been shown by sedimentation technique to be much smaller than those from other microorganisms. The suggestion that these particles form part of a delicate membrane system which may include mesosomes presents an interesting problem to be solved by electron microscopy.

Lysis in a bacterial population can be assessed from the amounts of intracellular material released into the suspending solution. Salton (182) proposed to measure the leakage of ultraviolet absorbing material from lysing cells, and this method has gained general acceptance. Under optical microscopic observation NRC-1004

cells suspended in salt solutions at pH values below 6.0 were seen to lyse. In these lysed cell suspensions, turbidities did not fall nor was there a measurable increase in the ultraviolet (260 mμ) absorbing material released. The discrepancy in the results from the different techniques used in the assessment of lysis were in all likelihood caused by the precipitation at low pH of ultraviolet absorbing material along with other cellular constituents which accounted for the high turbidity values. Ultraviolet absorbing material failed to appear in the supernatant as it was removed by centrifugation with the sedimented pellet. Acidification followed by centrifugation of distilled water lysed cell supernatants confirmed this hypothesis. The measurement of inorganic phosphate released by lysed cells previously used by Kushner et al. (115) on their investigations on Halobacterium cutirubrum was successfully applied in the present work.

NRC-1004 cells were suspended in various concentrations of mono- and divalent ions to study systematically the effectiveness of these ions in maintaining cellular integrity. The cells lysed in up to 3.0 M concentration of NaCl, LiCl, KCl, NH_4Cl, RbCl, and CsCl. The presence of 0.01 M Tris (tris(hydroxymethyl)aminomethane) buffer in the salt solution reduced the leakage of intracellular material. Turbidities of cell suspensions in monovalent salt solutions were higher in the presence of Tris buffer than in its absence. Glutamic acid, lysine, proline, methionine, arginine, cysteine, glycine, histidine, and

ethanolamine were tested at 0.1 M and 0.01 M concentrations in an attempt to relate the sparing effect of Tris buffer to an amino group, but the presence of these amino acids did not prevent lysis of NRC-1004 cells suspended in monovalent salt solutions.

The polyamine spermine which had a stabilizing effect on other bacteria and protoplasts (77) was equally ineffective. When cells were suspended in a solution of ashed Tris and NaCl the results of turbidimetric and intracellular material leakage measurements were very similar to those of cells suspended in Tris buffer-NaCl. By spectrographic analysis trace amounts of heavy metals were found in Tris buffer. Their implication in the sparing effect of Tris was demonstrated through the addition of the chelating agent EDTA to cell suspensions in Tris buffer and NaCl. This caused the cells to release the same amounts of intracellular material as when suspended in distilled water or NaCl alone. The turbidities of distilled water lysed cell suspensions fell in the presence of EDTA, an indication of the involvement of ions in the structure of NRC-1004 cell envelope.

Divalent salts alone, some in very low concentrations, could maintain cell integrity. Cupric ions were the most effective followed in decreasing order of effectiveness by Zn^{++}, Ni^{++}, Ca^{++}, Mn^{++} and Mg^{++}. Irving and Williams (94) had shown that these divalent cations followed the same scale in their ability to form organic ligands. The concentration of divalent ions which could prevent lysis

were too small to substantiate the belief that the effect of these ions on the cell envelope was through osmotic pressure.

This direct evidence for the role played by divalent cations in maintaining the structure of NRC-1004 cell envelope is of great relevance in the light of the observations made by Kushner and Onishi (116) and McClare (138) on the envelope of Halobacterium cutirubrum and Asbell and Eagon (9) and Eagon et al. (58) on the envelope of Pseudomonas aeruginosa.

At a concentration of 0.5 M, NaCl and to a lesser degree LiCl or RbCl maintained cellular integrity in the presence of 0.1 M $MgSO_4$. KCl had no effect on leakage of intracellular material or turbidity of NRC-1004 cell suspensions in 0.1 M $MgSO_4$. CsCl and NH_4Cl could not prevent cell lysis in the presence of 0.1 M $MgSO_4$.

The diversity and the many apparently conflicting results from electron microscopic, chemical and physiological studies on membrane preparations have induced numerous investigators to contest the generalization of the "unit membrane" concept.

The preponderant role played by divalent cations in the cell envelope of different bacteria such as the extreme halophile Halobacterium cutirubrum, the mesophilic human pathogen Pseudomonas aeruginosa, and the marine obligately psychrophile NRC-1004 provides another clue to the still mysterious structures of living membranes.

Studies on ionic requirements for growth of NRC-1004

showed that tryptone broth had to be supplemented by sodium, magnesium, and traces of calcium, potassium, and iron. In this respect NRC-1004 needs were characteristic of marine bacteria (32, 45, 125, 162, 205, 217). It has been pointed out in the literature (125) that marine bacteria, in addition to their salts requirement, generally showed a marked preference for amino acids as sources of carbon and nitrogen. Further investigations on the precise nutritional requirements of NRC-1004 should be of great help in the knowledge of marine bacteria. Other workers observed that active transport in a marine bacterium (125) and temperature growth characteristics of the psychrophile Vibrio marinus (205) were greatly affected by ions. The results of the work presented here, by stressing the role of ions in maintaining the integrity of NRC-1004 cell envelope and showing the effect of ions on the activity of envelope degrading enzymes in this microorganism, suggest that ions are possible determining factors in the physiology of NRC-1004.

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