Reprints of publications arising from this thesis

## Abstract

Three halococci, Sarcina morrhuae, S. littoralis and S. gigantea, obligate extremely halophilic bacteria, possessing a thick "cell wall" were shown to lack significant amounts of muramic acid and, therefore, peptidoglycan. In this respect and others they are similar to the fragile halobacteria. Cell walls of the halococci were found to be mostly carbohydrate and protein or peptide. The halobacteria and halococci differ from moderately halophilic bacteria which posses muramic acid. Several features (e.g. nucleic acid satellite bands, membrane permeability and cell walls of the halococci) of the extremely halophilic bacteria are similar to those found in some algae and it is suggested these similarities be investigated.

A brief survey of marine bacteria from the intertidal zone showed that many have a salt tolerance of 20 and 25% NaCl in contrast to previous reports. H5 Micrococcus, which contains muramic acid and peptidoglycan, is able to grow rapidly in media containing 0.5 to 25% NaCl. The unique features of the extremely halophilic bacteria are, therefore, unnecessary for growth at high salt concentrations.

H5 Micrococcus may possibly contain several cell populations exhibiting growth optima at different salt concentrations but this seems unlikely because populations of two moderately halophilic bacteria, Vibrio costicolus and Micrococcus halodenitrificans, which grow over the salt range 3 to 20% NaCl were shown to be homogenous in their growth response to salt by several methods. V. costicolus, which has known nutritional requirements, grows over a more limited range of salt concentration and initial pH in minimal medium. These factors must be taken into account when assessing the salt tolerance and requirements of organisms.

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## Abbreviations and Symbols used in Text

| | |
|---|---|
| ala | alanine |
| glu | glutamic acid |
| lys | lysine |
| DAP | diaminopimelic acid |
| TCA | trichloroacetic acid |
| EDTA | ethylenediaminetetraacetic acid |
| SDS | sodium dodecyl sulfate |
| MurNAc | N-acetyl muramic acid |
| GalN | galactosamine |
| GluN | glucosamine |
| CoA | co-enzyme A |
| UDP | uridinediphosphate |
| DNA | deoxyribonucleic acid |
| RNA | ribonucleic acid |
| G + C | guanine plus cytosine |
| A | angstrom |
| nm | nanometers |

## Preface

The purpose of the studies was to elucidate some of the relationships which exist between bacteria living in habitats of different salinity. These relationships can be characterized by physiological parameters and by peculiarities of chemical composition.

The study must begin by asking; what is already known of the organisms? This is answered in the first chapter. The answer naturally leads to specific questions about the organisms. Three questions, which are answered in chapters 2,3 and 4, are, could halophilic bacteria arise from existing sea and sea shore bacteria? how does salt concentration affect the nutritional requirements of a moderately halophilic organism? and do the extremely halophilic cocci lack muramic acid and therefore peptidoglycan as do the extremely halophilic bacilli? The answers naturally lead to a discussion of the ancestry of halophilic microorganisms.

## General Introduction to Halophilic Microorganisms

Halophilic bacteria originally aroused interest because of the economic loss caused to the salted fish, salted meat and leather tanning trade (Harrison and Kennedy 1922, Lochhead 1934, Petter 1932 and Flannery 1956). The bacteria were contaminants of the salt used as a preservative or curing agent in the trades. The salt implicated came from solar evaporation flats in various parts of the world. Naturally saline environments such as Great Salt Lake and the Dead Sea also have abundant flora of halophilic organisms (Baas-Becking 1928, Volcani 1940 and Eimhjellen 1964).

Recently, microbiologists have studied the halophilic bacteria because of the bacteria's ability to live in extreme environments (Kushner 1968). The halophilic bacteria, which survive in and depend on saline habitats, have many peculiarities. This thesis deals with some aspects of the distribution, growth and chemical specialization of these organisms.

Classification

Over the past thirty years various schemes have been proposed for classifying microorganisms by their salt tolerance. In the past, the simplest and most obvious was to define organisms as halophilic and non-halophilic; however, many organisms have a salt tolerance which overlaps the two categories. This compelled Flannery (1956) to propose subclasses. He divided "non-halophiles" into "salt sensitive" and "salt tolerant." All the non-halophiles grow best in salt (NaCl) concentrations of less than 2% but the salt tolerant ones can grow above 2%. The "halophiles" were divided into "facultative"and "obligate" depending on whether they grew in less than 2% NaCl. All "halophiles" grew best above 2% NaCl.

A complicated but useful scheme which has not come into general use was proposed by Ingram (1957) for classifying organisms with varying degrees of tolerance to high concentrations of sugar and salt. He listed organisms as follows,

1. Intolerant - do not require solute.
   - 1.1 Strongly - only grow in very low concentrations.
   - 1.2 Moderately - will grow in moderate concentrations.
   - 1.3 Slightly - grow in high concentrations.
2. Facultative - require some solute.
   - 2.1 Low optimum - grow best in low solute concentrations.
   - 2.2 Moderate optimum - grow best in moderate solute concentrations.
   - 2.3 High optimum - grow best in high solute concentrations.

3. Obligate - require a considerable amount of solute and grow well in high concentrations.

3.1 Weakly - slight growth in low solute concentrations.

3.2 Moderately - slight growth in moderate solute concentrations.

3.3 Strongly - grow only in high solute concentrations.

There are, therefore, nine possible categories in which an organisms ca be placed.

At present organisms are usually considered under the headings, no halophile, slight or marine, halotolerant, moderate halophile and extreme halophile (Larsen 1962). The boundaries like those of Ingram's scheme are, however, less well defined than the 2% salt boundary in Flannery's scheme. Various authors have introduced sub-headings principally by specifying the source of the organism; thus, headings such as "terrigenous halotolerant" are occasionally used. Considerable con fusion arises from this practice when "terrigenous halotolerants" are isolated from the sea and classified as such because of their salt response (Shah and deSa 1964).

The following terminology will be used here. It basically follows that of Baxter and Gibbons (1956) and Kushner (1968).

Non-halophiles - those organisms which grow without any added NaCl in the medium and do not grow in concentrations of NaCl above 0.5M. No doubt they often require some $Na^+$; a requirement which is easily fulfilled by $Na^+$ contaminating the medium (MacLeod 1965). Trypticase soy broth, for example, contains 0.09M $Na^+$.

Marine - those organisms which grow well in about 0.5M NaCl, poorly or not at all without added NaCl and poorly in about 1 M NaCl. This follows MacLeod (1965). Organisms isolated from the sea generally need 0.2 - 0.3 M NaCl for optimum growth but some require only low amounts of $Na^+$ (0.02 M or less).

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

Extremely halophilic - those organisms requiring 2.5 M NaCl for growth (and usually about 2M for survival) and which will grow in saturated or near saturated NaCl solutions (5.5-6.0 M).

Halotolerant - those organisms which have a low salt requirement, as do some of the marine bacteria, but have an extended upper salt tolerance range. The term "extremely halotolerant" is used to mean having the ability to grow in the high salt enviroment re-required by extremely halophilic bacteria (about 4 M).

This terminology is necessarily imprecise and reflects the fact that there is a gradation of organisms ranging from the salt intolerant to those dependant on high salt concentrations.

The non-halophiles, important in industry and medicine, are the best known and most numerous group (Brown 1964a). The halotolerants are probably the second most numerous group and have been isolated from soil, fresh water, sea water, fish and other animals (Shah and deSa 1964). Larsen (1962) and Ingram (1957) have reviewed the literature related to the salt tolerance of organisms isolated from

various sources.

The salt tolerance of some organisms isolated from the marine environment fits the "marine" classification; others fit in the less precise "halotolerant" category. Could the halophilic bacteria have arisen from marine bacteria trapped in evaporating sea water? The answer to this question can only come from studies of the bacteria of the sea and sea shore. Such a study is reported in chapter 2.

The few known moderately halophilic bacteria have been studied because of their economic importance. Vibrio costicolus for example is responsible for taint in ribs of pickled bacon (Smith 1938). In 1962 Larsen compiled a list of 18 moderate halophiles; most were isolated from salted products and brine, and others were isolated from Liman mud. Other moderate halophiles have been isolated from Soy sauce (Ueno 1964). While many cause spoilage others may contribute to the ripening of products (Flannery 1956).

The extreme halophiles include the rod shaped Halobacteria (or Halobacter) which are in the family Pseudomonadaceae and the cocci which are in the family Micrococcaceae (Breed et al. 1957). The cocci are classified as Micrococci or Sarcina although they are apparently Gram negative (Brown 1964a).The reclassification of the cocci into a new genus, Halococcus, proposed by Larsen (1967), is favoured by Gibbons (personal communication). Halococcus is used here and elsewhere (Moore and McCarthy 1969a). The extremely halophilic bacteria have been isolated from salt pans, salt lakes, salted fish, salted meats and salted hides. Many have bright carotenoid pigments located mainly in the envelopes thus accounting

for the"reddening" of their habitats. The carotenoids apparently protect the organisms from photochemical damage caused by bright sunlight to which they would be exposed in salt pans and salt lakes (Larsen 1962, Nandy and Sen 1967, Dundas and Larsen 1963). These organisms all grow well at temperatures of 35 to 40 $^{o}$C.

Most studies on halophiles are concerned with salt concentration but in food spoilage studies a variety of solutes are of interest. When salt, sugars, or other solutes dissolve in water they bind some water molecules (water of hydration) and thereby reduce the number of free water molecules present. This reduction in the available water is a common factor among various solutes. The availability of free water is expressed as the water activity $a_w$

$$a_w = \frac{P}{P_o} = \gamma_w C_w$$

where P is the vapour pressure of the solution and $P_o$ the vapour pressure of the pure solvent. $\gamma_w$ is the activity coeficient of water and C the concentration of the water. Unfortunately $\gamma_w$ is a variable and must be determined empirically for each solute and solvent concentration. Scott (1957) and Kushner (1970) in reviews on the water relations of organisms supplied some useful tables and graphs relating the concentration of various solutes to water activity. All solutes produce a lowering of the water activity but charged solutes (e.g.salts) may have a different physiological effect than do uncharged ones (e.g. sugars).

Some yeasts tolerate high concentrations of sugars and are called osmophilic. Many molds tolerate very dry locations and are called xerophilic. Molds can tolerate lower $a_w$ values than the yeasts and the yeasts tolerate lower $a_w$ values than the bacteria.

The term "osmophilic" has been criticized (Ingram 1957, Brown 1964a, Kushner 1970) because it implies without evidence that these organisms require the presence of non-penetrating solutes (osmotic protection). Yeasts that are osmophilic are often found to grow as well without the solute as with it and they are found to lose their tolerance for the solute when grown in media without it (Scarr and Rose 1966). Not all non-penetrating solutes have the same protective effect. One sugar tolerant yeast would not grow in glycerol or sucrose at an $a_w$ of 0.990 but did if polyethylene glycol was used, which suggests that lowering of the $a_w$ is not the only effect produced. The same yeast had an apparent requirement because it would not grow in basal medium ($a_w$ 0.997) but growth was poor at all $a_w$ values which suggested the medium was nutritionally poor (Anand and Brown 1968).

The term "osmophilic" is misleading in any event since osmo (from the greek osmé) means odor. The term should be osmotophilic, as suggested by Nickerson (1943).

Morphology

Pleomorphism has long been associated with the halophilic bacteria (see Flannery 1956 for an early review). The halobacteria which undergo dramatic changes in shape as the salt concentration is lowered, have been studied most extensively. Halobacterium cutirubrum which is a long rod in 4 M NaCl undergoes a change to transitional forms (swollen and distorted rods) then to spheres when the salt concentration is lowered. It disintegrates at about 1 M NaCl. Four molar $Na^+$ cannot be replaced by 4 M $K^+$, $Rb^+$, $Cs^+$, $Li^+$ or $NH_4^+$ in maintaining the rod shape (Abram and Gibbons 1961) although 3.5 M or higher $MgCl_2$, $CaCl_2$ and $CH_3COONa$ will maintain the rod shape (Boring et al. 1963). The hydrogen ion also has a protective effect. One tenth to one percent acetic acid prevents lysis but the cell becomes spherical. At above 1% the rod and transitional forms are retained. The cell surface which in the normal cell shows a regular hexagonal pattern with a center to center distance of 120 to 150 A becomes irregularly pebbled. The cells are not viable. Spheres and fragments can be obtained in 4.5 M NaCl if the pH is lowered to 4 although the surface is similar to that of normal cells and the cells are again not viable. Total disintegration in low salt solutions is characteristic only of the halobacteria although many marine bacteria show substantial cell wall loss and lysis (Buckmire and MacLeod 1965, Korngold and Kushner 1968). Moderate halophiles undergo substantial leakage in distilled water and consequent loss of viability. Micrococcus halodenitrificans loses DNA, presumably

by the lysis of some cells, below 0.7 M NaCl although it grows at 0.55 M (Takahashi and Gibbons 1957). Vibrio costicolus behaves similarly (Christian and Ingram 1959) but it also has a DNA slime layer at all concentrations which makes it especially interesting (Smithies and Gibbons 1954). Calcium and magnesium ions have substantial protective effects on M. halodenitrificans and allow growth at 0.3 M NaCl (Takahashi and Gibbons 1959). V. costicolus becomes pleomorphic at NaCl concentrations near 0.5 M but large fragments are still visible in distilled water.

The staining of the halophiles by standard bacteriological techniques is therefore rather difficult. Various workers have used methanol, acid alcohol and 4% formalin for fixing the halobacteria before staining (Flannery 1956). Phase or darkfield microscopy avoids the difficulties for morphological studies, but to classify organisms by the Gram stain reaction they must be fixed before staining. One method, using 1 to 2% acetic acid for 5 minutes, was devised by Dussault (1955) and was examined by Kushner and Bayley (1963). It is generally recognized that minor changes in the Gram stain procedure can influence the result (Conn and Pelczar 1957). Ingram (1957) cited some results of Petrowa (1935) as an example of Gram stain failure. Petrowa found that a salt tolerant coccus stained Gram positive when grown at low salt concentrations and stained Gram negative when grown at high salt concentrations. One must either decide that the Gram stain reaction is irrelevant for halophiles or classify them as Gram negative or positive indirectly, using chemical methods or electron microscopy. The chemical methods will be discussed later.

Early electron microscope studies of halobacteria failed to show an intact envelope until it was realized that the fixing and dehydrating solutions must contain salt to prevent dissolution of the cell (Brown and Shorey 1963). Suitable fixatives must of course be found for all electron and many optical microscope studies. The problems and results of electron microscope studies on halophilic bacteria have been reviewed by Glauert and Thornley (1969). After fixation with formaldehyde and permanganate (with salt) the halobacteria are seen to have a trilaminar membrane and a dense layer on the outside about 150 A thick. The outer edge of the dense layer has globules with a periodic spacing of 150A. These globules are believed to correspond to the hexagonal array seen in tangential sections and replicas. The wall appears to have fewer layers than most Gram negative organisms but more than many Gram positive organisms (see below). The only moderate halophile studied, M. halodenitrificans, has a typically Gram negative structure (Kocur et al. 1968).

Certain strains of halobacteria were seen to contain vacuoles by Petter (1934). She suggested that these were gas vacuoles similar to ones that had been observed in algae. Stoeckenius and Kunau (1967) showed that the gas vacuoles collapse on centrifugation but not on filtration. Hydrostatic pressure was also shown to collapse the vacuoles.

The halococci which do not lyse in distilled water have received little attention. Their morphology is known to be dependant on culture conditions as it also is for non-halophilic micrococci. For example, the number of cells present in a packet, the Gram reaction and colonial

morphology are dependant on the type of media and the age of the culture (Conn and Pelczar 1957). Brown (1964a) stated that there were no known Gram positive species of extremely halophilic bacteria. The halococci are Gram variable according to Larsen (1967) but the staining conditions were not specified.

Nutrition and Metabolism

In this section nutrition refers to the utilization of organic compounds and not to the need for specific ions.

Moderate halophiles

M. halodenitrificans and V.costicolus ferment sugars and have proteolytic activity (Flannery 1956). Flannery and Kennedy (1962) developed a synthetic medium for V.costicolus containing five amino acids, glucose, $NaNO_3$, $MgSO_4$ and NaCl. It is completely described in chapter 3. The nutritional requirements of other moderate halophiles are unknown.

Extreme halophiles

The nutrition of the halobacteria has been studied in greater detail than that of the moderate halophiles. Until recently the halobacteria were generally believed to use protein and amino acids but not carbohydrates as carbon sources (Gochnauer and Kushner 1969, Larsen 1967). Dundas et al. (1963) developed a synthetic medium for H. salinarium strains 1, 1M and 5 which contained ten amino acids, of which only four, valine, methionine, leucine and isoleucine were essential. The other six and cytidylic acid were stimulatory. H.cutirubrum, H.halobium, Micrococcus 9M and 49S also grew in the medium but H.salinarium CCNRLO 10 grew poorly. H.salinarium strains 53A and 53B and Micrococcus morrhuae would not grow in the medium. In a slightly more complex medium Onishi et al.(1965) obtained growth of various halobacteria and halococci. Their medium contained 15 amino acids, 2 nucleotides and glycerol. The medium was tested in detail on a strain of H.cutirubrum. Arginine, leucine, lysine and valine were

found to be essential and glycerol stimulatory. Onishi et al.(1965) suggested that glycerol was a readily utilizable precursor for lipid synthesis. Kates et al.(1968) showed this to be true. Gochnauer and Kushner (1969) have shown that the addition of $K^+$ to the medium of Onishi et al. (1965) stimulates growth and that the stimulatory effect of glycerol (0.1%) was even greater at the high $K^+$ level. Various carbohydrates, vitamins and metabolites also stimulated growth. No acid was produced from the carbohydrates but acid was produced from 2% glycerol, a concentration which inhibits growth. The standard carbohydrate utilization tests depend on the organisms metabolizing carbohydrates to acids which change the color of a pH indicator included in the medium. By such tests halobacteria would appear not to utilize carbohydrates.

Dundas and Halvorson (1966) studied the metabolism of arginine, an essential amino acid, in H.salinarium. They suggested that arginine is degraded to citrulline by arginine desimidase then to ornithine and carbamyl phosphate by ornithine transcarbamylase. The opposite biosynthetic pathway was found in non-halophilic bacteria.

Many of the halobacteria have extracellular proteolytic enzymes, as would be expected of organisms living in salted flesh. Norberg and Hofsten (1969) found a truely extracellular proteinase which degraded gelatine and casein, in several halobacteria. Proteolytic activity was not found in the wild strains of halococci investigated.

Gibbons (1957) investigated the hydrolysis of gelatine, casein and starch at various NaCl concentrations by 49 taxa of halophilic bacteria. Two were non-pigmented rods and 47 were pigmented bacteria

including 20 unnamed rods, 7 unnamed micrococci and 20 known species. Of the last, 14 were rods and the rest micrococci. Forty-five species hydrolysed gelatine but only 22, all rods, hydrolysed casein. Four rods and 5 cocci were able to hydrolyse starch. Most of the species were able to hydrolyse substrates best in 20-25% NaCl.

Petter (1931) showed that a red sarcina, probably Sarcina morrhuae, and S.gigantea could grow in an artificial medium if 1% asparagine or glycine was added as a nitrogen source.

As these examples show knowledge of the nutritional requirements of halophiles generally is fragmentary. One important question is, how do nutritional requirements of an organism depend on salt concentration? An answer to this question is reported in chapter 3.

Physiology

The halophilic bacteria are obviously different from other organisms in their ionic requirements. As has already been stated the morphology changes drastically when they are deprived of an adequate NaCl concentration. How specific is their requirement for NaCl?

In 1952, Flannery et al. investigated this question for Vibrio costicolus. This organism grew in 1% trypticase broth containing 0.4 to 3.5 M NaCl. If NaCl were replaced by $Na_2SO_4$ it grew from about 0.3 to 0.8 M ($Na_2SO_4$) and the addition of 0.2 M NaCl increased the growth range from about 0.2 to 0.8 M ($Na_2SO_4$). Slight growth was obtained when $MgCl_2$ replaced NaCl from 0.3 to 1.2 M ($MgCl_2$) and also when KCl and LiCl replaced NaCl. The addition of NaCl universally increased growth. Flannery et al. concluded that there was not a definite requirement for $Na^+$ but traces of $Na^+$ and $Cl^-$ might be necessary and that $Mg^{2+}$, $K^+$, and $Li^+$ could substitute but tended to be toxic. It would also seem that $SO_4^{2-}$ would also be toxic in high concentrations. $Na_2SO_4$ while satisfying the $Na^+$ requirement would not allow growth above 0.8 M $SO_4^{2-}$ even if NaCl was added. If NaCl is specifically eliminated from the medium no growth will occur in the presence of other salts (Christian 1956, Larsen 1962) confirming Flannery's suggestion that traces might be required. A similar situation exists with the marine bacteria (MacLeod 1965).

At the same time Robinson and Gibbons (1952) studied the effect of salt on the growth of Micrococcus halodenitrificans. They obtained

growth only if the medium contained NaCl and found an optimum concentration btween 4.4 and 8.8%. They also studied the effect of salt on nitritase activity and found the maximum nitrogen production (from nitrite) at 2.2% NaCl. This was an early indication that at least some enzymes had an activity maximum in a salt concentration below that required for growth.

Extremely halophilic bacteria require the same ions as do moderately halophilic bacteria. They require NaCl in high concentrations and only a small amount can be replaced by KCl or $MgCl_2$ since large concentrations of ions other than $Na^+$ and $Cl^-$ are toxic. Magnesium and potassium are, however, required in substantial amounts for growth (Larsen 1962). Potassium, especially, is often limiting in synthetic media (Gochnauer and Kushner 1969).

It is well known that high salt concentrations inhibit enzymes and "salt out" proteins from non-halophilic sources. This lead some early workers to consider the possibility that ions might be specifically excluded from the cell, but Ingram (1956) pointed out that if this were true the density of the halophilic cells would be so low that they could not be centrifuged down from the medium. Brown (1964a) stated that it was extraordinary for the ion exclusion theory to be considered in the first place since it requires a water impermeable membrane but just such a system seems to exist in the halophilic alga, Dunaliella viridis. Johnson et al. (1968) found that the maximum enzyme activity for four enzymes occurred at a salt concentration far less than that routinely employed for growth (3.75 M NaCl). $K^+$, $Li^+$ and $Cs^+$ were equally inhibitory. The enzymes appear to have

been completely inhibited at 2.0 M NaCl.

The measurement of internal ion concentration is of considerable importance. Shultz and Solomon (1961) developed a reliable method for the determination of intracellular ion concentrations in Escherichia coli and used it in a study of ion transport. The measurement of intracellular volumes depends on the accurate measurement of intercellular or interstitial space in the pellet and the pellet volume. Initially pellet volumes were determined by measuring the length of a pellet sedimented in special cytocrit tubes. Sedimentation of the pellet required a special centrifuge head. The intercellular space is usually measured by a dilution technique. Shultz and Solomon used labelled inulin and albumin for E.coli but as they point out large labelled molecules may not measure all the interstitial space because the large molecules could be excluded from much of the space that is available to extracellular water and other small molecules. It is difficult to find a small, non-penetrating, non-adsorbed, water soluble molecule to use for dilution. Assuming the molecule could be found then the volume of the pellet must be accurately determined. Usually this is done by weighing the pellet and making the assumption that the specific gravity of the interstitial water is unity because weights can be determined more accurately than volumes. For E. coli the assumption is essentially correct but it is not for halophiles because the specific gravity is changed by salt (e.g. 24% NaCl has a specific gravity of 1.1804). The specific gravity was taken into account by Christian and Waltho (1962).

Christian and Waltho (1962) determined the intracellular $Na^+$,

$K^+$, $Cl^-$, amino acid and phosphate concentrations in various bacteria, including two moderate halophiles, a halococcus and a halobacterium. In all cases the cellular $Na^+$ / medium $Na^+$ ratio was less than one but never less than 0.3 indicating substantial concentrations of $Na^+$ in the cell. $K^+$ on the other hand was highly concentrated in the cell. They considered that the halobacterium had a cellular $K^+$ / medium $K^+$ ratio of 4.75 M / 0.03M = 140. The medium $K^+$ concentration was determined before growth and therefore the ratio could be even higher since after growth most or all of this $K^+$ would be cell bound. The cellular $Cl^-$ was only slightly less than that in the medium for the two extreme halophiles and an order of magnitude less for the non-halophiles and moderate halophiles. The ratio of cellular ($Na^+ + K^+$) / medium $Na^+$ is greater than one for the non-halophiles and extreme halophiles but less than one for the moderate halophiles. These small differences for moderate halophiles are interesting but perhaps not meaningful since interstitial space was determined with phosphate which is known to be penetrating (see below) and based on figures with large standard errors. These assays were carried out only on stationary phase cells. The cellular $K^+$ in Halobacterium halobium has been shown to be even greater than Christian and Waltho's value during the mid log phase of growth (Gochnauer and Kushner 1971).

The measurement of interstitial space in an unclassified halobacterium was investigated by Ginzburg (1969). She found that sucrose, inulin ( MW 5,000-5,500), polyvinylpyrollidone (MW 30,000 - 40,000) and starch (MW 20,000 - 40,000) penetrated the cell membrane easily and that serum albumin (MW 65,000) and dextran (MW 80,000) were

excluded. Therefore measurement of the interstitial space of these organisms is to be undertaken cautiously. Ginzburg et al. (1970) determined the intracellular concentration of $Na^+$, $K^+$ and $Cl^-$ in the same halobacterium at various stages of growth. Care was taken to avoid breaking cells during routine handling. Separate aliquots of a carefully made suspension were used for protein, pellet volume, pellet weight and ions. The interstitial space was found to be 21.1% $\pm$ 0.5 when standard conditions were used. Cell densities were found by centrifuging cells through water immiscible layers of increasing density until only half of the cells were deposited below the layer. They found as did Christian and Waltho (1962) that $K^+$ was concentrated and maintained within the cell while $Na^+$ was extruded as growth progressed.

| | $K^+$ cell / $K^+$ medium | $Na^+$ cell / $Na^+$ medium |
|---|---|---|
| Early log phase | 940-790 | .307 - .77 |
| Late log phase | 3700- 1000 | .41 - .54 |
| Stationary phase | 4900-6700 | .138 - .18 |

The growth rate of the moderately halophilic bacteria is quite comparable to the growth rate of non-halophiles, but the extremely halophilic bacteria grow much more slowly. Larsen (1962) reported the shortest generation time of ten hours for the halobacteria and about double that for the halococci. Later, Larsen (1967) reported a generation time for halobacteria of 7 hours. The unidentified halobacteria studied by Ginzburg et al. (1970) had a generation time of 6.5 hours.

While most halophiles are strict aerobes, one, a moderate halophile, is known to be photosynthetic and a strict anaerobe (Raymond and Sistrom 1967). Four others isolated from soy sauce were also anaerobic (Ueno 1964). The matter of oxygen is of some importance because the concentration of dissolved oxygen decreases as the salt concentration increases (see Bunsen coefficients in Umbreit et al. 1957). This is probably one of the factors determining the growth rate of halophiles.

## Cell Walls and Cell Envelopes

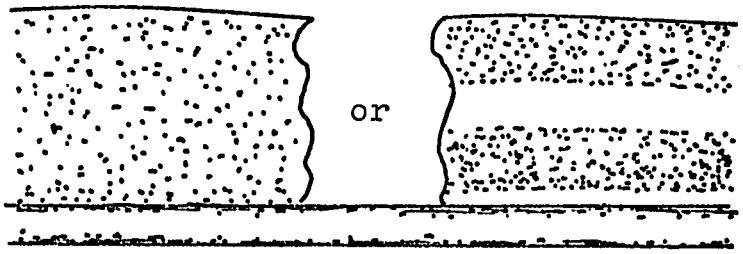
The properties of Gram negative cell envelopes were reviewed recently by Luderitz, Jann and Wheat (1968), Osborn (1969) and Rogers and Perkins (1968). Rogers and Perkins also reviewed the properties of Gram positive cell walls as did Ghuysen, Strominger and Tipper (1966). Glauert and Thornley (1969) reviewed "The Topography of the Bacterial Cell Wall".

### Non-halophiles

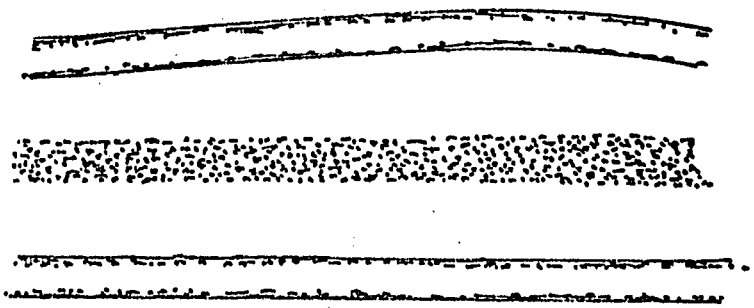
The entire continuous outer covering of a cell is called the cell envelope. It is comprised of both the cell wall and cell membrane (Salton 1964). The various appendages, capsules and amorphous structures which are found in some bacteria are not considered to be a part of the cell wall or membrane. The cell membrane is dealt with in the next section.

Figure 1 shows a diagramatic representation of Gram positive and Gram negative cell envelopes. The Gram positive cell envelope consists of basically two structures, the plasma membrane and an outer dense band. The latter is sometimes seen in the electron microscope as several bands, depending on the species examined and the preparative methods. Sometimes a space exists between the dense outer band and the plasma membrane. The Gram positive cell wall, that is, the outer band, is fairly easy to isolate (see below).

The Gram negative cell envelope is much more difficult to take apart because there is strong adhesion between the plasma membrane and the rest of the envelope and because of the complex topography. The




Gram positive cell wall and membrane




Gram negative cell envelope

Figure 1

cell wall (fig.1) is usually considered to consist of the dense band and the outer membrane (Glauert and Thornley 1969). The use of the term "membrane" was criticized by Salton (1967) because it should be reserved for the trilaminar structure 75A thick next to the cytoplasm which functions as a selectively permeable barrier. He contends that the description of the outer layer as a "membrane" came about accidentally and should be discouraged. The dense band is also referred to as a rigid layer or R-layer and is probably exclusively peptidoglycan. As isolated by Weidel and Pelzer (1964) it is referred to as the sacculus which Weidel considers to be one large bag shaped macromolecule. The outer 'membrane' or layer is a flexible layer of lipid, protein and polysaccharide, which is responsible for many of the antigenic and endotoxic properties of the cell. The antigens from this layer are called the somatic "O" antigens.

Gram Positive Cell Walls

The preparation of cell walls has been reviewed by Rogers and Perkins (1968). The cell wall of the Gram positive bacteria were the first ones to yield their basic structure. To prepare such walls, cells were disrupted by shaking a cell suspension with small glass beads (ballotini), by passing through a French pressure cell or "X" press, by sonication or by grinding with sand or alumina. The cell walls were separated and washed free of debris by differential centrifugation. The preparation of cell walls of some bacteria by this method often leaves some protein adhering to the peptidoglycan, which is removed by incubation with one or more proteolytic enzymes.

Shaking with ballotini beads is the usual method for obtaining the entire wall (Salton and Horne 1951, Cummins and Harris 1956).

Initially the entire wall was thought to be exclusively peptidoglycan but later work showed other components in small amounts such as teichoic acids, teichuronic acids and protein if it was not removed by treatment with proteolytic enzymes.

Peptidoglycan

Peptidoglycan is also referred to in the literature as mucopeptide, murein, mucopolymer, glycosaminopeptide and glycopeptide. Peptidoglycan is, however, the term favoured at present and Osborn (1969) considers it to be the most chemically descriptive; it will, therefore, be used here. Mucopeptide is used widely in England (Rogers 1970). Peptidoglycan structure and biosynthesis have been studied most extensively in Staphylococcus aureus and Micrococcus lysodeikticus.

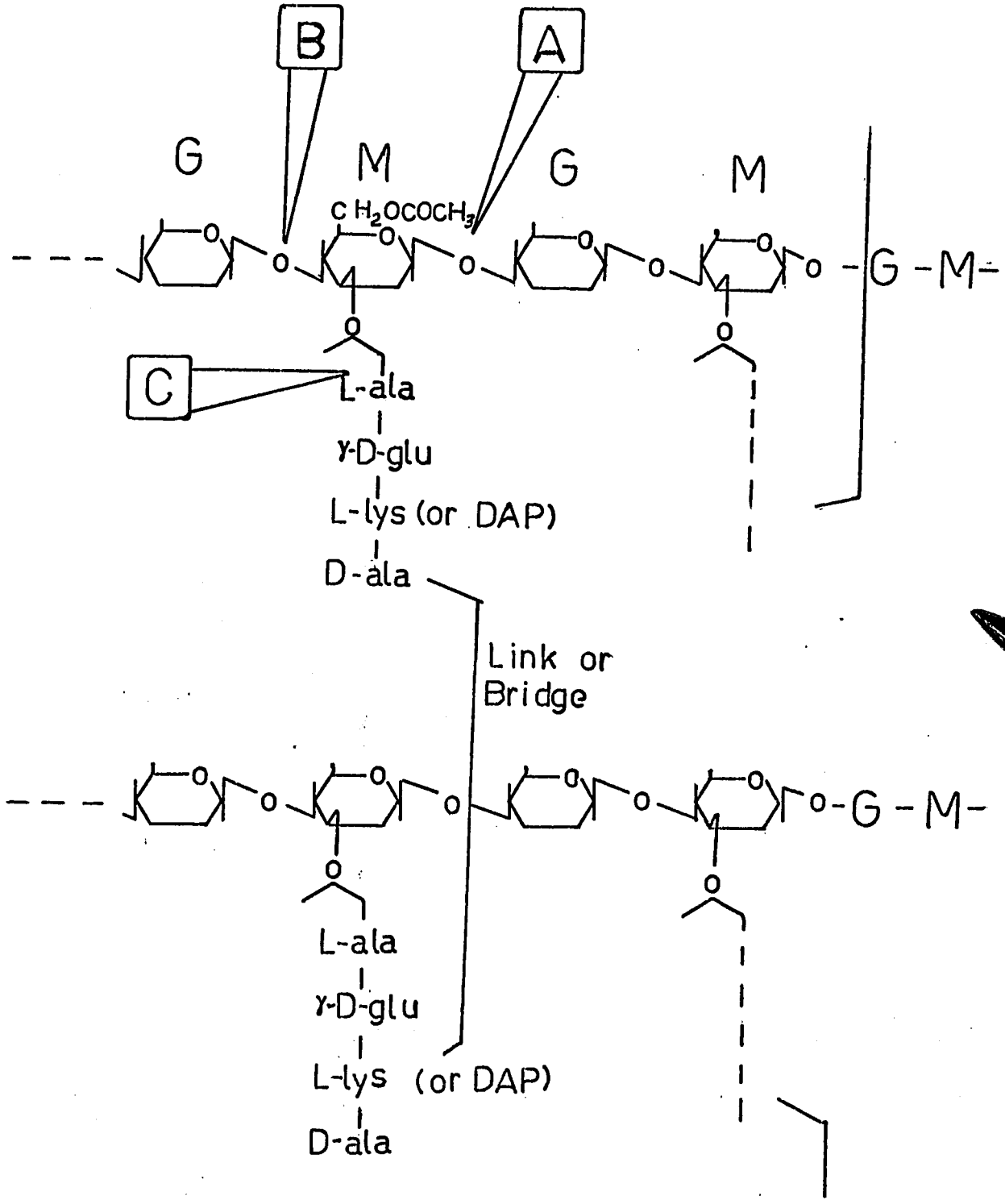
The peptidoglycan fraction is generally considered to give form to the cell and to prevent lysis by osmotic rupture of the membrane. That this is true can be demonstrated by degrading the layer with lysozyme. The cell then becomes a protoplast if it is protected from osmotic rupture, or lyses completely if it is not protected. Peptidoglycan can be isolated in various ways. Fuller (1938) isolated peptidoglycan from hemolytic streptococci with hot formamide although the separation wasn't recognized until Krause and McCarty (1960) studied the method. Park and Hancock (1960) developed an extraction technique for Staph. aureus peptidoglycan. The cells were washed

with cold 5% trichloroacetic acid (TCA) to remove low molecular weight compounds soluble in TCA such as pool amino acids, 75% ethanol to remove lipid and some amino acid containing material, hot 5% TCA to remove nucleic acids and teichoic acids and finally digested with trypsin to remove any residual protein. Hatton (1968) has since shown that the method of Park and Hancock is a very reliable and versatile technique for isolating the peptidoglycan of M. lysodeikticus.

On acid hydrolysis peptidoglycan yields glucosamine ( 2-amino-2-deoxy-D-glucose), muramate (3-O-lactyl-D-glucosamine), alanine, glutamate, glycine, lysine or diaminopimelate (DAP) and in some cases serine, 2 homoserine and D-ornithine.

The amino sugars, glucosamine (G) and muramate (M), have been shown to alternate in chains joined by β-1,4 linkages (Fig.2). In Staph. aureus the amino groups are all acetylated as is shown by the Morgan-Elson reaction after enzymatic degradation ( the acetyl groups are lost on acid hydrolysis). Some N-acetyl muramate residues also have O-acetyl groups on the 6 carbon. In M.lysodeikticus the sugars do not have O-acetyl groups and some muramate residues do not have N-acetyl groups. These glycans are joined together through peptides attached to the lactyl groups of muramate. Thus it is that muramate, which is only known to occur in bacteria, is a key component of the cell walls.

The cross linking peptide chains are usually based on the tetra-peptide L-ala-γ-D-glu-L-lys(or DAP)-D-ala which is attached to muramate by the amino group of L-alanine. In a few organisms the L-lysine (or DAP) is substituted by other amino acids and the α-carboxyl group




Peptidoglycan structure and sites of attack of enzymes.

Figure 2

of D-glutamate is also sometimes substituted. A second outstanding feature of the cell walls is the occurence of both D and L amino acids.

The peptides are linked through the terminal carboxyl group of D-alanine and the ε-amino group of L-lysine (or DAP). The manner in which they are joined differs in various organisms. In Staph. aureus the groups are joined by a pentaglycine peptide while in M. lysodeikticus it is suggested that four more tetrapeptides, in addition to the two present, form the bridge.

Various lytic enzymes are now used for highly specific degradations of peptidoglycan. Their use in structural studies was reviewed by Ghuysen (1968). The best known, egg white lysozyme, hydrolyses the glycosidic linkages at the reducing end of N-acetylmuramate (A in fig.2). Another, the glycosidase of lysostaphin (endo-N-acetylglucosaminidase) hydrolyses the glycosidic linkage at the end of N-acetylglucosamine (B in fig.2). The peptides can be cleaved from the lactyl group of muramate by Streptomyces N-acetylmuramyl-L-alanine amidase if the glycan is first split (C in fig.2). The remainder of the enzymes are peptidases except for Myxobacter AL-1 protease which degrades peptide bridges in various organisms and slowly hydrolyses N-acetylmuramyl-L-alanine linkages. This makes it especially useful for isolating glycan chains.

Peptidoglycan Biosynthesis

The biosynthetic pathways leading to the formation of peptidoglycan are known (figure3) and have been reviewed by Osborn (1969). Fructose-6-phosphate is aminated to glucosamine-6-phosphate with an amide group ($-NH_2$) from glutamine. The amino group is then acetylated

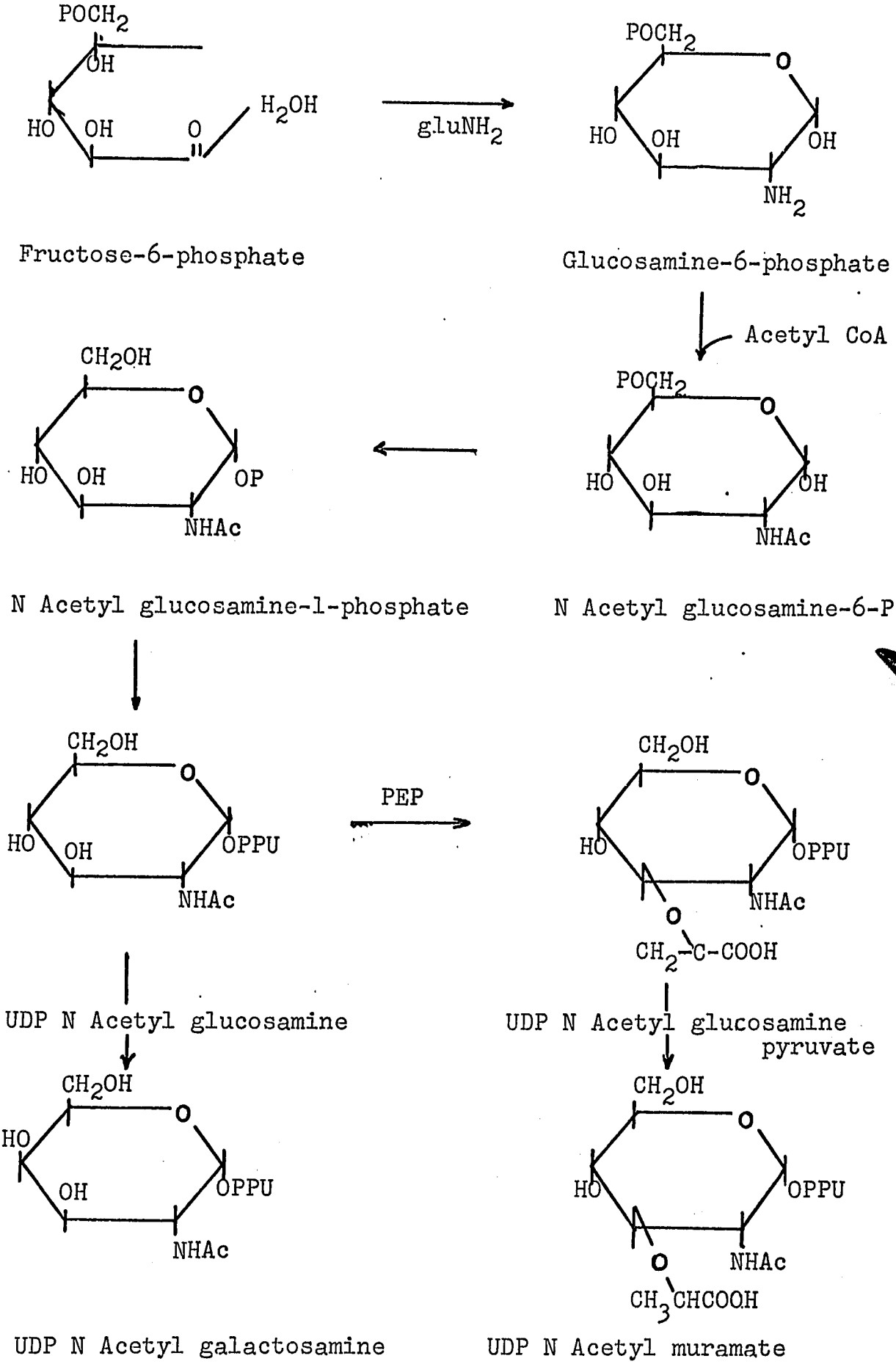
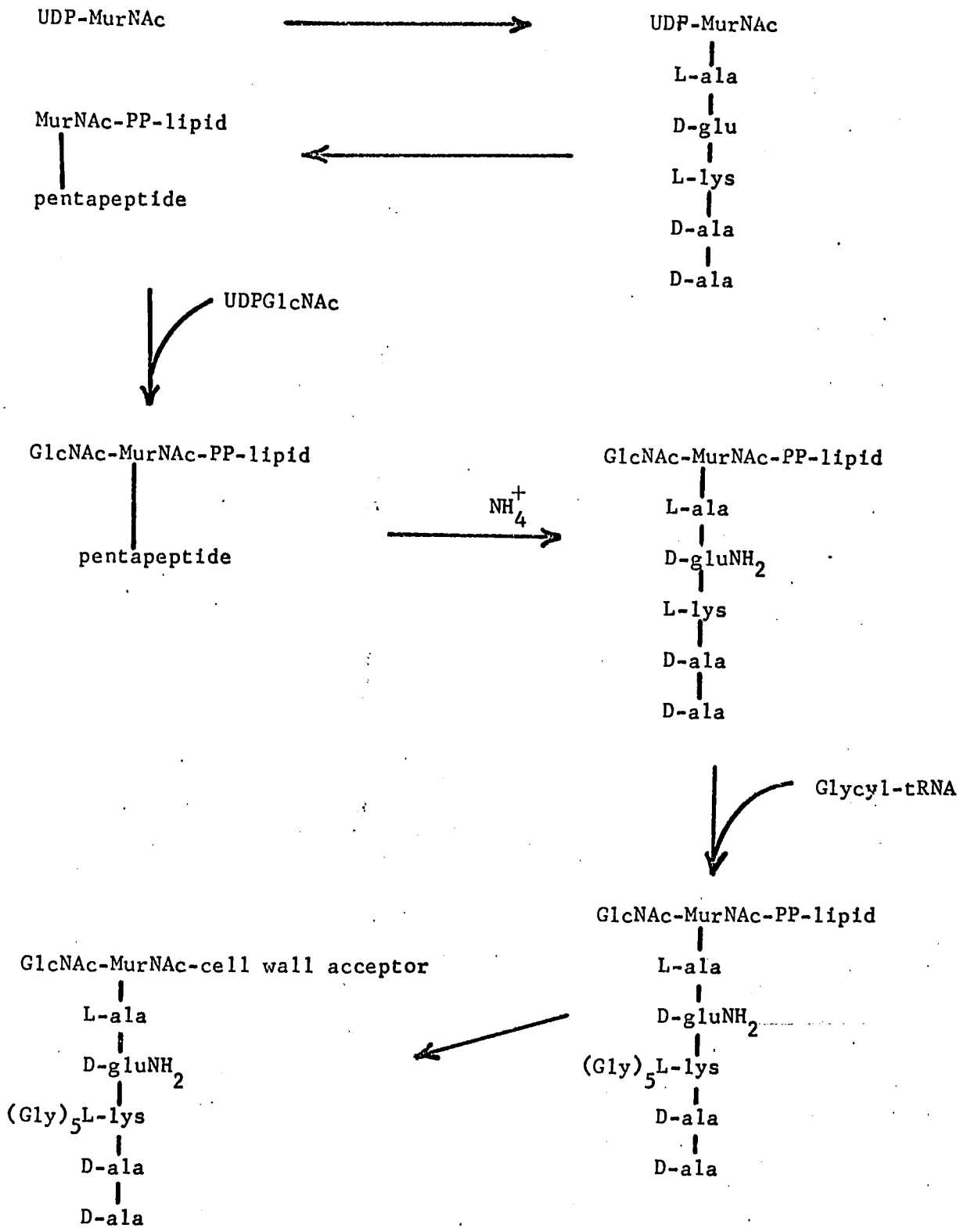

POCH2
OH
HO
OH
O
H2OH
gluNH2
POCH2
O
HO
OH
OH
NH2
Fructose-6-phosphate
Glucosamine-6-phosphate
Acetyl CoA
CH2OH
O
HO
OH
OP
NHAc
POCH2
O
HO
OH
OH
NHAc
N Acetyl glucosamine-1-phosphate
N Acetyl glucosamine-6-P
CH2OH
O
HO
OH
OPPU
NHAc
PEP
CH2OH
O
HO
OPPU
NHAc
O
CH2-C-COOH
UDP N Acetyl glucosamine
UDP N Acetyl glucosamine
pyruvate
CH2OH
O
HO
OH
OPPU
NHAc
CH2OH
O
HO
OPPU
O
NHAc
CH3CHCOOH
UDP N Acetyl galactosamine
UDP N Acetyl muramate

Figure 3

with acetyl CoA to give N-acetylglucosamine-6-phosphate. The phosphate is then transferred to the 1 position and the molecule is coupled to uridinediphosphate (UDP). UDP-N-acetylglucosamine is the precursor of N-acetylgalactosamine and N-acetylmuramate. To UDP-N-acetylglucosamine is added phosphoenolpyruvate (PEP) yielding UDP-N-acetylglucosaminyl pyruvate which on rearrangement becomes UDP-N-acetylmuramate (UDP-MurNAc) (Mahler and Cordes 1966). The biosynthesis of the peptidoglycan proper starts here with the addition of amino acids to the lactyl group of muramate to give UDP-MurNAc-pentapeptide (figure 4). This is transferred to a lipid carrier along with pyrophosphate, and N-acetylglucosamine is added to the 4 carbon position of MurNAc. If the glutamate is to be converted to glutamine it occurs here, before the addition of crosslinks. The amino acids of these links or bridges are species specific. When the bridge is complete the whole unit is transferred to the cell wall acceptor site. The final step is the linking of the bridges between units and the release of the terminal D-alanine to give the typical tetrapeptide (Osborn 1969).

Other Components

The other components of the Gram positive cell walls are teichoic acids, polysaccharides, protein antigens and lipids. The protein antigens are of considerable interest in medicine and in the typing of species but will not be considered further here. The lipid components have only been studied in mycobacteria and corynebacteria, being virtually absent in other species (Ghuysen et al.1968). They too will not be considered further.




Biosynthesis of Peptidoglycan in Staphylococcus aureus

Figure 4

The polysaccharides of the cell walls have been known for a long time and they have been used as a basis of classification for hemolytic streptococci. They are composed of glucosamine, galactosamine and various neutral sugars which are usually attached by covalent bonds to the peptidoglycan as is shown by the difficulty of extraction but the nature and location of the bonds is not known (Rogers and Perkins 1968). The teichoic acids on the other hand have been studied extensively by Baddiley and co-workers (Baddiley 1968). They are polymers of ribitol phosphate and glycerophosphate and are thus characterized by the large amount of organically bound phosphate and also by D-alanyl ester groups. The subunits are held together by phosphodiester bonds. Other carbohydrates are attached to the backbone.

Gram Negative Cell Walls

Gram negative cell walls are different from Gram positive walls. They contain only 0.1 to 10% peptidoglycan (Luderitz et al. 1968) as compared to 50 to 90% peptidoglycan from Gram positive walls (Ghuysen et al. 1968). Electron microscope studies have shown that a thin layer of peptidoglycan is sandwiched between the cytoplasmic membrane and an out membrane-like layer (Osborn 1969). The outer layer is composed of lipopolysaccharide (1-5% cell dry weight) with some phospholipid (5-15% cell dry weight) and protein. Because of the important endotoxic and antigenic properties of the lipopolysaccharide it has been well studied as the source of somatic "O" antigens (Luderitz et al. 1968). The lipopolysaccharides are long chain heteropolymers with a polysaccharide core and covalently attached lipid and oligosaccharide

units. In some organisms the lipopolysaccharide layer may be further surrounded by loosely bound hydrophilic acid polysaccharide (capsules or capsular antigens). Techniques for selectively taking apart the cell wall are still being devised. Westphal and Jann (1965) recommended 45% phenol at 68°C as being a generally useful agent for the extraction of lipopolysaccharide. The capsular material, lipopolysaccharide and nucleic acids are found in the aqueous layer on cooling. The nucleic acids may be removed by precipitation with cetyltrimethylammonium bromide (Cetavlon) or differential centrifugation. The centrifugation also removes capsular polysaccharide.

Weidel et al. (1960) developed a method for isolation of the R-layer (peptidoglycan) from Escherichia coli. The cells were first extracted with 0.1 N NaOH several times, centrifuged, taken up in 0.4% sodium dodecylsulfate (SDS) and shaken with ballotini beads in a Mickle disintegrator for one hour. After slow centrifugation, cell envelopes were sedimented at 22,000 xg and washed exhaustively. Cells were extracted further with 90% phenol, centrifuged at 35,000 xg and reextracted thrice with phenol. The cells were dialysed, centrifuged and washed exhaustively. The pellet was reextracted with SDS as before, centrifuged and washed. The procedure was controlled by electron microscope examination at each step and repeated if necessary.

This very time-consuming procedure gave better purity than two other methods which were also used to isolate peptidoglycan. Mandelstam (1962) used organic solvent extraction, disulfide bond-breaking agents, pepsin, 90% formic acid, 90% phenol and a copper

sulfate EDTA mixture. Schocher et al. (1962) used saturated SDS for long periods after first obtaining cell walls by the method of Salton and Horne (1951) or Cummins and Harris (1956). In neither study were the envelopes examined under the electron microscope. The peptidoglycan of Gram negative cells is clearly much more difficult to obtain than that of Gram positive cells.

Peptidoglycan

The basic structure of peptidoglycan in Gram negative bacteria is similar to that of Gram positive bacteria. The mechanism of attachment of the remainder of the wall to peptidoglycan is unknown (Luderitz et al. 1968).

Lipopolysaccharide

The numerous components of this fraction have been studied in detail only in the Enterobacteriaceae. Generally five sugars are present; glucose, galactose, glucosamine, and two sugars which are unique to the lipopolysaccharides, L-glycero-D-mannoheptose and 3-deoxy-D-manno-octulosonic acid (KDO) (Osborn 1969). It has been suggested that these sugars make up a "core" of the polysaccharide. Other sugars which occur in the lipopolysaccharide of some bacteria are mannose, galactosamine, mannosamine, rhamnose, fucose, ribose, xylose, the rare sugars 3,6 dideoxyhexoses and the rare hexosamines 2-amino-2,6-dideoxyhexoses and 3-amino-3,6-dideoxyhexoses (see Osborn 1969 for a review).

The many partial structures found and methods for degrading this fraction cannot be treated here. Luderitz et al.(1968) have catalogued the known information and discussed the proposed structures for

the lipopolysaccharide fraction. Adams and Singh (1970) recently proposed a structure for Lipid A based on the most recent evidence.

Moderately Halophilic Bacterial Cell Walls

The structure of the cell walls of moderately halophilic bacteria have not been studied extensively. The chemical composition of the cell walls of Micrococcus halodenitrificans and Vibrio costicolus was determined by Smithies et al.(1954). The amount of nitrogen was higher (about 13%) than in the non-halophiles which have a nitrogen content of 10%. The lipid content was similar to that of the Gram negative non-halophiles.

The cell wall carbohydrate (reducing substance) was unusually low; that of M. halodenitrificans was 0.5% and that of V. costicolus was 1.5% (including hexosamine). Most Gram positive and Gram negative non-halophilic cell walls have carbohydrate contents from 15 to 50%. During cell wall preparation, water washes could have removed some wall material as occurs in some marine organisms (Buckmire and MacLeod 1964) but the 3% carbohydrate content found for the whole cell is accounted for mainly as nucleic acid pentose which suggests the low value of the cell wall carbohydrate is correct. Smithies et al. (1954) pointed out that a low carbohydrate content may not be unique to halophiles because M. pyogenes also has a carbohydrate content of less than 2%.

Electron micrographs taken by Smithies et al.(1954) show that both the coccus and the vibrio yield round or elliptical cell walls with

much electron dense material. The addition of $Ca^{2+}$ to the culture media increased the thickness and toughness of the wall.

Takahashi and Gibbons (1959) extended the analysis of M. halodenitrificans cell walls. They modified the cell envelope isolation technique by heat-treating the cells before shaking with ballotini to avoid fragmentation which occurs with untreated cells. This treatment was shown not to affect the analysis of cells grown in 1.0 M NaCl.

The walls contained 10.5 to 12% nitrogen and 2.9 to 3.2% reducing sugar which contradicts the values found by Smithies et al. (1954) but Takahashi and Gibbons (1959) used a dairy grade NaCl containing small amounts of $Mg^{2+}$ and $Ca^{2+}$. This might have stimulated a an increase in carbohydrate content as the walls are known to be thicker when $Ca^{2+}$ is present (see above). The reducing sugars found were glucose, galactose, mannose, arabinose, xylose and hexosamine. They also found 16 amino acids including DAP and proline. The DAP:N ratio decreased with salt concentration in the media, which Takahashi and Gibbons suggested meant less cell wall material was produced at low salt concentration but it could also mean the cell wall chemistry had changed. The cell walls were shown to be chemically similar to those of other Gram negative bacteria.

Muramic acid has apparently not been specifically sought in these two organisms. However Kushner (1968) reports that Masui and Ohtani (personal communication) found muramic acid in a moderately halophilic coccus and in a pseudomonad.

Extremely Halophilic Bacterial Cell Walls

The halobacteria have a cell envelope that is different from those typical of both Gram positive and Gram negative bacteria (see Morphology). The outer layer disintegrates rapidly when the NaCl concentration is lowered.

The surface of Halobacterium cutirubrum (Kushner and Bayley 1963), H. halobium (Houwink 1956) and H. salinarium (Mohr and Larsen 1963) cells have a regular hexagonal pattern with a center to center distance of 120 - 150 A in shadowed preparations. In thin sections a trilaminar membrane is seen inside next to the cytoplasm and a dense border 130 - 150 A thick on the outside (Stoeckenius and Rowen 1967, Cho et al. 1967). Sometimes a regularly beaded appearance is seen on the edge of the outer border but the edge is often ragged. In the same study, Stoeckenius and Rowen found cells which had become spherical on lowering the salt (NaCl) concentration lost their surface pattern at about 1.6 M and started losing material from the envelope at 1.4 M. The loss of material is complete at 1.0 M but the membrane is still intact. Below 1.0 M the membrane starts to disintegrate. Similar results were found for H. salinarium (Steensland and Larsen 1969). The loss of material in low salt solution caused some difficulties in early electron microscope studies (Brown and Shorey 1963) until salt was added to the fixing and dehydrating solutions (Stoeckenius and Rowen 1967).

Brown and Shorey (1963) investigated the chemical composition of H. halobium and H. salinarium envelopes. Kushner et al. (1964) investigated H. cutirubrum envelopes. The envelopes were found to be mostly

lipid and protein. Envelopes of H. cutirubrum contained 45 - 57% protein and about 22% lipid. H. halobium envelopes had a protein content of 65% and H. salinarium envelopes a protein content of 75%. Reducing substances accounted for 6.2% of the envelopes of H. halobium, 5.2% of H. salinarium envelopes and 7.0% of H. cutirubrum envelopes. A large portion of the reducing substance can be accounted for as hexose and a small amount as hexosamine. Muramic acid and DAP were not detected. The envelopes of H. cutirubrum were also shown not to contain any D amino acids or teichoic acids (Kushner and Onishi 1968).

Since these envelopes do not posses several components of peptidoglycan several questions arise: 1.What are the structural components? 2. How is salt involved in maintaining the envelopes? 3. What gives form (rod shape) to the cell? The last question has not been definitely answered yet but enough information is available to give partial answers to the first two questions.

In 1963 Brown showed that the material obtained by dissolution of cell envelopes in water would sediment in an ultracentrifuge with a $s_{20,w}$ of 4 and that this material could be sedimented in half saturated ammonium sufate. Onishi and Kushner (1966) showed that on sedimentation in the presence of 0.1 M NaCl or KCl two components could be obtained. The amount of the second component increased with increasing salt concentration suggesting that it was an aggregate of material from the first component. Larsen (1967) reported purification of similar material on Sephadex and carboxymethyl cellulose columns. It migrated with one electrophoretic band and under the electron microscope appeared as particles with diameters of 100 - 200 A. The

composition was similar to that of cell envelopes (70 -75% protein and 20% lipid). Larsen (1967) reported the presence in this material of a 2- amino sugar, derived in the 3 carbon position which gave the Rondle - Morgan color of muramic acid but which did not behave chromatographically as muramic acid.

More recently Marshall et al. (1969) have used the disintegration caused by 1.0 M NaCl or 0.02 M $MgCl_2$ followed by differential centrifugation for isolating the outer layer of H. halobium cell envelopes. They reported that their preparation is almost exclusively protein (85.8 - 86.3%) and carbohydrate (3.9 - 8.2%). The preparation also contained 1.7% amino sugar and 3.1 - 3.2% nucleic acid but no lipid. Their preparation lacked glucosamine, galactosamine and muramic acid but contained another hexosamine. It contained uracil but not thymine.

Later Steensland and Larsen (1969) showed that H. salinarium strain 1 ( and other halobacteria) rapidly reforms closed vesicles when disrupted with glass beads in 4.3 M salt trapping some cytoplasm inside them. Thus considerable caution should be exercised in interpreting results from cell envelope preparations disrupted in high salt (4.3 M) concentrations. The amount of substances released and the changes which take place under various conditions depend on the strain being investigated. Corner and Marquis (1969) transferred Bacillus subtilis protoplasts made in 2.38 osmolal sucrose to solutions of lower osmolality by dialysis and by direct transfer. The protoplasts remained intact at much lower osmolality when transferred by dialysis. Thus, membrane failure was related to brittle fracture ( rate dependant) rather than to the ultimate tensile strength being exceeded. The usual methods of

transferring cells from high concentrations to low is to mix a small volume of cells at high concentrations with a large volume of diluent at the desired concentration but this could result in lysis by brittle fracture and should be taken into account.

Halophilic envelope proteins are acidic (Brown 1963, Kushner and Onishi 1966, McClare 1967, Steensland and Larsen 1969, Marshall et al. 1969) and it is accepted that salt is required to shield mutually repulsive groups. Brown (1965) titrated electrometrically for the available groups both in their native state (4.3 M NaCl) and in their dissociated state (in water). He found that all the carboxyl groups which were exposed in water could be accounted for in salt as well but many basic groups could not be detected in their native state. This suggested that the basic groups were"buried" in the native envelope and supported the idea that ions are necessary for shielding and conformation.

Brown(1964b) showed that a marine bacteria, whose envelopes normally disintegrated at about 1 - 2% NaCl, required 5% NaCl to prevent lysis after it was succinylated. Thus the addition of carboxyl groups to the surface made the envelope halophilic.

Several arguments have been presented for and against the use of terms such as "cell wall" and " cell membrane" in reference to the halobacteria. Stoeckenius and Rowen (1967) and Cho et al. (1967) referred to the morphological outer layer of the envelope as a cell wall but Marshall et al. (1969) consider the term inconsistent with the instability of the layer. It lyses before the membrane disintegrates. When the conformation of this layer is lost at lower NaCl concentrations

the organism becomes spherical. Therefore, it seems reasonable that the outer layer is required for the rod shape while the membrane maintains the cellular integrity ( but not viability). The ions $Na^{+}$, $K^{+}$, and $Mg^{2+}$ seem to be indispensible in maintaining the proper orientation of the parts.

The halococci are different from the halobacteria. They do not lyse in distilled water although they do leak. Light microscope observations indicate no change takes place when the cells are suspended in distilled water. That they are difficult to break mechanically is known (Dr. R.R.Colwell personal communication in Kushner 1968, Ueno 1964). The composition of the wall is unknown. It has been suggested (Brown 1964a) that the lack of muramic acid, and thus peptidoglycan, may be characteristic of extreme halophiles but whether muramic acid exists in the halococci is unknown. It was of special interest to compare these walls to those of halobacteria because of the unique chemical features of the latter (Table 1).

Chapter 4 describes an examination of halococci cell walls. A preliminary study of the walls begins by trying some relatively easy extraction methods. Their effectiveness is determined by chemical composition and where possible electron microscope examination. The chemical composition of the unextracted cell is determined for reference. Measurements of muramic acid in cells and walls are made so that the relationship of halococci to halobacteria, moderate halophiles, halotolerants and non-halophiles can be determined.

Table 1

Some important components of cell walls.

| | GluN | GalN | Mur | Hexose | Pentose | D-ala | Techoic | DAP | Lys | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Gram positive | | | | | | | | | | |
| Staph.aureus | + | +* | + | + | | + | + | – | + | Rogers and Perkins 1968 |
| M.lysodeikticus | + | | + | + | | + | – | – | + | Salton 1953 |
| B.subtilis | + | + | + | + | | + | + | + | – | " |
| Gram negative | | | | | | | | | | |
| E.coli | + | ± | + | + | ± | + | +? | + | + | " |
| Salmonella | + | ± | + | + | ± | + | | ± | + | " |
| Halophiles | | | | | | | | | | |
| M.halodenitrificans | + | | | + | + | | | + | + | Takahashi and Gibbons 1959 |
| H.cutirubrum | + | | – | + | + | – | – | – | + | Kushner and Onishi 1968 |
| H.halobium | + | + | – | + | | | | – | + | Kushner et al.1964<br>Steensland and Larsen 1969 |
| Acholeplasma (membranes)<br>(Mycoplasma) | + | + | – | + | + | | | – | + | Stoeckenius and Kunau 1967<br>Engelman and Morowitz 1968 |

* Stewart-Tull 1968

± depends on species or strain

Membranes

Membranes are the trilaminar structure which, in bacteria, encloses the cytoplasm and lies beneath the cell wall. They have been reviewed by Salton (1967), Rogers and Perkins (1968) and Korn (1969). Only halophilic membranes are considered here.

Moderately halophilic bacteria have lipids similar to those of non-halophilic bacteria but extremely halophilic bacteria have unique diether lipids (Kates et al. 1966). Those of Halobacterium cutirubrum have been extensively studied by Kates and co-workers. The major component of the lipids is the diether analogue of phosphatidyl glycerophosphate and the minor component is the diether analogue of phosphatidyl glycerol. Other diether components are a glycolipid and a glycolipid sulfate ester (Kates et al. 1967). The fatty acids of non-halophilic bacteria are replaced almost completely in extremely halophilic bacteria by dihydrophytol groups linked by diether bonds to glycerol. Sarcina littoralis, a halococcus, has more fatty acids (2.7%) than the halobacteria. The taxonomic importance of the lipids was discussed by Kushner (1968), who suggested that the diether lipids and the phytol chains be used as taxonomic characters. These lipids should be sought in a larger number of microorganisms in order to confirm the apparent uniqueness.

McClare (1967) investigated the bonding between lipid and protein complexes from envelopes of a strain of H. halobium. At the time of the experiments he was not aware of the presence of a cell wall but he contends that it only complicated the interpretation and did not invalid-

ate his conclusions. He separated envelopes into two fractions, one set by dialysis and another by lipid solvent extraction. He analysed the four fractions for amino acids, phosphorus, nitrogen and lipid. From solubility and recombination of components experiments he concluded that lipid is bound to one group of protein with hydrophobic interactions and to another with polar bonds and that these seem to be in the same complex. He proposed a tentative model based on chelation of magnesium by ε groups of protein and lipid phosphate. The model also included an ionic link between terminal phosphate of the lipid and arginyl groups of protein. The model accounted for the'buried'groups found in Brown's titration experiments (see above). His most controversial suggestion was that the membrane has only one layer and is therefore asymetric, as required for active transport.

Stoeckenius and Kunau (1967) devised a procedure for isolating membranes from halobacteria. After lysis of cells or envelopes by dialysis the membranes were separated by centrifugation. The membrane pellet was further separated into two fractions, the gas vacuole membranes and purple membranes on a sucrose gradient. The cell membrane as isolated from the sucrose gradient contained 40% lipid, 60 % protein and no hexosamine.

Ginzburg (1969) determined the permeability of a halobacterium to various solutes ( see above). She postulated that large pores exist in the membrane because large molecules (MW 40,000) penetrate easily and that the membrane must be asymetric in order to retain small molecules.

Stevenson (1966) showed that the uptake of L-glutamate by H. salinarium required an energy supply and high external $Na^+$ concentrations. The uptake was retarded by anaerobic conditions and dinitrophenol. KCl can replace NaCl in maintaining respiration but glutamate is not taken up. It was suggested that sodium plays a role in oxidative phosphorylation.

Enzymes

In early physiological studies on halophilism, enzymes were of particular interest. It was well known that enzymes from non-halophilic sources were rapidly "salted out" or inactivated by salt. If halophilic enzymes were the same, it was reasoned, then the intracellular salt concentration must be low. Robinson et al. (1952) studied nitrate reductase activity of Micrococcus halodenitrificans and found that the optimum salt concentration for enzyme activity was 0.4 M NaCl in whole cells and about half that for cell free extracts. Other studies have shown that this is not true for all enzymes. From Larsen's (1962) catalogue of studies on halophilic enzymes certain generalizations can be stated. The enzymes of halophilic bacteria are more salt tolerant than enzymes of non-halophiles and sometimes quite halophilic. The salt concentration for maximum enzyme activity is rarely the same as salt concentration for maximum growth. For example H. salinarium grows best and has maximum lactic dehydrogenase activity in 4 M NaCl but it has maximum desulfhyrase activity at 0.5 M. Enzymes of moderate halophiles are less salt tolerant and less salt dependant than those for extreme halophiles.

Larsen (1967) noted that potassium was usually at least as effective in activating the enzymes as sodium and often more so but Norberg and Hofsten (1968) found the opposite to be true for an extracellular proteinase. This is as expected because the internal potassium concentrations are high and sodium is low whereas in the medium the reverse is true. Halophilic enzymes also require salt for stability. They are denatured by lowering the salt concentration and generally

cannot be renatured by adding salt back quickly. Many enzymes can be restored to at least partial activity by dialysis against high salt concentrations. The ability to reactivate enzymes has contributed significantly to the purification of halophilic enzymes (Holmes and Halvorson 1965).

Ribosomes

Ribosomes from non-halophilic sources require small concentrations (0.001 M) of $Mg^{2+}$ for stability and are unstable in high concentrations of alkali halides (see review by Osawa 1968). This prompted an examination of ribosomes from Halobacterium cutirubrum (Bayley and Kushner 1964). The ribosomes were found to require 3 to 4 M KCl and 0.1 M $MgCl_2$ for stability (70s form) and they dissociated (31s and 52s form) if KCl was lowered to 2.0 M or $MgCl_2$ to 0.01 M. If the concentrations are lowered further more dissociation occurs. The protein components were found by electrophoresis to be acidic as with halophilic proteins rather than basic as with non-halophilic ribosomal proteins. Salt is necessary as with other halophilic proteins to shield repulsive groups. One fraction, rich in RNA, did, however, contain a basic protein. This fraction bound $K^+$ weakly and $Mg^{2+}$ stongly suggesting $Mg^{2+}$ is bound to RNA (Bayley 1966a).

Partial reconstitution of the dissociated ribosomal particles could be achieved by dialysing back the salts (Bayley 1966b). Bayley and Griffiths (1968a) prepared and studied a cell free peptide synthesizing system. As in non-halophilic systems it required $NH_4^+$ and $K^+$ for binding transfer (t) RNA but the system was truly halophilic since it required 3.8 M KCl, 1.0 M NaCl and 0.4 M $NH_4Cl$ for maximum activity. The aminoacyl t-RNA synthetase has been found to prefer but not require high KCl concentrations; NaCl and $NH_4Cl$ permit a lower activity (Griffiths and Bayley 1969). One molar NaCl is required for efficiency (Bayley and Griffiths 1968a) and fidelity of translation (Bayley and Griffiths 1968b).

## Nucleic Acids

The nucleic acids of the halobacteria and halococci are unusual because they contain a satellite DNA band, first shown by Joshi et al. (1963) and later investigated by Moore and McCarthy (1969a,b). The latter showed that the satellite bands were not of episomal origin nor were they the result of mixed cultures. Satellite DNA bands were found in six extreme halophiles, including a halococcus, but not in four moderately halophilic organisms.

The six extreme halophiles (one coccus and 5 rods) investigated by Moore and McCarthy had a G+C content of 66-68% in the major component and 57 -60% in the minor component which accounted for 11-36% of the total DNA. SL-1, the anaerobic photosynthetic obligate halophile of Raymond and Sistrom(1967), had a G+C of 70%. The base compositions are in good agreement with those of Joshi et al.(1963), Marmur et al.(1963) and Larsen (1967). G+C contents of 59.5 for Sarcina littoralis and 62% for S. gigantea were determined by Dr R.R.Colwell (personal communication) and they are also in close agreement with other halophilic bacteria considering that the two DNA bands (assumed to be present) were not separated in this study.

Base sequence homology was investigated by Moore and McCarthy (1969b). The five halobacteria were found to be closely related as indicated by DNA-DNA duplex formation and DNA-RNA hybridization. Two moderate halophiles and the halococcus showed little hybridization or duplex formation with DNA from the halobacteria.

The introduction has briefly dealt with classification of halophilic organisms, their morphology, nutritional requirements and physiology. The cell envelopes have been discussed and compared to the cell walls and envelopes of some non-halophilic bacteria. The membranes and the uniqueness of the extreme halophile lipids were touched on briefly. The enzymes and ribosomes and their salt requirements were also dealt with briefly. The nucleic acid, their unusual satellite bands and the G + C content were briefly reviewed. Some of the unusual properties probably are the result of physiological adaptation to an extreme environment but others do not seem to be.

Aim of Research

The remainder of the thesis deals with certain physiological and chemical properties of bacteria living in saline environments; presented in three parts. The first part (chapter 2) deals with the salt tolerance of organism present in and near the sea. It attempts to answer the question; are there potentially halophilic bacteria present in the marine environment? The second part (chapter 3) concerns the effect of salt concentration on the nutritional requirements of a moderately halophilic bacteria but before the effect was determined it was necessary to show that a population of moderately halophilic bacteria were genetically homogeneous in their salt response. The third part (chapter 4) deals with the questions; do the halococci lack muramic acid as do the halobacteria? and if not, what is the chemical composition of the halococcal cell walls?

# Chapter 2

## Salt Tolerance of Bacteria from the Sea and Seashore

This chapter reports a study of the distribution of salt response in bacteria from the sea and sea shore environment. The physical and microbiological survey was undertaken by Mr. D.B.Shindler and myself at the Fisheries Research Board Station in St. Andrews, New Brunswick. Under Dr. M. Gochnauer's guidance we investigated the environment and bacteria with the hope of finding a clue to the ancestry of halophilic bacteria.

Various investigators have attempted to 'train' non-halophilic bacteria to grow in high salt concentrations. Conversely, other workers have attempted to 'train' halophiles to grow in lower and lower salt concentrations. Some of the workers also used mutagenic agents (Larsen 1962). Larsen (1967) has, for example, attempted to grow two marine bacteria, one a Gram negative rod, the other a micrococcus in a high salt environment, with and without mutagenic agents. Despite the large populations used, he, as have other investigators, met with no success. As Larsen says "The most carefully conducted experiments rather support the contention that extreme halophilism is a quite permanent character and also that it is a character not easily acquired."

Extremely halotolerant organisms are known (Ingram 1957). Many micrococci, for example, are able to grow slowly in the presence of 25% NaCl (Larsen 1962). The isolation of halophilic and halotolerant organisms has been reviewed by Larsen (1962) and Ingram (1957). Both authors have been justifiably critical of workers who claim to have isolated obligate extremely halophilic bacteria from soil and fresh water sources because it is unlikely that the bacteria could survive

exposure to these environments and because of lack of detail in the earlier reports.

Since many extremely halophilic microorganisms are found in salt pans, salt lakes, which are sedimentary deposits of early seas, and other sources that are indicative of a marine origin it seems reasonable to postulate that halophiles evolved from marine species.

Shah and deSa (1964) isolated 217 halotolerant and halophilic bacteria from sea water, solar salt, soil, salted fish, dried fish, pickled fish and decomposed fish. A sample from the source was inoculated into an enrichment medium composed of aged sea water, peptone, 12% NaCl and ferric phosphate. From sea water, they isolated 6 terrigenous halotolerants (which grow well in nutrient agar containing up to 12% NaCl), 18 marine halotolerants ( which grow well in sea water agar but not in nutrient agar made with distilled water) and 5 moderate halophiles (which grow well in agar containing 6 to 9% NaCl) but they failed to isolate extremely halotolerant and obligately halophilic bacteria (which only grow in 15% NaCl and above). The classification of an organism was based on colony formation after 2 - 6 days incubation.

Some other less extensive investigations have been undertaken. MacLeod and Onofrey (1957) reported that three marine bacteria were inhibited by 0.8 M NaCl. Tyler et al.(1960) investigated 15 bacteria isolated from the sea. They found that all grew at 0.8 M NaCl, 9 grew at 1.4 M NaCl and none grew at 2.6 M NaCl within 48 hours in a medium composed of 1% Trypticase, aged sea water and NaCl. Brown and Turner (1963) examined 12 marine bacteria and found that none grew above 8%

NaCl in 6 days in a medium composed of peptone and NaCl.

These previous studies strongly suggest that extremely halotolerant bacteria are not present in the marine environment. However, none of the investigators used media which fulfilled the optimal requirements for growth of halophiles nor did any of them incubate their cultures long enough for the extremely halotolerant or halophilic organisms to develop. Most of the extremely halophilic bacteria require rich medium (such as yeast extract and Casamino acids) and additional $Mg^{2+}$ and $K^{+}$ for good growth but many, especially the halococci, still require several weeks for growth (see below).

Since laboratory strains of many marine bacteria when kept for long periods on enriched media have been shown to lose their requirement for NaCl (see MacLeod 1968 for a review), recent isolates from the marine environment were necessary.

It seemed possible that tidal pools might have a higher salt concentration than the sea and be conducive to the development of potential halophiles. Work was carried out on such pools near the St. Andrews Fisheries Research Board Station. This work, which included measurement of the pool ionic content, was primarily concerned with measuring the relative abundance of organisms of various salt tolerance in these ecological niches. Previous studies, which used enrichment cultures do not provide such information.

Materials and Methods with Notes

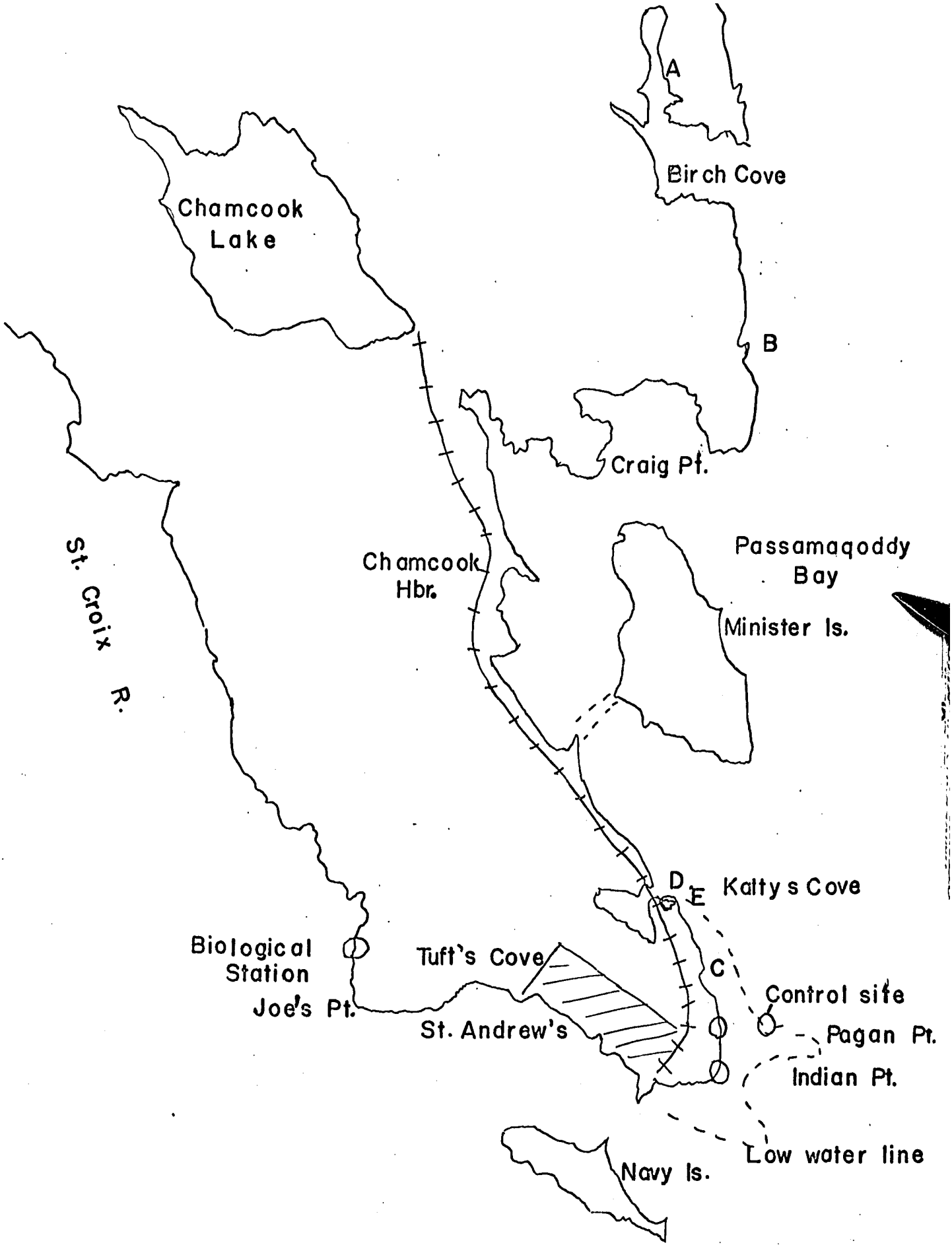
Geographical Survey. The entire area shown on Mapl was explored. The coast from the biological station around Navy Island and up to Wheaton Lake was examined in detail for likely tidal pools. The east side of Passamaquoddy Bay was not examined as closely since it was less accessible.

Sites. The selected pools were chosen because they had sufficient volume for sampling; could be reached relatively easily; were reasonably close to the high tide line, where maximum evaporation might occur and; were least subject to dilution by rain water run off. They gave a diversity of environments by chance. The chosen pools were at Birch Cove (A), "Yellow Cottage Site" (B), "Farmer's Cove" (C) and Katy's Cove (D and E).

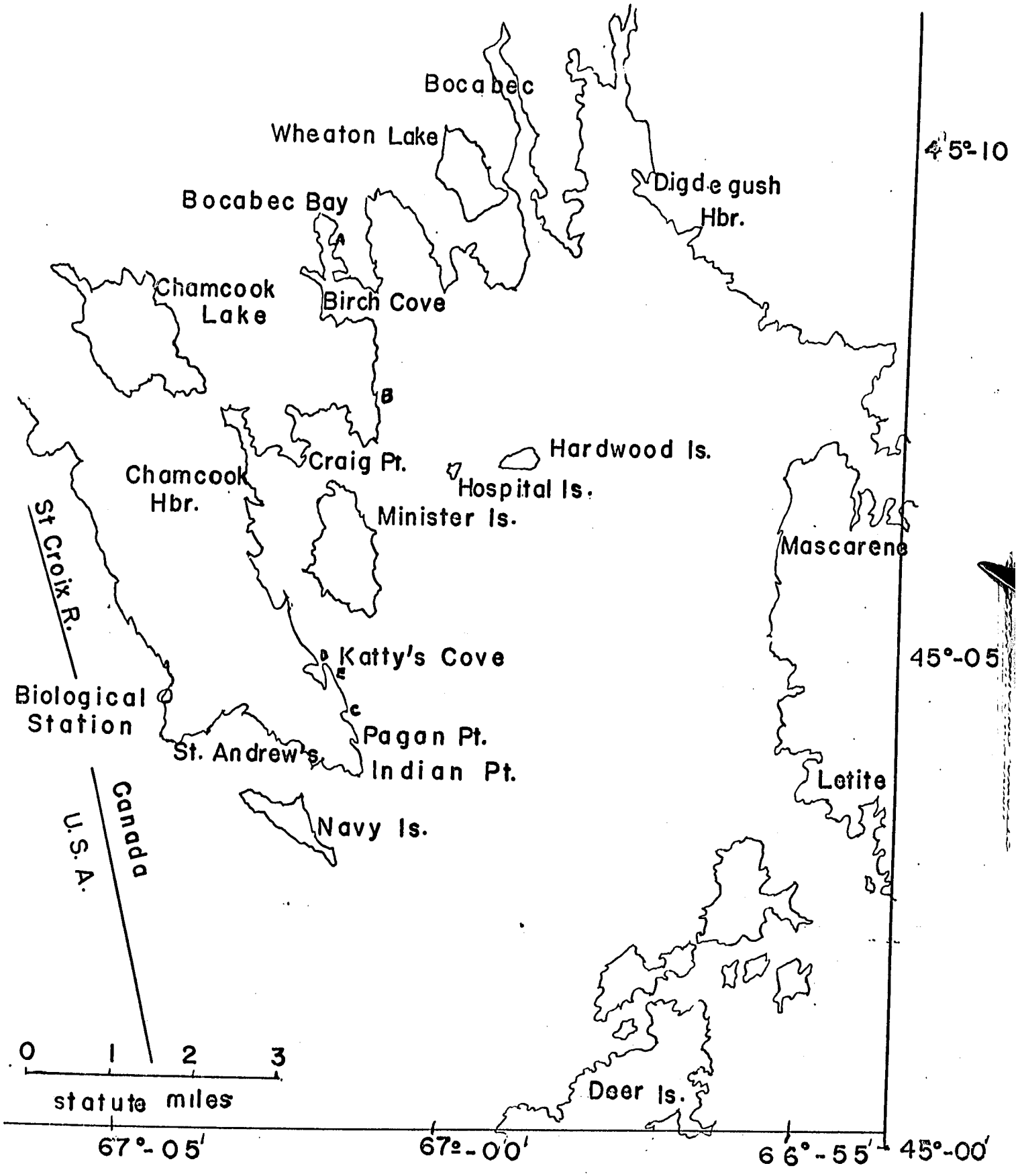
Site A was a marsh pool at the high tide line about 3 m in diameter and about 15 cm deep. It was isolated on rocks from the surrounding shore.

Site B was a true rock pool located on a rocky point. It was about 2 m diameter and about 15 cm deep and located at about the three-quarter tide line.

Site C was composed of many pools, some muddy, some rocky, invaded by the high tide. Near the ocean inlet a pool which was about 20 m diameter and 30 cm deep was sampled. The south side was a flat wooded area, the west a railroad track. The north was a long sloping hill with a farmhouse on the crest. The east side was a natural stone ridge erected by the sea with a gap 3 - 4 m wide.


A
Birch Cove
Chamcook
Lake
B
Craig Pt.
St. Croix R.
Chamcook
Hbr.
Passamaqoddy
Bay
Minister Is.
D
E
Katty's Cove
Biological
Station
Tuft's Cove
C
Joe's Pt.
St. Andrew's
Control site
Pagan Pt.
Indian Pt.
Low water line
Navy Is.

Map 1


Bocabec
Wheaton Lake
Bocabec Bay
Digdegush Hbr.
Chamcook Lake
Birch Cove
A
B
Craig Pt.
Hardwood Is.
Hospital Is.
Chamcook Hbr.
Minister Is.
Mascarene
St Croix R.
Katty's Cove
D
E
C
Biological Station
Pagan Pt.
St. Andrew's
Indian Pt.
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Navy Is.
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Canada
0 1 2 3
statute miles
Deer Is.
45°-10
45°-05
45°-00
67°-05'
67°-00'
66°-55'

Map 2

Sites D and E were in a large flat area similar to Site C. They were also surrounded by a stone ridge on the north and east sides, a steep hill on the south and a railroad track on the west. It is filled by a roaring torrent for about an hour at high tide. Site D was a marsh pool 3 m diameter and 10 cm deep which was always full and site E was a pool with a stony bottom about 400 m diameter and 1 - 2 m deep which loses volume, probably by seepage, after high tide.

The "control" site was located about 5 m off shore at Pagan Point on the side opposite the garbage dump effluent.

Sampling Procedure. Sampling was done on warm sunny days with high tide in the afternoon (preferably as late as possible). Weather and tide conditions permitted only a few samplings to be taken in the three week period available. Sites A and B were sampled twice on different days, the others were sampled only once.

At the site the air temperature, water temperature and salinity were recorded. Six hundred ml of raw water was collected in clean polyethylene bottles. A 3x3 sample (see Bacteriological Samples) was taken and the filtered water samples saved for analysis in clean polyethylene bottles. All samples were kept out of sunlight and as cool as possible.

Salinity was measured with a hydrometer calibrated in "%NaCl" (Fisher "Salimeter"). Temperature was taken with standard alcohol field thermometers. Air temperatures were taken in the shade provided by a convenient object. The sizes of the pools were estimated on site.

Bacteriological Samples. Just prior to going in the field, sterile Millipore pads were dampened with 1 ml of the nutrient broth and placed in 5 cm diameter petri dishes. These were placed in plastic bags for each site. Samples were taken on sterile Millipore HA filters using a Millipore water analysis filtration apparatus (Millipore Ltd., Montreal, P.Q.). Three 1 ml samples were taken and placed on nutrient pads, one at each salt concentration. Three 10 ml and three 100 ml samples were taken similarly. These nine samples (3x3) were taken at each site. All handling was done as quickly and cleanly as conditions would permit. The apparatus was washed with distilled water between sites. The petri dishes containing samples were re-sealed in plastic bags with masking tape and kept as upright and as cool as possible until returned to the laboratory. On return an additional 1 ml of nutrient broth was added to the petri plates and they were incubated in bags at 30°C to prevent evaporation over the long incubation period. Thirty degrees centigrade was thought to be the most likely temperature at which the desired organisms would develop. Most marine bacteria are probably psychrophilic and grow best at 20 - 25°C and do not grow above 30°C (MacLeod 1965). The known extremely halophilic bacteria require temperatures of 37 - 40°C for optimum growth but will grow slowly at 30°C. The final and determining factor was that none of the pools were above 30°C but some were very close to it.

The incubation time is probably a critical factor in the results that other workers have obtained. Six days of incubation, while being adequate for most marine bacteria, are inadequate for halophilic

organisms. The halobacteria under optimum conditions (37°C) require several days to grow and the halococci often require 7 days before growth is visible. At lower temperatures (30°C) these halophiles take longer. Some of the moderate halophiles while having an optimum temperature of 30°C require considerable time to grow when the salt concentration is higher than optimum (Forsyth and Kushner 1970). Therefore, a month was allowed for the organisms to form colonies and to grow in liquid media.

Media. The media was also particularly important. Halophiles are known to be fastidious, requiring both yeast extract and casein hydrolysate as used in this work for good growth. Both marine organism and halophilic organisms require higher concentrations of ions (other than NaCl) than most bacteria. The synthetic sea water, "Instant Ocean" (Aquarium Systems Inc.,Wickliffe, Ohio, USA.) presumably fulfills the ionic requirements because it is suitable for many fastidious organisms (Segedi and Kelley 1964). It contains (w/v) NaCl, 2.65%; $MgSO_4$, 0.66%; $MgCl_2$, 0.52%; $CaCl_2$, 0.13%; KCl, 0.071%; $NaHCO_3$, 0.02%; and traces of other ions. Aged sea water used by other investigators is variable in composition and may contain toxic substances (see ZoBell 1946 for a discussion).

The medium (broth) contained 0.1% yeast extract (Difco) and 0.4% Casamino Acids technical (Dico) made up in regular strength "Instant Ocean" sea water mix (about 3% NaCl). Additional NaCl (technical) was added to make the 10, 20, 25 and 30% NaCl concentrations. The "0" % NaCl media was made up with a salt solution (but not NaCl) prepared by Mr. D.B.Shindler to closely approximate "Instant Ocean". It contained

(w/v): $KHCO_3$, 0.025%; $CaSO_4 \cdot 7H_2O$, 0.0069%; $MgCl_2 \cdot 6H_2O$, 0.0305%; $MgSO_4$, 0.012%; $Ca(NO_3)_2 \cdot 4H_2O$, 0.00006% and the trace elements which were supplied from the trace element vial included in the " Instant Ocean" package. Agar (2%) was added if required. The medium was dispensed and autoclaved. The final pH was 7.5. Because sterilization facilities were not readily available at St. Andrews the medium was made in Ottawa, packaged in convenient bottles and tubes and shipped to St.Andrews (except for "0"%).

Measurements. Chloride was measured by titrating with mercuric nitrate. An excess of mercury forms a colored complex with diphenylcarbazone to indicate the end point (Schales and Schales 1941).

Sodium and potassium ion concentrations were measured with a Jarell-Ashe flame photometer (We are indebted to the Biochemistry Dept., Faculty of Medicine, University of Ottawa, for the use of the instrument).

Available phosphate was determined by the standard method for sea water analysis (Strickland and Parsons 1964) which depends on production of molybdenum blue, measured colorimetrically.

Bacteriological counts were made using a dissecting microscope when definite colonies had developed on the Millipore filter. Pure cultures were obtained by making a streak plate from each desired colony. These streak plates contained the same medium solidified with 2% agar at the same concentration used to isolate the organism. All of the purification and many of the counts were done on our return to Ottawa.

Samples from an isolated colony were streaked on an agar slant at the same concentration of salt, inoculated into a series of tubes to test for salt tolerance and smeared on a slide for Gram staining by the Kopeloff and Beerman modification of the Gram stain using Burke's counterstain. In this procedure acetone-ether is used for decolorizing (Conn and Pelczar 1957). Salt tolerance was tested by inoculating organisms into a series of broth tubes at "0", 3, 10, 20, 25,and 30% NaCl and incubating at 29-30°C. They were checked for turbidity visually at 2, 4, 7, 14, 21 and 28 days. Each tube was rated as "—" no growth, "(+)" possible growth, "+" slight growth up to "++++" very heavy growth. Only tubes rated + or higher were considered to have grown.

Lipids were extracted by the method of Bligh and Dyer (1959) and the infrared spectrum recorded on a Perkin-Elmer 457 spectrophotometer after drying the lipids on NaCl windows.

## Results

Physical Data. Table 2 shows that the water temperature in the tidal pools was higher than or the same as in the "control" site (sea water offshore), as would be expected in relatively shallow pools on a sunny day. The pH values were all similar to the "control". The salinity and the $Na^+$, $K^+$, $Cl^-$ and $PO_4^{3-}$ concentrations are either lower than or the same as the control. This indicates that some of the pools were probably diluted with fresh water. Sites C, D and E could all have been diluted by runoff from the surrounding land. Sites A and B were physically isolated and the mechanisms of dilution are unknown. Also included in the table are some standard values for sea water. The sea water values for $Na^+$, $K^+$ and $Cl^-$ are higher than the pools as are the $K^+$ and $Cl^-$ values for the control site but $Na^+$ for the control site is higher than that for sea water. The differences might be attributable to a difference in methods and to normal variation. The physical data indicate that halophilism or even extreme halotolerance would not be a selective advantage in these pools because even the moderate halophiles Vibrio costicolus and Micrococcus halodenitrificans require 0.5 M (3%) NaCl for survival and would probably not withstand the fluctuating salinity (Christian and Ingram 1959, Takahashi and Gibbons 1959). Slight halotolerance might, however, be an advantage.

Microbiological Data. Table 3 shows the number of bacteria in the environment that have various levels of salt tolerance. The number growing at 3% NaCl were those bacteria which would grow on our medium at the same ionic strength as sea water. The number growing at 10% NaCl were those which were salt tolerant up to at least 10% NaCl on

Table 2

Physical and chemical data on sea and tidal pools*

| Site | $A_1$ | $A_2$ | $B_1$ | $B_2$ | C | D | E | 'Control' | Sea water** |
|---|---|---|---|---|---|---|---|---|---|
| Water temperature °C | 25 | 24 | 21 | 17 | 28 | 29 | 25 | 17 | |
| pH | 8.8 | 8.5 | 8.2 | 8.1 | 8.4 | 9.0 | 8.6 | 8.3 | 8 |
| Salinity expressed as % NaCl | 3.7 | 3.5 | 3.7 | 3.5 | <2 | 2.5 | 3.0 | 3.7 | 3.77 |
| $Na^+$ mM | 460 | 465 | 465 | 445 | 370 | 410 | 450 | 530 | 469 |
| $K^+$ mM | 9.5 | 8.7 | 9.5 | 9.4 | 7.2 | 8.4 | 9.1 | 9.6 | 9.9 |
| $Cl^-$ mM | 470 | 470 | 490 | 480 | 330 | 410 | 460 | 500 | 543 |
| $PO_4^{-3}$ nM | 320 | 340 | 370 | 465 | 210 | 60 | 80 | 500 | < 33 - 3,300 |

* $A_1$, $A_2$, and $B_1$,$B_2$ represent samples taken on separate days from pools A and B between 1200 and 1400 hours (on flood tide) August 5 - 9 1969, weather sunny with no rain since last high tide.

** Values calculated from data in Handbook of Chemistry and Physics 40th edition.

Table 3

Number of colony forming units per milliliter in samples from various sites able to grow at 3, 10 and 20% NaCl.*

| %NaCl \ Site | A | $A_2$ | $B_1$ | $B_2$ | C | D | E | Control |
|---|---|---|---|---|---|---|---|---|
| 3 | 42 | 350 | 200 | >400 | 90 | 200 | 53 | 90 |
| 10 | 16 | 33 | 60 | >400 | 35 | 48 | 15 | 7 |
| 20 | 0.2# | 1 | 11 | 10 | 6 | 20 | 4 | 0.35# |

* colonies were allowed 1 month to grow.

# used 100ml. sample.

our medium. Similarly the number growing at 20% NaCl had a salt tolerance of at least 20% NaCl. The time required for colonies to develop at 20% NaCl was longer by several weeks than the time required for colony formation at 3% NaCl. It appears from the table that there is a higher percentage of salt tolerant organisms in tidal pools than in the ocean but this conclusion cannot be statistically justified because only one control sample was taken. The number of organisms tolerant to 20% NaCl is indeed higher than expected in all samples. High salt tolerance was not expected because it does not appear to be an ecological advantage except under special circumstances (e.g. on drying sea weed).

Table 4 shows the salt tolerance of certain selected bacteria. As many visually different colony types as possible were selected, therefore this sampling was not random. All of the bacteria whether they were isolated on 3, 10 or 20% NaCl grew at 3% NaCl; therefore, they were not more halophilic than marine bacteria. All the bacteria grew at 10% NaCl which was not expected on the basis of colony counts at 3, 10 and 20% NaCl, this perhaps emphasizes the non-random nature of the selection. Seventy one percent grew at 20% NaCl and about 11% grew at 25% NaCl and 3 of the 69 at 30%, albeit slowly. The incubation times at 25% and 30% NaCl were two weeks or more, as expected. Group I were the organisms isolated at 3% NaCl and they were never exposed to higher salt concentrations. Nine of the 21 tested were able to grow in 20% NaCl but not higher. GroupII organisms were isolated at 10% NaCl and they possessed slightly higher salt tolerance than group I organisms. Seven of the 22 tested were able to grow at 25% NaCl and two could grow

Table 4

Salt tolerance of isolates by site.

| | Site | A | A2 | B | B2 | C | D | E | 'Control' | Total number |
|---|---|---|---|---|---|---|---|---|---|---|
| I | Number of organisms isolated at 3% NaCl | 4 | 2 | 1 | - | 3 | 4 | 3 | 4 | 21 |
| | Number which grew at 0% NaCl | 2 | 0/1 | - | - | 2 | 3/3 | 2 | 1/3 | - |
| | 10% | 4 | 2 | 1 | - | 3 | 4 | 3 | 4 | 21 |
| | 20% | 0 | 0 | 0 | - | 2 | 3 | 2 | 3 | 9 |
| | 25% | - | 0/1 | - | - | 0/2 | 0/3 | 0/2 | 0/2 | - |
| | 30% | 0 | 0 | 0 | - | 0 | 0 | 0 | 0 | 0 |
| II | Number of organisms isolated at 10% NaCl | 5 | 1 | 2 | 1 | 3 | 5 | 6 | 3 | 26 |
| | Number which grew at 0% | 2/4 | 1 | 2 | 1 | 3 | 4 | 2/3 | 2 | - |
| | 3% | 5 | 1 | 2 | 1 | 3 | 5 | 6 | 3 | 26 |
| | 20% | 1 | 0 | 2 | 1 | 3 | 3 | 6 | 2 | 18 |
| | 25% | 0/2 | - | 0 | 0 | 1 | 1/4 | 4 | 1 | - |
| | 30% | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 2 |
| III | Number of organisms isolated at 20% | 3 | 3 | 2 | 1 | 3 | 6 | 2 | 2 | 22 |
| | Number which grew at 0% | 2/3 | 1/1 | - | 0 | 2 | 0/1 | 2 | 2 | - |
| | 3% | 3 | 3 | 2 | 1 | 3 | 6 | 2 | 2 | 22 |
| | 10% | 3 | 3 | 2 | 1 | 3 | 6 | 2 | 2 | 22 |
| | 25% | 0 | - | - | 0 | 0 | 1/2 | 1 | 0 | - |
| | 30% | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |

read 2/4 as 2of the 4 tested etc.

at 30% NaCl. Group III organisms were isolated at 20% and did not apparently possess higher salt tolerance than group II organisms. The most halotolerant bacteria came from sites C, D, E and the offshore site. This finding was surprising since sites C, D and E were the least saline sites at the time they were sampled (Table 2).

The times required before growth (lag phase) of bacteria isolated on various salt concentrations were roughly determined at various salt concentrations (Table 5). A few of the bacteria required more than a week before growth was visible in 0, 3 and 10% NaCl. At 20% NaCl a higher proportion required more than one week while at 25% none grew in the first week. At 30% only two grew and they required more than two weeks before growth was visible. This shows that adequate time must be allowed for growth. Those that required more than four weeks were considered to be unable to grow at that salt concentration.

To demonstrate that halophilic bacteria required more time to grow under our test conditions than is usually allowed, six extremely halophilic bacteria, Sarcina morrhuae, S. littoralis, S.gigantea, Halobacterium halobium, H. salinarium and H. cutirubrum and a very halotolerant H5 Micrococcus were grown under the same conditions but in their own medium (see Chapter 4). The halotolerant organism showed signs of growth in two days (it normally grows more rapidly than the others). S.littoralis showed some growth in about two weeks at which time S. morrhuae and S.gigantea showed no growth by the criterion used to judge the isolated bacteria. All three halobacteria showed growth on the sixth day.

Because S. gigantea was classified by Petter (1931) as a moderate

Table 5

Number of cultures showing visible turbidity (ca.0.05 OD at 660nm) in specified time periods when grown in various NaCl concentrations.

| NaCl concentration of growth medium,% | Lag period (days) | | | | | |
|---|---|---|---|---|---|---|
| | 0-8 | 9-14 | 15-21 | 22-28 | >28* | not tested** |
| "0" | 33 | 1 | 1 | 1 | 17 | 16 |
| 3 | 68 | 2 | | | | |
| 10 | 68 | 2 | | | | |
| 20 | 29 | 19 | 1 | 2 | 19 | |
| 25 | 0 | 7 | 0 | 1 | 35 | 26 |
| 30 | 0 | 0 | 2 | 0 | 67 | |

* i.e. no growth in 28 days

** cultures died or lost.

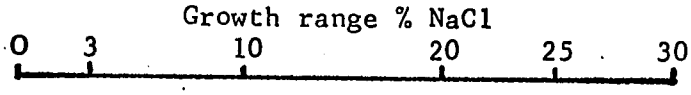
halophile capable of growth from 3 to 30% NaCl on agar at an optimum temperature of 20°C, it was decided to investigate S. gigantea, S. morrhuae and H5 Micrococcus further. Petter grew them on a medium composed of $MgSO_4$, 0.02%; $K_2HPO_4$, 0.02%; Peptone "Poulenc" 1% and NaCl. An identical medium, except that Difco Peptone was substituted, was used here. The NaCl concentrations were 0.5, 3, 6, 9, 12, 24 and 30%. S. gigantea grew on 12% NaCl on agar at 20 and 30°C but only on 24% at 37°C. H5 Micrococcus grew on 0.5 - 24% at 20°C and up to 30% at 30°C. At 37°C it grew on 3 to 24% NaCl. All three organisms seem to be more halophilic at higher temperatures. If this S.gigantea is also the same as that found by Petter then it seems to have developed a higher salt requirement (now 12% instead of 3%) over the 40 years since it was isolated (i.e. it has become an extreme halophile). Further work is necessary to trace the history of this culture to its original source, if possible.

Of the 69 bacteria isolated (Table 6) 52 were Gram negative rods, 9 were Gram positive rods, 4 were Gram negative cocci, 2 appeared to be Gram variable rods, 1 a Gram variable coccus and 1 a Gram negative vibrio. The Gram variable bacteria could possibly be classified as Gram negative or positive if specially cultured and processed. It is of interest that the organisms growing at 25% NaCl were 1 coccus, 1 vibrio, 7 Gram negative rods and 2 Gram positive rods. This showed that no particular Gram stain reaction is associated with halotolerance as it seems to be with halophilism (Brown 1964a). The large number of Gram negative rods isolated is probably a reflection of the relatively high numbers of Gram negative rods present in the marine environment (MacLeod 1965).

Table 6

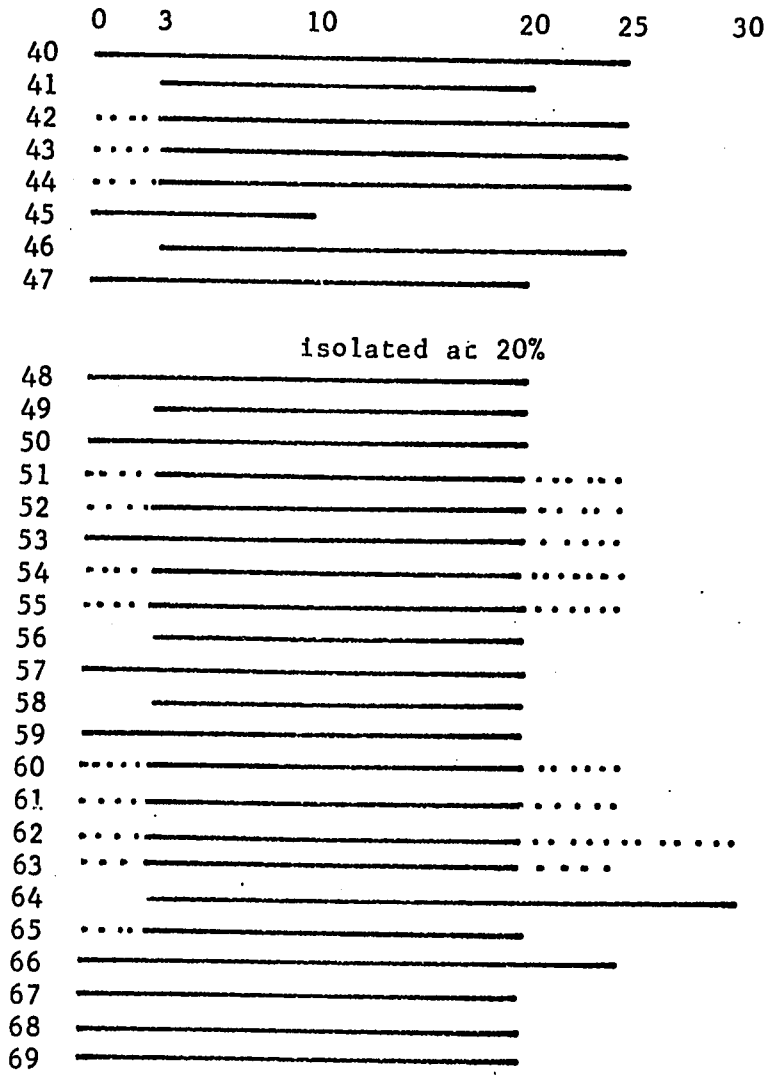
Salt tolerance and morphology of isolates

by NaCl concentration in isolation medium.




| | Pigment | Morphology Shape | Morphology Gram |
|---|---|---|---|
| **isolated at 3%** | | | |
| 1 | - | R | - |
| 2 | - | R | - |
| 3 | + | C | - |
| 4 | - | R | - |
| 5 | + | R | - |
| 6 | - | R | - |
| 7 | - | R | - |
| 8 | - | C | - |
| 9 | - | R | - |
| 10 | + | R | + |
| 11 | + | R | + |
| 12 | + | C | ± |
| 13 | - | R | - |
| 14 | - | R | - |
| 15 | - | R | - |
| 16 | + | R | ± |
| 17 | - | R | - |
| 18 | - | R | - |
| 19 | - | R | - |
| 20 | - | R | - |
| 21 | - | R | - |
| **isolated at 10%** | | | |
| 22 | - | R | - |
| 23 | - | R | - |
| 24 | - | R | - |
| 25 | + | R | - |
| 26 | - | R | - |
| 27 | - | R | - |
| 28 | - | R | - |
| 29 | - | R | - |
| 30 | - | C | - |
| 31 | + | R | + |
| 32 | - | C | - |
| 33 | + | R | - |
| 34 | + | R | + |
| 35 | + | V | - |
| 36 | - | R | - |
| 37 | - | R | - |
| 38 | - | R | - |
| 39 | - | R | - |

Table 6 (continued)




| | | | |
|---|---|---|---|
| 40 | - | R | - |
| 41 | - | R | + |
| 42 | - | R | - |
| 43 | - | R | - |
| 44 | - | R | - |
| 45 | - | R | - |
| 46 | - | R | - |
| 47 | - | R | - |
| 48 | - | R | - |
| 49 | - | R | - |
| 50 | - | R | - |
| 51 | - | R | + |
| 52 | - | R | - |
| 53 | - | R | - |
| 54 | - | R | - |
| 55 | - | R | - |
| 56 | - | R | - |
| 57 | - | R | - |
| 58 | - | R | - |
| 59 | - | R | - |
| 60 | - | R | - |
| 61 | - | R | - |
| 62 | - | R | + |
| 63 | - | R | - |
| 64 | - | R | - |
| 65 | - | R | + |
| 66 | - | R | - |
| 67 | - | R | + |
| 68 | - | R | - |
| 69 | - | R | + |

dotted line means not tested

R = bacillus C = coccus V = vibrio

Two of the bacteria showing high salt tolerance (numbers 33 and 40) were examined for the lipids characteristic of extreme halophiles. Kates et al. (1966) showed that the lipids extracted from extreme halophiles had weak ester-carbonyl bands at 1725 $cm^{-1}$ and a doublet at 1385-1375 $cm^{-1}$ attributed to $(CH_3)_2C$ groups. They showed that the lipids of non-halophiles and moderate halophiles had strong ester-carbonyl bands and the doublet at 1385-1375 $cm^{-1}$ is replaced by a single peak at 1387 $cm^{-1}$. The infrared spectra for the lipids examined here were of the latter type (non-halophilic or moderately halophilic) and were virtually indistinguishable from those of Kates et al. (1966).

Discussion

This brief study showed that a small percentage of the bacteria present in or near the ocean were able to grow in high salt concentrations. Previous studies have not shown this because the bacteria used were taken from enrichment cultures instead of directly from the environment.

The high salt tolerance of selected isolates was also shown. This study differs from previous studies because nutritionally richer medium was used and a greater time allowed for growth. These two factors, time allowed and nutritional adequacy of the medium, can account for the different conclusions of this and previous studies (see introduction). Christian (1955) reported that Salmonella oranienberg, a non-halophile, had a reduced salt tolerance range in nutritionally poor medium and that it grew very slowly in high salt concentrations. Forsyth and Kushner (1970) (and chapter 3) reported that Vibrio costicolus, a moderate halophile, behaved similarly.

These findings are even more remarkable when it is remembered that the tidal pools investigated were less saline than the sea. The low salt concentrations of the tidal pools is in keeping with the general concept of a salinity gradient from fresh to salt water. The pools were chosen because it was thought that they should be more saline than the sea, an assumption that was obviously incorrect.

# Chapter 3

# Study of Growth Response in Populations of two Moderately Halophilic Bacteria to Various Salt Concentrations and Influence of Salt Concentration on Nutrition of a Moderately Halophilic Bacterium.

Interest in the nutrition of halophilic bacteria arises from the idea that peculiarities in nutrition might lead to a possible explaination of their relationship to other bacteria. The nutrition of extremely halophilic organism has been studied but the small amount of information available has not yet yielded any clues. The nutrition of a moderate halophile has been investigated by Flannery and Kennedy (1962). None of the studies have used salt concentration as a parameter. Salt concentration is important because it is possible that an organism found to be halotolerant under nutritionally rich conditions may become halophilic when deprived of certain nutrients. An organism with the salt sensitive metabolic pathways suggested here has never been reported and probably has never been sought. Indeed the halophilic organisms already known have not been investigated as to the effect of salt concentration on their metabolic requirements.

Moderately halophilic bacteria grow over a wide range of salt concentration (0.5 - 3.5 M) and are therefore suited to this study and others which compare chemical or physiological properties at different salt concentrations. Extremely halotolerant bacteria would also have been suited but their existence was not known until the study reported here was complete. The nutrition of Vibrio costicolus had already been investigated by Flannery and Kennedy (1962) and it was chosen for further study. A detailed knowledge of this organism can also contribute to other studies such as the ribosomal studies by Dr. D.J.Kushner (unpublished).

Before undertaking any studies it was necessary to decide if all

the cells in the population could grow over the entire salt range or if some cells can grow better at higher or lower salt concentration. This question was examined in two moderate halophiles V. costicolus and Micrococcus halodenitrificans. The nutrition of the latter has not been investigated but knowledge of the genetic homogeneity of the culture will be useful in later work.

Materials and Methods

Organisms. Cultures of Vibrio costicolus No.508 and Micrococcus halodenitrificans No.509 obtained from the National Research Council of Canada, Ottawa, were maintained on slants containing 0.5% proteose peptone, 0.5% tryptone, 1 M NaCl and 1.5% agar at pH 7.0 and were transferred at monthly intervals. The cultures were grown for 24-48 hours at room temperature and then refrigerated until used to inoculate precultures.

Growth was measured turbidimetrically at 660 nm on a Coleman Junior spectrophotometer in 19 mm precalibrated tubes. A blank value was obtained from an uninoculated tube of the appropriate medium. Viable counts were made with drop plates (Campbell and Konowalchuk 1948). Cells were grown in tubes 19x150 mm inclined at 30° to the horizontal and shaken on a reciprocating shaker at 100 cycles per minute.

Precultures were grown in the medium to be tested or, where specified, in a simpler medium to avoid the carry over of nutrients from the complex medium. A 10 ml tube was shaken 24-48 hours at 30°C. Precultures used for determining amino acid requirements were washed twice and resuspended in 1 M NaCl.

Media. Salts glucose medium contained, per liter: $K_2HPO_4$, 3.12 g; $KH_2PO_4$, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NH_4Cl$, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; glucose, 10 g (autoclaved separately); NaCl as required, pH 7.6-7.7. The total concentration of salts other than NaCl is 0.073 M.

The medium developed by Flannery and Kennedy (1962) contains, per liter: glucose, 1.0 g; $K_2HPO_4$, 3.12 g ; $KH_2PO_4$, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaNO_3$, 1.0 g; NaCl, 69 g (1.2 M); L-cysteine, 0.1 g; L-glutamic acid, 1.0 g; L-arginine, 0.2 g; DL-valine, 0.2 g; DL-isoleucine, 0.2 g.

The total concentration of salts other than NaCl is 0.036 M. In Flannery and Kennedy's work the pH of this medium was adjusted to 7.0 and in this work to pH 7.5 Better growth was obtained at the higher pH (see Results). In both cases cysteine was sterilized by filtration. Glucose (1%) was autoclaved separately.

In complex media various combinations of vitamin-free casamino acids (Difco), proteose peptone (Difco) and tryptone (Difco) were used at a concentration of 1% each unless otherwise specified. It was found necessary to add 0.1 g/l $MgSO_4 \cdot 7H_2O$ to the casamino acids when used alone to obtain the best growth.

Trypticase soy broth (Baltimore Biological Laboratories) already contained .09 M NaCl: specified NaCl concentrations were added to this.

Replica Plates. Both organisms were grown until just turbid in proteose peptone + tryptone (0.5% each) broth containing 0.6 or 3.0 M NaCl. Cultures were diluted and plated out to yield separate colonies. Twenty to thirty colonies were transferred with a needle to other plates at the same NaCl concentration to yield good separation. This plate, when grown, was used to inoculate sterile velvet secured to a sterile wood block. Four plates (0.6, 1.0, 2.0 and 3.0 M NaCl) were inoculated by placing them on the velvet. The orientation of the plate was marked on the plate and maintained in the same position relative to the camera during photography. The plates were photographed with a 35 mm camera using Kodak Plus-X Pan Professional film at EI 320 (Development in Acufine). The plates were back lighted, the cover removed and a picture taken as quickly as possible to reduce the chance of contamin-

ation. Pictures were taken at 1, 2, 3,4, 6, 8 and 10 days after inoculation.

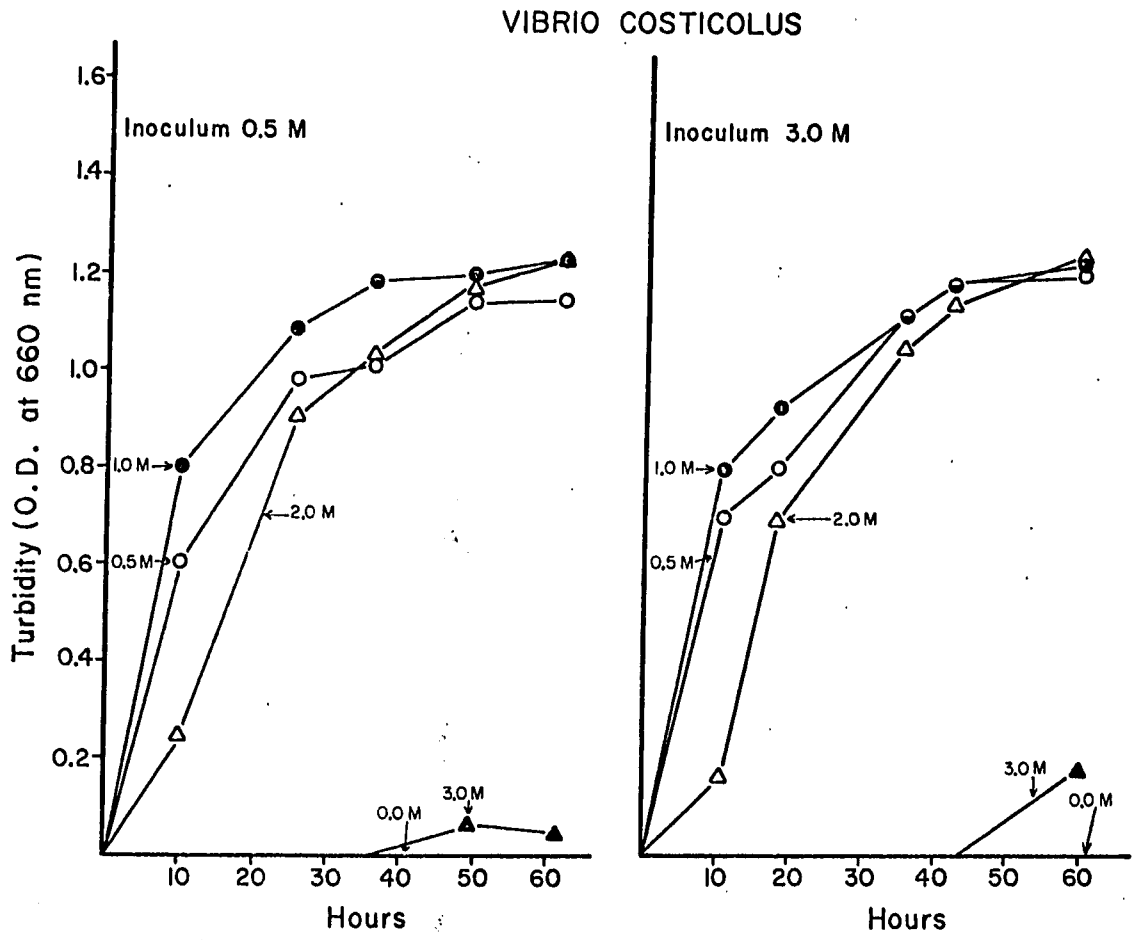
## Results

### Levels of Response to Salt in Populations of Moderate Halophiles

In order to test if all the cells in a culture of moderately halophilic bacteria had the same response to salt attempts were made to select populations that could grow best at the extremes of salt concentration. Both Vibrio costicolus and Micrococcus halodenitrificans were grown in trypticase soy broth with 0.5 M or 3.0 M NaCl added. Each culture was inoculated into a separate duplicate series of trypticase soy broth tubes with from 0 to 3.5 M NaCl added. For each microorganism the same type of growth curve was obtained, whether the inoculum consisted of cells grown in high or low salt concentrations (Figures 5 and 6). The same results as shown in these figures were also obtained if cultures were transferred twice at the high or low salt concentration before being inoculated into the series.

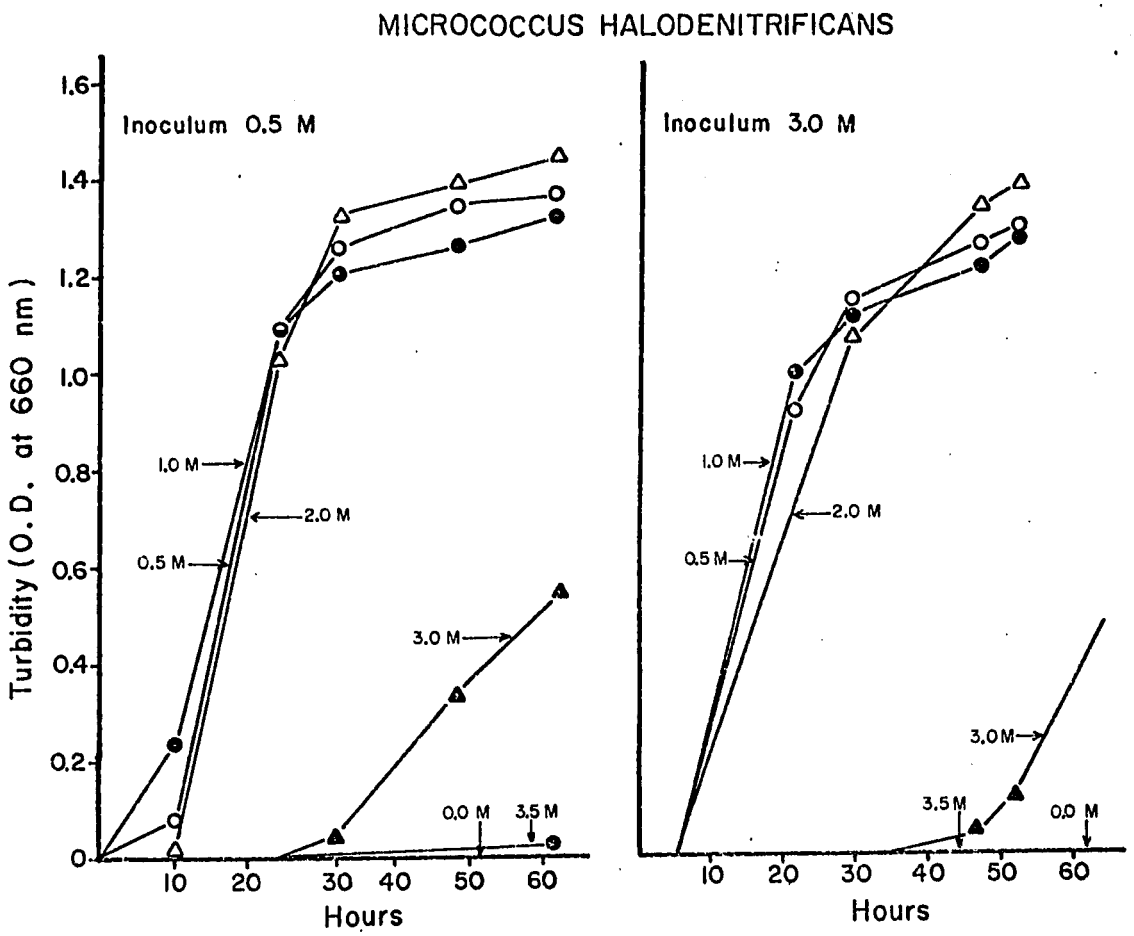
Another way of learning if the population is homogenous in its salt response is to find if all the cells can grow at all salt concentrations. To test this, cells were grown 24 hours in proteose peptone + tryptone (0.5% each) broth containing 0.6 M NaCl or 3.0 M NaCl. Cultures were diluted with the same NaCl concentration as in the growth medium and plated in triplicate on proteose peptone + tryptone (0.5% each) agar containing different concentrations of NaCl.

No colonies of M. halodenitrificans developed in agar containing 0.1 or 4.0 M NaCl (Table 7). Between 0.4 and 2.5 M NaCl about the same number of colonies developed from M. halodenitrificans grown in 0.6 M or in 3.0 M NaCl. On agar containing 3.0 M NaCl the same number of colonies of M. halodenitrificans precultured in 3.0 M NaCl




Effect of preculture conditions on salt response of V.costicolus. For results on the left, inocula (0.02ml) from a 22 hour preculture in trypticase soy broth + 0.5 M NaCl were added to tubes containing 10 ml trypticase soy broth and NaCl at the concentrations shown by each curve. After 62 hours 0.02ml inocula of the culture containing 3.0 M NaCl were added to a fresh series of tubes, to give the results shown on the right.

Figure 5




Effect of preculture conditions on salt response of M.halodenitrificans. See figure 5 for details.

Figure 6

Table 7

Viable counts on agar containing different salt concentrations

| NaCl concentration in agar, M | NaCl concentrations at which inoculum was grown: 0.6 M — Dilution | 0.6 M — Mean number of colonies ± S.E. | 0.6 M — Development time, days | 3.0 M — Dilution | 3.0 M — Mean number of colonies ± S.E. | 3.0 M — Development time, days |
|---|---|---|---|---|---|---|
| | | *Micrococcus halodenitrificans* | | | | |
| 0.1 | $10^{-4}$ | 0 | 20 | $10^{-4}$ | 0 | 14 |
| 0.4 | $10^{-7}$ | 31 ± 1.9 | 2 | $10^{-6}$ | 118 ± 4.3 | 2 |
| 0.5 | " | 32 ± 1.6 | 2 | " | 121 ± 8.0 | 2 |
| 1.0 | " | 34 ± 1.8 | 2 | " | 102 ± 0.5 | 2 |
| 1.5 | " | 36 ± 1.4 | 2 | " | 108 ± 2.6 | 2 |
| 2.0 | " | 34 ± 4.9 | 2 | " | 105 ± 3.3 | 3 |
| 2.5 | " | 39 ± 6.7 | 4 | " | 108 ± 1.0 | 14 |
| 3.0 | " | 20 ± 2.9 | 10 | " | 119 ± 2.8 | 14 |
| 3.5 | " | 6.4 ± 4.0 | 20 | " | 78 ± 7.0 | 14 |
| 4.0 | $10^{-4}$ | 0 | 20 | $10^{-4}$ | 0 | 14 |
| | | *Vibrio costicolus* | | | | |
| 0.1 | $10^{-4}$ | 0 | 14 | $10^{-6}$ | 15 ± 0.3 | 3 |
| 0.4 | $10^{-5}$ | 91 ± 2.4 | 5 | " | 23 ± 2.2 | 3 |
| 0.5 | " | 116 ± 2.6 | 2 | " | 26 ± 1.6 | 3 |
| 1.0 | " | 114 ± 5.1 | 2 | " | 29 ± 1.5 | 3 |
| 1.5 | " | 58 ± 4.1 | 3 | " | 22 ± 0.6 | 3 |
| 2.0 | " | 38 ± 2.0 | 5 | " | 19 ± 3.1 | 3 |
| 2.5 | " | 20 ± 0.3 | 5 | " | 17 ± 1.3 | 5 |
| 3.0 | " | 18 ± 1.7 | 14 | " | 13 ± 1.1 | 14 |
| 3.5 | $10^{-4}$ | 14 ± 2.5 | 14 | $10^{-5}$ | 37 ± 7.0 | 14 |
| 4.0 | $10^{-4}$ | 0 | 14 | $10^{-4}$ | 0 | 14 |

developed as at lower salt concentrations; however, relatively fewer colonies developed from cultures grown on 0.6 M NaCl medium. On agar containing 3.5 M NaCl fewer colonies of both cultures developed, the relative drop in numbers being greater in cells cultured in 0.6 M NaCl.

Cultures of V. costicolus (Table 7) grown in 3.0 M NaCl produced very small colonies on agar containing 0.1 M NaCl. These probably grew in the local high salt environment created by the inoculum, an interpretation strengthened by the fact that cultures grown in 0.6 M NaCl produced no colonies on 0.1 M NaCl.* V. costicolus grown in 3.0 M NaCl developed about the same number of colonies on agar containing 0.4 to 2.5 M NaCl. Less than one half as many developed at 3.0 M NaCl as at 1.0 M. V. costicolus grown in 0.6 M NaCl developed the most colonies at 0.5 and 1.0 M NaCl. The number decreased as the salt concentration in the agar increased; approximately one sixth as many colonies developed at 3.0 M as at 1.0 M NaCl.

These results suggest that some cells are damaged on being transferred from a lower to a higher salt concentration but not on

* To check this point, drops (0.025 ml as used in our plating method) of 3 M NaCl were placed on the dry surface of 1.5% agar. At intervals of time part of the surface was flooded with 5% $AgNO_3$. For comparison, a series of drops of different NaCl concentration was placed on agar and the agar flooded with $AgNO_3$ immediately after the drops had sunk in. Relative intensities of the precipitates indicated that a concentration corresponding to at least 0.5 M NaCl, precipitated immediately, remained beneath the 3.0 M drops for more than 2 days.

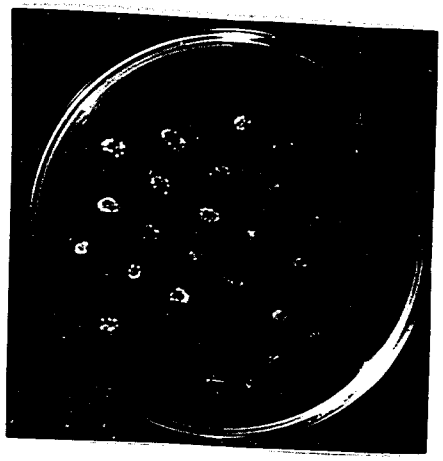
being transferred from a higher to a lower. The smaller number of colonies appearing in higher salt concentrations may be correlated with the slower growth rate, in that the progeny of some cells may have died before forming visible colonies.

Homogeneity of salt response was also tested by the replica plating technique (Plates 1 - 8 and Table 8).

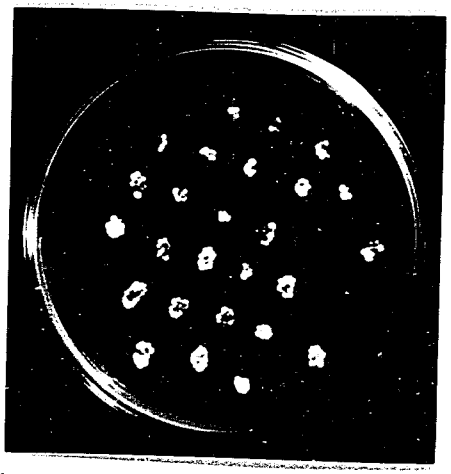
V. costicolus replicated from plates containing 0.6 M salt (Plates 1 and 2) grew best in the salt concentration range 0.6 - 2.0 M and slightly more slowly in 3.0 M; in all salt concentrations growth was complete in ten days (Table 8). Cells replicated from plates containing 3.0 M NaCl (Plates 3 and 4) gave the same results, except that colonies in 0.6 M salt developed more slowly during the first day and cells in 3.0 M grew faster than 0.6 M inoculum cells on 3.0 M.

The results with M.halodenitrificans (Plates 5 - 8) were similar to those found by viable counts (Table 7). All colonies from 3.0 M inoculum grew completely in 3 days in 1.0, 2.0 and 3.0 M salt as did colonies from 0.6 M inoculum on 1.0 M salt (Table 8). Growth in 0.6 M salt was slower. Cells grown on 0.6 M salt developed colonies very slowly in 2.0 and 3.0 M salt; at 10 days one-third of the colonies had not developed at all on 3.0 M whereas those originally grown on 3.0 M NaCl were completely grown by the third day.

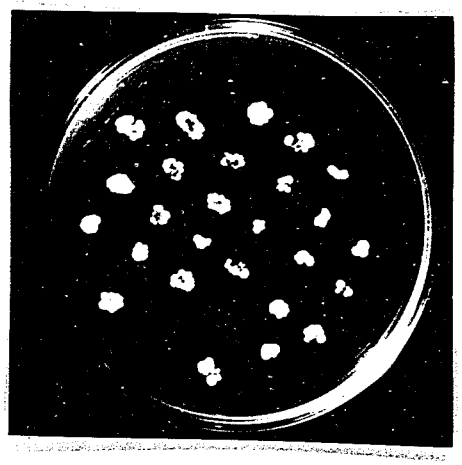
In summary the replica plates show that: In almost all cases every colony in the parent plate gave rise to colonies in the plates of agar at different salt concentrations. The rate of growth of all colonies on a given plate appeared the same. It was difficult, however, to be certain of this, because replication did not transfer the same



1 day

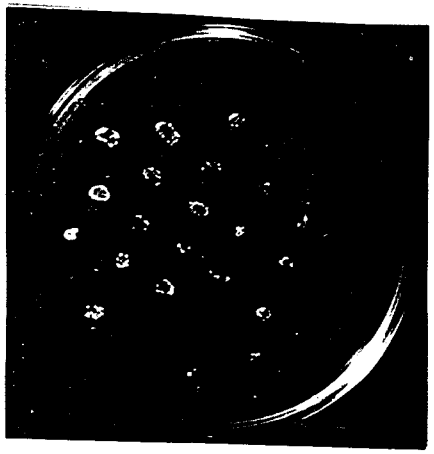


2 days

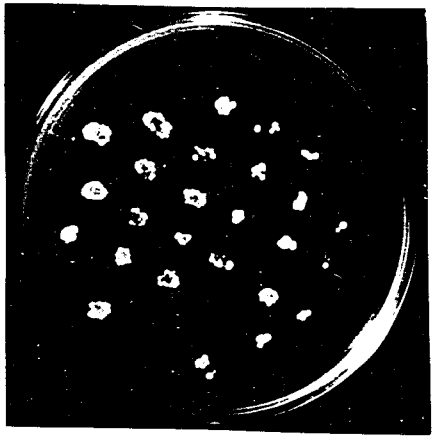


3 days

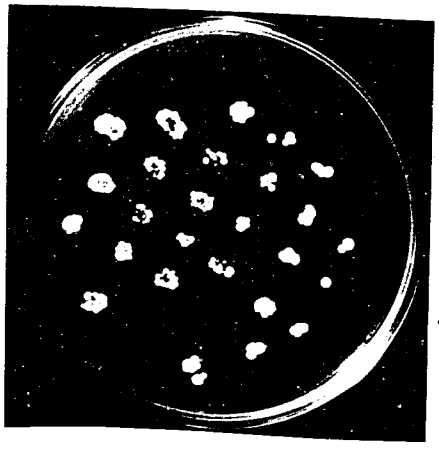
Vibrio costicolus grown in 0.6M NaCl transferred to 0.6 M.



1 day



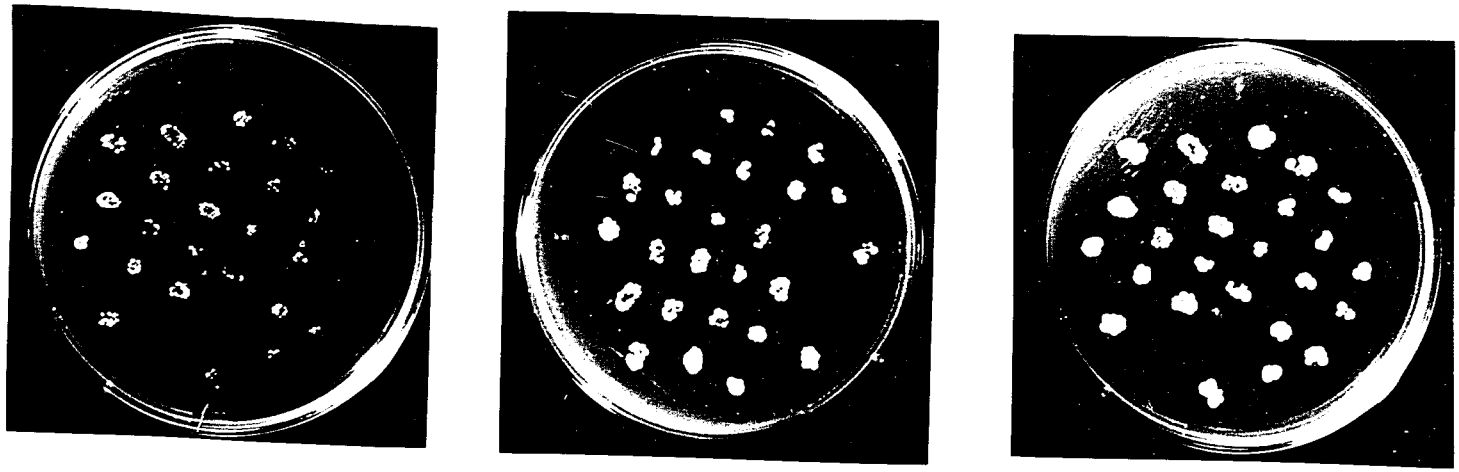
2 days



3 days

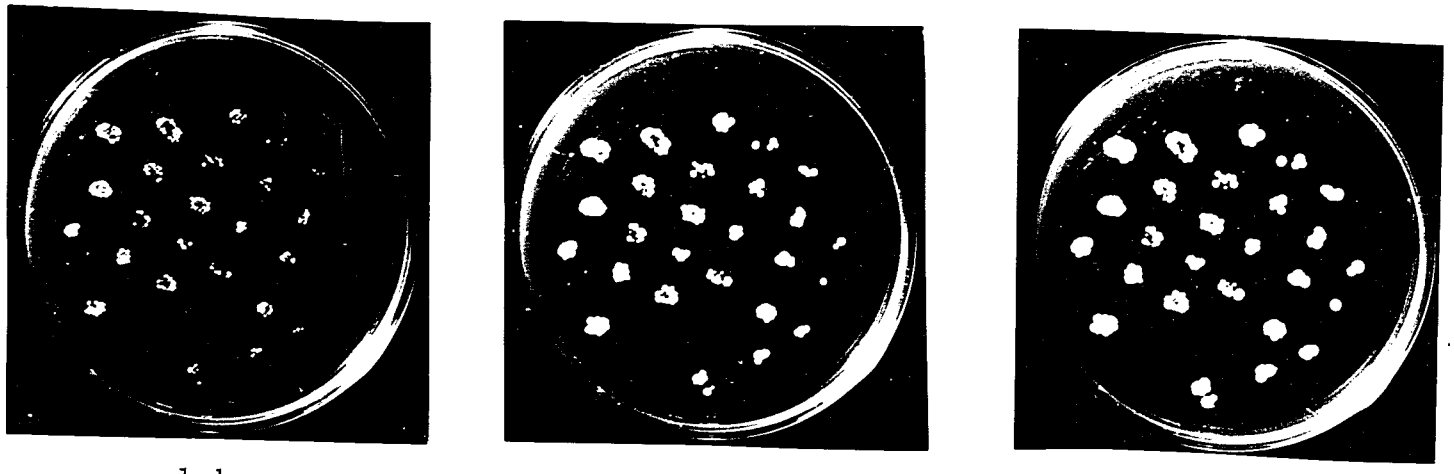
Vibrio costicolus grown in 0.6 M NaCl transferred to 1.0 M.

Plate 1.



1 day  2 days  3 days

Vibrio costicolus grown in 0.6M NaCl transferred to 0.6 M.

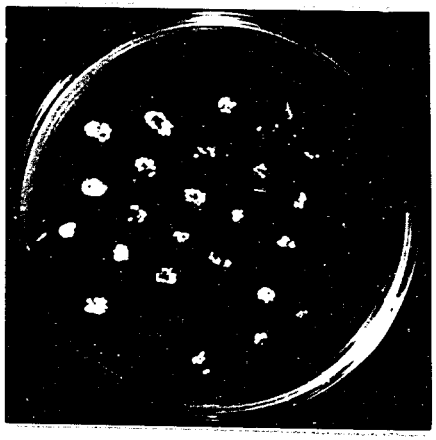


1 day  2 days  3 days

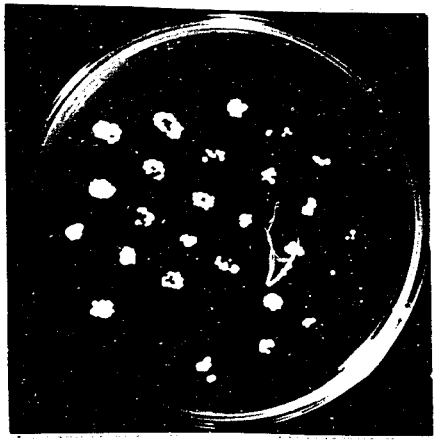
Vibrio costicolus grown in 0.6 M NaCl transferred to 1.0 M.

Plate 1.

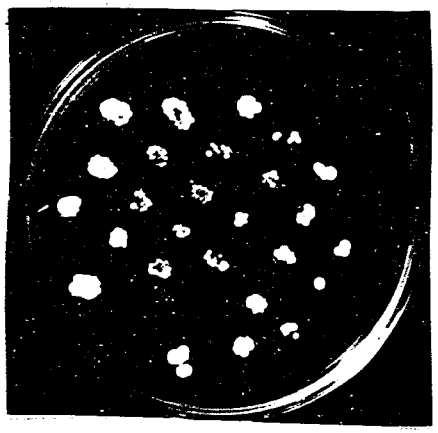
1 day



2 days

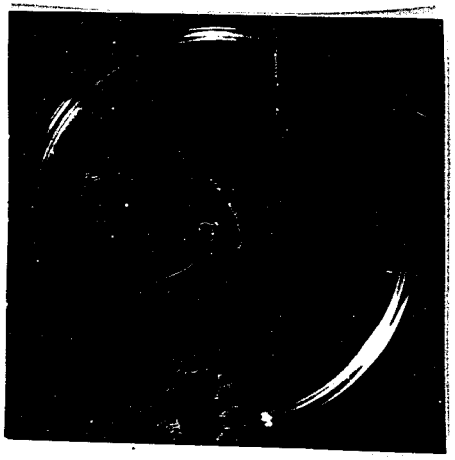


3 days



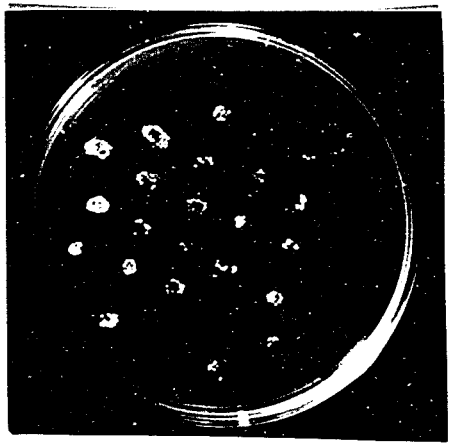
10 days

Vibrio costicolus grown in 0.6 M NaCl transferred to 2.0 M.

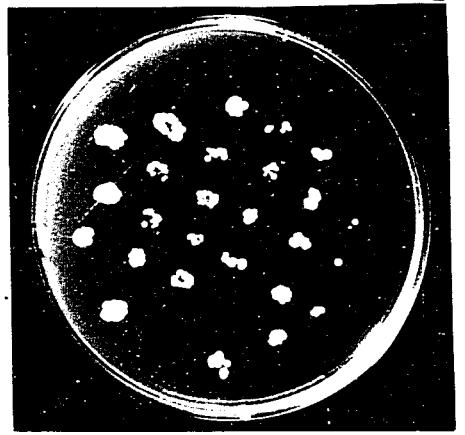


1 day

2 days



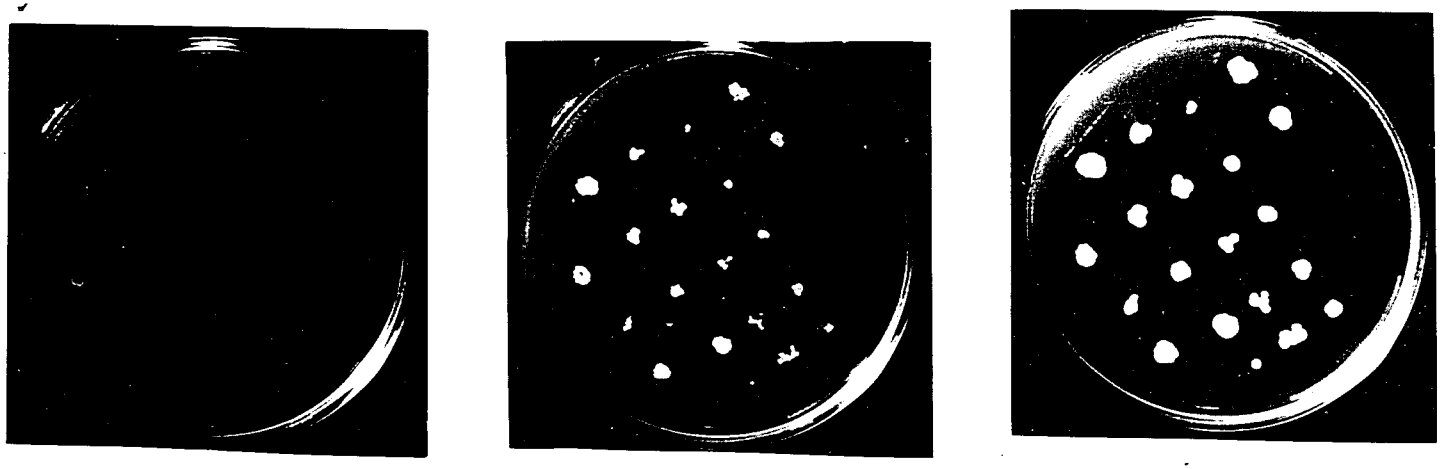
3 days



10 days

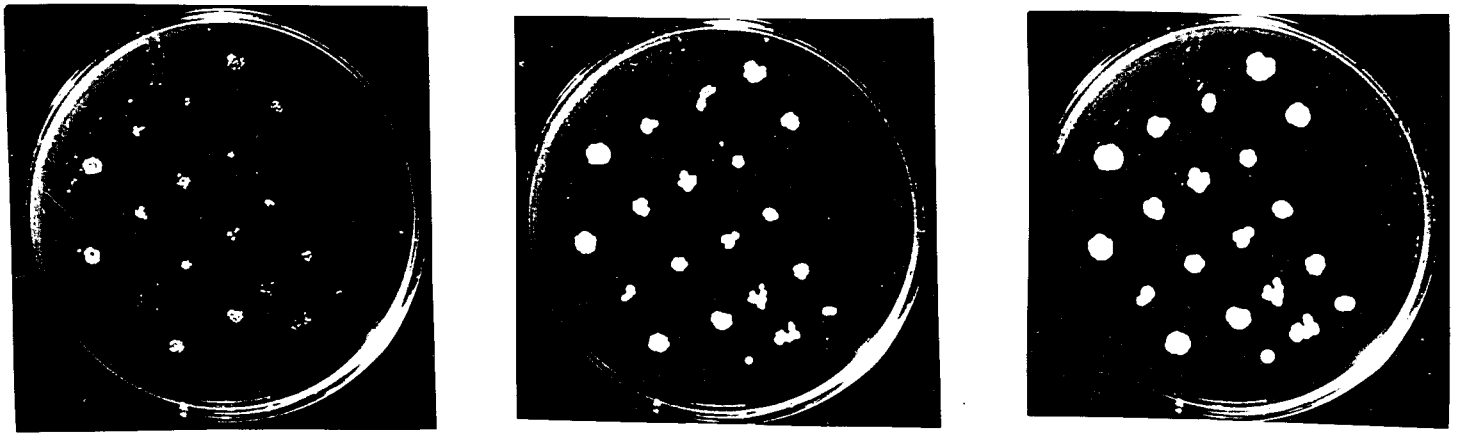
Vibrio costicolus grown in 0.6 M NaCl transferred to 3.0 M.

Plate 2



1 day 2 days 3 days

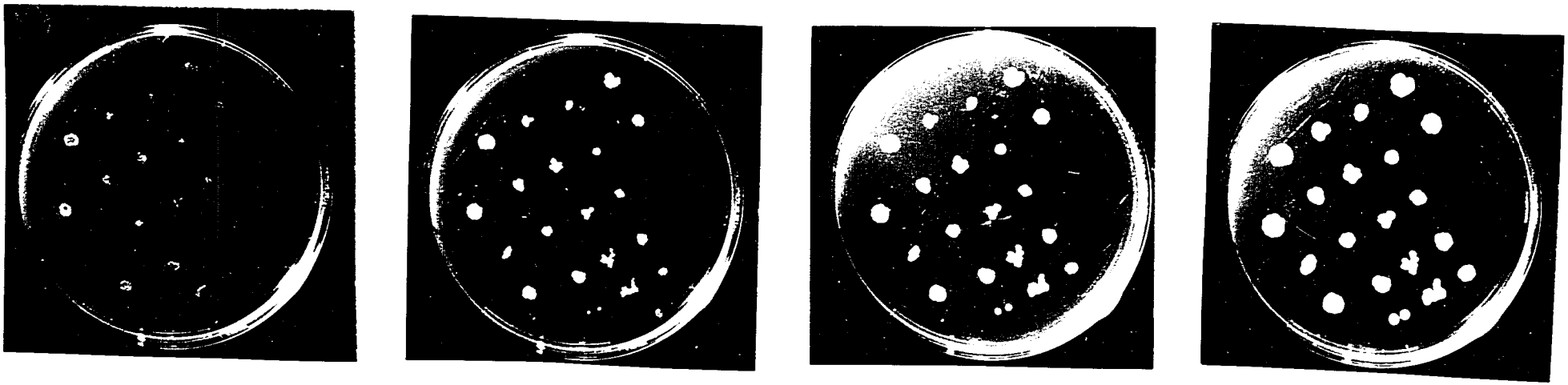
Vibrio costicolus grown in 3.0 M NaCl transferred to 0.6 M.



1 day 2 days 3 days

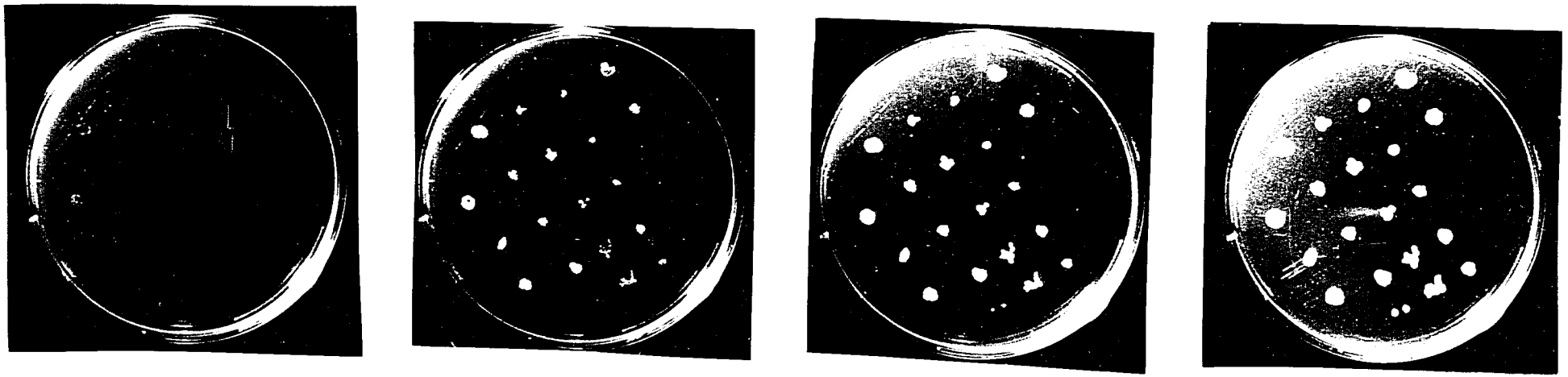
Vibrio costicolus grown in 3.0 M NaCl transferred to 1.0 M.

Plate 3



1 day 2 days 3 days 8 days

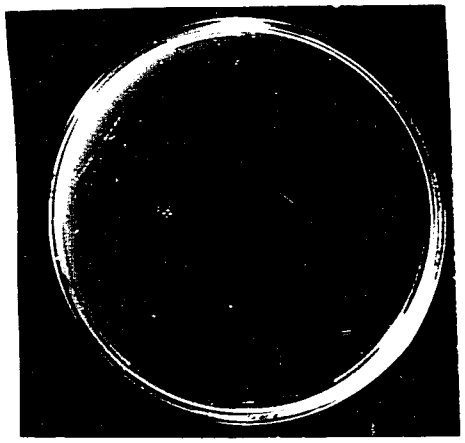
Vibrio costicolus grown in 3.0 M NaCl transferred to 2.0 M.



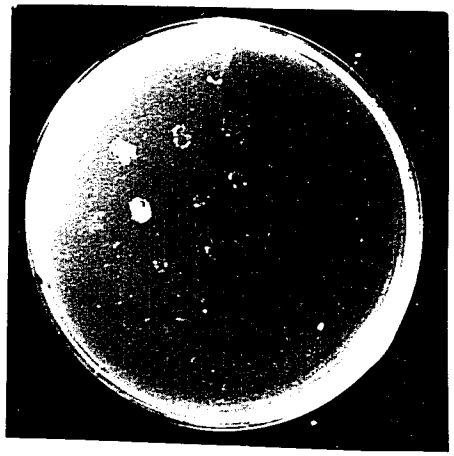
1 day 2 days 3 days 8 days

Vibrio costicolus grown in 3.0 M NaCl transferred to 3.0 M.

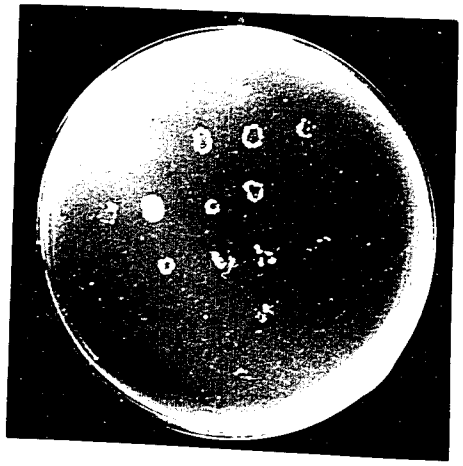
Plate 4



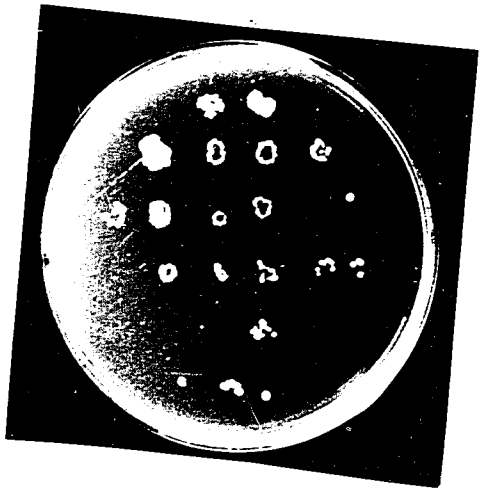
1 day



2 days

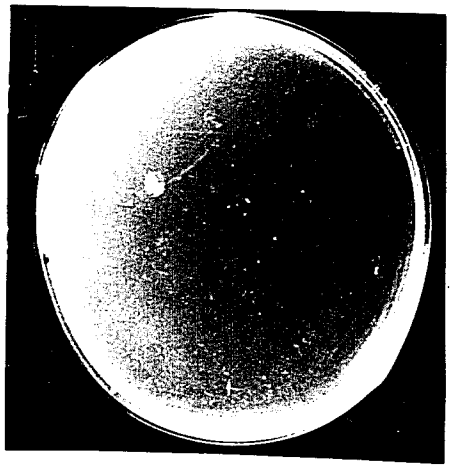


3 days

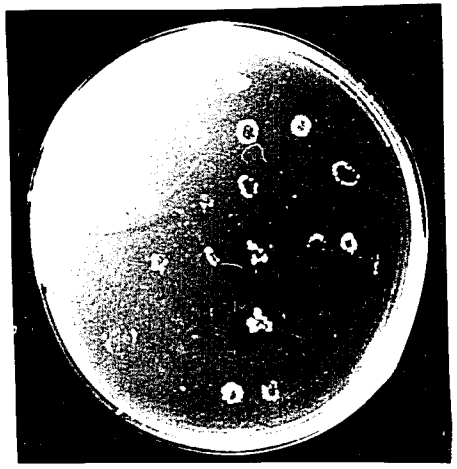


10 days

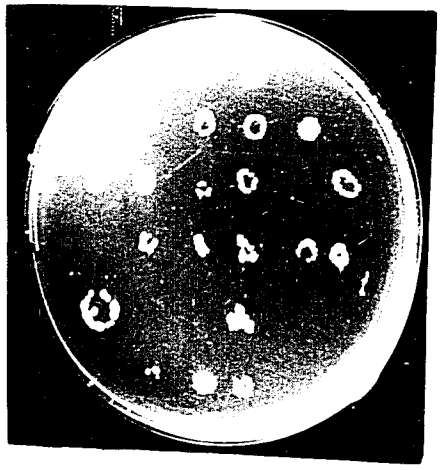
Micrococcus halodenitrificans grown in 0.6 M NaCl transferred to 0.6 M.



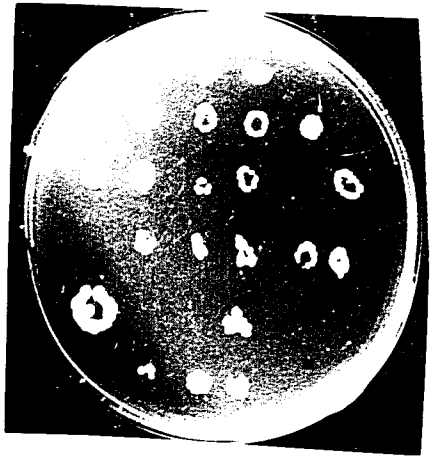
1 day



2 days



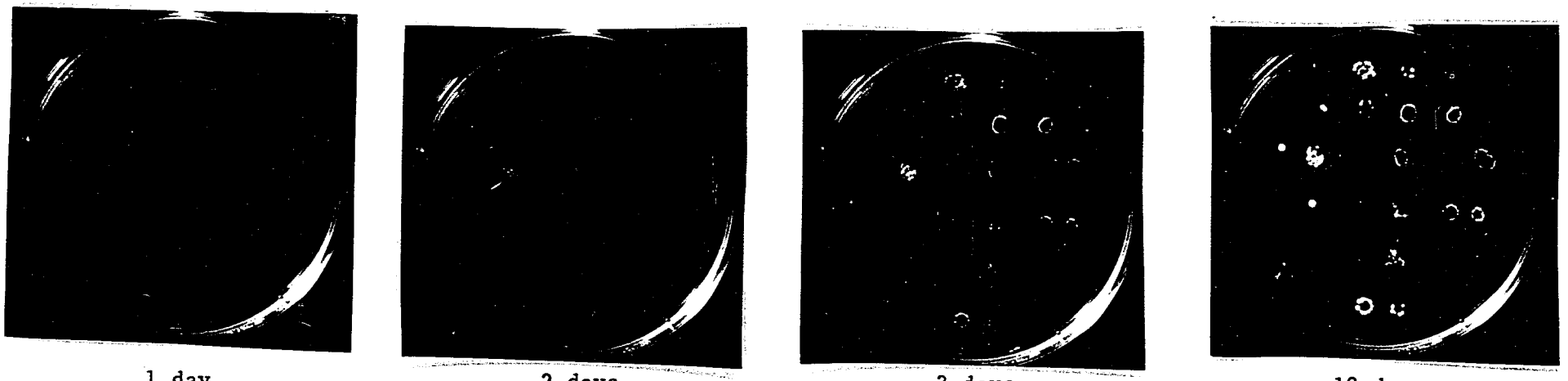
3 days



10 days

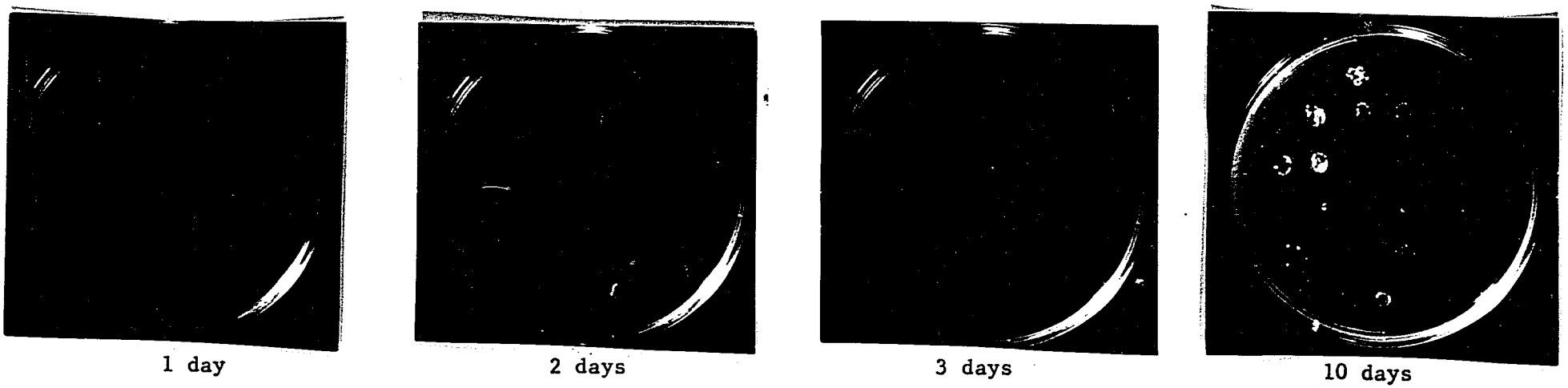
Micrococcus halodenitrificans grown in 0.6 M NaCl transferred to 1.0 M.

Plate 5



1 day 2 days 3 days 10 days

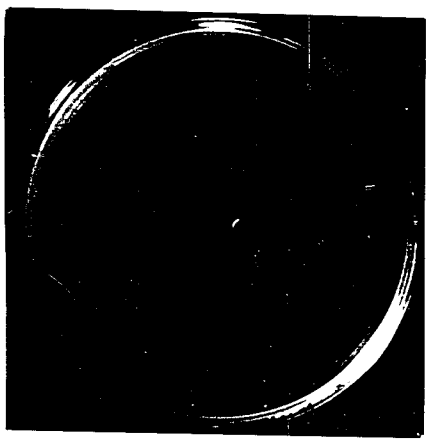
Micrococcus halodenitrificans grown in 0.6 M NaCl transferred to 2.0 M.



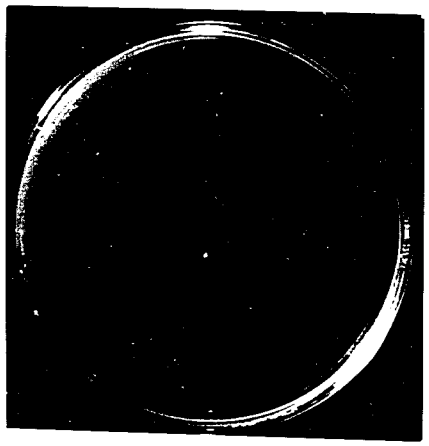
1 day 2 days 3 days 10 days

Micrococcus halodenitrificans grown in 0.6 M NaCl transferred to 3.0 M.

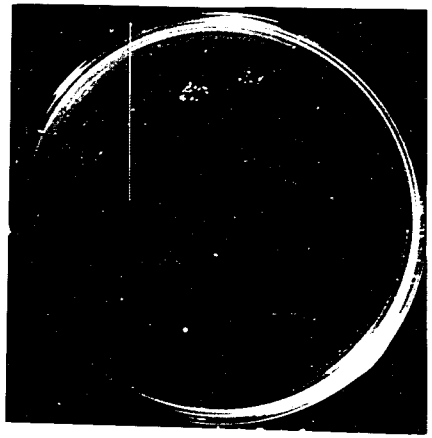
Plate 6



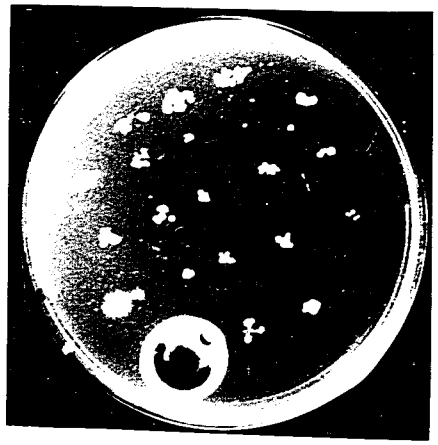
1 day



2 days

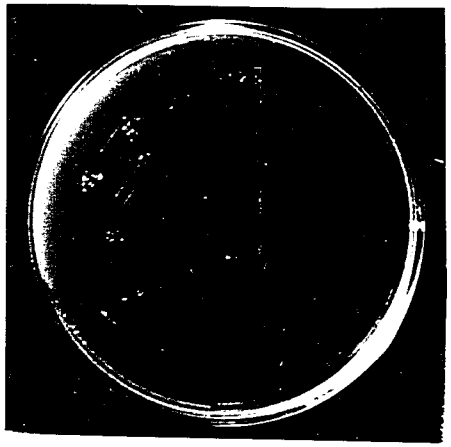


3 days

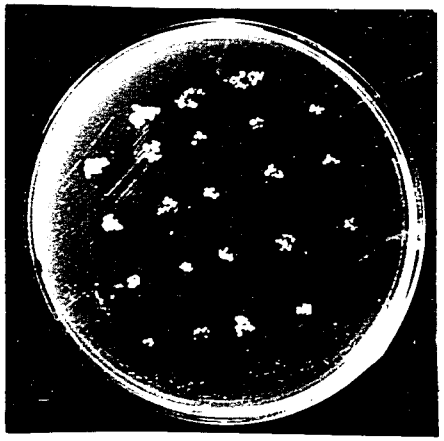


8 days

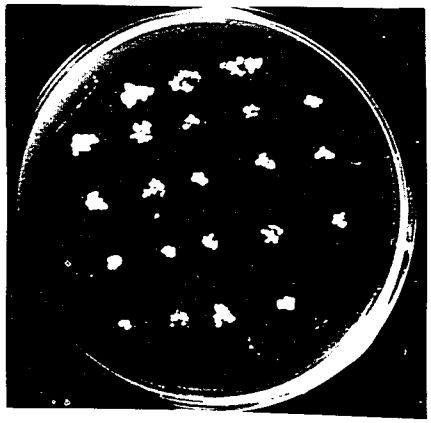
Micrococcus halodenitrificans grown in 3.0 M NaCl transferred to 0.6 M.



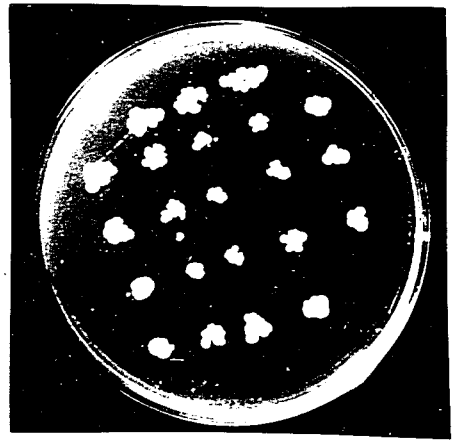
1 day



2 days



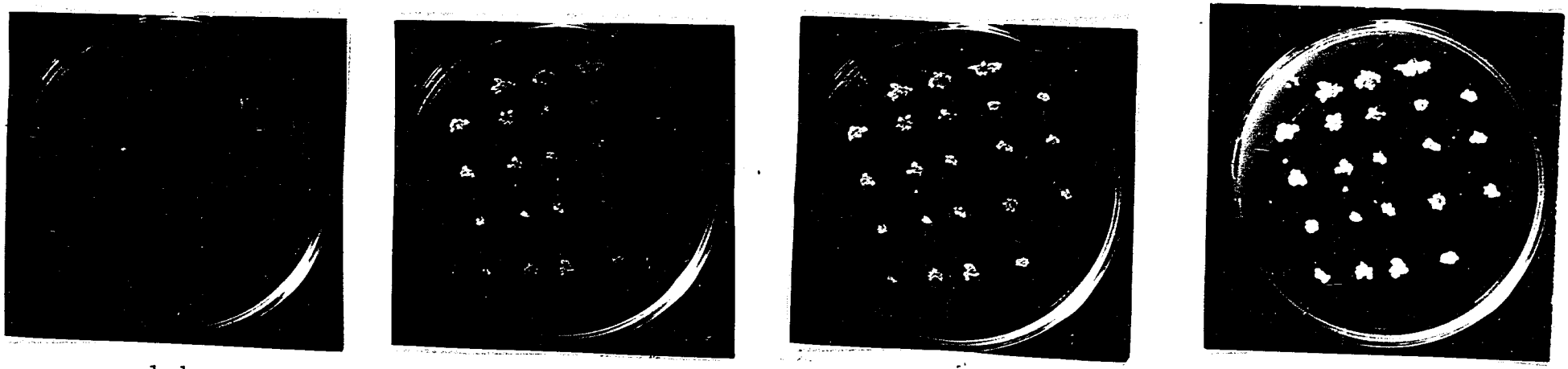
3 days



8 days

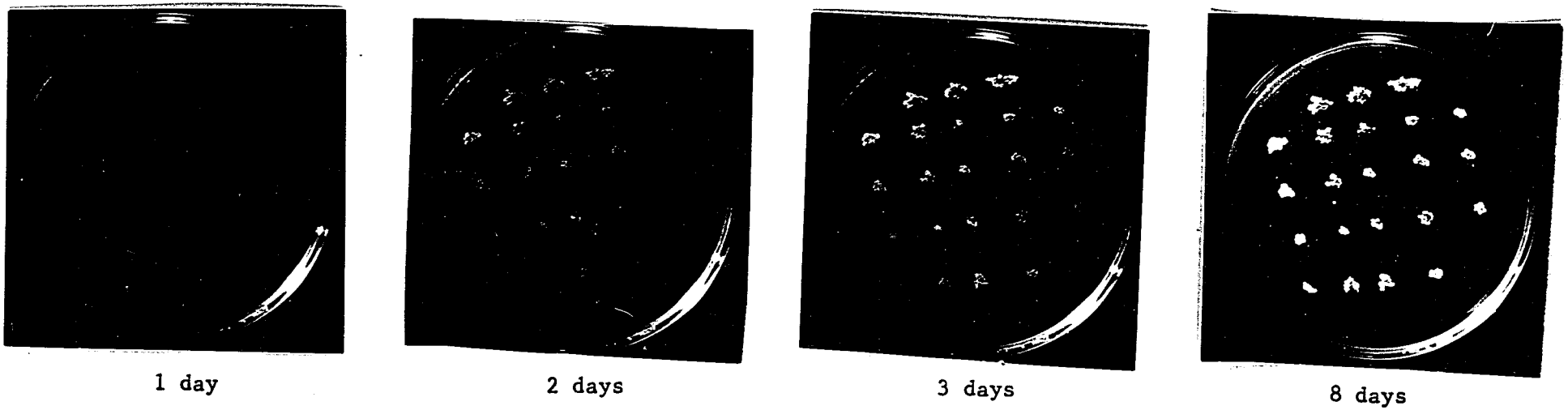
Micrococcus halodenitrificans grown in 3.0 M NaCl transferred to 1.0 M.

Plate 7



1 day 2 days 3 days 8 days

Micrococcus halodenitrificans grown in 3.0 M NaCl transferred to 2.0 M.



1 day 2 days 3 days 8 days

Micrococcus halodenitrificans grown in 3.0 M NaCl transferred to 3.0 M.

Plate 8

Table 8

Summary of results shown in Plates 1 to 8.

| Inoculum NaCl concentration M | Plated on NaCl concentration M | Time required for all colonies to develop days | Number of colonies | Comments |
|---|---|---|---|---|
| | | Vibrio costicolus | | |
| 0.6 | 0.6 | 2 | 26 | control |
| " | 1.0 | 2 | 26 | |
| " | 2.0 | 2 | 26 | |
| " | 3.0 | 10 | 26 | |
| 3.0 | 0.6 | 2 | 20 | |
| " | 1.0 | 2 | 20 | |
| " | 2.0 | 2 | 20 | |
| " | 3.0 | 3 | 20 | control, grew faster than 0.6 M inoculum at 3.0M |
| | | Micrococcus halodenitrificans | | |
| 0.6 | 0.6 | 3-10 | 21 | control |
| " | 1.0 | 3 | 21 | |
| " | 2.0 | 10 | 18 | |
| " | 3.0 | 10 | 14 | |
| 3.0 | 0.6 | 8 | 22 | mold contaminant on 8 d.plate |
| " | 1.0 | 3 | 23 | |
| " | 2.0 | 3 | 23 | |
| " | 3.0 | 3 | 22 | control, grew faster than 0.6 M inoculum at 3.0 M |

number of cells from every colony. Cells transferred from a lower to a higher salt concentration (0.6 to 3.0 M) appeared to have a longer lag phase than cells transferred from high to high (3.0 to 3.0 M). Also shown is the apparent sensitivity of M. halodenitrificans cells to transfers going from low to high salt concentration but not from high to low.

The three types of experiments indicate that cultures of V. costicolus and M. halodenitrificans are genetically homogenous with respect to their response to salt concentrations. That is, each cell in the culture can grow over the whole range of salt concentration at which the culture grows.

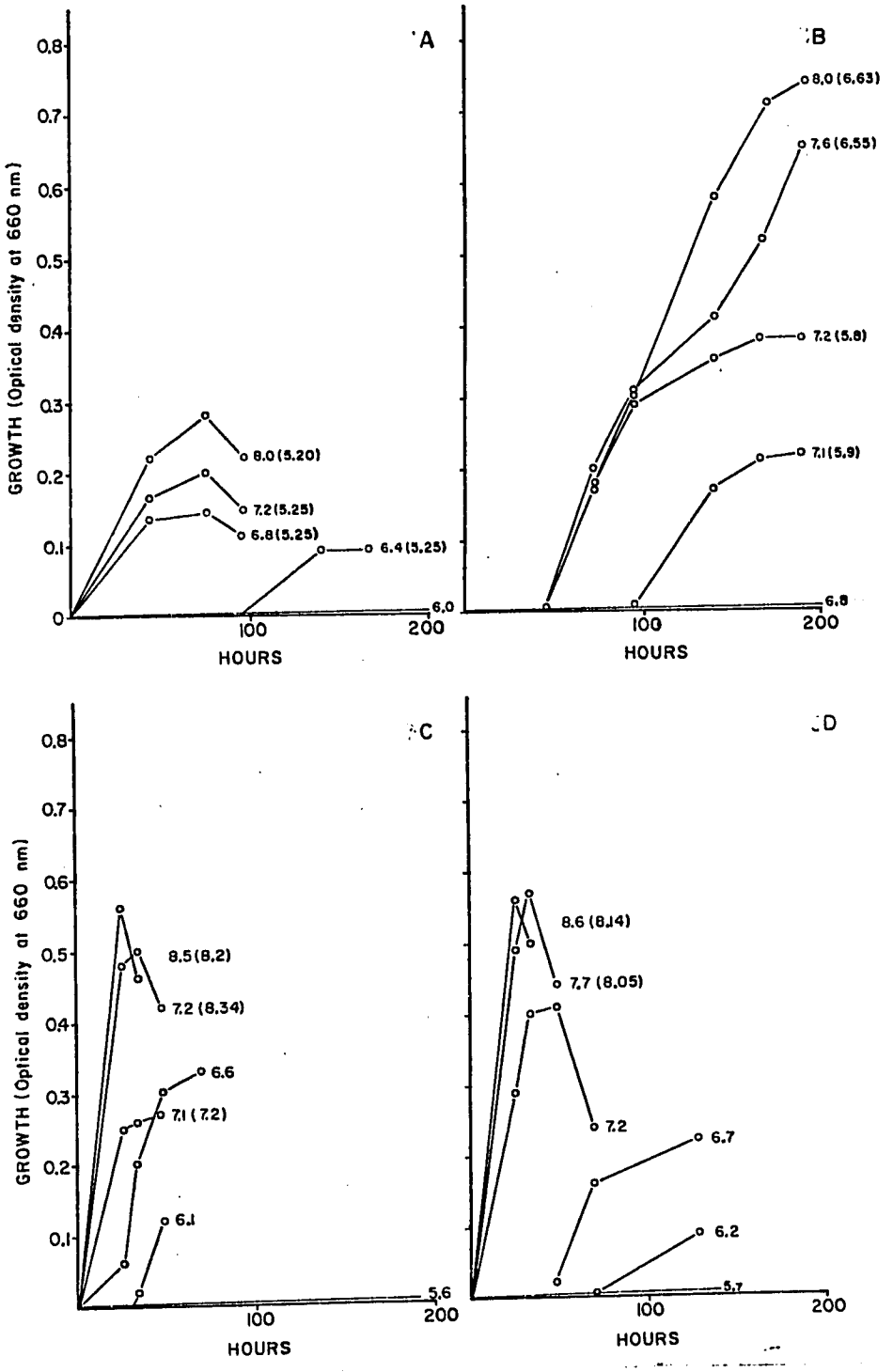
Growth Requirements of Vibrio costicolus at Different Salt Concentrations. Comparison of defined growth media. Flannery and Kennedy (1962) developed a simplified synthetic medium for V. costicolus from the much more complex medium developed by Herbst and Snell (1949) for Hemophilus parainfluenzae. The latter medium contained 18 amino acids, vitamins, purine and pyrimidine bases and certain salts, including $NaNO_3$ but no $NH_4^+$ salts. It supported growth of V. costicolus if 1.2 M NaCl was added. By omitting different constituents of this medium, singly and in groups, Flannery and Kennedy developed a simplified medium containing five amino acids (L-cytine or cysteine, L-glutamic acid, L-arginine, DL-valine and DL- isoleucine) glucose and salts (see Materials and Methods section). Glucose, cyst(e)ine and NaCl were essential for growth; the other amino acids stimulated growth, except that valine and isoleucine antagonized each other. They stimulated growth if added together but inhibited it if added separately.

The nutritional requirements of V. costicolus were approached differently in this study, beginning with a minimal medium suitable for the growth of Escherichia coli. This, when supplemented with 1 M NaCl, also supported the growth of V. costicolus.

The growth in minimal medium and in the medium of Flannery and Kennedy were compared at different pH values and the effects of adding more phosphate buffer to each medium were studied (Figure 7). In the amino acid medium, growth took place after a shorter lag period than in minimal medium and the cell yield was higher. Growth occurred at a lower starting pH value in the amino acid medium. Adding 0.4 M phosphate buffer to this medium lengthened the lag period and decreased the rate of growth at pH values below 7.

In minimal medium addition of extra buffer increased the lag period but also greatly increased the final yield at most pH values. In terms of total cell yield, buffered minimal medium is better than amino acid medium. The stimulatory effect of phosphate buffer is probably due to its control of pH, since, as will be seen, the changes in salt concentration it causes would probably not affect growth. For unknown reasons, cultures could grow at lower initial pH values in the absence of added phosphate than they grew at in its presence

Even though the amino acid medium also contained glucose, the pH rose during growth. The presence of amino acids may have influenced glucose catabolism , or else enough basic substances (e.g. amines) were produced to mask the effects of organic acids produced from glucose.

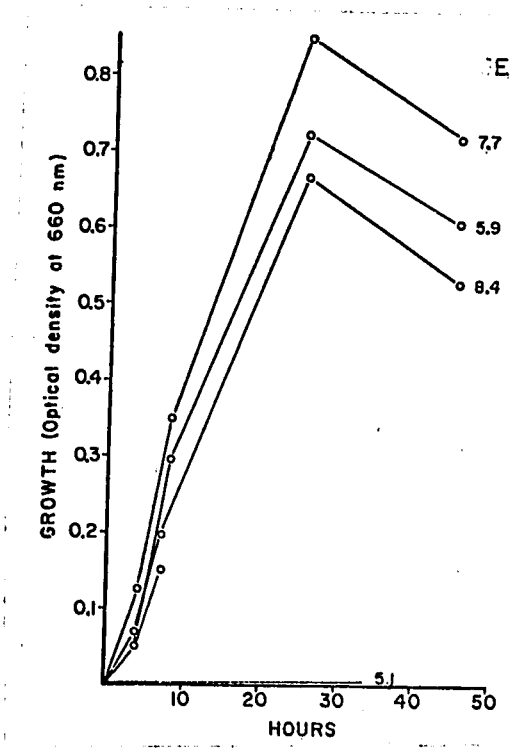



Growth of V.costicolus at various initial pH values in media containing 1 M NaCl. Number in parentheses is final pH. Optical density figures are averages of duplicate cultures.

A. Salts-glucose. B. Salts-glucose with four times the normal phosphate concentration.

C. Flannery and Kennedy medium. D. C with four times the normal phosphate concentration.

Figure 7.




E. Proteose peptone (1%) and tryptone (1%).

Figure 7 continued.

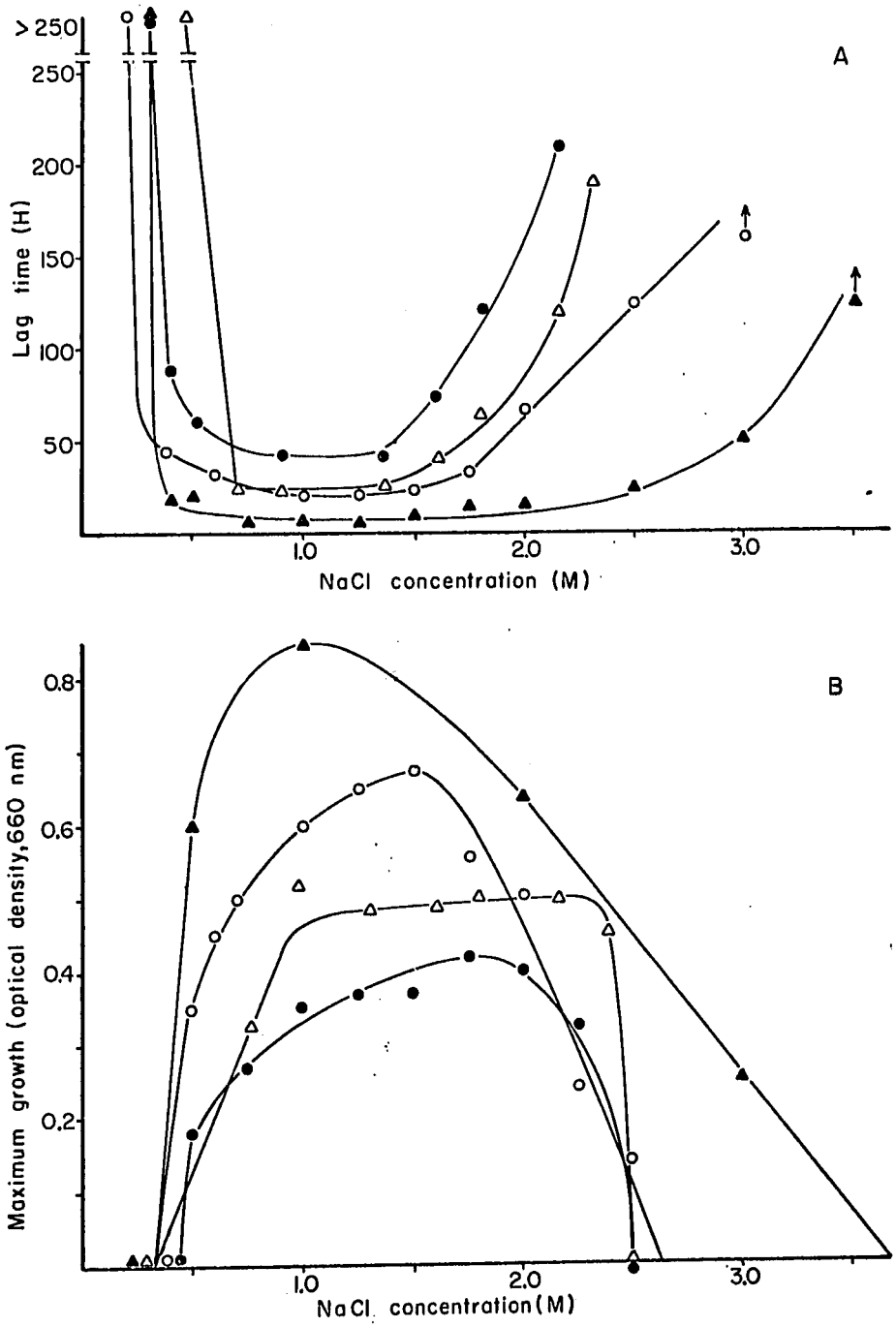
In a complex medium (proteose peptone + tryptone, containing 1 M NaCl) growth was less pH-sensitive in the range 6.0 - 8.4 than in defined media(Figure 7E) though at pH 5.0 cells did not grow.

Some studies, similar to those of Flannery and Kennedy (1962) were carried out to determine which amino acids in their medium were necessary for growth. In this study cysteine or cystine were not found to be essential, though cysteine stimulated growth 10 to 20%. All amino acids could be replaced by ammonium salts but if ammonia was not added arginine and cysteine must be present for growth. Neither arginine nor cysteine alone allowed growth but arginine could be replaced by glutamate or cysteine could be replaced by glutamate, isoleucine and valine (vis. growth occurred in arginine,cysteine; cysteine,glutamate:arginine, glutamate, isoleucine,valine). When a higher number of amino acids were present growth increased. The medium of Flannery and Kennedy always gave more growth. The reasons for the differences between their results and those found here are not clear nor were they investigated because amino acids had already been shown to be unnecessary if another ammonium source was included. In this study growth was measured at slightly different pH values and salt concentration than they used (pH 7.2- 7.6 and 1.0 M NaCl as compared to pH 7.0 and 1.2 M NaCl). Growth in amino acid medium is very sensitive to pH (see Figure 7 C and D) and a small change in pH could conceivably alter nutritional requirements. This possibility has not been investigated further.

Effect of NaCl Concentration on Nutritional Requirements. Previous workers studied the growth requirements of V. costicolus only in media containing 1.0 to 1.2 M NaCl. The effect of salt concentration on nutritional requirements was examined by comparing growth in a variety of media over a wide range of NaCl concentrations. Growth was measured by two parameters: the length of the lag period and the maximal cell yield, expressed as optical density. Viable counts could not be used, since at the highest and lowest salt concentrations at which growth was possible cells did not separate after dividing but formed long filaments.

The widest growth range, extending from 0.4 M to 3.5 M NaCl was obtained with the complex medium, proteose peptone + tryptone (Figure 8) and with proteose peptone + casamino acids or tryptone + casamino acids (not shown). Casamino acids alone gave a smaller growth range (usually 0.4 M to 2.5 M ; in some experiments cells grew in 0.3 M NaCl, but these cultures contained large amounts of cell debris). As the medium was simplified this range was further reduced. The range in the amino acid medium of Flannery and Kennedy was 0.6 to 2.4 M NaCl, and in the minimal medium between 0.4 and 0.7 M (in different experiments) to 2.3 M NaCl.

These results suggested that at the highest salt concentrations enzymes responsible for forming certain amino acids and growth factors were inhibited. Adding amino acids extended the growth range slightly, but the greatest extension occured when complex media were added. In a search for the growth factors responsible we added the following vitamins and bases (1 mg/ml) singly and in combination to the casamino




Change in length of lag phase of growth (A) and of the maximum growth response (B) with different NaCl concentrations.

● Salts-glucose medium.

o Casamino acids and $Mg^{2+}$

Δ Medium of Flannery and Kennedy.

▲ Proteose peptone and tryptone.

Figure 8 (A and B)

acid medium: riboflavine, riboflavine-5-phosphate, nicotinic acid, choline chloride, biotin, D-araboascorbic acid, ascorbic acid, calcium pantothenate, thiamine hydrochloride, pyridoxine phosphate, folic acid, uracil, guanine, adenine, cytosine and thymine. None of the additions permitted cells to grow in as high a salt concentration as they could in complex media.

Effect of Potassium on the Sodium Requirement. Work with extreme halophiles has indicated that their growth may be limited by the availability of potassium (Gochnauer and Kushner 1969). This ion is found in very high intracellular concentrations in extreme halophiles and also in the moderate halophile V.costicolus, (Christian and Waltho 1962). It was possible that a competition existed between $Na^+$ and $K^+$ so that at high NaCl concentrations a requirement for $K^+$ might limit growth. To test this, KCl was added to the amino acid medium of Flannery and Kennedy containing different NaCl concentrations. In the experiment shown (Table 9) cells did not grow in 0.5 M NaCl with 0.065 M $K^+$ (the amount of $K^+$ normally present in this medium). As more KCl was added cells grew. In 1.0 M NaCl, adding up to 0.3 M KCl had little effect on growth. Adding more than 0.1 M KCl to 2.4 M NaCl inhibited growth. The growth response is about that expected if extra NaCl instead of KCl had been added. Thus, at high NaCl concentrations there is no extra requirement for KCl, though at low NaCl concentrations $K^+$ can stimulate growth.

Table 9

Growth in medium of Flannery and Kennedy at various concentrations of $Na^+$ and $K^+$.

| NaCl concentrations (M) | KCl concentrations (M) | Incubation time (hours) | Average maximum O.D.* |
|---|---|---|---|
| 0.5 | 0.056 | 192 | 0.00 |
| 0.5 | 0.1 | 192 | 0.08 |
| 0.5 | 0.3 | 95 | 0.20 |
| 1.0 | 0.056 | 48 | 0.25 |
| 1.0 | 0.1 | 48 | 0.27 |
| 1.0 | 0.3 | 48 | 0.34 |
| 2.4 | 0.056 | 168 | 0.41 |
| 2.4 | 0.1 | 192 | 0.42 |
| 2.4 | 0.3 | 192 | 0.00 |

* Results are averages of triplicate samples. In all cases incubation was continued at least until successive readings showed no further increase in optical density (660nm).

Discussion

It is helpful to compare Vibrio costicolus, which requires a substantial salt concentration for growth with microorganisms that do not need salts for growth but can also tolerate high concentrations of salts and other solutes. Christian (1955) found that Salmonella oranienberg could grow in a complex medium adjusted with salts or sucrose to a water activity ($a_w$) of 0.94 to 0.95, but in a minimal medium the limiting $a_w$ for growth was between 0.96 and 0.97. Adding five amino acids and eight water-soluble vitamins to the salts-adjusted medium extended the growth range to an $a_w$ of 0.95, that of 1.4 M NaCl.

Dulaney et al. (1967) found that 10% sucrose (0.19 M) or high concentrations of other sugars inhibited the growth of Vibrio percolans and Erwinia carotovora in defined media, and that this inhibition could be reversed by yeast extract or by betaine, one of its components.

Rafaeli-Eshkol (1968) and Rafaeli-Eshkol and Avi-Dor (1968) studied an extremely halotolerant, obligately aerobic, Gram negative rod ($B_{a1}$) isolated from crude solar salt. They found that regulation of intracellular ion content did not account for halotolerance but that the salt resistance of the respiratory system of cells grown in low salt, or grown in high salt and washed in water could be increased by betaine, choline and other low molecular weight substances. Labelled choline was shown to be converted to betaine by the organism. Betaine is an important methyl group donor but its role in halotolerance is unknown.

Examples of the influence of $a_w$ on the nutritional requirements of molds are discussed by Scott (1957).

V. costicolus can grow at a much higher salt concentration than S. oranienberg (3.5 M NaCl; $a_w$ 0.86) and cannot grow below 0.4 - 0.5 M NaCl, that is above an $a_w$ value of 0.98. Growth at the highest salt concentrations was found to occur only in complex media. V.costicolus could also grow in a slightly lower salt concentration in a mixture of amino acids than in minimal medium. Enzymes of moderate halophiles are known to require some salt for activity (about 0.5 M) and to be inhibited at higher salt concentrations (Flannery and Durio 1964, Robinson et al. 1952). Our results suggest that at unfavourable salt concentrations enzymes necessary for the formation of amino acids and growth factors are inhibited; however, the components present in tryptone or peptone that permitted the extended range of growth were not identified. Some "training" experiments have usually involved attempts to change the reactions of populations of cells, but so far there have been few attempts to study variations of salt response within populations. Results of this study indicate that cultures of M. halodenitrificans and V. costicolus are homogenous in their response to salt; that is, that each cell can grow over the entire salt range in which the culture can grow. In comparing the physiological and chemical properties of cultures of these bacteria grown in different salt concentrations, any changes can be interpreted as due to a response to salt rather than a selection of certain members of the population.

Staphylococcus aureus grows in relatively high salt concentrations, though it does not need salts for growth. The fact that the same number of colonies form in agar containing about 2.2 M salt as in the absence of salt (Scott 1957) argues for the homogeneity of the salt

response of these cells. Scott also found that at the highest solute concentrations in which cells could grow in liquid media they could not form colonies in agar.

Little enough is known of the nutritional requirements of any halophilic bacteria. The nutritional requirements of some extreme halophiles have now been worked out (Dundas et al. 1963, Onishi et al. 1965, Gochnauer and Kushner 1969), though only at one salt concentration of the narrow range in which these organisms can grow. The results of this study show that the nutrition of V. costicolus is simpler than was previously thought, since it can grow well on a minimal medium. This work also emphasizes the necessity of considering the effects of salt concentration and pH in measuring the nutritional requirements of any salt tolerant or halophilic microorganism.

The principal conclusions of this study are that all cells in the population of V. costicolus and M. halodenitrificans are able to grow over the same salt range and that the salt range over which growth occurs is dependent on the nutrients supplied. The pH range over which growth occurs is also dependant on the nutrients supplied.

The moderate halophiles do not seem to be very different from non-halophiles in composition (see Chapter 1) but it would be helpful to know if V. costicolus has muramic acid. This question will be answered in the next chapter. The same question asked of the halococci and an extremely halotolerant coccus will also be answered.

# Chapter 4

# Study of

# Cell Walls of three Halococci

# and

# a Halotolerant coccus

## Introduction

The nature of the cell envelopes of non-halophilic bacteria and the halobacteria were discussed in Chapter 1.

The halobacterial cell envelopes and the outer layers in particular have certain chemical peculiarities. The most prominent is the absence of muramic acid from the envelope. The outer layer of Halobacterium halobium prepared by Marshall et al. (1969) which are almost exclusively protein and carbohydrate, not only lacked muramic acid but also glucosamine and galactosamine. The hexosamine content, as determined by the method of Gatt and Berman (1966) after exhaustive dialysis, was 1.7%. Although Marshall et al. apparently did not separate the amino sugars from other non-amino sugar chromogens (see Gatt and Berman) only an unidentified amino sugar was found. Stoeckenius and Kunau (1967) using an amino acid analyser found both glucosamine and galactosamine in a cell envelope fraction of H. halobium which apparently derived from the "cell wall" (" outer layer" in the terminology of Marshall et al.).

The absence of muramic acid and the conflicting results obtained for other hexosamines naturally raises questions about the presence and identity of amino sugars in halophilic bacterial cell envelopes. The lack of muramic acid has been suggested as a characteristic of extreme halophiles but the halococci have not been examined for muramic acid. Indeed the only thing known about the halococcal cell envelopes is that they are difficult to break, that is, they are resistant to the disruptive methods used on other bacteria. In this they contrast

greatly to the fragile halobacteria. In this chapter the chemical composition of the halococci and their envelopes are examined. The structure of general interest is the envelope minus the cell membrane, specifically, the layer responsible for the shape of the cell. In non-halophiles this is the wall or R-(rigid) layer, but to avoid confusion it will be called the S-(shape) layer. The use of "wall" in this chapter does not mean to imply that this layer has the same composition as in non-halophiles.

Materials, Methods and Notes

Organisms. Escherichia coli ATCC 11303 and Staphylococcus aureus Duncan were used in some experiments to provide a comparison to the halophiles.

H5 Micrococcus sp (Venkataraman) was obtained from the National Research Council of Canada. It was reported to be an extremely halophilic coccus but it grew equally well in 0.5% or 25% NaCl. Therefore, it is an extremely halotolerant organism. It is unusual because it grows much faster than the three halococci and faster than other halotolerant organisms in high salt concentrations (see Chapter 2).

The halococci, Sarcina morrhuae Delft, S. gigantea Delft, and S. littoralis Lochhead were also obtained from the National Research Council of Canada.

H5 Micrococcus, S. littoralis and S. morrhuae are all cocci about 1 - 1.5 microns in diameter occurring in packets of 2, 4 and sometimes 8 cells. S. gigantea is much larger, about 3-6 microns diameter, occurring frequently in irregular masses. It forms dry granular colonies on agar while the others all have mucoid colonies. S. morrhuae and S. littoralis are both red pigmented while the others are buff. S. littoralis and S. gigantea grew on "regular halophile" agar (see below) at 37°C on 15, 20 and 25% NaCl but not on 10% while S. morrhuae only grew at 20 and 25% NaCl; therefore, these three organisms are extremely halophilic bacteria. Larsen (1962) lists a S. gigantea as a moderate halophile. Petter (1931) described a moderately halophilic S. gigantea she isolated. Presumably the one

used here is a different strain or a mutant (see Chapter 2).

The moderate halophile, Vibrio costicolus was the same organism used in Chapter 3. It was used for comparison and to see if it contained muramic acid.

Halobacterium cutirubrum, obtained from the National Research Council of Canada, was also used for comparison to the halococci.

Growth Media. E. coli was grown in Trypticase Soy Broth (Baltimore Biological Laboratories), Staph. aureus was grown in m-Staphylococcus Broth (Difco). V. costicolus was grown in Proteose Peptone (Difco) and Tryptone (Difco) 0.5% each in 1 M NaCl at pH 7.2. S. morrhuae, S. littoralis, S. gigantea, H5 Micrococcus and H. cutirubrum were grown in "regular halophile" medium which contained per liter; yeast extract (Difco) 10g, Casamino Acids technical (Difco) 7.5g, KCl 2g, $MgSO_4 \cdot 7H_2O$ 20g, Na citrate 3g, NaCl 250 g and 1 ml of 4.98% $FeSO_4 \cdot 7H_2O$ solution in 0.001 M HCl. The medium was adjusted to pH 6.8 - 6.9 with NaOH. Yeast extract and Casamino Acids solutions were autoclaved separately and mixed on cooling with the autoclaved salt.

The E.coli was grown in 5 l of medium with vigorous aeration at 37°C. Staph. aureus was grown in 800 ml lots on a reciprocating shaker at 100 - 110 cycles per minute at 37°C. Both were harvested just prior to the stationary phase of growth, washed 3 times with 0.25 volumes of distilled water and frozen until required. The three extreme halophiles and H5 Micrococcus were grown in 10 or 15 l lots at 37°C either in a 5 gallon polypropylene bottle with stirring and aeration or in a New

Brunswick fermentor. They were harvested just before the stationary phase of growth with a Sharples centrifuge and stored at -5°C until washed for use. They were washed three times with a basal salt solution containing per liter; KCl 2g, $MgSO_4 \cdot 7H_2O$ 20g, Na citrate 3g and NaCl 250g. The cells were washed in bulk and apportioned for the various experiments to minimize variations.

Dry Weight. The unextracted cell dry weights were determined by drying portions in tared crucibles in an oven at 105 - 110°C to a constant weight. This was usually obtained in 48 hours. The ash free dry weights were determined by subtracting the weight of the ash (see below) from the total dry weight. Extracted cells were dried in tared tubes under vacuum over freshly heated NaOH pellets or under vacuum at 55°C over silica gel. The two methods gave comparable results. Lipid dry weights in extracted cells were determined by removing water from the chloroform-methanol fraction as a benzene azeotrope by adding a half volume of benzene and evaporating to dryness under vacuum. The dried material was taken up in chloroform and evaporated to dryness under vacuum in tared tubes over silica gel.

Ash Weight. The ash weight was determined after heating the crucible used for dry weights to dull red over a bunsen burner and cooling in a desiccator. The procedure was repeated until a constant weight was obtained.

Salt Content. Salt was determined as chloride in the ash. After dissolving the ash in a known volume of water the concentration was determined by the method of Schales and Schales (1941). The chloride

arose from NaCl in the intercellular space and from the intracellular salt, probably KCl and NaCl in a 1:1 ratio. Assuming NaCl was the only chloride present, it accounted for 73.6 $\pm$ 0.7% (SE) of the ash for all microorganisms. Clearly all the chloride is not NaCl. Presumably a substantial fraction is KCl (Christian and Waltho 1962) and chlorides of other cations heavier than sodium. Because of the uncertainty about internal ionic concentration, it was more accurate to determine the ash content directly.

Nitrogen. Samples were digested with 1 ml .2N $H_2SO_4$ and 0.02% $CuSeO_4$ on a digestion cabinet until fuming ceased and until completely clear. A drop of $H_2O_2$ (30%) was added and the samples reheated if necessary. The overnight digestion in an oven as in the original Johnson method was inadequate but the same reagents and ammonia assay were used (Umbreit et al. 1957). Ammonia in the digest was assayed for directly with Nessler's reagent. The optical density of the colored product was measured at 490 nm on a Coleman Jr spectrophotometer in precalibrated tubes. At least one standard and a blank were run with each assay. Protein was determined by multiplying N x 6.25 after correction for non-protein nitrogen.

Phosphate. Phosphate was determined after digestion in $H_2SO_4$ and $H_2O_2$ by the method of Chen et al. (1956). The optical density of the molybdenum blue which was formed was read at 700 nm in a Coleman Jr spectrophotometer in precalibrated tubes or for maximum accuracy in a Beckman DU spectrophotometer at 820 nm. A set of standards and a blank were run with each assay.

Carbohydrate and other Reducing Substances. They were determined after hydrolysis in 2 N HCl for 2 hours in a boiling water bath. Evaporation was prevented by a'cold finger'. The hydrolysates were freeze-dried, water was added and the hydrolysates redried several times to remove HCl and resuspended in water.

Reducing Substances were determined by the method of Nelson-Somogyi (Hodge and Hofreiter 1962) which gives a reaction with any α-hydroxy-ketone. This microcolorimetric method depends on the reduction of cupric salts to cuprous oxide. The cuprous oxide is then used to reduce arsenomolybdate to molybdenum blue. The optical density was read at 500nm on a Coleman Jr spectrophotometer in precalibrated tubes. Glucose was used as a standard and 100 g ribose was found to have a reducing power of 92 g of glucose while 100 g of D-2-deoxyribose has a reducing power of 9.2 g. Unhydrolysed RNA has no reducing power. Glucosamine has the same reducing power as glucose while muramic acid has a reducing power one-third that of glucose.

Carbohydrates were determined by the phenol-sulfuric acid reaction of Dubois et al. (1956). Five carbon and higher sugars form a furfural derivative in the presence of concentrated sulfuric acid and heat. The furfural derivative presumably reacts with phenol to give a chromophore with an absortion maximum at 480 nm. The optical density was measured on a Coleman Jr spectrophotometer in precalibrated tubes. Reactions will also occur with tetroses if the breakdown products of higher sugars are present. Glucose was used as a standard and blanks were included with each assay. Glucosamine and muramic acid gave no color in this assay presumably because they would not form the furfural derivative.

Another method which was used to determine the presence of pentose or hexuronic acids, hexoses, 6-deoxyhexoses, heptoses and 2 deoxypentoses is the cysteine-sulfuric acid method (Dische 1962a). The five classes of sugars are differentiated by small shifts in the absorption maximum and by changes which occur with the addition of water and with time. The absorption spectra were run on a Beckman DB recording spectrophotometer. 2-deoxypentoses produced a measurable color by this method only if present in ten times as high a concentration as other sugars. Glucosamine and muramic acid only contribute to the spectra when present in a ten-fold excess.

The diphenylamine reaction (Burton 1956) is very sensitive and specific for 2-deoxyribose. It was used to determine this sugar and thus the DNA content.

RNA was determined by Bial's orcinol reagent modified according to Mejbaum (Dische 1962a). The reaction is not specific. Color due to D-glucose, D-mannose and D-fructose can be corrected for (Brown 1946) but those due to D-galactose, hexuronic acids and other sugars cannot (see Dische 1962a for a critical discussion). Dichromatic readings were taken at 670 and 580 nm on a Coleman Jr spectrophotometer. If the ratio for the sample departed significantly from the standard (RNA or ribose 580/670 = 0.51 - 0.61) it was discarded as with the hydrolysates of whole cells. Hot trichloroacetic acid (TCA) extracts were used for determining RNA contents of cells and the color was assumed to be due to RNA alone (see Webb and Levy 1958 for discussion). Despite the lack of specificity of the method it served to confirm the absence of RNA and hence of cytoplasmic contaminants in extracted cells.

Total nucleic acid was determined by the optical density at 260 and 280 nm using the method of Warburg and Christian (1941) as described in Conn and Pelczar (1957).

Glucose and galactose were determined with the highly specific enzymatic Glucostat and Galactostat reagents (Worthington Biochemicals, Freehold, N.J.).

For the determination of neutral sugars the hyrolysed material was passed through a mixed bed ion exchange resin. A column of equi-equivalent amounts of Dowex 50 $H^+$ and Amberlite IRA-400 $COO^-$ removes all the charged molecules including amino sugars (but not N-acetyl amino sugars) without destruction of the sugars as recommended by Hough and Jones (1962). Destruction of the sugars might occur if a hydroxide form column was used (Kazal 1967). Reducing substances and carbohydrates were determined as described previously.

Sugar Chromatography. The hydrolysates were first deionized on a mixed bed resin (see above) and concentrated by freeze-drying before spotting on the appropriate support. Initially two descending chromatograms were run on Whatman 3MM paper, one with ethyl acetate:pyridine: water (8:2:1 v/v/v) and the second with benzene: 1-butanol:pyridine: water (1:5:5:3) for 16 to 18 hours. The sugars were detected with the $AgNO_3$ reagent of Trevelyan et al. (1950). Later cellulose thin layer plates and the same mobil phases were adopted because of the increased speed and sensitivity. On thin layers ammoniacal silver was used to detect the sugars since a permanent record was not desired (Hough and Jones 1962).

A third useful technique was chromatography on kieselguhr thin layer plates with a mobile phase composed on 35 ml ethyl acetate and 65 ml of 66% isopropanol in water. This system seems to be less salt sensitive than cellulose thin layer plates. Layers were sprayed with anisaldehyde-sulfuric acid or anisidine phthalate (Randerath 1964). The latter reagent should give a red spot for pentoses and a yellow spot for hexoses but the colors were not to be depended on and the method was less sensitive than $AgNO_3$.

Amino sugars. Glucosamine, galactosamine and muramic acid were determined by the methods of Stewart-Tull (1968). These methods were a combination of earlier methods by Cessi and Pilego (1960) and Cessi and Serafini-cessi (1963). Glucosamine plus galactosamine and muramic acid were determined by the first method. Under alkaline conditions, in the presence of acetyl acetone, chromogens were formed which were separated by distillation. The chromogens for glucosamine and galactosamine were in the distillate and were reacted with a p-dimethylaminobenzaldehyde reagent (A) to give a chromophore which has an absorption maximum at 545 nm after 30 minutes. The chromogens of muramic acid and other substances, such as amino acids and sugars were left behind to be reacted with a second p-dimethylaminobenzaldehyde reagent (B). The chromophores were read at 510 nm after 24 hours. The color formed by the usual interfering substances, sugars and amino acids was reported to fade rapidly (Schloss 1951). Non-specific absorption was encountered in applying this method to the halococci.

Galactosamine was determined by converting it to a pyrrole derivative in the presence of methanol:triethylamine:acetyl acetone:pyridine (7.3:2:0.6:0.1 v/v/v/v) at 55°C for 16 hours (Cessi and Serafini-cessi 1963) where only galactosamine readily forms this compound. After evaporating the organic solvents under vacuum the compound was converted to a chromogen (2-methylpyrrole) in a borate buffer pH 8.0 at 100°C for 20 minutes, distilled off and reacted with reagent A as for glucosamine. The absorbance was measured at 550 nm after 30 minutes. Salt interferes with the determination of galactosamine presumably because the sample deposit is insoluble in the organic solvents.

Glucosamine HCl (Eastman Chemicals), galactosamine HCl (Sigma Chemicals) and muramic acid (Sigma chemicals) were used as standards. Stewart-Tull (1968) reported a small amount of contaminating hexosamine in the muramic acid which he obtained from the same source. The optical density measurements at 550, 545, and 510 nm were determined on a Beckman DU spectrophotometer. The Beckman DU was chosen for these measurements because of its high resolution. In other determinations where the absorption band is less sharp, such as that of molybdenum blue, the wider bandwidth of the Coleman Jr was quite acceptable and the same results were obtained as with Beckman instruments. Because of the use of tubes in the Coleman Jr the light path is slightly longer and therefore the Coleman Jr offers a slight increase in sensitivity. The spectra, when required, were run on a Beckman DB recording spectrophotometer.

The separation of hexosamines and muramic acid was also carried out on a charcoal-Celite column by the method of Park, described in Perkins and Rogers (1959). Columns of 0.25 g Norit A and 0.25 g Celite were prepared in 2 N HCl in Pasteur pipettes. After a preliminary wash with at least 10 ml of 2 N HCl the samples were added to the column in 2 N HCl. The column was eluted with 10 ml of water which removed glucosamine and galactosamine and then with 10 ml of 5% ethanol which removed the muramic acid. The presence of salt in the hydrolysates of whole cells prevented retention of amino sugars on the column. These columns could, however, separate muramic acid and hexosamines in the hydrolysates of extracted cells.

Amino sugars were chromatographed on silica gel G layers using n-propanol:ethyl acetate:water (5:1:4 v/v/v) in one direction and n-propanol:water (7:1.5 v/v) in the second according to the method of Gal(1968). They were detected by the Elson-Morgan reagent described by Gal which is relatively specific for amino and acetylamino sugars. This method was also relatively insensitive to salt.

Amino sugars were also chromatographed on Whatman 3MM paper using n-butanol:acetic acid:pyridine:water (60:3:40:30 v/v/v/v) as a solvent for 16 hours. These early chromatograms were sprayed with ninhydrin 0.5% in butanol which detected amino acids and amino sugars. Ninhydrin is more sensitive and dependable than the Elson-Morgan reagent because the volatile chromogens of glucosamine and galactosamine formed in the Elson-Morgan reaction may be lost on heating.

Amino Acids. Samples were hydrolysed in 6 N HCl at 105°C for 14 hours in sealed tubes. The HCl was removed by freeze-drying, resuspending in water and redrying several times. The amino acid analysis was carried out by M. Yves Patenaude on a Technicon amino acid analyser. The amino acid analyser can be used for the quantitative determination of muramic acid, glucosamine and galactosamine if the instrument is properly calibrated and has sufficient resolution. The instrument was used here for muramic acid on a qualitative and roughly quantitative basis and not at all for glucosamine and galactosamine. Muramic acid was eluted from the Chromabead column slightly ahead of and merging with glutamic acid. It was seen as a shoulder on the glutamic acid peak. Glucosamine gave a peak following the peak for alanine. In some determinations amino acids were chromatographed on mixed layers of cellulose and silica gel according to Turner and Redgwell (1966). They were detected with ninhydrin which also detects amino sugars including muramic acid. This method yields qualitative and roughly quantitative results.

Lipids. Lipids were extracted by a modification of the Bligh and Dyer (1959) method as suggested by Dr. M.Kates (personal communication). A concentrated cell suspension (0.8 V) was mixed with 3 volumes of chloroform:methanol (1:2 v/v) and left overnight at room temperature in a flask with a ground glass stopper. After centrifugation at 3,000xg the supernatant was removed and the pellet resuspended in 3.8 volumes of chloroform:methanol:water (1:2:0.8) as before and shaken intermittently for an hour. After centrifugation the supernatants were combined and a further 2 volume each of chloroform and water were added.

The chloroform phase was removed and a half volume of benzene added to it. It was evaporated to dryness under vacuum and weighed.

The extracted cell pellet was mixed with 1 volume of methanol and evaporated to dryness under vacuum. After hydrolysis of the pellet in 6 N HCl for 3 hours at 100°C, the fatty material was extracted with 3 volumes of petroleum ether 3 times. The petroleum ether extracts were combined, evaporated to dryness under vacuum and weighed.

Cell Extraction Procedures

Method 1. This is the method of Park and Hancock (1960) but scaled up and slightly modified. The entire procedure was carried out in 30 ml Correx centrifuge tubes.

1. About 250 mg dry weight of cells were suspended in 40 ml of cold water (0-4°C) in tubes and mixed with 10 ml of cold 25% trichloroacetic acid (TCA). After ten minutes the tubes were centrifuged at 4,000 xg for 5 minutes and the supernatant decanted. The inside of the tube was wiped dry.

2. The pellet was resuspended in 50 ml 75% ethanol with the aid of a Branson Sonifier at the minimum power that would produce a homogenous suspension in 30 seconds (about 4 amps) and held at room temperature at least 10 minutes, then centrifuged and dried as before.

Extracted halococci and H5 Micrococcus could not be readily resuspended by shaking or pipetting. Hence ultrasound was used to resuspend them, even though it was realized that small particles might be removed from the extracted material. Though an intensive search for such particles was not made, supernatants, after centrifuging

resuspended cells, were clear. Intact cells were not disrupted by 10 minutes exposure to ultrasound at maximum power.

3. The pellet was resuspended in 50 ml 5% TCA with a sonifier and heated 6 minutes at 90°C. The tubes were cooled, centrifuged and dried as before.

4. The pellet was resuspended with a sonifier in 14.25 ml of 0.05 N $NH_4HCO_3$ and 0.005 N $NH_4OH$ buffer pH 8.3 to which 0.75 ml of trypsin 1 mg/ml was added. The tubes were incubated at 37°C for 2 hours. The optical density was recorded at 500 nm before and after incubation. The tubes were centrifuged at 6,000 xg for 10 minutes, decanted and the material resuspended in distilled water. They were centrifuged and the pellet taken up in distilled water as desired.

Method 2. This is a modification of method 1. Chloroform:methanol: water (1:2:0.8 v/v/v) was substituted for 75% ethanol in step 2. The material prepared by this method was examined under the electron microscope by M. Jean-Yves D'Aoust.

Method 3. This was a modification of method 2. Ten percent TCA was substituted for 5% TCA at step 3 and the cells were heated in a boiling water bath for 10 minutes. It was used primarily for determination of total DNA and RNA which are extracted in the hot TCA.

Method 4. The material from the first two methods was further extracted with formamide to try to prepare an S-layer (Fuller 1938, Krause and McCarty 1961). The pellet was heated in 2 ml of formamide for 20 minutes at 170°C in an oil bath. The tubes were cooled and 5 ml of 3% HCl in ethanol was added. After centrifugation at 30,000xg for 10 minutes the sediment was saved for analysis. One volume of acetone was added to

the supernatant and it was recentifuged at 3,000xg for 10 minutes. A white precipitate was obtained. In the hemolytic streptococci the first sediment was peptidoglycan and the second a polysaccharide (Krause and McCarty 1961).

Method 5. Ten grams of solid phenol was added to the pellet (about 1 ml) from method 2 and shaken for 45 minutes at room temperature. The solution was centrifuged at 40,000 xg for 45 minutes at 16°C. The pellet obtained was washed twice with 10 ml of water, centrifuged at 30,000 xg for 30 minutes each time.

Lytic Agents

Maxted's enzyme, a mixture of enzymes produced by Streptomyces albus (McCarty 1952 a and b), was obtained from Colab Laboratories, Chicago Heights, Illinois. As purchased it is the spent culture medium and is used for lysing bacteria, particularly the hemolytic streptococci.

Lysozyme was obtained from Sigma Chemicals, St. Louis, Mo.

Sonication was carried out in 30 ml Correx centrifuge tubes or in half of a polycarbonate centrifuge tube, with a Branson Sonifier.

Electron Microscopy

The following methods were used by M. Jean-Yves D'Aoust to obtain the electron micrographs shown here.

For thin sectioning small pellets of cells or extracted cells were fixed overnight in the cold in 3% formaldehyde and 3% gluteraldehyde in basal salt solution (final pH 6.7). After staining for one hour in cold 1% aqueous uranyl acetate they were dehydrated in a cold alcohol series. Infiltration was carried out with acetone and Epon 812 resin (1:1 v/v) for one hour at room temperature. The pellets were finally polymerized in fresh resin for 2 days at 55 °C. Gold sections were collected on carbon-formvar coated copper grids.

Surface replicas were prepared on microscope slides by shadowing at an angle of 25° with platinum-carbon. The replicas, strengthened with a carbon film, were collected on grids for examination.

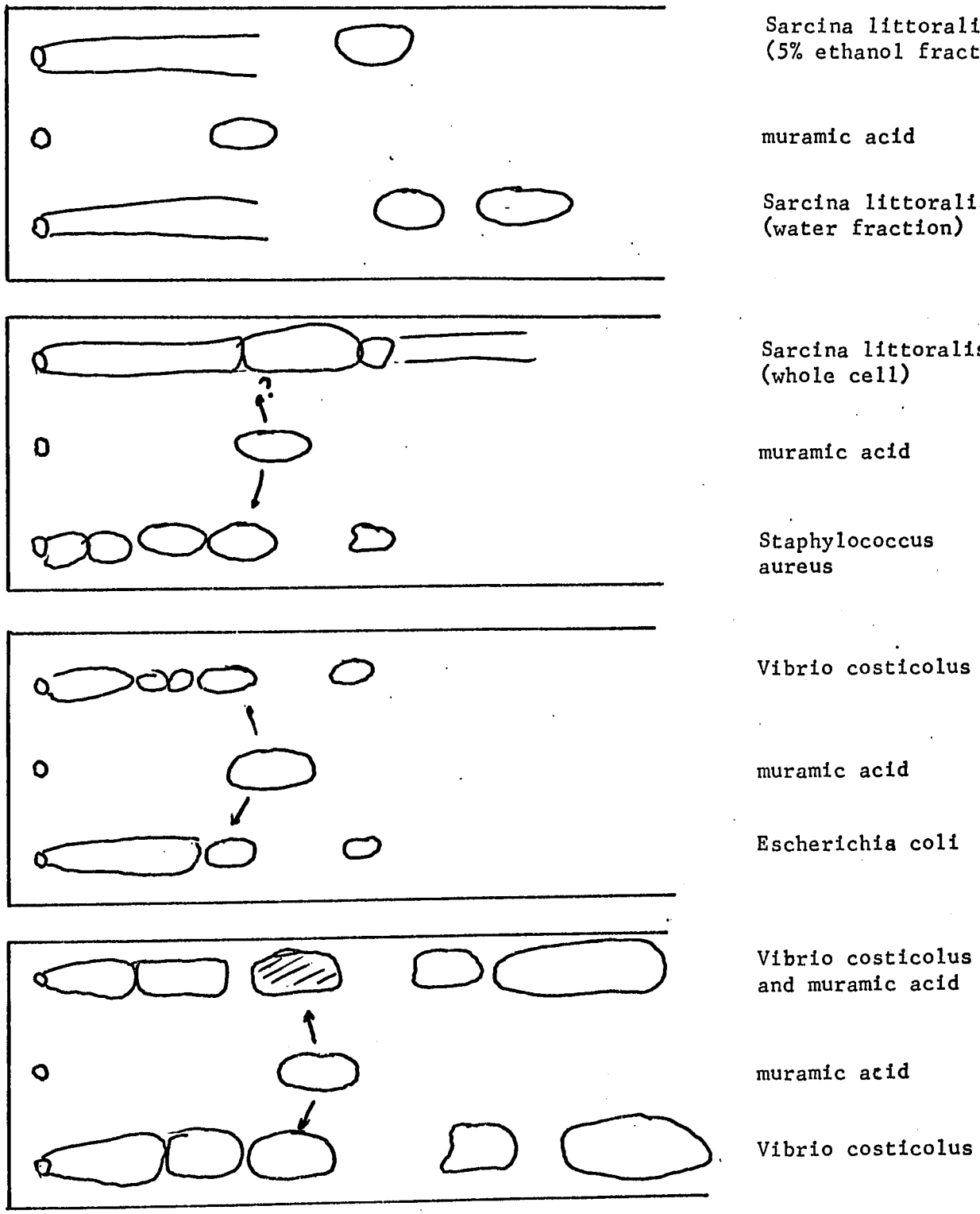
## Results

### Muramic Acid in Whole Cells

The first aim of this work was to establish unequivocally whether cell walls of the halococci contained muramic acid, the key substance that forms an essential part of the walls of all other bacteria with the exception of the halobacteria and the mycoplasma (Salton 1964).

As a first approach muramic acid was looked for in whole cells using paper chromatography. Whole cell hydrolysates of Vibrio costicolus were run with and without muramic acid added to the hydrolysate (Figure 9). The muramic acid spots in hydrolysates were easily seen and were slightly retarded in relation to pure muramic acid. Similar retardation occurs with Escherichia coli and Staphylococcus aureus hydrolysates. Sarcina littoralis chromatograms were seen as long smears with inadequate separation and therefore they failed to show the presence or absence of muramic acid. Whole cell hydrolysates of S. littoralis were separated on charcoal-Celite columns. Both the water and 5% ethanol fractions failed to show a definite muramic acid spot but did show long heavy trailing. Muramic acid and salt were placed on a column in concentrations similar to what would be expected in the cell (1% muramic acid and 25% salt). Assay of the fractions by the Stewart-Tull (1968) method showed that muramic acid was not retained on the column.

The second approach was to assay for muramic acid chemically using the method of Stewart-Tull (1968). Although this method is not definitive, a lack of color would show the absence of muramic acid.

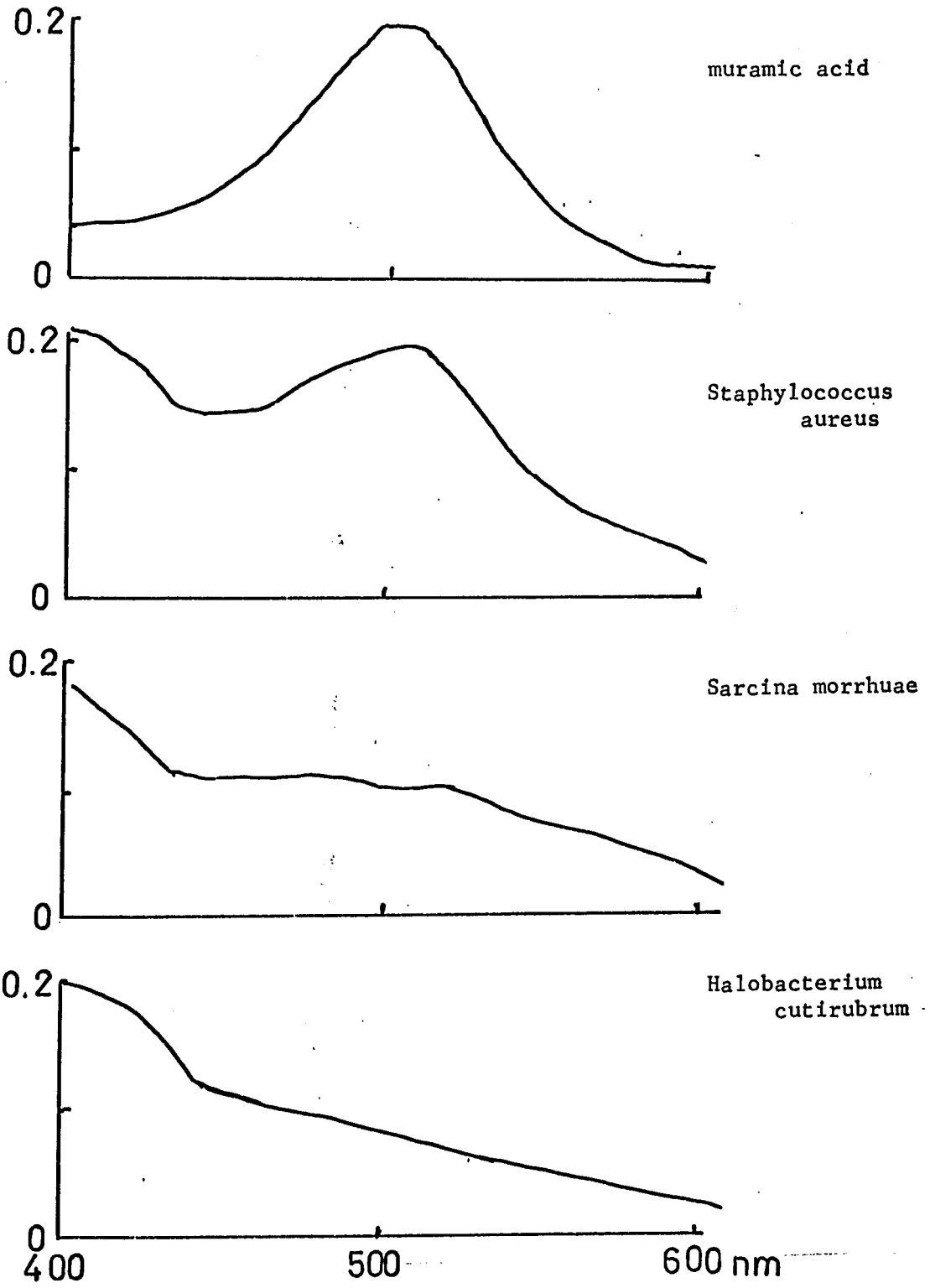



Amino sugar chromatograms detected with ninhydrin

Figure 9

A chromophore was formed which absorbed strongly at 510 nm (Figure 10), the maximum for muramic acid chromophore but its color appeared different from that of pure muramic acid chromophore and also from the Staph. aureus sample treated in the same way. When spectrograms were made definite peaks at 510 nm appeared for Staph. aureus and the muramic acid standard but not for S. littoralis and S. morrhuae; with the latter organisms a non-specific absorption occurred. Later the same non-specific absorption occurred in a hydrolysate of Halobacterium cutirubrum assayed for muramic acid. H. cutirubrum has been shown not to contain muramic acid (Kushner 1964) as has H. salinarium (Brown and Shorey 1963).

Sephadex G-10 is recommended by the manufacturer for desalting small molecules such as glucose. A 45 cm column was found to separate pure muramic acid from basal salt with 50 - 75% loss of muramic acid on the column. A muramic acid fraction was not recovered from Staph. aureus hydrolysates to which salt was added, nor was it recovered from a S. morrhuae hydrolysate. Because of the many difficulties encountered, muramic acid could not be demonstrated unequivocally using the methods above. Therefore, it was decided to use a cell fraction which retained the shape of the cell and which was salt free.




Spectra of non-volatile chromophores found by Elson- Morgan reaction in various cell hydrolysates and from muramic acid.

Figure 10

Attempts at Cell Lysis and Breakage

Some preliminary experiments were carried out to affirm or deny the difficulties which other investigators reported in breaking the halococci.

Cells were suspended (10 mg wet weight/ ml) in 10% sodium lauryl sulfate, 10% sodium deoxycholate and in both for several hours with no apparent change in optical density or microscopic appearance. In contrast, such treatment of other cells (Bacillus subtilis and E. coli) immediately transformed the turbid suspension to clear opalescent solutions. Another set of cells were incubated for 45 minutes at 37°C in saline (0.15 M NaCl) EDTA (0.1 M) pH 8 with 1 mg/ml lysozyme. H5 Micrococcus showed a substantial breakdown under these conditions as judged by a decrease in density and an increase in viscosity. The halococci showed no change in density or viscosity. Incubation at 37°C with Maxted's enzyme (half strength) for 8 hours did not affect the turbidity or viscosity of the suspensions.

An attempt was made to break H5 Micrococcus and S. littoralis cells with ballotini beads in a Mickle tissue disintegrator. One gram of cells, 5 ml of basal salt, 2.5 g of ballotini, with and without 2.5 g of glass powder were shaken in a Mickle disintegrator at 4°C for 45 minutes and then diluted with about 2 volumes basal salt. After removal of the beads and glass powder the supernatant was spun at 3,000 xg for 5 minutes to remove cells. The supernatant and the gelatinous layer on the pellet were spun at 17,000 xg for 25 minutes to collect cell walls. This crude cell wall pellet volume was estimated to represent less than one tenth of the cell pellet volume and was not

further investigated.

Sonication for 10 minutes at full power, with ice water cooling the tube had no visible effect on S. littoralis or H5 Micrococcus cells suspended in basal salt. In contrast, less than one minute exposure is sufficient to disrupt V. costicolus or M.halodenitrificans .

Cells (4-6 g wet weight) suspended in cold basal salt with 1% phenol were passed through a French pressure cell. DNA was then extracted from the suspension by the method of Marmur (1961) and determined by its optical density at 260 nm in 0.015 M NaCl 0.0015 M Na citrate pH 7.0. Forty-five µg/ml of calf thymus DNA gave an optical density of 1.0. About 30 µg of H5 Micrococcus DNA was recovered. S. gigantea gave about 6 mg DNA, S. littoralis about 3 mg and no DNA was recovered from S. morrhuae in two attempts. The DNA recovered from S.gigantea and S. littoralis is only about one tenth of their total DNA (see Table 12). These experiments tend to confirm the reported difficulties encountered in breaking these cells.

Some enzymes of S. morrhuae have been studied but the method of cell breakage, described as "Aasmundrud unpublished" by Larsen (1962, 1967) is not given. Moore and McCarthy (1969 a,b) stated that DNA was obtained by the method of Marmur (1961) from Halococcus morrhuae strain 24 but this method (SLS or Lysozyme-EDTA) was found to be ineffective with the halococci studied here.

Cell Envelopes

The difficulties encountered in preparing cell envelopes by mechanical methods left only chemical methods as an alternative. The Park and Hancock (1960) method of preparing cell walls was adopted as a starting point.

Table 10 shows the percentage of ash-free dry weight of cell material left after extraction by the various methods. Method 1 removes considerable material. Method 2 reduces the halococci by roughly similar values and H5 Micrococcus to a much lower value. Method 3, which was used primarily to extract nucleic acid, liberates even more material except from H5 Micrococcus .

Table 11 shows the effect of trypsin on the optical density of the suspensions during the last step of extraction methods 1, 2 and 3. The 50 minute and 110 minute figures for method 1 showed that the action is essentially complete by 110 minutes. Staph. aureus, for which the method of Park and Hancock (1960) was originally devised, served as a control. The optical density decreases for cells extracted by methods 2 and 3 are larger, especially for the halococci, than for method 1 probably because more protein was exposed to the action of trypsin by the chloroform-methanol-water solvent. Hot formamide (method 4 Table 10) extracted even more material from the cells but method 5, which was a phenol extraction following method 2, failed to decrease the weight.

One criterion for assessing whether a cell envelope or wall preparation is free of cytoplasmic contamination is to examine it for

Table 10

The effectiveness of various cell extraction methods in terms of weight loss.

| | Percent of the ash free dry weight of cell remaining after extraction | | | | |
|---|---|---|---|---|---|
| Method* | 1 | 2 | 3 | 4 | 5 |
| H5 Micrococcus | 29 | 8.9 | 15 | - | - |
| Sarcina morrhuae | 42 | 23 | 9.4 | 1.9 | 24 |
| S.gigantea | 31 | 27 | 4.0 | 3.7 | 26 |
| S.littoralis | 36 | 22 | 13 | - | - |

* treatment:

Method1 ; a.cold TCA b.ethanol c.hot 5% TCA 90 C for 6 min. d.trypsin.

Method 2: a.cold TCA b.$CHCl_3$:MeOH:$H_2O$ c.hot 5% TCA d.trypsin.

Method 3: a.cold TCA b.$CHCl_3$:MeOH:$H_2O$ c.hot 10% TCA 100 C 10 min. d.trypsin.

Method 4: After method 2 extract with hot formamide.

Method 5: After method 2 extract with 90% phenol.

see materials and methods for details.

Table 11

Cell digestion by trypsin

| | Percent decrease in optical density (700nm) during trypsin digestion | |
|---|---|---|
| | 50 minutes | 110 minutes |
| Method 1 | | |
| Staphylococcus aureus | 60 | 61 |
| H5 Micrococcus | 65 | 67 |
| Sarcina morrhuae | 15 | 24 |
| S.gigantea | 19 | 19 |
| S.littoralis | 22 | 26 |
| Method 2 | | |
| H5 Micrococcus | | 75 |
| S.morrhuae | | 65 |
| S.gigantea | | 64 |
| S.littoralis | | 57 |
| Method 3 | | |
| H5 Micrococcus | | 75 |
| S.morrhuae | | 74 |
| S.gigantea | | 49 |
| S.littoralis | | 55 |

the presence of nucleic acids. The DNA content of a hydrolysate of cells extracted by method 1 was measured (Table 12) but to assess the effectiveness of extraction, the total DNA content of the cells was needed. Methods 1 and 2 use hot 5% TCA to extract nucleic acids as does Schneider's (1945) method. Extraction with hot 10% TCA (method 3) removed more nucleic acid during the exposure to hot TCA than was removed by hot 5% TCA. It is possible, however, that all the DNA was not extracted even with hot 10% TCA, although the DNA contents are comparable to those reported for other bacteria.

RNA is another cytoplasmic marker but it appears to constitute a part of the envelope of H. halobium (Marshall et al. 1969) and is therefore not as reliable. The orcinol reaction used for RNA is not as specific as the diphenylamine reaction is for DNA. Any pentose will react as will hexoses (see methods). Method 3 liberates the most orcinol-reacting material, which is called RNA in the table. Ribose was easily found in chromatograms of hydrolysates of unextracted cells but it was not detected in hydrolysates of cells extracted by method 1. Calculations based on the smallest amount of ribose detectable chromatographically showed that less than 1% of the original orcinol reacting material was present.

U.V. spectroscopy on TCA extract, although not noted for specificity, yielded the total nucleic acid values and indicated that the RNA values were probably too high. As will be shown later arabinose is probably present in the cell wall and may account for some of the orcinol reacting material. The total nucleic acid values used in later tables are therefore values found by U.V. absorption. The RNA values are

Table 12

Nucleic acid content of hot TCA extracts, cells extracted by method 1 and intact cells.

| | Nucleic acids in hot TCA extracts as a percent of cell dry weight (ash free) from | | | | | | Intact cells | | DNA content of cells following extraction by Method 1 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Method 2 | | | Method 3 | | | | | | |
| | DNA | RNA | total NA | DNA | RNA | total NA | DNA | total NA | Wt% | % total NA in cell** |
| H5 Micrococcus | 0 | 4.1 | 3.6 | 1.5 | 11.5 | 7.8 | | | .053 | 1.0 |
| Sarcina morrhuae | 0.5 | 7.2 | 7.0 | 4.1 | 18.2 | 11.9 | | | .048 | 2.0 |
| S.gigantea | 0.8 | 12.8 | 9.2 | 5.6 | 19.3 | 14.9 | | | .067 | 3.7 |
| S.littoralis | 3.3 | 13.2 | 6.4 | 3.0 | 18.1 | 8.7 | | | .02 | 2.2 |
| Vibrio costicolus* | | | | | | | 3 | 25 | | |
| Micrococcus halodenitrificans* | | | | | | | 2.5 | 18 | | |
| Halobacterium salinarium* | | | | | | | 4.3 | 15 | | |

* Smithies et al. 1954 by a spectrophotometric method on suspensions of whole cells.

** this value may be in error because it is based on the DNA values obtained from the hot TCA extracts of methods 2 and 3 which may not contain all the DNA.

DNA was determined by the diphenylamine reagent. RNA by orcinol. Total NA by UV spectra.

for Methods 2 and 3 see table 10.

total nucleic acid minus the DNA.

Measurements of DNA and RNA contents of cells extracted by method 1 shows that they are essentially free of cytoplasmic constituents. Cells extracted by method 2, which extracts more material, should also be free of cytoplasmic constituents.

Unextracted cells and cells extracted by method 2 and 4 were examined with the electron microscope by M. Jean-Yves D'Aoust. Plates 9 - 13 show cross sections of cells. Plate 9 (S. littoralis) shows a cell wall about 500 A thick and a well defined nuclear area. Plate 10 (S. gigantea) shows the typical irregular arrangement of cells, one of which appears to be without a cell wall. Some of the nuclear areas are filamentous. S. morrhuae (Plate 11) is similar to S. littoralis. Plates 12 and 13 represent H5 Micrococcus; in 13 the wall is less defined and a large number of spherical (or possibly hexagonal) particles can be seen outside the nuclear area.

Plates 14 - 17 show cells extracted by method 2. In S. littoralis (Plate 14) the cell walls are well defined and the cytoplasm appears to be reduced but densely staining particles can be seen in the nuclear area. S. gigantea (Plate 15) also has densely staining particles in the nuclear area but the fibrous appearance seen in the whole cells is no longer visible. Some S. morrhuae cells (Plate 16) appear to be empty but most still have some material present. H5 Micrococcus (Plate 17) was also not completely empty.

Plates 18-22 show replicas of whole cells and 23 - 26 replicas of extracted cells. S. littoralis (Plates 18, 19 and 23) appear to have a smooth surface. The extracted cells appeared to be flatter but

Plate 9. Sarcina littoralis cells c.s. x 48,000.

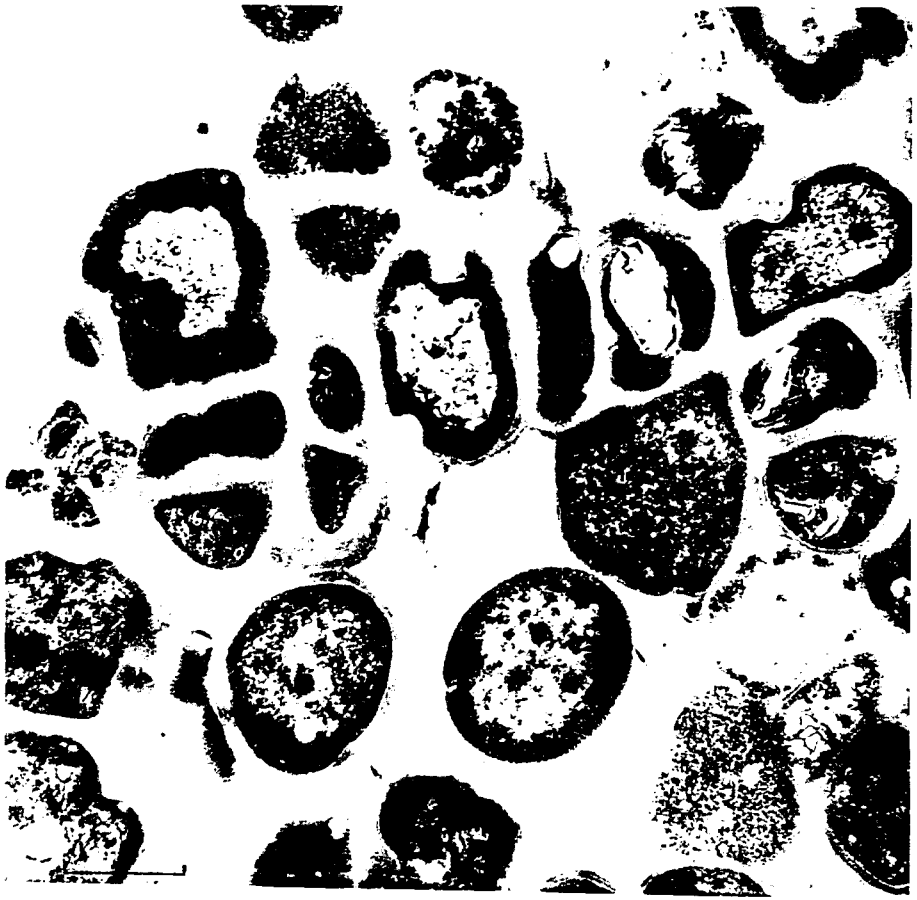


Plate 10. Sarcina gigantea c.s. x 12,000.

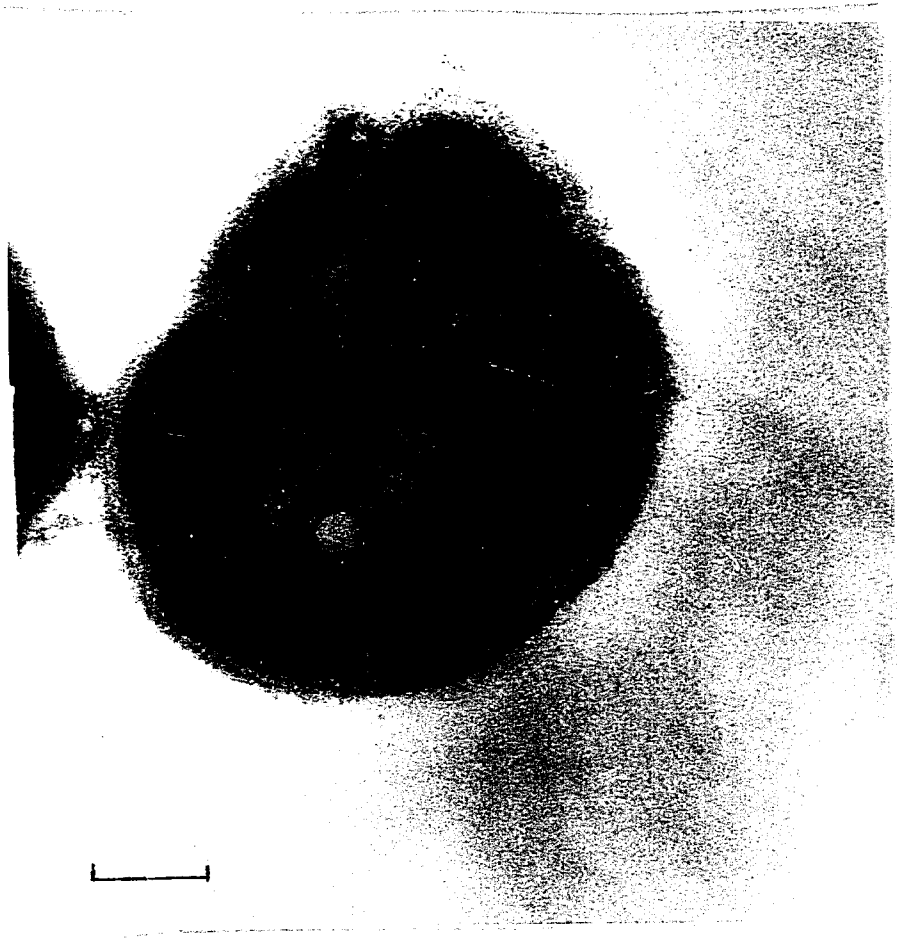


Plate 11. Sarcina morrhuae cells c.s. x 64,000.

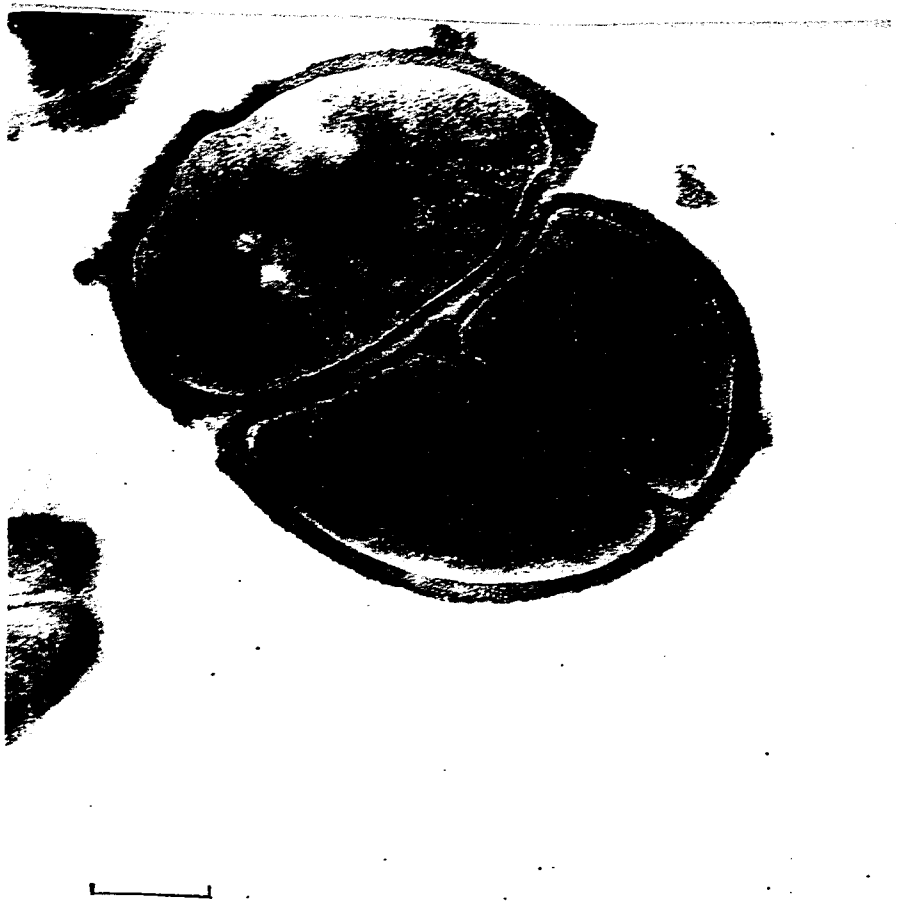
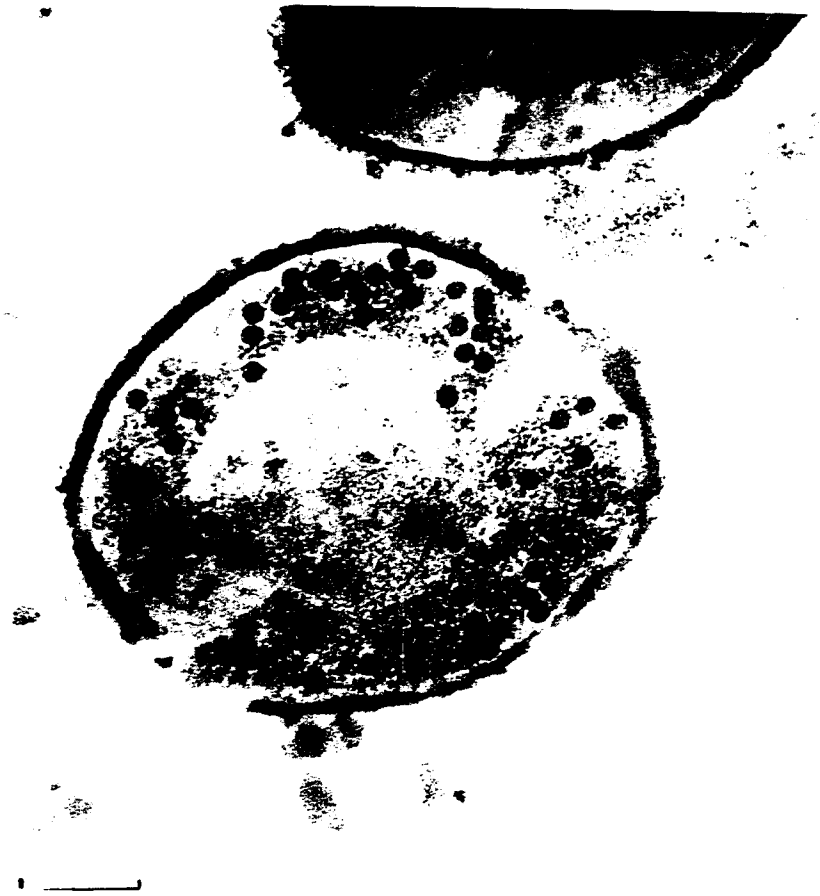


Plate 12. H5 Micrococcus cells c.s. x 64,000.



Place 13. H5 Micrococcus cells c.s. x 64,000.

Plate 14. Extracted Sarcina littoralis cells c.s. x 16,000.

Plate 15. Extracted Sarcina gigantea cells c.s. x 32,000.

Plate 16. Extracted Sarcina morrhuae cells c.s. x 16,000.

Plate 17. Extracted H5 Micrococcus cells c.s. x 48,000.

Plate 18. Replica of Sarcina littoralis cells x 16,000.

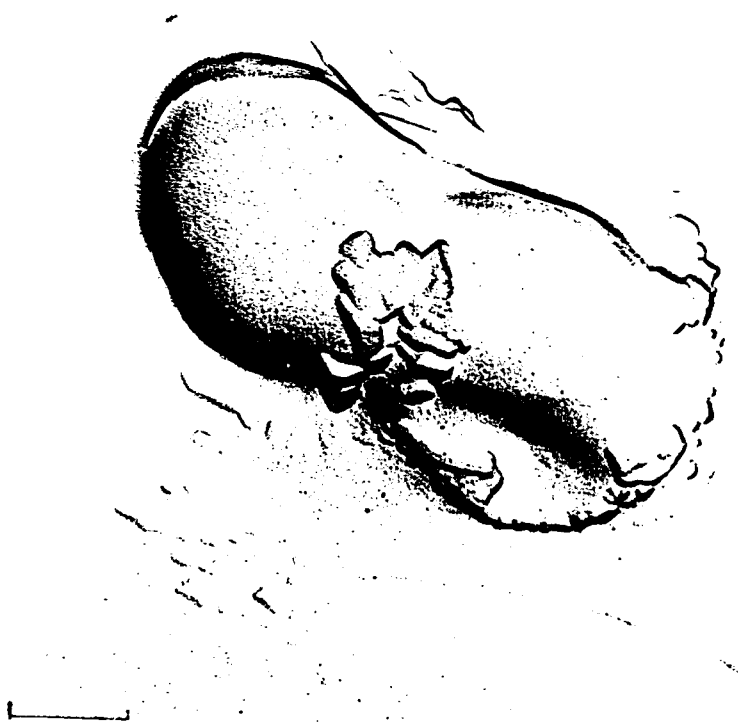


Plate 19. Replica of Sarcina littoralis cells x 32,000.

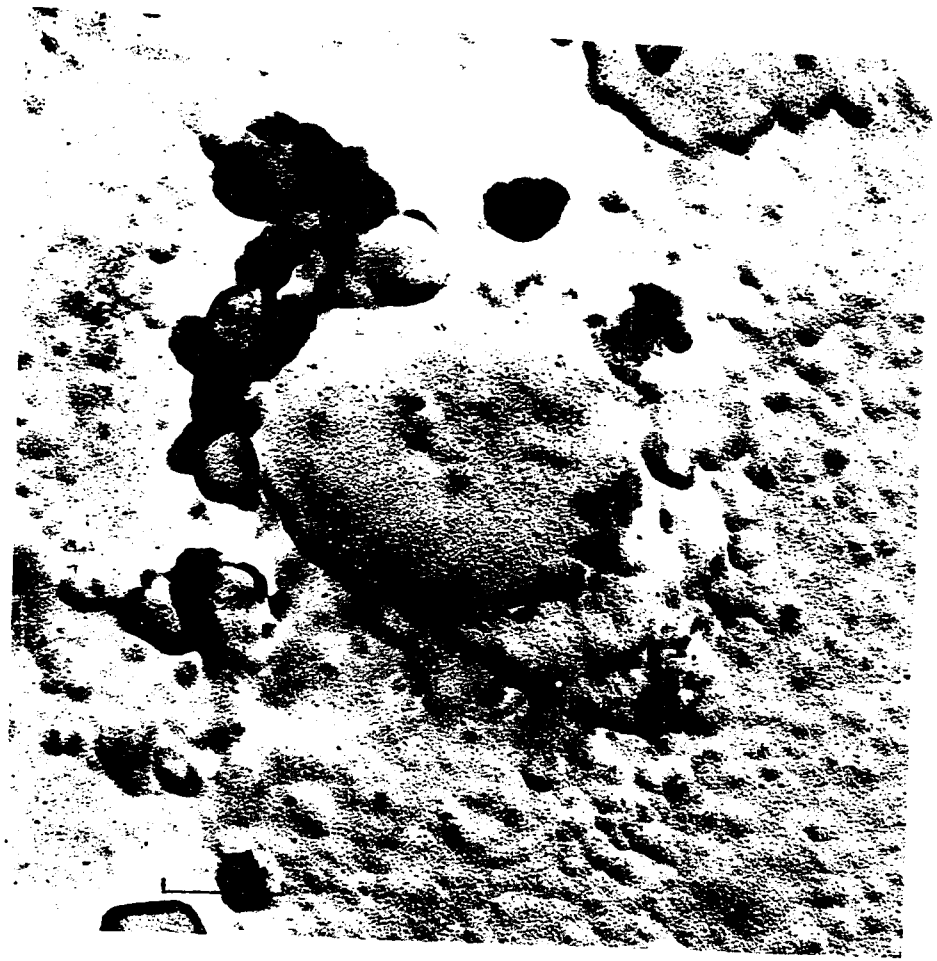


Plate 20. Replica of Sarcina gigantea cells x 32,000.

Plate 21. Replica of Sarcina morrhuae cells x 12,000.

Plate 22. Replica of H5 Micrococcus cells x 24,000.

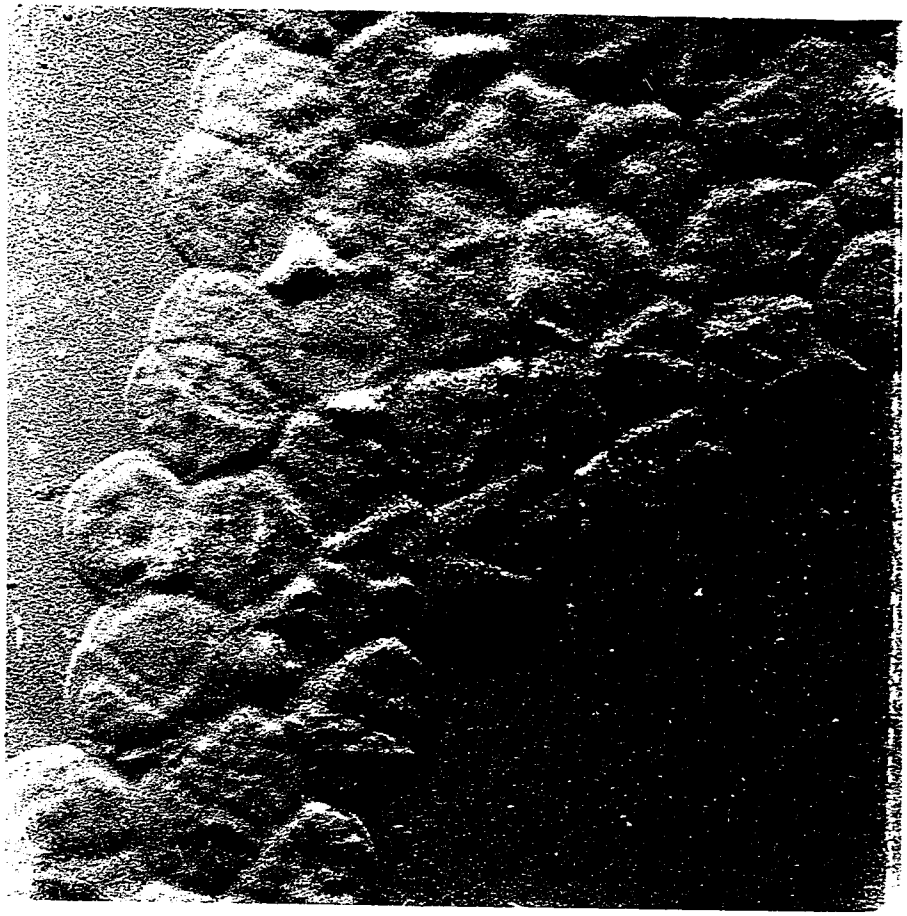


Plate 23. Replica of extracted Sarcina littoralis cells x 12,000.

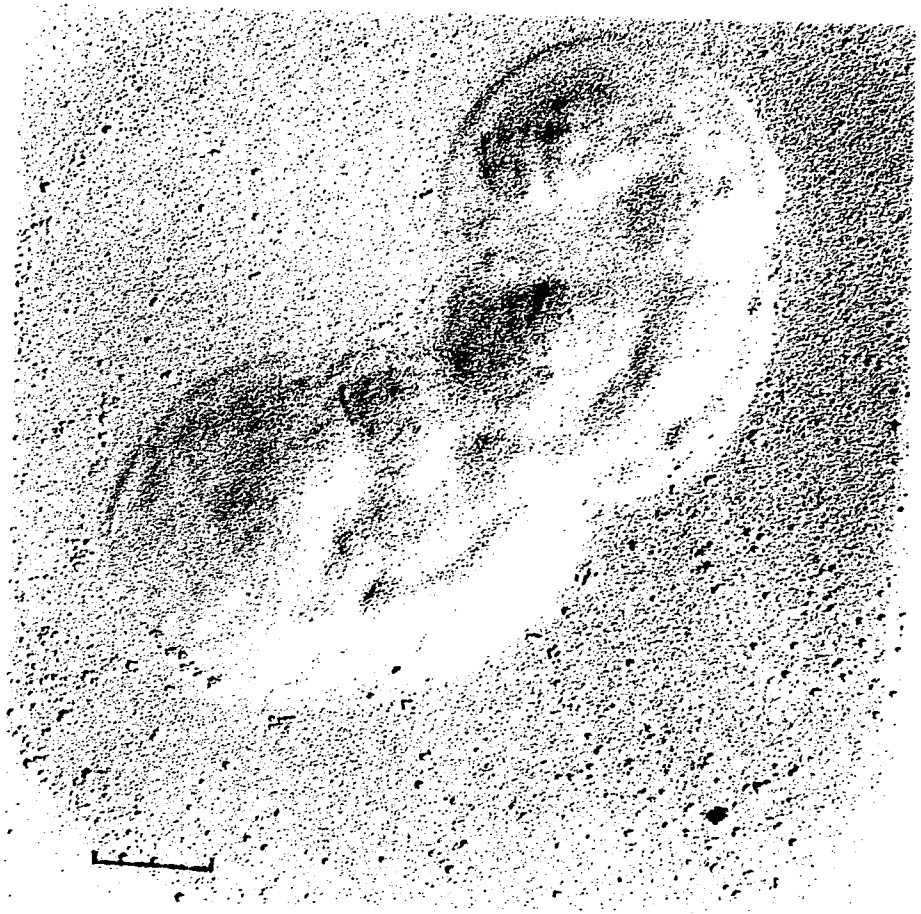


Plate 24. Replica of extracted Sarcina gigantea cells x 24,000.

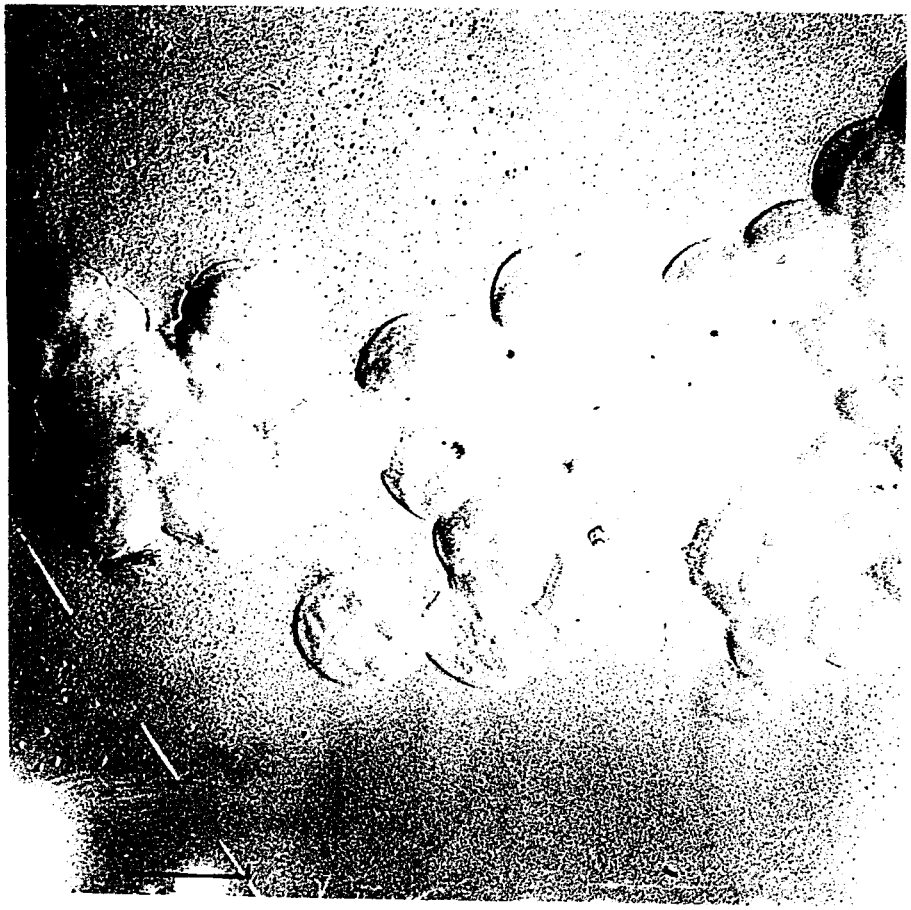


Plate 25. Replica of extracted Sarcina morrhuae cells x 8,000.

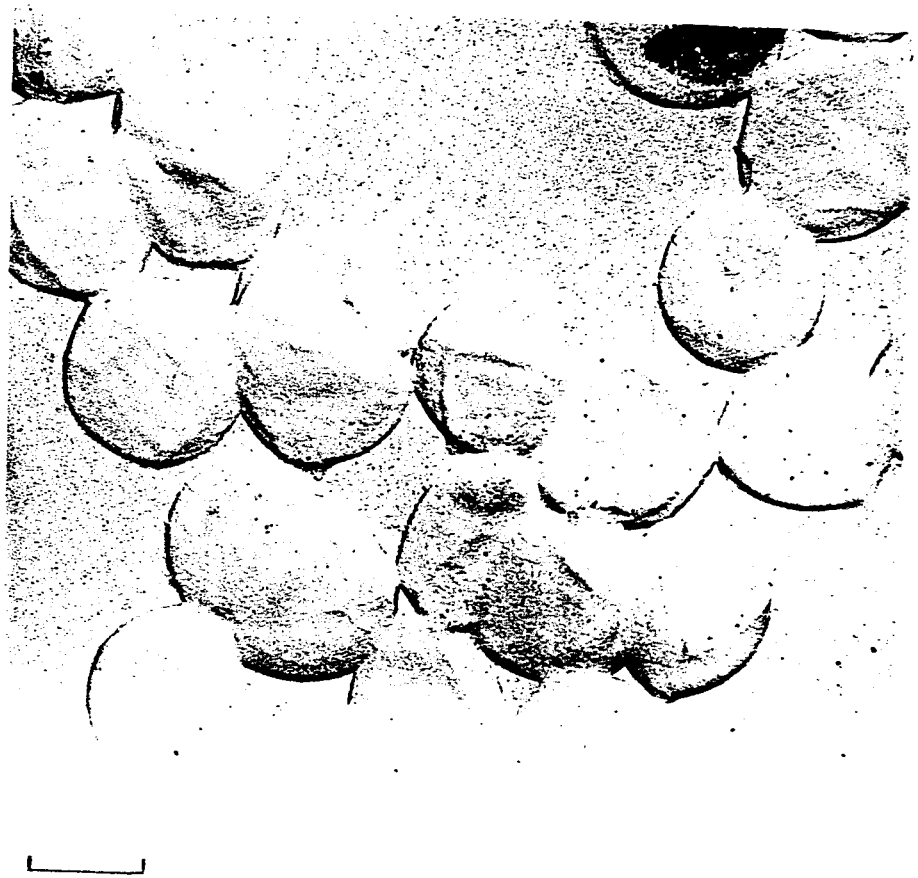


Plate 26. Replica of extracted H5 Micrococcus cells x 16,000.

slightly convoluted. S. gigantea whole cells (Plate 20) were difficult to replicate. The extracted cells (Plate 24) appear as flattened spheres. S. morrhuae (Plates 21 and 25) seems to be similar to S. littoralis. H5 Micrococcus (Plate 24) cells appear to be much flatter than the whole cells of halococci. The extracted cells (Plate 26) were convoluted.

Tables 13 through 18 are comparisons of the chemical constituents in unextracted cells with cells extracted by methods 1 and 2. Other bacteria and their cell envelopes are included for comparison.

Table 13 shows the nitrogen content. More nitrogen was removed by method 2 than by method 1. If the nitrogen that remains mainly represents protein, then protein contents range from 32% of the weight in extracted S. littoralis cells to 79% of the weight in extracted H5 Micrococcus cells. Amino sugars, as measured later, account for less than 6% of the nitrogen and contribute only a small error. Nucleic acid probably accounts for much of the nitrogen in the unextracted cells, but for little or none of the nitrogen in extracted cells.

Table 14 shows the phosphorus content. Most of the phosphorus present in the unextracted cells was removed by the extraction methods and the phosphorus removed probably accounted for the nucleic acid and phospholipid. Method 2 removed about the same percentage from each of the halococci. The residual phosphorus is similar in amount to that found in the cell envelopes of E. coli and H. cutirubrum. Curiously, more phosphorus was removed from S. gigantea by method 1 than by method 2. Both unextracted and extracted cells of S. littoralis have lower phosphorus contents than the other halococci..

## Table 13

Nitrogen content of unextracted cells, extracted cells and envelopes.

as a percent of ash free dry weight

| | Unextracted | NA%N | method 1 | | method 2 | | Envelopes |
|---|---|---|---|---|---|---|---|
| | | | wt% | % total | wt% | %total | |
| H5 Micrococcus | 7.1±0.8 (2) | 16 | 7.0 | 29 | 12.6 | 16 | - |
| Sarcina morrhuae | 6.8±0.5 (2) | 26 | 4.8 | 49 | 7.3 | 25 | - |
| S.gigantea | 5.7±0.3 (2) | 39 | 4.5 | 65 | 9.5 | 45 | - |
| S.littoralis | 6.8±0.3 (2) | 20 | 6.4 | 48 | 5.1 | 16 | - |
| E.coli | 10.8 | | | | | | 10* |
| Halobacterium salinarium** | 12.7 | | | | | | - |
| H.cutirubrum *** | 65-71% protein | | | | | | 45-57% protein |

* Salton 1953

**Smithies et al. 1954

***Kushner et al. 1964

Table 14

Phosphorus content of unextracted cells, extracted cells, and envelopes.

as a percent of ash free dry weight

| | Unextracted | NA%P | method 1 | | method2 | | Envelopes |
|---|---|---|---|---|---|---|---|
| | | | wt% | % total | wt% | %total | |
| H5 Micrococcus | 3.4±.07 (2) | 22 | 0.83 | 7.1 | 1.3 | 3.0 | - |
| Sarcina morrhuae | 4.3±.77 (2) | 27 | 0.74 | 7.2 | 1.4 | 7.5 | - |
| S.gigantea | 5.1±.63 (2) | 28 | 0.92 | 5.5 | 1.4 | 7.6 | - |
| S.littoralis | 1.4±.14 (2) | 61 | 0.48 | 12 | 0.50 | 7.9 | - |
| Escherichia coli | 3.85 | | | | | | 1.5* |
| Halobacterium salinarium** | 2.1 | | | | | | - |
| H.cutirubrum*** | 2.1 | | | | | | 1.0 |

for explainations and footnotes see table 13.

Results obtained by the Nelson-Somogyi assay for reducing substances (Table 15) must be interpreted cautiously. The presence of protein interferes with its use in determining blood glucose levels, though it is still suitable for pure sugar solutions. This assay was used by Smithies et al. (1954) and Salton (1953) for carbohydrates contained in wall preparations which, however, are known to contain protein (Rogers and Perkins 1968). As will be seen even extracted and trypsin digested halococci contained residual amino acids. Acid hydrolysed casein (Difco - Casamino acids vitamin free) has a reducing power 2.7% that of glucose. If 50% of the wall was protein then there would be about 1 or 2% non-carbohydrate reducing substance in the wall. This would be less than 10% of the reducing substance. It is possible that some substances interfere with the reducing substance and result in low values, although this did not occur with glucose and Casamino acids since the reducing powers were additive. The reducing substance then must be mostly carbohydrate.

Extracted cells of halococci have somewhat higher carbohydrate contents when they are measured by the phenol-sulfuric acid assay (Table 16), than when they are measured as reducing substances. H5 Micrococcus and Staph. aureus, however, have much lower contents. This may be explained by the amino sugars in the latter. Amino sugars were higher in H5 Micrococcus than in the halococci and Staph. aureus was found to contain 3.3% amino sugars which do not react in the phenol-sulfuric acid assay. Under less vigourous hydrolytic conditions the amount is undoubtedly higher. While Stewart-Tull (1968) has shown a similar low value for M. lysodeikticus Perkins and Rogers (1959) had

Table 15

Reducing substances content of unextracted cells, extracted cells and envelopes as a percent of ash free dry weight.

| | Unextracted | NA%RS# | method 1 | | method 2 | | Envelopes |
|---|---|---|---|---|---|---|---|
| | | | wt% | %total | wt% | %total | |
| H5 Micrococcus | 3.8±.04 (2) | 33 | 11.2 | 85 | 16.1 | 38 | - |
| S.morrhuae | 10.4±0.6 (2) | 12 | 13.6 | 55 | 24.3 | 54 | - |
| S.gigantea | 6.9±.04 (2) | 21 | 11.1 | 50 | 19.6 | 77 | - |
| S.littoralis | 10.0±0.7 (2) | 9 | 13.4 | 48 | 24.8 | 55 | - |
| Escherichia coli | 3.3±0.3 (2) | | | | | | 16* |
| Halobacterium salinarium** | 3.0 | | | | | | -- |
| H.cutirubrum*** | 2.94 | | | | | | 7.0 |
| Staphylococcus aureus | 11.9 | | 27.6 | | | | |

# Only ribose in RNA has a significant reducing power since the reducing power of DNA is one tenth that of the RNA. The ribose content was determined with orcinol.

for other footnotes and explanations see table 13.

Table 16

Carbohydrate content of unextracted and extracted cells.

Phenol-sulfuric acid assay expressed in grams

of glucose/100g. ash free dry weight.

| | Unextracted | Method 1 | | Method 2 | |
|---|---|---|---|---|---|
| | | wt% | %total | wt% | %total |
| H5 Micrococcus | 3.1±0.2 (2) | 1.48 | 14 | - | - |
| Sarcina morrhuae | 13.5±0.8 (2) | 18.8 | 59 | 46.8 | 80 |
| S.gigantea | 10.6±0.4 (2) | 20.0 | 58 | 33.6 | 86 |
| S.littoralis | 11.3±0.5 (2) | 19.6 | 62 | 46.5 | 90 |
| Escherichia coli | 6.3±.07 (2) | | | | |
| Staphylococcus aureus | 3.9 | 0.86 | - | | |

for explainations of headings and footnotes see table 13

shown that about one-third of the wall could be accounted for as glucosamine and muramic acid if the hydrolysis was carefully controlled. Carbohydrate (measured by the phenol-sulfuric acid assay) accounts for a third to almost half of the weight of halococci cells extracted by method 2. Eighty to 90% of the carbohydrate in the unextracted cells is present in halococci cells extracted by method 2. Halococci extracted by methods 1 and 2 were assayed for hexoses and pentoses by the cysteine- sulfuric acid assay (Table 17). Hexoses represent about 30% and pentoses about 3% of the carbohydrate present in the halococci after extraction by method 1. In halococci extracted by method 2 hexoses account for 64 to 95% of the carbohydrate and pentoses 6 -17%. Cells extracted by method 2 were also assayed for pentose with the orcinol reaction. The values shown are uncorrected for hexoses and other substances which contribute to the color; nevertheless uncorrected pentose accounts for about 8% of the total carbohydrate in the halococci.

The neutral sugars (Table 18) account for 25 - 38 % of the carbohydrates and seem to be mostly hexoses. Glucose and galactose account for about one third of the hexoses in the column eluates. Column losses were not determined but some of the carbohydrate may still have been bound to ionic groups, such as amino acids, and therefore retained on the column.

Paper chromatograms of neutral sugars (Figure 11) yielded five spots for each of the halococci. Thin layer chromatograms and three detection reagents did not yield any additional spots. All the halococci showed the same pattern. Two major spots were readily identified as

Table 17

Hexose and pentose contents of extracted cells.

| | Method 1 | | | | Method 2 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Hexose | | Pentose | | Hexose | | Pentose | | Pentose* | |
| | wt% | % total | wt% | % total | wt% | % total | wt% | % total | wt% | % total |
| H5 Micrococcus | 0 | - | 0.4 | .27 | - | - | - | - | 1.3 | - |
| Sarcina morrhuae | 6.1 | 32 | .47 | 2.5 | 30 | 64 | 2.8 | 6 | 3.8 | 8.1 |
| S.gigantea | 7.6 | 38 | .66 | 3.3 | 32 | 95 | 4.4 | 13 | 2.8 | 8.3 |
| S. littoralis | 6.2 | 32 | .59 | 3.0 | 39 | 84 | 8 | 17 | 3.6 | 7.7 |

* by orcinol all others by cysteine-sulfuric acid, glucose and ribose used as standards.

% total means the percent of total carbohydrate (by phenol-sulfuric acid) in extracted cells. accounted for as hexose or pentose.

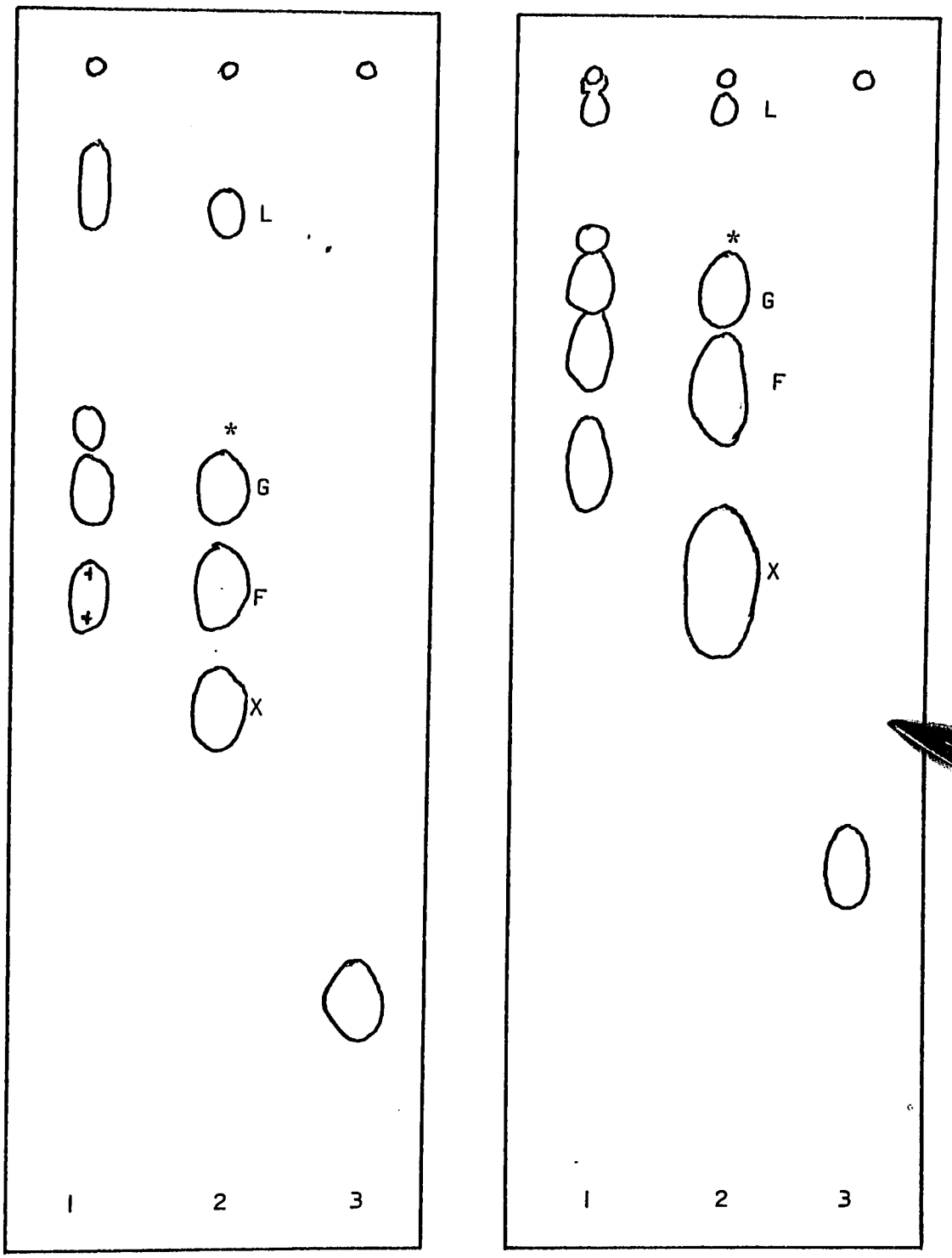
wt % means the percent of hexose or pentose in extracted cells (as glucose or ribose).

Table 18

Neutral sugars in cells after extraction by method 1.

| | Reducing substances* | Carbohydrates* | | Glucose | Galactose |
|---|---|---|---|---|---|
| | Wt % | Wt% | %total | Wt% | Wt% |
| H5 Micrococcus | 1.9 | .006 | - | - | - |
| Sarcina morrhuae | 2.3 | 4.6 | 25 | 1.2 | 0.26 |
| S. gigantea | 4.0 | 5.1 | 26 | 1.3 | 0.43 |
| S. littoralis | 5.4 | 7.4 | 38 | 2.0 | 0.54 |

* as glucose




Benzene:BuOH:Pyridine:$H_2O$ Ethyl acetate:Pyridine:$H_2O$

Sugar chromatograms of cells after extraction by method 2.

1.Sarcina littoralis 2.Lactose (L), Glucose (G), Fructose (F), Xylose (X), * position of galactose found on earlier chromatograms of known sugars. 3.H5 Micrococcus.

Figure 11

glucose and galactose, a finding confirmed by the respective oxidase tests. Two minor spots, which on the ethyl acetate:pyridine:water solvent corresponded to arabinose ($R_{glu}$ 2.0)* and mannose ($R_{glu}$ 1.45), are combined into a single elongated spot on benzene:butanol:pyridine: water solvent (arabinose 1.21, mannose 1.29); therefore they are tentatively identified as arabinose and mannose. The fifth small spot which had a low $R_{glu}$ value in all solvents behaved as lactose but lactose would probably have been destroyed during hydrolysis; therefore, this spot remains unidentified. A small amount of material also remained at the origin in the ethyl acetate: pyridine: water solvent on paper; it was not investigated. The unidentified spots could be carbohydrate polymers.

H5 Micrococcus sugars had a quite different pattern; they contained one fast running spot. A comparison of its $R_{glu}$ value in two solvents with that in the literature (Hough and Jones 1962) suggested it is probably glucuronolactone, which would be formed from glucuronic acid on acid hydrolysis and drying. A less likely possibility would be 6-deoxy-L-mannose but 6-deoxyhexoses were shown not to be present in the cysteine-sulfuric acid assay,whereas glucuronolactone could be present and undetected.

It was initially thought that the 75% ethanol in the Park and Hancock (1960) method would extract lipid from these cells as it does

* $R_{glu}$ is the distance from the origin of "X" divided by the distance from the origin of glucose.

for Staph. aureus but a considerable amount of petroleum ether extractable material was found after hydrolysis of method 1 extracted cells (Table 19). On the suggestion of Dr. M. Kates repeated (2X) extractions by the Bligh and Dyer method were used to get the total lipids. Kates et al. (1966) had found only 0.7% lipid in S. littoralis which Dr. Kates thought might be low because of incomplete extraction (Personal communication). After such repeated extraction,(Column A Table 19) hydrolysis and petroleum ether extraction of cells (E) yielded very little more material. The chloroform:methanol:water extractant was substituted for 75% ethanol, thus creating method 2. The lipid extracted by petroleum ether from hyrolysates of method 1 extracted cells is greater than that found by the Bligh and Dyer method in whole cells suggesting some lipid may be lost in the water phase of the Bligh and Dyer extraction (see discussion).

It was thought that perhaps amino acids were extracted with petroleum ether and contributed to the weight but when acid hydrolysed casein is extracted by the same procedure only 0.1% of the material is extracted showing that this source of error is unlikely. The matter was not investigated further as the lipid was not considered important to the investigation of the envelope and the S-layer.

The lipid extracted from method 1 extracted cells is similar in amount to that found in E.coli and H. salinarium envelopes which were prepared mechanically. Salton (1953) also extracted envelopes with ether but obtained only 36% of the total E. coli lipids which were extractable after hydrolysis. Similarly only 12% of the Bacillus

Table 19

Lipid content of unextracted cells, cells after extraction by method 1 and cell envelopes. g/100g ash free dry weight

| | Unextracted | | Extracted cells | | Envelopes | | | |
|---|---|---|---|---|---|---|---|---|
| | A | E | B | | C | D | B | A |
| H5 Micrococcus | 3.0 | .053 | 17.7 | (5.1) | | | | |
| Sarcina morrhuae | 1.1 | .047 | 9.9 | (4.2) | | | | |
| S.gigantea | 1.4 | .077 | 17.0 | (5.3) | | | | |
| S.littoralis | 1.7 | .068 | 13.0 | (4.7) | | | | |
| Escherichia coli* | | | | | 8.2 | 8.6 | 22.5 | |
| Bacillus subtilis* | | | | | 0.3 | 1.6 | 2.5 | |
| Halobacterium salinarium | 3.6** | | | | | | 11.0*** | |
| H.cutirubrum | 3.5** | | | | | | | 22**** |

A by Bligh and Dyer (1959)

B ether extractable after hydrolysis

C ether extractable

D ether extractable after reflux with methanol

E ether extractable after hydrolysis of residue pellet from Bligh and Dyer (not including aqueous phase).

* Salton 1953 ** Kates et al. 1964 *** Smithies et al.1954 ****Kushner et al.1964

( ) lipid in extracted cell expressed as a percentage of the unextracted cell weight (ash free).

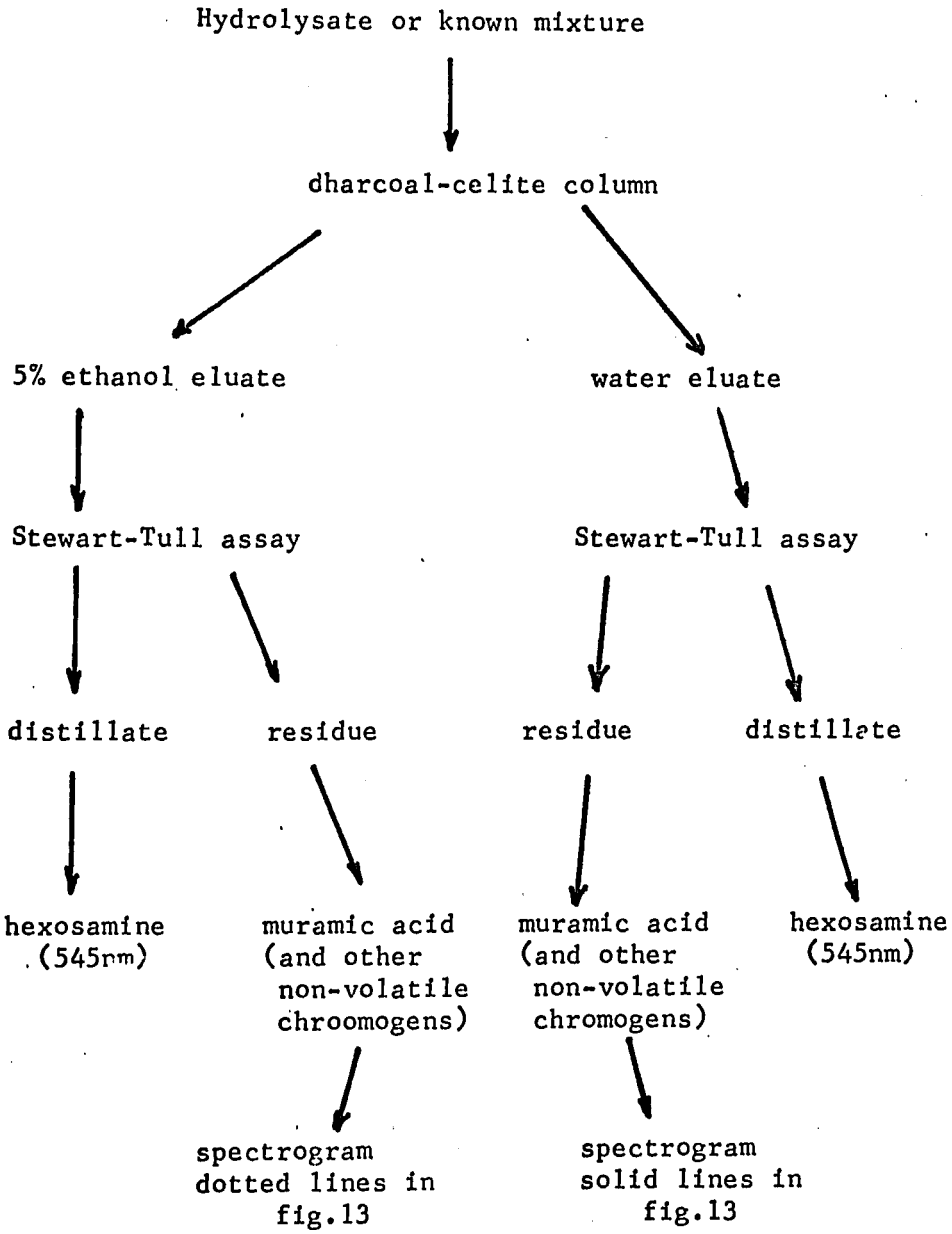
*subtilis* lipids were extractable without hydrolysis.

Search for Muramic Acid in Extracted Cells

Some substances still present in the extracted cell residue gave colored products which interfered with the determination of muramic acid chromophore in the Stewart-Tull assay. The hydrolysates were therefore separated on charcoal-Celite columns. The water and 5% ethanol eluates were assayed for hexosamine and muramic acid (Figure 12). In Figure 13A are spectrograms of the muramic acid assays of the water and ethanol eluates of the standard solution containing muramic acid. The 5% ethanol fraction shows the characteristic absorption maximum at 500-510 nm. The muramic acid was completely recovered from the column in the 5% ethanol fraction. The rather high extinction (83% of that in the ethanol fraction) at 500-510 nm of the water fraction suggests that the column contributed a chromogenic substance.

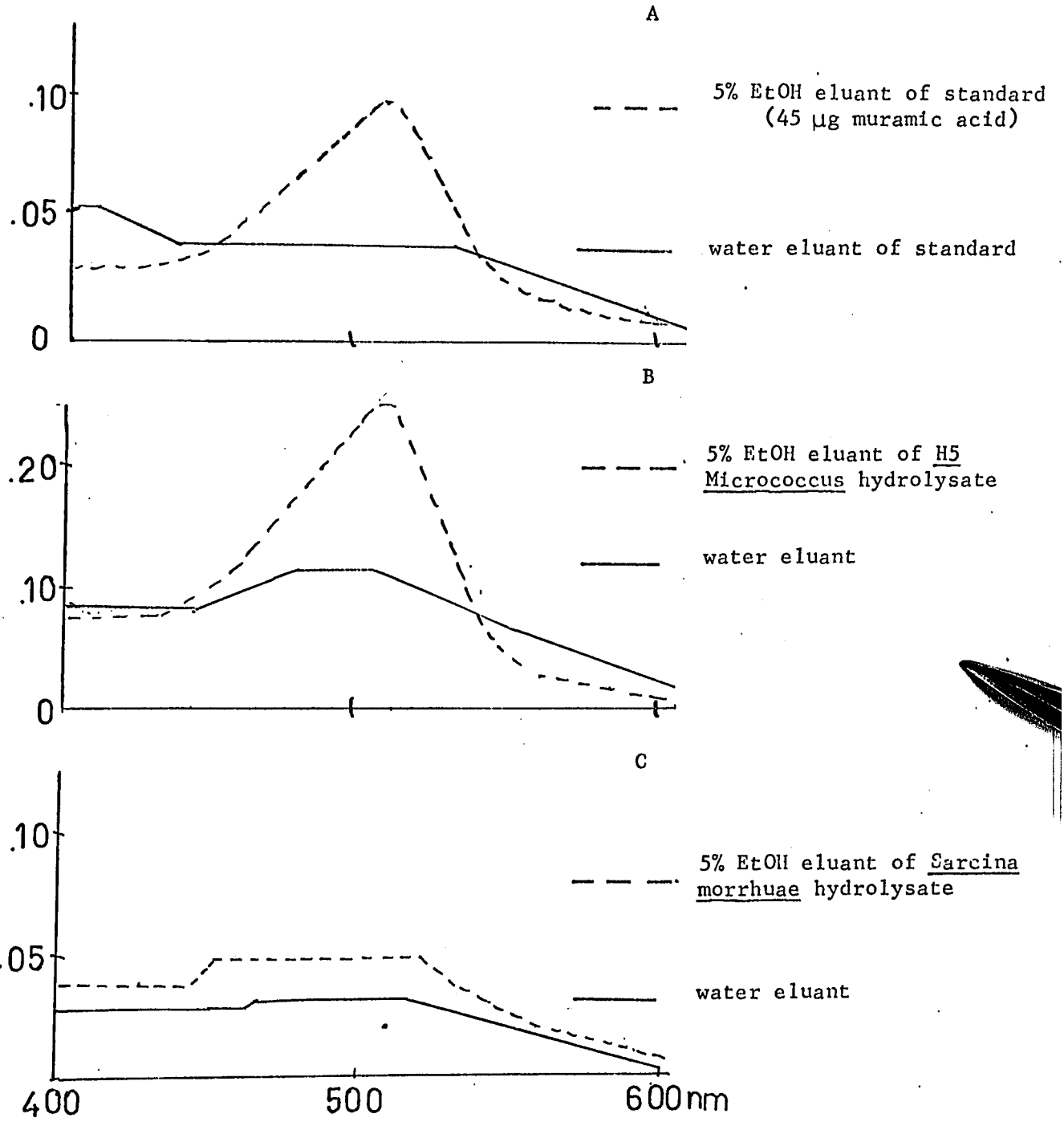
Figure 13B is the spectrogram of H5 Micrococcus eluants. The ethanol eluant showed an obvious muramic acid peak but the water eluant also showed a peak with an absorption maximum at 490 nm. Figure 13C is the spectrogram of S. morrhuae eluants. This spectrogram was the same as S.littoralis and S. gigantea spectrograms. The center of the flat topped peak is 485 nm.

Because of the difference in spectra it seemed unlikely that the material absorbing at 510 nm was truely muramic acid. Even if it was, it was present in very low concentrations. Values for the cocci studied here and values obtained for other bacteria are given in Table 20 and they show that if 510 nm absorbing material from the halococci is muramic acid then it is present in less than one tenth the concentration




Fractionation procedure used to obtain spectra of amino sugar chromophores

Figure 12




Spectra of muramic acid assay of charcoal-Celite column eluates (see figure 12). Optical densities were read against an identical assay using water in place of the eluant.

Figure 13

Table 20

Amino sugar content of cells extracted by method 1.

| | g/100g dry weight | | | molar ratio | | |
|---|---|---|---|---|---|---|
| | Glucosamine | Galactosamine | Muramic acid | Glucosamine | Galactosamine | Muramic acid |
| H5 Micrococcus | 3.3 | 0.17 | 1.0 | 1 | 0.052 | 0.22 |
| Sarcina morrhuae | 2.3 | 0.92 | $\leq$ 0.04 | 1 | 0.41 | $\leq$ 0.013 |
| S. gigantea | 1.6 | 0.56 | $\leq$ 0.08 | 1 | 0.35 | $\leq$ 0.037 |
| S.littoralis | 1.7 | 0.85 | $\leq$ 0.08 | 1 | 0.51 | $\leq$ 0.034 |
| Halobacterium halobium envelopes | hexosamine | 0.5-1.2* | | 1 | 0.87** | - |
| Pseudomonas aeruginosa envelopes*** | 0.56 | 0.18 | 0.36 | 1 | 0.32 | 0.45 |
| Staphylococcus aureus envelopes*** | 0.55 | 0.03 | 0.48 | 1 | 0.054 | 0.63 |
| Staph.aureus Duncan | 1.7 | | 1.58 | | | |
| Staph.aureus Duncan | 0.932**** | | | | | |

*Brown and Shorey (1963), Brown et al.(1965).

**Stoeckenius and Kunau (1967) Total of amino sugars in fractions

*** Stewart-Tull (1968).

****calculated from Park and Hancock (1960)

found in the H5 Micrococcus and in substantially lower concentration than is found in the other bacteria.

Muramic acid gives a characteristic peak on an amino acid analyser. When hydrolysates of the different bacteria studied were investigated in this way, only small, and possibly insignificant, peaks which might correspond to muramic acid could be found in the halococci (Figure 14), but E. coli, Staph. aureus and H5 Micrococcus showed definite peaks. Because of the lack of separation between muramic acid and glutamic acid the concentration cannot be determined by integration. Using the peak height to determine concentration is less accurate than using the peak area but it is a reasonable alternative. This technique gave a muramic acid concentration in extracted H5 Micrococcus of 0.93% and concentrations of muramic acid in the halococci of less than 0.03% (compare to Table 20). The shoulder used to determine the value in the halococci was a slight deviation of the glutamic acid peak near the base. It was not regarded by the operator of the amino acid analyser as being significant. The shoulder on H5 Micrococcus glutamic acid was quite obvious and definite.

The analyser also yielded rough amino acid values for the material remaining in the extracted bacteria (Table 21); None appeared to dominate. In contrast, Staph aureus treated in the same way contained only a large amount (88%) of characteristic peptidoglycan amino acids (glycine, alanine, lysine, glutamic acid). These results show that some protein (10-20% of weight) remained.

Amino acid analyser plots around glutamic acid peak

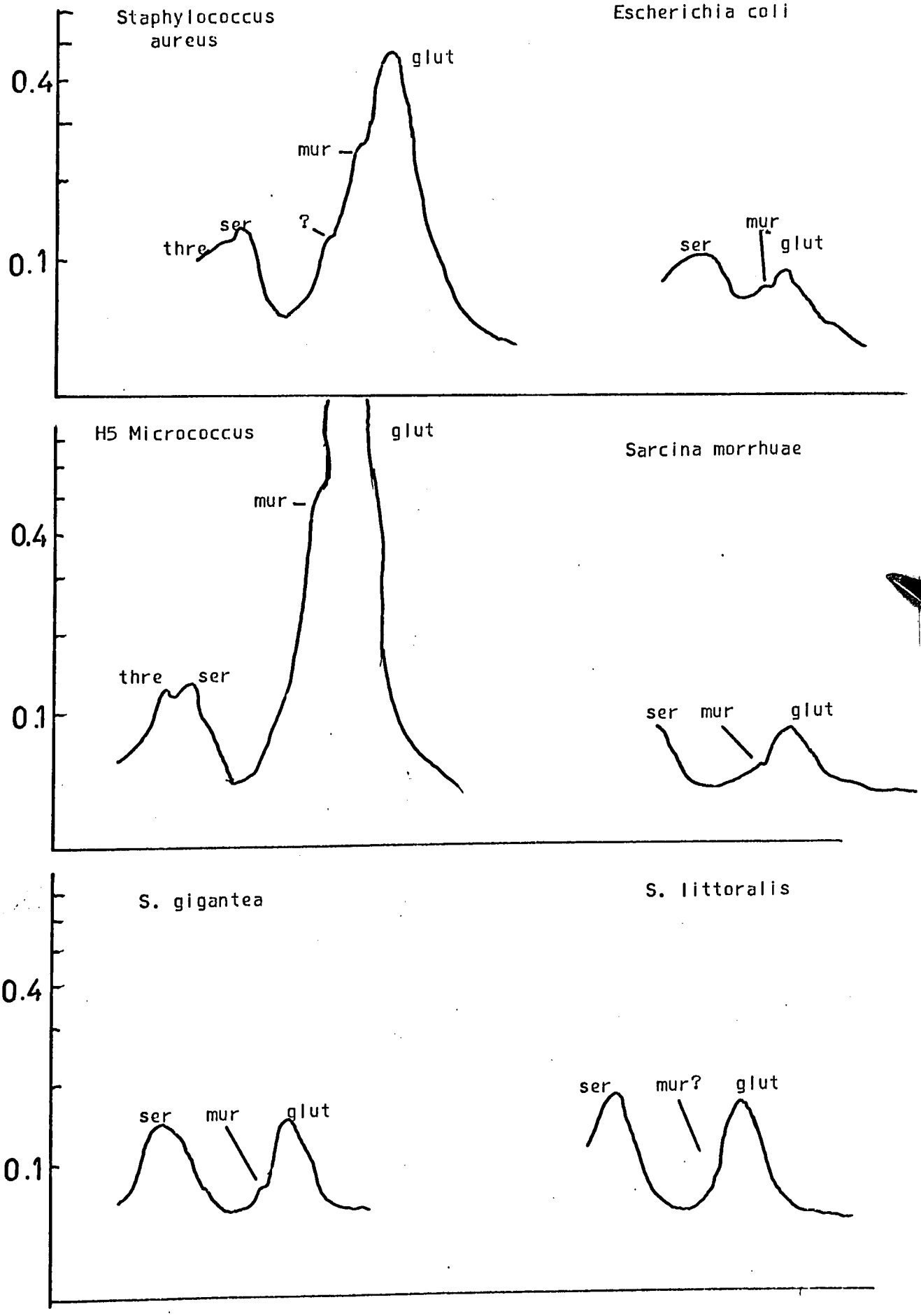



Figure 14

Table 21

Amino acid analysis of cells after extraction by method 1

hydrolysed 14 hours 110°C 6N HCl

expressed as mole %.

| | H5 Micrococcus | Sarcina morrhuae | Sarcina gigantea | Sarcina littoralis |
|---|---|---|---|---|
| Aspartic | 3.2 | 9 | 7.5 | 8.5 |
| Serine | 2.5 | 6.7 | } 12 | } 10.7 |
| Threonine | 2.3 | 5.9 | | |
| Muramic | 476** | - | - | -- |
| Glutamic | | 7.8 | 9.7 | 8.4 |
| Proline | - | - | trace | - |
| Glycine | 3.5 | 12.3 | 14.9 | 10.8 |
| Alanine | 15.7 | 8.3 | 11.2 | 13.7 |
| Valine | 1.8 | 5.5 | 5.0 | 6.1 |
| Methionine | 4.1 | 4.7 | 15 | 15.6 |
| Isoleucine | 11.5 | 4.3 | 3.0 | 2.5 |
| Leucine | 2.9 | 8.0 | 8.4 | 8.6 |
| Tyrosine | trace | 2.7 | 0.9 | 1.4 |
| Phenylalanine | 2.4 | 7.5 | 3.9 | 3.5 |
| Ammonia * | 69 | 148 | 69 | 74 |
| Lysine | 1.5 | 7.8 | 3.7 | 3.3 |
| Histidine | 0.7 | 4.8 | 2.2 | 2.5 |
| Arginine | 0.9 | 5.6 | 2.7 | 4.5 |
| uM/mg | - | .64 | .70 | 1.1 |

* not included in total

** this value was determined as if the peak were due to glutamic alone and it was also too high for an accurate determination, see text for discussion.

Further Chemical Degradation of the Cell Walls

The most detailed chemical work was carried out on material extracted by various modifications of the Park and Hancock procedure. Electron microscope and chemical studies showed that they were relatively free from cytoplasmic contaminants and suggest that it represents a good first approximation to the cell walls. Some further extractions have also been carried out using hot formamide (method 4) and phenol (method 5).

After hot formamide treatment the density was reduced by adding acid alcohol and the residue spun down. Electron micrograph replicas show that the residue still resembles the cells. This material and material which can be precipitated from the formamide-acid alcohol mixture with acetone were analysed for reducing substances and hexosamine. The total content was distributed as shown in Table 22.

The cell residue contains about one-third of the total hexosamine and reducing substances. This means that method 4 extracted S. morrhuae cells contain 88% reducing substances (expressed as glucose), and 26% hexosamine (as glucosamine) by weight. Formamide extracted cells of S. gigantea contain 28% reducing substances and 6% hexosamine. Because of the small amount of material remaining the weights and chemical determinations were done on two separate extractions. The weight of one of the acetone precipitates was also determined and amounted to more than the total beginning extracted cell weight. The other appeared to behave similarly. After hydrolysis, method 4 extracted cells were chromatographed for the amino acids but heavy trailing and spot distortion suggested extreme care is necessary in interpreting results.

Table 22

Content of formamide extracted material (method 4) expressed as a percentage of the amount present before formamide treatment.

| | Reducing substances (as glucose) | | Hexosamine (as glucosamine) | |
|---|---|---|---|---|
| | Cell residue | Acetone precipitate | Cell residue | Acetone precipitate |
| H5 Micrococcus | 31 | 5.5 | 41 | 18 |
| Sarcina morrhuae | 29 | 4.3 | 35 | 18 |
| S. gigantea | 30 | 22 | 32 | 36 |
| S. littoralis | 36 | 17 | 36 | 32 |

Amino sugars from hydrolysates of S. littoralis crude S-layers were chromatographed in 2 dimensions by Gal's method and sprayed with Elson-Morgan reagents (Gal 1968). On these preliminary chromatograms spots were obtained, none of which corresponded well enough with glucosamine, galactosamine or muramic acid to permit ready identification. Five spots were found in H5 Micrococcus extracted cell hydrolysates. One corresponded fairly well with muramic acid and the other did not correspond with glucosamine or galactosamine. Two unknown spots in H5 Micrococcus correlated with two unknown spots in S. littoralis. The difficulties encountered were not unexpected as formamide probably changes sugars and amino sugars because of the high pH (11) and high temperature (170°C).

Discussion

In 1963 Brown and Shorey and in 1964 Kushner stated that muramic acid was not found on paper chromatograms of halobacterial cell envelope hydrolysates. Some muramic acid might be present in small, structurally unimportant, amounts. Similar results could not be readily demonstrated using hydrolysates of halococci cells so a chemical method was sought for extracting salt free walls. A relatively simple procedure based on Park and Hancock's (1960) peptidoglycan extraction method, yielded material which represents a first approximation to the cell wall.

Hydrolysates of extracted cells contained a chromogenic material in a quantitative test for muramic acid. The chromophore did not have the same spectra as a muramic acid chromophore. After separation by a proven method a very small amount of chromogenic material remained in a fraction which would contain muramic acid. The chromogenic material could represent a small amount of muramic acid. Similar conclusions can be drawn from amino acid analyser results. An amount of muramic acid as small as this would not be readily detected by paper chromatography. These results generally confirm the absence of muramic acid in extremely halophilic bacteria.

It is also possible that another substance similar to muramic acid may be present. Such a substance was found by Larsen (1967). He reported a 2-amino sugar substituted in the 3 carbon position which yielded color in the Rondle-Morgan reaction similar to muramic acid but the amino sugar did not behave chromatographically as muramic acid.

Although it seemed that muramic acid might be replaced by another substituted amino sugar in peptidoglycan some factors suggest that this is unlikely. Peptidoglycan from other bacteria contain reducing amino sugars which fail to yield color with the phenol-sulfuric acid reaction for carbohydrates. In the halococci walls large amounts of phenol-sulfuric acid positive carbohydrates are present. Indeed, the walls as prepared by Method 2 are almost completely carbohydrate and protein (or peptide). The carbohydrate is almost exclusively hexose (notably glucose and galactose). Further preliminary extraction with formamide suggests that carbohydrate and a lesser amount of hexosamine comprise the structure conferring S-layer.

One of the aims of this study was to determine the similarity of these organisms to Gram negative and Gram positive bacteria but this is rather meaningless with an organism which apparently lacks a peptidoglycan layer. In this and other factors the halococci are similar to the fragile halobacteria.

The lack of a demonstratable peptidoglycan in halobacteria and halococci seems to be related to the high salt requirements of these organisms. The relation has been elucidated in the halobacteria but not in the halococci. The salt may simply be required to maintain conformation of the halococci cell walls as it is for halophilic enzymes.

The content of the crude cell walls is summarized in Table 23. The halococci have a cell wall containing mostly carbohydrate and probably protein or peptide. The carbohydrates and amino sugars form

Table 23

Composition of crude "cell walls".

( Cells extracted by method 2 are assumed to be crude "cell walls").

| | H5 Micrococcus | Sarcina morrhuae | Sarcina gigantea | Sarcina littoralis |
|---|---|---|---|---|
| % of cell wt. | 8.9 | 23 | 27 | 22 |
| Nitrogen | 12.6 | 7.3 | 9.5 | 5.1 |
| Phosphorus | 1.3 | 1.4 | 1.4 | 0.5 |
| Reducing substances | 16.1 | 24.3 | 19.6 | 24.8 |
| Carbohydrate | nil | 46.8 | 33.6 | 46.5 |
| Hexose | - | 30 | 32 | 39 |
| Pentose | - | 2.8 | 4.4 | 8 |
| Lipid* | .053 | .047 | .077 | .068 |
| DNA** | .17 | .088 | .077 | .033 |
| RNA*** | $<.1$ | $<.2$ | $<.2$ | $<.2$ |
| Muramic acid** | 3.25 | $\leq.073$ | $\leq.09$ | $\leq.13$ |
| glucosamine** | 10.7 | 4.2 | 1.84 | 2.8 |
| galactosamine** | .55 | 1.7 | .64 | 1.4 |

all values except % cell wt. are g/100g dry wt of extracted cell

* see table 19

**assumes that all the compound found in method 1 extracted cells is present in method 2 extracted cells.

*** based on the non appearance of ribose on chromatograms

an S-layer. The H5 Micrococcus has muramic acid and therefore peptidoglycan. The high nitrogen content suggests much protein. The lipid contents shown in the table are those which are ether extractable after hydrolysis. Most of the lipid was removed by the Bligh and Dyer(1959) extraction but much of it did not stay in the chloroform phase suggesting a lipoprotein or lipopolysaccharide soluble in chloroform:methanol: water (1:2:0.8) but not in the chloroform methanol phase. It was not important in maintaining shape.

These results have been very recently confirmed in a note by Brown and Cho (1970) for S. morrhuae and S. littoralis who found that an X press was able to break some cells.

Thesis Summary and Discussion.

These studies have shown that there exist in and near the sea bacteria capable of growth at quite high salt concentrations. If an essential structure changed and failed to function at low salt concentration then the organism would be moderately halophilic at least under some salt concentrations.

The nutritional requirements of a moderately halophilic bacteria were found to be dependant on salt concentration as are some non-halophiles. This organism and an extremely halotolerant coccus were found to contain muramic acid and are therefore similar to the non-halophiles.

A lack of significant amounts of muramic acid in the halococci confirm the generalization that extremely halophilic bacteria lack the peptidoglycan R-layer of non-halophiles. Studies of the lipid by Dr. M.Kates also confirm that the special lipids of the halobacteria are also found in the halococci and so far have been found nowhere else. The lack of peptidoglycan and the presence of unusual lipids may be related because the biosynthesis of peptidoglycan requires a lipid carrier which may not be present or functioning in the extreme halophiles. Clearly the extreme halophiles are a very special group of organisms difficult to relate to other bacteria.

The unique properties of the extreme halophiles, however, are not required for growth at high salt concentration. H5 Micrococcus for example grows rapidly at high salt and at low salt concentrations. The extreme halophiles must have been trapped in their ecological niches by their special structures.

Could these organisms have arisen spontaneously in their environment? The presence of a fatty acid synthetase with maximum activity at low salt concentration (Pugh et al. 1971) suggests that they arose from a pre-existing less halophilic organism.

It seems possible that the extreme halophiles are not closely related to the bacteria. If they are then they should have the enzymes necessary for the synthesis of muramic acid. The salt concentration for maximum activity (if present) would be an important clue to the ancestry of the extreme halophiles. Brown and Cho (1970) stated that the extreme halophiles were incapable of synthesizing muramic acid because it did not occur in the cell envelopes. Such conclusions are not justified because these organisms may be capable of synthesis but not of transporting the peptidoglycan subunit across the membrane.

If the extremely halophilic bacteria are incapable of synthesizing muramic acid then they may be of non-bacterial origin. They could be closely related to the algae. Some circumstantial evidence is available for this hypothesis. Some green algae have DNA satellite bands with G + C contents (64% and 56%) similar to the extreme halophiles (Gense-Vimon 1971). Gas vacuoles, found by Stoeckenius and Kunau (1967), are unusual in bacteria but not in algae (see Petter 1931). The lack of a rigid cell wall in the halobacteria has also been reported in the green halophilic alga, Dunaliella parva which also has unusual permeability (Ginzburg 1969). Lastly, the walls of the halococci seem to be more like those of plants than of bacteria. Obviously, an algal ancestry is not proved by these factors but they do make the hypothesis worth investigating.

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## Nutrition and distribution of salt response in populations of moderately halophilic bacteria

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# Nutrition and distribution of salt response in populations of moderately halophilic bacteria

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Cultures of *Micrococcus halodenitrificans* and *Vibrio costicolus* can grow in the presence of 0.4–3.5 *M* NaCl. Three lines of investigation: attempts to select for more or less salt-tolerant cells; colony counts at different salt concentrations; and replica plating experiments, suggested that populations of these organisms were genetically homogeneous in their salt response. That is, each cell in a population could grow over the whole range of salt concentration in which the culture grew.

The nutritional requirements of *V. costicolus* were studied. This organism can grow in a minimal (salts–glucose) medium at pH values above 6.0 and in a salt concentration range 0.5–2.2 *M*. Increasing the concentration of phosphate buffer greatly increased cell yield. The range of salt concentration in which *V. costicolus* could grow depended on the nutrient supply, being widest in complex media. A wider range was possible in the presence of amino acids than in a salts–glucose medium. All growth factors permitting growth to occur at high salt concentrations were not identified.

## Introduction

The extremely halophilic bacteria, which require NaCl concentrations of more than 15% for growth and can grow in saturated NaCl, are especially interesting because of the unusual nature of their enzymes, membranes, and ribosomes (see references 12–14 for reviews of the physiology of extreme halophiles). The existence of moderately halophilic bacteria, able to grow in from 3 to 20% NaCl, has also been known for a number of years (reviewed in 13 and 16). The moderate halophiles have been less studied, possibly because they appear to be less unusual organisms. They do not undergo the same dramatic lysis as the halobacteria when placed in distilled water. Their enzymes are more active at moderate (about 1.0 *M*) salt concentrations than enzymes from non-halophiles. At higher salt concentrations the enzymes of moderate halophiles are less active than those of extreme halophiles.

However, in one respect the moderate halophiles are very attractive cells to study: the extremely halophilic rods are remarkable for the acidity of the proteins of their membranes, ribosomes, and—probably—their enzymes. The same may be true of the extremely halophilic cocci, though only their enzymes have thus far been studied. Brown (1) suggested that one of the characteristic responses of bacteria to growth at high salt concentrations is acidity of their proteins. The moderately halophilic bacteria, which can grow over an eightfold range of salt concentration, may offer us the possibility of studying how growth at different salt concentrations affects the cell's chemical and physiological properties. Before undertaking such studies it is necessary to answer the question: Can all cells in a population of moderately halophilic bacteria grow over the entire salt range at which the population grows or is there a distribution in salt response, with some cells being able to grow best at higher and others at lower salt concentrations? We have studied this question with two moderate halophiles, *Micrococcus halodenitrificans* and *Vibrio costicolus*. Some of the nutritional requirements of the latter have already been worked out (8). These requirements and their changes with changing salt concentrations have now been further studied.

## Materials and Methods

### *Organisms*

Cultures of *Vibrio costicolus* No. 508 and *Micrococcus halodenitrificans* No. 509 obtained from the National Research Council of Canada, Ottawa, were maintained on slants containing 0.5% proteose peptone, 0.5% tryptone, 1 *M* NaCl, and 1.5% agar at pH 7 and were transferred at monthly intervals. The cultures were grown for 24–48 h at room temperature and then refrigerated until used to inoculate precultures.

### *Growth*

Growth was measured turbidimetrically at 660 nm on a Coleman Junior spectrophotometer in 19-mm precalibrated tubes. A blank value was obtained from an uninoculated tube of the appropriate medium. Viable counts

were made with drop plates (2). Cells were grown in tubes (19 × 150 mm) inclined at 30° to the horizontal and shaken on a reciprocating shaker at 100 oscillations per minute.

*Precultures*

Precultures were grown in the medium to be tested or, where specified, in a simpler medium to avoid the introduction of unwanted nutrients. A 10-ml tube was shaken 24–48 h at 30 °C. Precultures used for determining amino acid requirements were washed twice and resuspended in 1 *M* NaCl.

*Media*

Salts–glucose medium contained, per liter: $K_2HPO_4$, 3.12 g; $KH_2PO_4$, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NH_4Cl$, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; glucose, 10 g (autoclaved separately); NaCl as required, pH 7.6–7.7. The total concentration of salts other than NaCl is 0.073 *M*.

The medium developed by Flannery and Kennedy (8) contains, per liter: glucose, 1.0 g; $K_2HPO_4$, 3.12 g; $KH_2PO_4$, 0.28 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaNO_3$, 1.0 g; NaCl, 69 g (1.2 *M*); L-cysteine, 0.1 g; L-glutamic acid, 1.0 g; L-arginine, 0.2 g; DL-valine, 0.2 g; DL-isoleucine, 0.2 g. The total concentration of salts other than NaCl is 0.036 *M*. In Flannery and Kennedy's work the pH of this medium was adjusted to 7.0 and in our work to 7.5. Better growth took place at the higher pH (see Results). In both cases cysteine was sterilized by filtration. Glucose (1%) was autoclaved separately.

In complex media various combinations of vitamin-free casamino acids (Difco), proteose peptone (Difco), and tryptone (Difco) were used at a concentration of 1% each unless otherwise specified. When casamino acids were used alone, addition of 0.1 g/l $MgSO_4 \cdot 7H_2O$ was necessary for best growth.

Trypticase soy broth (Baltimore Biological Laboratories) already contained 0.09 *M* NaCl; specified NaCl concentrations were added to this.

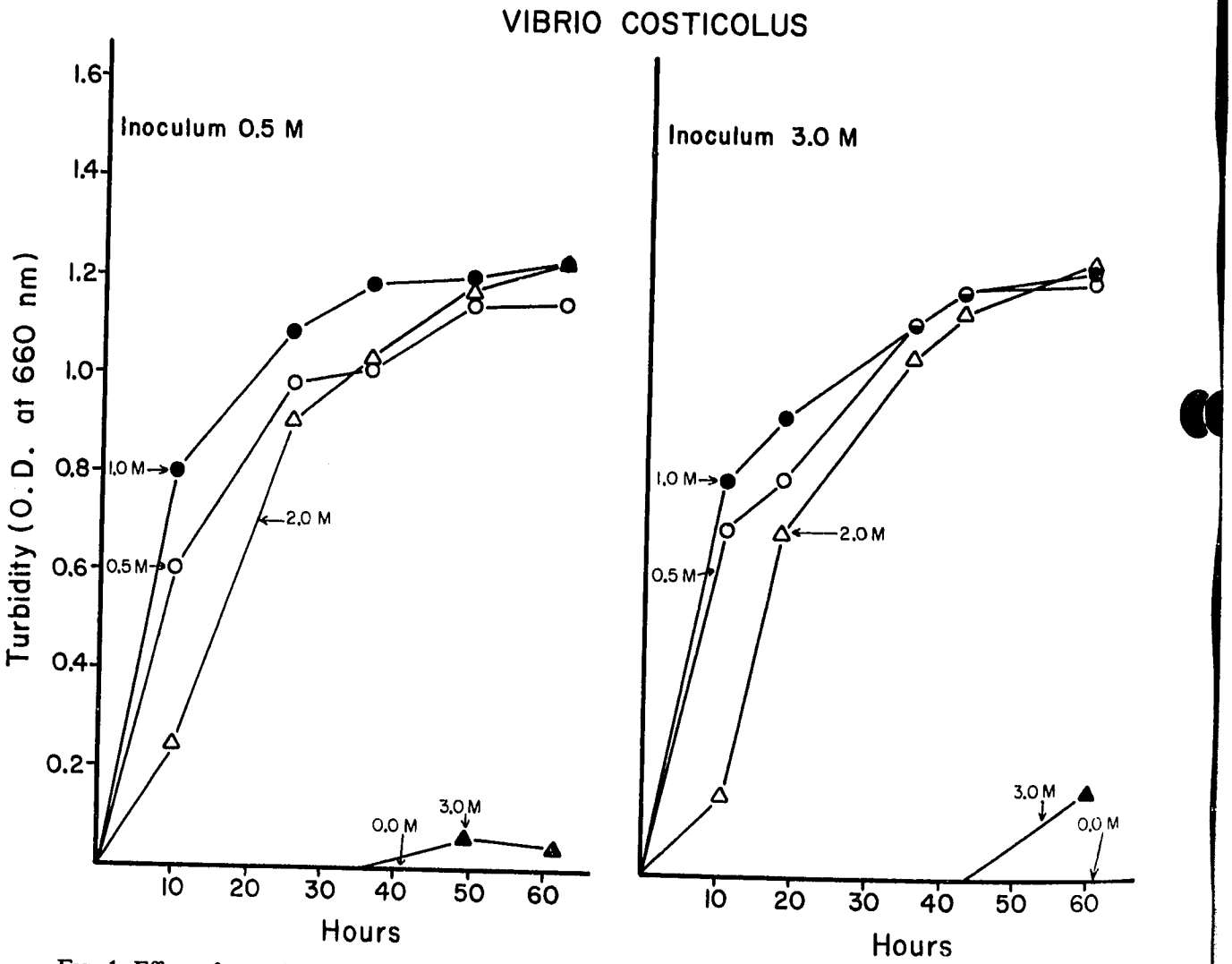


FIG. 1. Effect of preculture conditions on salt response of *V. costicolus*. For results on the left, inocula (0.02 ml) from a 22-h preculture in trypticase soy broth + 0.5 *M* NaCl were added to tubes containing 10 ml trypticase soy broth and NaCl at the concentrations shown by each curve. After 62 h 0.02 ml inocula of the culture containing 3.0 *M* NaCl were added to a fresh series of tubes, to give the results shown on the right.

## Results

*Levels of Response to Salt in Populations of Moderate Halophiles*

To test if all the cells in a culture of moderately halophilic bacteria had the same response to salt, we tried to select populations that could grow best at the extremes of salt concentrations. Both *Vibrio costicolus* and *Micrococcus halodenitrificans* were first grown in trypticase soy broth with 0.5 *M* or 3.0 *M* NaCl added. Each culture was then inoculated into a duplicate series of trypticase soy broth tubes to which from 0 to 3.5 *M* NaCl was added. For each microorganism the same type of growth curve was obtained, whether the inoculum consisted of cells grown in high or low salt concentrations (Figs. 1 and 2). The same results were also obtained if cultures were transferred twice at the high or low salt concentration before being inoculated into the series.

Another way of learning if the population is homogeneous in its salt response is to find if all cells can grow at all salt concentrations. To test this, cells were grown for 24 h in proteose peptone – tryptone (0.5% each) broth containing either 0.6 *M* NaCl or 3.0 *M* NaCl. Cultures were diluted with the same NaCl concentration as in the growth medium and plated in triplicate on proteose peptone – tryptone (0.5% each) agar containing different concentrations of NaCl.

No colonies of *M. halodenitrificans* developed in agar containing 0.1 or 4.0 *M* NaCl (Table I). Between 0.4 and 2.5 *M* NaCl, about the same number of colonies developed from *M. halodenitrificans* previously grown in either 0.6 *M* or in 3.0 *M* NaCl. On agar containing 3.0 *M* NaCl

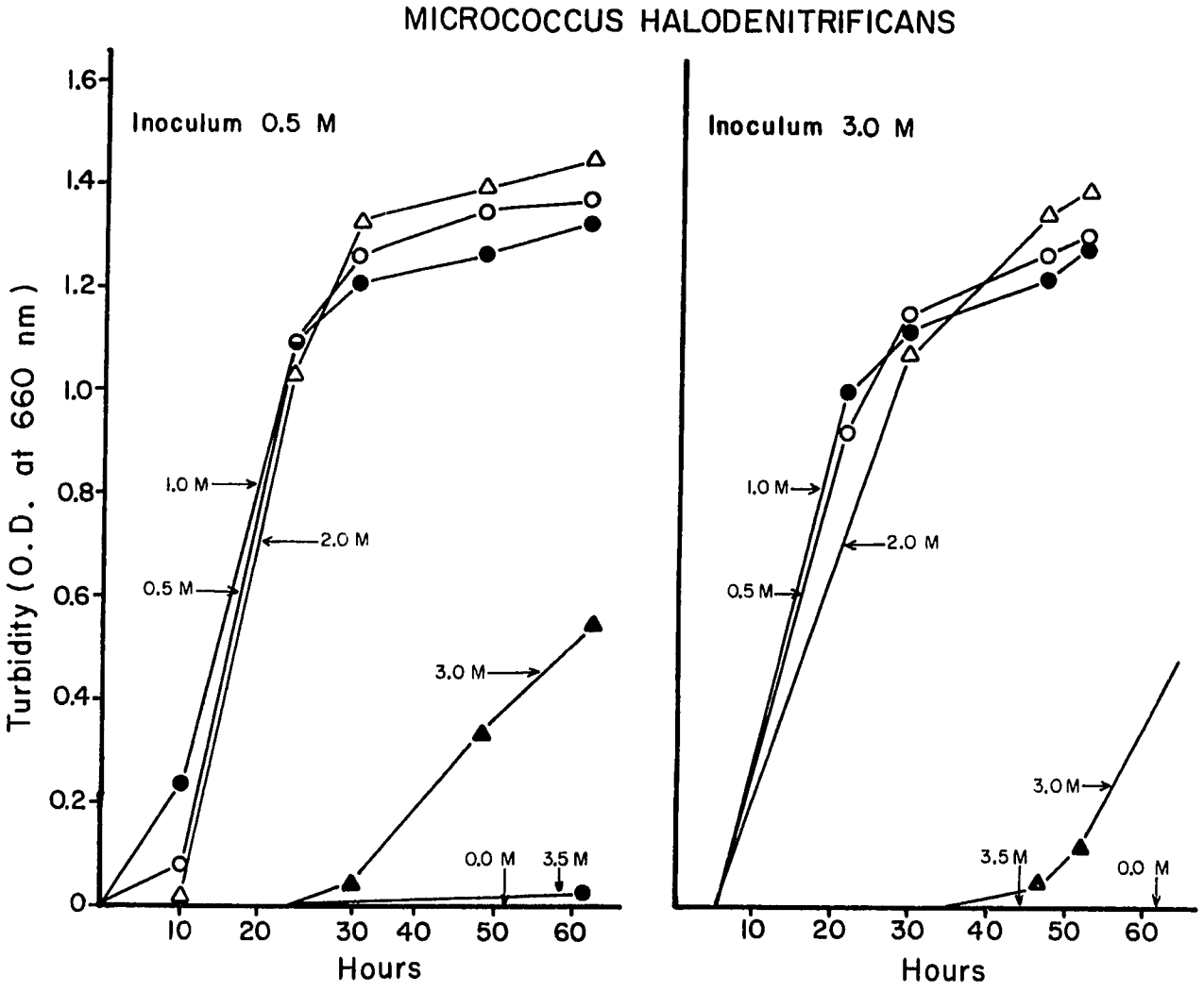


FIG. 2. Effect of preculture conditions on salt response of *M. halodenitrificans*. (Details as in Fig. 1.)

the same number of colonies of *M. halodenitrificans* precultured in 3.0 *M* NaCl developed as at lower salt concentrations; however, relatively fewer colonies developed from cultures grown on 0.6 *M* NaCl medium. On agar containing 3.5 *M* NaCl fewer colonies of both cultures developed, the relative drop in numbers being greater in cells cultured in 0.6 *M* NaCl.

Cultures of *V. costicolus* grown in 3.0 *M* NaCl produced very small colonies on agar containing 0.1 *M* NaCl. These probably grew in the local high salt environment created by the inoculum, an interpretation strengthened by the fact that cultures grown in 0.6 *M* NaCl produced no colonies on 0.1 *M* NaCl.[1] *V. costicolus* grown in 3.0 *M* NaCl developed about the same number of colonies on agar containing 0.4 to 2.5 *M* NaCl. Less than one-half as many developed at 3.0 *M* NaCl as at 1.0 *M*. *V. costicolus* grown in 0.6 *M* NaCl developed most colonies at 0.5 and 1.0 *M* NaCl. The number decreased as the salt concentration in the agar increased; about one-sixth as many colonies developed at 3.0 *M* as at 1.0 *M* NaCl.

Homogeneity of salt response was also tested by replica plating. *M. halodenitrificans* and *V. costicolus* were grown until just turbid in broth (proteose peptone – tryptone) containing 0.6 *M* or 3.0 *M* NaCl. Cultures were diluted and enough plated on proteose peptone – tryptone agar at the same NaCl concentration at which they were grown to yield separate colonies. Twenty to 30 colonies were inoculated with a needle on other plates of the same medium to obtain good separation. After the new colonies had grown they were replica plated on proteose peptone – tryptone agar containing 0.6, 1.0, 2.0, and 3.0 *M* NaCl. Growth was observed daily for 10 days and recorded photographically. In almost all cases every colony in the parent plate gave rise to colonies in the plates of agar at different salt concentrations, indicating that populations were

[1]To check this point, drops (0.025 ml, as used in our plating method) of 3 *M* NaCl were placed on a dry surface of 1.5% agar. At intervals part of the surface was flooded with 5% $AgNO_3$. For comparison, a series of drops of different NaCl concentrations was placed on agar and the agar flooded with $AgNO_3$ immediately after the drops had sunk in. Relative intensities of the precipitates indicated that a concentration corresponding to at least 0.5 *M* NaCl, precipitated immediately, remained under the 3.0 *M* drops for more than 2 days.

TABLE I

Viable counts on agar containing different salt concentrations

| NaCl concentration in agar, *M* | NaCl concentration at which inoculum was grown: 0.6 *M* Dilution | 0.6 *M* Mean number of colonies ± S.E. | 0.6 *M* Development time, days | 3.0 *M* Dilution | 3.0 *M* Mean number of colonies ± S.E. | 3.0 *M* Development time, days |
|---|---|---|---|---|---|---|
| | | *Micrococcus halodenitrificans* | | | | |
| 0.1 | $10^{-4}$ | 0 | 20 | $10^{-4}$ | 0 | 14 |
| 0.4 | $10^{-7}$ | 31±1.9 | 2 | $10^{-6}$ | 118±4.3 | 2 |
| 0.5 | " | 32±1.6 | 2 | " | 121±8.0 | 2 |
| 1.0 | " | 34±1.8 | 2 | " | 102±0.5 | 2 |
| 1.5 | " | 36±1.4 | 2 | " | 108±2.6 | 2 |
| 2.0 | " | 34±4.9 | 2 | " | 105±3.3 | 3 |
| 2.5 | " | 39±6.7 | 4 | " | 108±1.0 | 14 |
| 3.0 | " | 20±2.9 | 10 | " | 119±2.8 | 14 |
| 3.5 | " | 6.4±4.0 | 20 | " | 78±7.0 | 14 |
| 4.0 | $10^{-4}$ | 0 | 20 | $10^{-4}$ | 0 | 14 |
| | | *Vibrio costicolus* | | | | |
| 0.1 | $10^{-4}$ | 0 | 14 | $10^{-6}$ | 15±0.3 | 3 |
| 0.4 | $10^{-5}$ | 91±2.4 | 5 | " | 23±2.2 | 3 |
| 0.5 | " | 116±2.6 | 2 | " | 26±1.6 | 3 |
| 1.0 | " | 114±5.1 | 2 | " | 29±1.5 | 3 |
| 1.5 | " | 58±4.1 | 3 | " | 22±0.6 | 3 |
| 2.0 | " | 38±2.0 | 5 | " | 19±3.1 | 3 |
| 2.5 | " | 20±0.3 | 5 | " | 17±1.3 | 5 |
| 3.0 | " | 18±1.7 | 14 | " | 13±1.1 | 14 |
| 3.5 | $10^{-4}$ | 14±2.5 | 14 | $10^{-5}$ | 37±7.0 | 14 |
| 4.0 | $10^{-4}$ | 0 | 14 | $10^{-4}$ | 0 | 14 |

homogeneous in their salt response. The rate of growth of all colonies on a given plate appeared the same. However, it was difficult to be certain of this, because replication did not transfer the same number of cells from every colony.

*V. costicolus* replicated from plates containing 0.6 *M* salt grew best in the salt concentration range 0.6–2.0 *M* and slightly more slowly in 3.0 *M*; in all salt concentrations growth was complete in three days. Cells replicated from plates containing 3.0 *M* NaCl gave the same results, except that colonies in 0.5 *M* salt developed more slowly during the first day.

The results with *M. halodenitrificans* were similar to those found by viable counts (see Table I). All colonies grew completely in 3 days in 1.0 and 2.0 *M* salt. Growth in 0.6 *M* salt was slower. Cells grown on 0.6 *M* salt developed colonies very slowly in 3.0 *M* salt; at 10 days, one-third of the colonies had not developed at all, whereas those originally grown on 3.0 *M* NaCl were completely grown by the third day.

### *Growth Requirements of Vibrio costicolus at Different Salt Concentrations: Comparison of Defined Growth Media*

Flannery and Kennedy (8) developed a simplified synthetic medium for *V. costicolus* from the much more complex synthetic medium developed by Herbst and Snell (10) for *Hemophilus parainfluenzae*. The latter medium contains 18 amino acids, vitamins, purine and pyrimidine bases, and certain salts, including $NaNO_3$ but no $NH_4^+$ salts. It supported growth of *V. costicolus* if 1.2 *M* NaCl was added. By omitting different constituents of this medium, singly and in groups, Flannery and Kennedy reduced it to five amino acids (L-cystine or cysteine, L-glutamic acid, L-arginine, DL-valine, and DL-isoleucine), glucose, and salts (see Materials and Methods section). Glucose, cyst(e)ine, and NaCl were essential for growth; the first three amino acids stimulated growth; valine and isoleucine stimulated growth if added together but inhibited it if added separately.

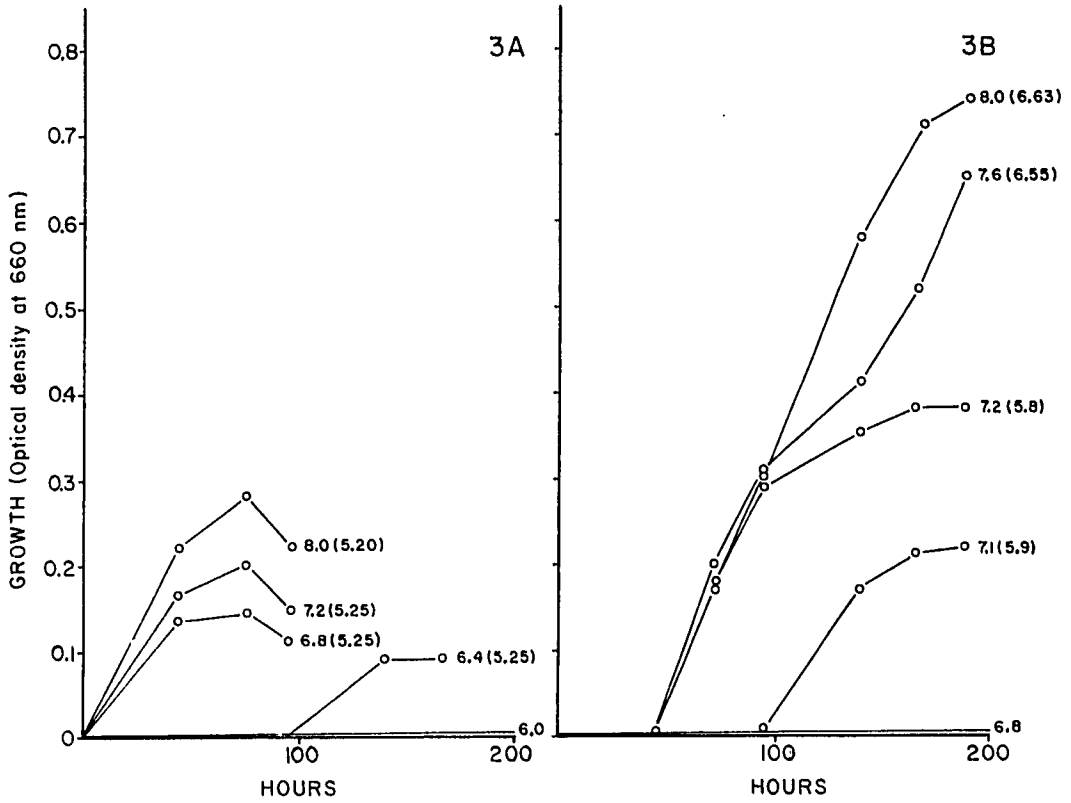


FIG. 3, A–E. Growth of *V. costicolus* at various initial pH values in media containing 1 *M* NaCl. Number in parentheses is final pH. (A) Salts–glucose; (B) salts–glucose with four times the normal phosphate concentration; (C) Flannery and Kennedy medium; (D) Flannery and Kennedy medium with four times the normal phosphate concentration; (E) proteose peptone (1%) + tryptone (1%). Optical density figures are averages of duplicate cultures.

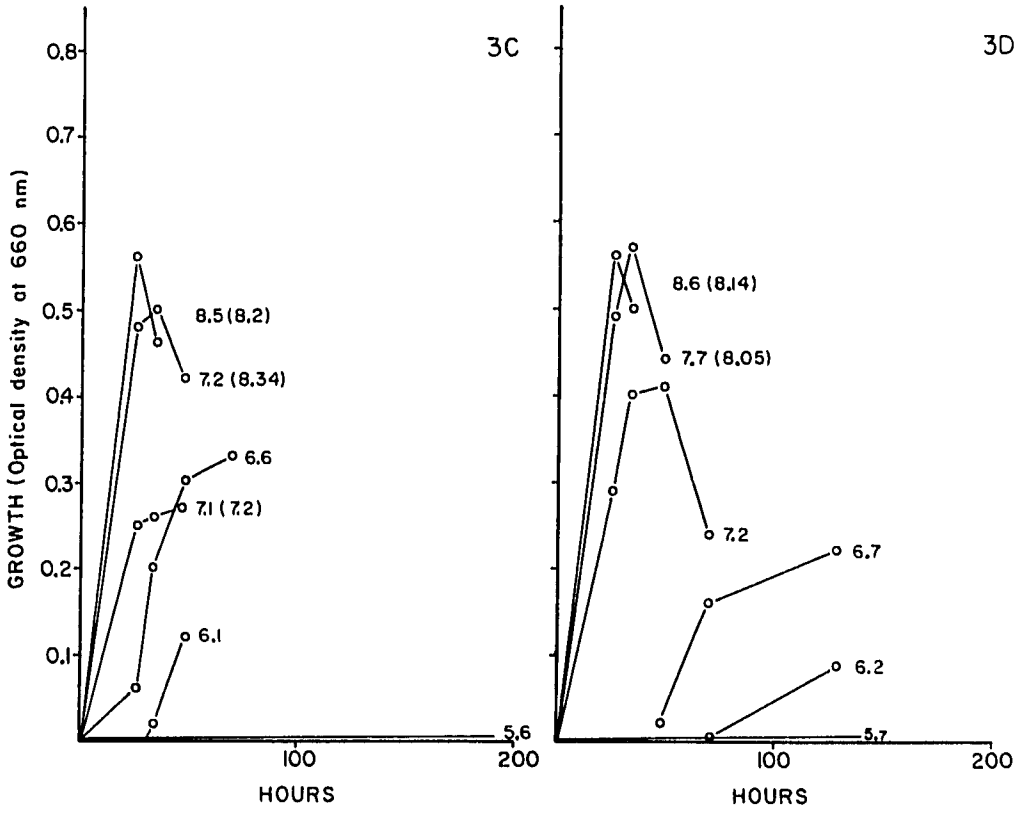


FIG. 3, C and D.

We approached the nutritional requirements of *V. costicolus* differently, and found that a minimal (glucose–ammonia salts) medium supplemented with 1 *M* NaCl also supported growth. The growth in minimal medium and in the medium of Flannery and Kennedy was compared at different pH values and the effects of adding more phosphate buffer to each medium were studied (Fig. 3). In the amino acid medium, the lag period was shorter and the cell yield higher. Growth occurred at a lower starting pH value in the amino acid medium. Adding 0.4 *M* phosphate buffer to this medium lengthened the lag period and decreased the rate of growth at pH values below 7.

In minimal medium addition of extra buffer increased the lag period but, at most pH values, also greatly increased the final yield to a level higher than that attained in the amino acid medium.

The pH fell during growth in minimal medium but remained relatively constant or rose during growth in the amino acid medium of Flannery and Kennedy (which also contained glucose), presumably as the result of products of amino

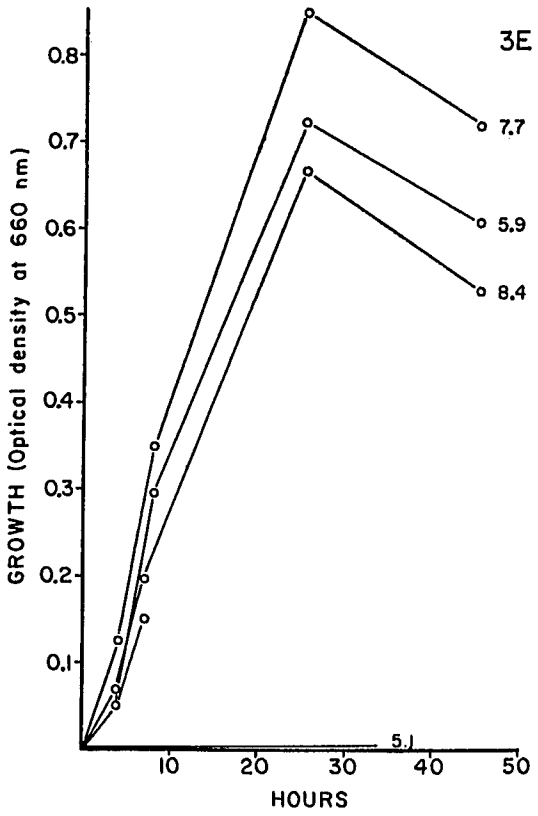


FIG. 3, E.

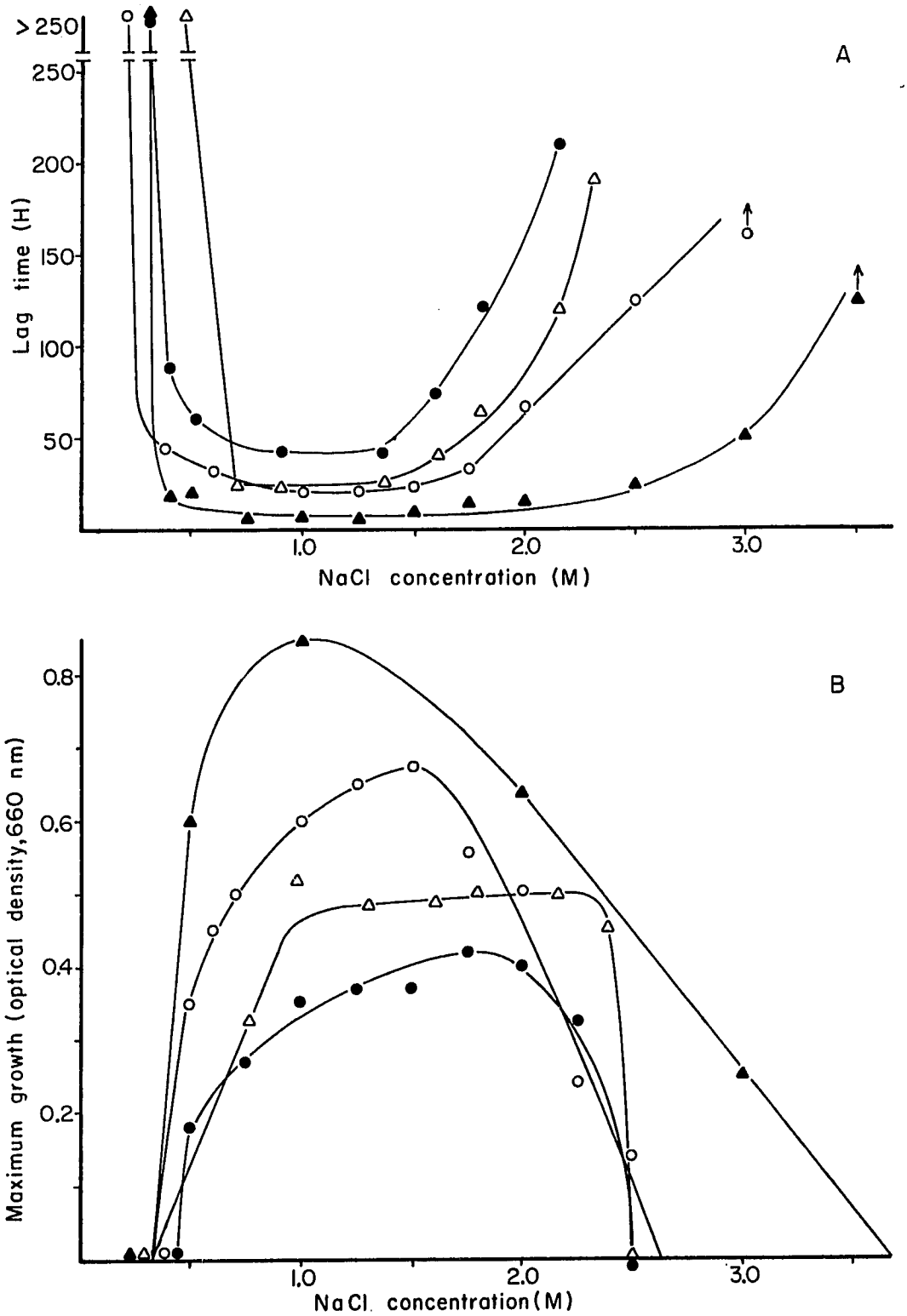


FIG. 4, A and B. Change in length of lag phase of growth (A) and of maximum growth response (B) with different NaCl concentrations in (●) salts–glucose; (△) medium of Flannery and Kennedy (8); (○) casamino acids and $Mg^{2+}$; (▲) proteose peptone and tryptone.

acid metabolism. For unknown reasons, cultures could grow at lower initial pH values in the absence of added phosphate than in its presence.

In a complex medium (proteose peptone + tryptone, containing 1 *M* NaCl) growth was less pH-sensitive in the range 6.0–8.4 than in defined media (Fig. 3E); at pH 5.0 cells did not grow.

Studies were carried out to determine which amino acids in the medium of Flannery and Kennedy (8) were necessary for growth. Unlike these workers, we did not find cysteine or cystine essential, though cysteine stimulated growth 10 to 20%. Though the reasons for the different results obtained are not clear, we measured growth at slightly different pH values and salt concentrations (pH 7.2–7.6 and 1.0 *M* NaCl as compared to pH 7.0 and 1.2 *M* NaCl). Growth in amino acid medium is very sensitive to pH (see Fig. 3, C and D) and a small change in pH could conceivably alter nutritional requirements. This possibility has not been investigated further.

*Effect of NaCl Concentration on Nutritional Requirements of V. costicolus*

Previous workers studied the growth requirements of *V. costicolus* only in media containing 1.0 to 1.2 *M* NaCl. We examined these requirements over a wide range of salt concentrations. Growth was measured by both the length of the lag period and the maximal cell yield, expressed as optical density. Viable counts could not be used, since, at the highest and lowest salt concentrations at which cells grew, they did not separate after dividing, but formed long filaments.

The widest growth range (0.4 *M* to 3.5 *M* NaCl) was obtained with proteose peptone + tryptone (Fig. 4) and with proteose peptone + casamino acids or tryptone + casamino acid (not shown). Casamino acids alone gave a smaller growth range (usually 0.4 to 2.5 *M* NaCl; in some experiments cells grew in 0.3 *M* NaCl, but these cultures contained large amounts of cell debris). As the medium was simplified this range was further reduced. The range in the amino acid medium was from 0.4–0.7 *M* (in different experiments) to 2.5 *M* NaCl. In the completely synthetic media growth did not occur above 2.2–2.3 *M* NaCl.

These results suggested that at the highest salt concentrations enzymes responsible for forming certain amino acids and growth factors were inhibited. Adding amino acids extended the growth range slightly, but the greatest extension occurred in complex media. In a search for the growth factors responsible we added the following vitamins and bases (1 mg/ml), singly and in combination, to the casamino acid medium: riboflavine, riboflavine-5-phosphate, nicotinic acid, choline chloride, biotin, D-araboascorbic acid, ascorbic acid, calcium pantothenate, thiamine hydrochloride, pyridoxine phosphate, folic acid, uracil, guanine, adenine, cytosine, and thymine. None of the additions permitted cells to grow in as high a salt concentration as they could in complex media.

## Discussion

Attempts to train non-halophilic microorganisms to grow in much higher salt concentrations or to train halophiles to grow without salt have had little or no success (11, 12, 14, 15). This indicates that the salt response of populations of cells is genetically stable, but so far there have been few attempts to study variations of salt response *within* populations. Our results suggest that cultures of *M. halodenitrificans* and *V. costicolus* are homogeneous in this respect, that is, that each cell can grow over the entire salt range in which the culture grows. Any changes in the properties of these bacteria on growth at different salt concentrations are probably not due to selection.

This interpretation is based on experiments showing that no selection of more or less salt-resistant cells occurred if cells were precultured in different salt concentrations, and salt response then measured in liquid medium. Most replica-plating experiments also supported the genetic homogeneity of salt response in these populations.

The situation became more complex when growth was measured by colony formation at higher salt concentrations (3.0–3.5 *M*). At these concentrations colonies grew very slowly and fewer developed; possibly, because of the slow growth rate the progeny of a fraction of the cells died before forming visible colonies. Some of the cells precultured in 0.6 *M* NaCl failed to form colonies at as high a salt concentration as cells precultured at 3.0 *M* NaCl. This *could* be taken to show that the latter medium had selected

more salt-tolerant cells. In view of our other results we think a more likely explanation is that some cells are damaged on being transferred from a lower to a higher salt concentration, but not on being transferred from a higher to a lower.

*Staphylococcus aureus* tolerates, but does not need, relatively high salt concentrations. Scott reported that the same number of colonies form in agar containing about 2.2 *M* salt as in the absence of salt (18). This suggests that these cells are also homogenous in their salt response.

The nutritional requirements of some extreme halophiles, which grow in a narrow range of high salt concentration, have now been worked out (5, 9, 16). Our present results show that the nutritional requirements of *Vibrio costicolus* are simpler than previously thought. Within a three- to four-fold range of salt concentration this organism grows well on a minimal medium.

Solute concentration affects the nutritional requirements of several microorganisms. Christian (3) found that *Salmonella oranienburg* could grow in a complex medium adjusted with salts or sucrose to a water activity ($a_w$) of 0.94 to 0.95, but in a minimal medium the limiting $a_w$ for growth was between 0.96 and 0.97. Adding five amino acids and eight water-soluble vitamins to the salts-adjusted medium extended the growth range to an $a_w$ of 0.95, that of 1.4 *M* NaCl.

Dulaney *et al.* (4) found that 10% sucrose (0.19 *M*) or high concentrations of other sugars inhibited the growth of *Vibrio percolans* and *Erwinia carotovora* in defined media, and that this inhibition could be reversed by yeast extract or by betaine, one of its components.

Examples of the influence of $a_w$ on the nutritional requirements of molds are discussed by Scott (18).

*V. costicolus* can grow at a much higher salt concentration than *S. oranienburg* (3.5 *M* NaCl; $a_w$ 0.86) and cannot grow below 0.4–0.5 *M* NaCl, that is above an $a_w$ value of about 0.98. We found that growth at the highest salt concentrations took place only in complex media. *V. costicolus* could also grow in a slightly *lower* salt concentration in a mixture of amino acids than in minimal media. Some enzymes of moderate halophiles are known to require some salt for activity (about 0.5 *M*) and to be inhibited at higher salt concentrations (7, 17). Though the component(s) of peptone or tryptone that permitted the extended growth range were not identified, our results suggest that, at unfavorable salt concentrations, enzymes responsible for forming amino acids and other growth factors are inhibited.

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# Salt tolerance of intertidal marine bacteria

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# Salt tolerance of intertidal marine bacteria

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A large proportion of bacteria isolated from the intertidal zone could grow in higher salt concentrations than found in seawater, and some could grow in saturated or near-saturated NaCl. Half the bacteria isolated could grow in media very low (0.06%) in NaCl; most of the Gram-positive organisms belonged to this group. There was no correlation between pigmentation and salt tolerance.

The most detailed studies of marine bacteria deal with their requirement for, rather than with their tolerance of, NaCl (4, 5). The limited data available suggest that salt concentrations a few times higher than those found in the sea inhibit growth of these bacteria. Zobell stated in 1946 that "very few marine bacteria grow in sea water media to which 12 per cent NaCl has been

added.... Virtually no marine bacteria... grow in sea water to which 24 per cent NaCl has been added" (10). Though Shah and de Sa (7) found that a few marine bacteria could grow slowly in media containing 21% NaCl, other surveys, involving a total of 27 species of marine bacteria, showed that 8–12% NaCl were the highest concentrations that permitted growth (1, 9). Thus, salt tolerance of marine bacteria seems limited and indeed, it has been pointed out that halotolerance is more common in terrestrial bacteria (4). However, our results, arising from work concerned with the origin of halophilic bacteria, show that extremely halotolerant bacteria do exist in marine environments and that they can be demonstrated under appropriate culture conditions.

Samples of intertidal waters were taken near St. Andrews, N.B., from five tidal pools and a location in the bay 5 m from shore, not affected by runoff but exposed at low tide. The temperature of the pools at the time of sampling varied from 17 to 29°C; that of the bay was 17°C. Salinity measurements showed that the pools had been diluted with fresh water to 70–86% the concentration of seawater. Triplicate sets of 1-, 10-, and 100-ml water samples were filtered through a Millipore® portable water filtration apparatus equipped with 0.45-μ pore size HA filters. One set of filter membranes was placed on Millipore® pads saturated with nutrient medium at each of three different final NaCl concentrations (see Table 1 for composition of the media). Petri dishes containing the pads and filters were placed in plastic bags to prevent drying and incubated with periodic examination up to 1 month at 30°C. (In contrast, in the work cited above (1, 7, 9) incubations were carried out at this temperature for, at most, 6 days.) After enumeration, colonies were selected from each salt concentration to give a variety of morphological colony types and purified by plating on 2% agar containing the same medium as that on which they were isolated.

TABLE 1
Colony counts of water and mud samples

| NaCl, %, in Millipore plate pad | Colony-forming units per milliliter | |
|---|---|---|
| | Water samples | Mud samples |
| "0" | Not tested | 17 850±5 500 |
| 3 | 146±52 | 53 500±6 700 |
| 10 | 31±7.3 | 33 200±9 750 |
| 20 | 6.1±1.7 | 3 060± 610 |

NOTE: Figures show mean values ± standard errors for seven water samples (one pool was sampled at two different times) and two mud samples. The selective medium consisted of 0.1% Difco yeast extract, 0.4% Difco casamino acids, and regular strength "Instant Ocean," an artificial seawater mixture supplied by Aquarium Systems, Inc., Wickliffe, Ohio, U.S.A., described in ref. 6. This solution contains NaCl, 2.65% (w/v); $MgSO_4$, 0.66%; $MgCl_2$, 0.52%; $CaCl_2$, 0.13%; KCl, 0.071%; $NaHCO_3$, 0.02%, and traces of other ions. The *final* NaCl concentrations in the selective media were adjusted to 3, 10, and 20%. The medium for testing growth in "0%" NaCl contained $KHCO_3$, 0.025% (w/v); $CaSO_4 \cdot 2H_2O$, 0.0069%; $MgCl_2 \cdot 6H_2O$, 0.0305%; $MgSO_4$, 0.012%; $Ca(NO_3)_2 \cdot 4H_2O$, 0.00006%, in addition to yeast extract and casamino acids as above. The last two components contributed about 0.06% NaCl. Final pH of all media after autoclaving at 15 lb pressure for 15 min was 7.6 ± 0.1.

All samples contained bacteria that were able to grow at 20% NaCl as well as a larger number able to grow at 10% NaCl (Table 1). The largest number of bacteria, in all samples, grew at 3% NaCl. After a year's storage at 4°C samples of mud taken from the bottom of two pools were diluted 100-fold with sterile artificial seawater and centrifuged 10 min at 125 × *g*; aliquots of the supernatant were filtered and tested as above. The results (Table 1) showed that the mud was quite rich in halotolerant bacteria. Considerably fewer organisms from mud developed in the "0 NaCl" medium than in 3% or 10% NaCl media.

After 2 months' storage at 4°C, tests were carried out on the salt response of strains isolated from water, using liquid media containing 3, 10, 20, 25, and 30% NaCl. One year after isolation, growth was examined in a medium very low in NaCl ("0 NaCl"; see Table 1).

All strains grew on 3% and 10% NaCl media, regardless of the salt concentration on which they were isolated (Table 2). Cultures selected on media containing 10 and 20% NaCl were able to grow in the highest NaCl concentrations; eight of these grew in 25% NaCl and two in 30% NaCl media. About one-quarter of the strains tested failed to grow in the medium very low in NaCl. Ten of the latter strains were tested and grew rapidly if 3% NaCl was added to the "0 NaCl" medium. They had, therefore, a specific requirement for NaCl rather than for any of the other constituents of "Instant Ocean" mixture (see Table 1).

The 69 isolates shown in Table 2 consisted of 52 Gram-negative rods, 9 Gram-positive rods, 2 Gram-variable rods, 4 Gram-negative cocci, 1 Gram-variable coccus, and 1 Gram-negative vibrio. The seven cultures growing in 25% NaCl were Gram-negative rods. One Gram-positive and one Gram-negative rod grew in 30% NaCl. Those growing in 20% NaCl included cocci and a vibrio, as well as a preponderance of Gram-negative

rods. Thus, tolerance to 20% NaCl is not limited to one morphological type. The prevalence of rods among bacteria able to grow at the highest salt concentrations may only reflect the general prevalence of Gram-negative rods in populations of marine bacteria (10).

The 35 strains growing in the "0 NaCl" media included 7 Gram-positive rods and one Gram-variable coccus. This supports other observations that most Gram-positive bacteria isolated from the sea can grow in media low in NaCl (5). However, the ability to grow in such media was certainly not limited to Gram-positive cells.

Of the strains shown in Table 2 only six isolated on 3% NaCl and five isolated on 10% NaCl formed yellow, pink, or reddish-orange colonies. There was no correlation between pigmentation and ability to grow in higher salt concentrations. In this respect these bacteria differ from *Bacillus* strains from soil, in which pigmentation accompanied increased salt tolerance (8).

Our studies of halotolerant marine bacteria were prompted by an interest in the origin of extremely halophilic bacteria, whose relation to the rest of the microbial world remains obscure (2, 3). It is sometimes thought that extreme halophiles are derived from marine bacteria. The

TABLE 2

Salt response of cultures isolated at different salt concentrations

| % NaCl in isolation medium | % NaCl in test medium: 0 | 3 | 10 | 20 | 25 | 30 | No. strains showing growth pattern indicated |
|---|---|---|---|---|---|---|---|
| | Growth patterns | | | | | | |
| | +++* | +++ | +++ | 0 | 0 | 0 | 3 |
| | NT | +++ | +++ | 0 | 0 | 0 | 3 |
| | 0 | +++ | ++ | 0 | 0 | 0 | 1 |
| | 0 | +++ | +++ | 0 | 0 | 0 | 4 |
| 3 | +++ | +++ | +++ | ± | 0 | 0 | 1 |
| | NT | +++ | +++ | ++ | 0 | 0 | 1 |
| | +++ | +++ | +++ | ++ | 0 | 0 | 2 |
| | +++ | +++ | +++ | +++ | 0 | 0 | 3 |
| | ± | +++ | +++ | ++ | 0 | 0 | 1 |
| | 0 | +++ | +++ | +++ | 0 | 0 | 2 |
| | | | | | | | Total = 21 |
| | +++ | +++ | +++ | 0 | 0 | 0 | 4 |
| | NT | +++ | +++ | 0 | 0 | 0 | 1 |
| | 0 | +++ | +++ | 0 | 0 | 0 | 2 |
| | +++ | +++ | +++ | ± | 0 | 0 | 2 |
| | +++ | +++ | +++ | ++ | 0 | 0 | 3 |
| | +++ | +++ | +++ | +++ | 0 | 0 | 5 |
| 10 | 0 | +++ | +++ | ++ | 0 | 0 | 1 |
| | 0 | +++ | +++ | +++ | 0 | 0 | 1 |
| | +++ | ++ | +++ | ++ | ++ | 0 | 1 |
| | +++ | +++ | +++ | +++ | ++ | 0 | 1 |
| | 0 | ++ | +++ | +++ | ± | 0 | 1 |
| | NT | +++ | +++ | +++ | ++ | 0 | 3 |
| | +++ | +++ | +++ | +++ | ++ | + | 1 |
| | | | | | | | Total = 26 |
| | ++ | +++ | +++ | ++ | 0 | 0 | 1 |
| | +++ | +++ | ++ | ++ | 0 | 0 | 1 |
| | NT | +++ | +++ | ++ | 0 | 0 | 4 |
| | +++ | +++ | +++ | ++ | 0 | 0 | 2 |
| | NT | +++ | +++ | +++ | NT | 0 | 5 |
| 20 | +++ | +++ | +++ | +++ | 0 | 0 | 3 |
| | + | +++ | +++ | +++ | 0 | 0 | 1 |
| | 0 | +++ | +++ | ++ | 0 | 0 | 1 |
| | 0 | +++ | +++ | +++ | 0 | 0 | 2 |
| | +++ | +++ | +++ | ++ | ++ | 0 | 1 |
| | 0 | +++ | +++ | +++ | ++ | + | 1 |
| | | | | | | | Total = 22 |
| | | | | | | | Grand total = 69 |

*Salt response: +++ growth before 8 days (usually 1–2 days), ++ growth in 9–14 days, + growth in 15–21 days, ± growth in 22–28 days, 0 no growth in 4–5 weeks and NT, not tested.

salterns, in which so many extreme halophiles live, arise by evaporation of seawater. It now appears that seawater, near the shore and in tidal pools, contains an impressive number of bacteria able to grow in salt concentrations approaching saturation. Such bacteria have also been found in samples of Caribbean seawater (Gochnauer and Kushner, unpublished). As seawater evaporates we would expect that the concentrated saline environment would have a progressively higher proportion of halotolerant bacteria. Possibly, on prolonged exposure to such an environment some members of this population would lose the ability to grow at lower salt concentrations.

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