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ABSTRACT

The major lipids of Vibrio costicola membranes and whole cells are phosphatidylglycerol and phosphatidylethanolamine. Lesser amounts of lysophosphatidylethanolamine, cardiolipin, a glycolipid and two other phospholipids, tentatively identified as lysocardiolipin and lysophosphatidylglycerol, are also present. Phosphatidylglycerol and lysocardiolipin contents markedly increase, whereas that of phosphatidylethanolamine decreases with increasing salt concentration (0.5M to 3.0M NaCl) in the growth medium. These changes result in an increase in the mole ratio of negatively charged polar lipids to neutral polar lipids. Little effect of growth phase on the proportions of the lipid components or their fatty acids was observed in cells grown in 1.0M salt. The major fatty acids found were 16:0, 16:1 and 18:1; minor amounts of 14:0, 17:1 and traces of 12:0, 12:1, 14:1, 15:0, 15:1 and 17:0 were also found. The proportions of certain fatty acids also changes with changing salt concentration in the medium.

The relative amounts of a major outer membrane protein of molecular weight 58,000 greatly increases in response to increasing salt concentration (0.2M to 3.0M) in the growth media. No observable effect of growth phase on the relative amount of the salt-regulated protein was observed in cells grown in media containing 0.5 or 1.0M NaCl. This protein was shown to be a glycoprotein, transmembrane and bound to peptidoglycan.

The salt-response and/or specific activity of the F_0F_1 ATPase was affected be the NaCl concentration of the growth media, phase of growth in which cells were harvested, freezing and thawing, and by the method of preparation of cell material (sonication or pressure bomb) prior to assaying for ATPase activity. Similarly the membrane bound 5'-nucleotidase activity also seemed regulated by the above factors and thus understanding of both ATPase and 5'-nucleotidase activity was not clear due to the complexity of the system.

Examination of intracellular solutes revealed virtually no intracellular glycerol in V. costicola for cells grown in the range of 0.2 to 3.0M NaCl. The most important changes in intracellular amino acids of V. costicola grown in media containing 0.5M NaCl and then transfered to medium containing 2.0M NaCl was an increase in the percent of glutamine and glutamic acid whereas the percentage of glycine and alanine decreased. Both the total negatively charged free amino acids and the total amino acid content increased as the NaCl concentration of the medium was raised.

RESUME

La phosphatidylglycérol et la phosphatidyléthanolamine sont les lipides majeurs des membranes et des cellules entières de Vibrio costicola. Des quantités moindres de lysophosphatidyl-éthanolamine, de cardiolipine, un glycolipide et deux autres phospholipides que nous avons temporairement identifiés comme étant la lysocardiolipine et la lysophosphatidylglycérol sont aussi présents. La teneur en phosphatidylglycérol augmente de façon marquée, tandis que la teneur en phosphatidyléthanolamine diminue lorsque nous augmentons la concentration de sel (0.5 à 3.0M NaCl) dans le milieu de culture. Ces changements se traduisent par une augmentation du ratio moléculaire des lipides polaires négatifs en regard des lipides polaires neutres. Nous avons observé que la phase de croissance avait peu d'effect sur la proportion des composés lipidiques ou des acides gras chez les cellules cultivées en présence de 1.0M de sel. Les acides gras majeurs que nous avons trouvés sont 16:0, 16:1 et 18:1 des quantites mineures de 14:0, 17:1 et 18:0 ainsi que des traces de 12:0, 12:1, 14:1, 15:0, 15:1 et 17:0 ont également été trouvées. Les proportions de certains acides gras changent aussi lorsque nous modifions les concentrations de sel dans le milieu de culture.

Les quantites relatives de la principale proteine de la membrane externe de poids moleculaire 58,000 augmente fortement en reponse à l'augmentation en sel (0.2M a 3.0M) du milieu de culture. Aucun effet

notable de la phase de croissance sur la quantité relative de la protéine reglée par le sel fut observé dans les bactéries cultivées dans des milieux contenant 0.5 ou 1.0M NaCl. Cette protéine a été identifée comme étant une glycoprotéine, transmembrane et liée au peptidoglycane.

La réponse au sel et/ou l'activité spécifique de la F_0F_1 ATPase fut affectée par la concentration en NaCl du milieu de culture, la phase de croissance à laquelle les bactéries sont recueillies, congelées et dégelées et par la méthode de préparation du matériel cellulaire (sonication ou bombe à pression) avant d'essayer l'activité de l'ATPase. De manière similaire, l'activité de la 5'-nucléotidase membrane semblait aussi réglée par les facteurs cités ci-dessus, rendant ainsi la compréhension de l'activité de l'ATPase et de la 5'-nucléotidase pas claire, à cause de la complexité du système. L'examination des solutes intracellulaires n'a révélé aucun glycérol intracellulaire chez V. costicola pour les cellules cultivées entre 0.2 et 3.0M NaCl.

Les changements les plus importants en acides aminés intracellulaires lorsque V. costicola cultivé en présence du 0.5M NaCl fut transféré en milieu contenant 2.0M NaCl, furent une augmentation de glutamine et d'acide glutamique et une diminution de glycine et d'alanine. Chaque acides aminés libres charges négativement et le pourcentage total en acide amines augmentait lorsque la concentration en NaCl du milieu augmentait.

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ABBREVIATIONS

AMP	Adenosine 5'-monosposphate
ATP	Adenosine 5'-triphosphate
ATPase	Adenosine triphosphatase
b	Bacteria
CL	Cardiolipin
CM	Cell membrane
CW	Cell wall
DAP	Diaminopimelic acid
DCCD	N,N-Dicyclohexylcarbodimiide
12:0	Dodecanoic acid
12:1	Dodecenoic acid
EDTA	Ethylenediaminetetraacetate
GABA	Alpha-aminobutyric acid
17:0	Heptadecanoic acid
17:1	Heptadecenoic acid
KDO	2-Keto-3-deoxyoctonate
LPS	Lipopolysaccharide
lyso-PE	Lyso-phosphatidylethanolamine
lyso-PG	Lyso-phosphatidylglycerol
n	Nucleoplasm
18:0	Octadecanoic acid
18:1	Octadecenoic acid
OM	Outer membrane
16:0	Palmitic acid
16:1	Palmitoleic acid
15:0	Pentadecanoic acid
15:1	Pentadecenoic acid
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PL	Phospholipid
PPT	Proteose peptone tryptone
PS	Periplasmic space
SDS	Sodium dodecyl sulfate
sarcosyl	Sodium-lauryl sarcosinate
TCA	Trichloroacetic acid
14:0	Tetradecanoic acid
14:1	Tetradecenoic acid
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

1. Life in High Solute Concentrations.

Many bacteria and fungi inhabit environments considerably harsher than those inhabited by other known life forms. There are many examples of microorganisms that have adapted to such environments; under high pressure within the ocean depths, acid and alkaline conditions, at high temperatures, high levels of radiation, high concentrations of heavy metals or other strong chemicals and lastly under conditions of high solute concentrations such as in salt and sugar (see reviews by Heinen, 1974; Heinreich, 1976; Kushner, 1978, 1980; Shilo, 1978).

All microorganisms require water for growth; however, the exact amount needed for growth varies from one microorganism to another. This water requirement is expressed in terms of available water or water activity (a_w). Solutes and ions tie up water in solution. Therefore, an increase in the concentration of dissolved solutes such as salts and sugars reduces the a_w and the bacteria via osmosis lose cellular water. The water activity (a_w) can be defined by the equation:

$$a_w = \frac{p}{p_o} = \frac{n_2}{n_1 + n_2}$$

where P is the vapor pressure of the solution and P_o that of the solvent, and n_1 and n_2 are the number of moles of solute and solvent respectively (Frazier and Westhoff, 1978). Table 1 presents values of limiting a_w for growth of various groups of microorganisms over the entire a_w range in which life is possible.

Fungi capable of growing in extremely dry environments (low a_w) have been categorized as "xerophilic". Yeasts growing in high salt or sugar concentrations are called "osmophilic" (Corry, 1973). The lowest a_w capable of supporting growth has been reported for a xerophilic and an osmophilic microorganism, Xeromyces bisporus and Saccharomyces rouxii respectively (Table 1). Furthermore, Table 1 shows that among the prokaryotes, the extremely halophilic bacteria are capable of growth at the lowest a_w value. Larsen (1967) defined these extreme halophiles as any organism which grows best above concentrations of 20% NaCl.

The extreme halophiles and similarly all other bacteria can be categorized according to the range of salt concentrations which permits growth (Table 2). Interest in studying the extreme halophiles is not solely because they are limited to an extreme environment, but also because they belong to the Archaebacteria, together with the methanogens and thermoacidophiles whose members are supposedly of ancient lineage and which also contain structural differences of certain cell components common to all bacteria (Woese, 1981). Extreme halophiles also contain a unique light energy-transducing pigment, bacteriorhodopsin, so far not found in other prokaryotes (Stockenius and Bogomolni, 1982).

TABLE 1

Limiting water activities (a_w) for microbial growth

Organism	a_w lower limit	References
BACTERIA		
non-halophiles:		
Bacillus mycoides	0.97-0.99 (salt)	Burcik (1950)
Bacillus subtilis	0.90 (NaCl)	Gould & Measures (1977)
Escherichia coli	0.95 (NaCl) 0.935 (glycerol)	Gould & Measures (1977)
Lactobacillus	0.90	Brown (1976)
Clostridium botulinum	0.945 (NaCl)	Gould & Measures (1977)
Enterobacter aerogenes	0.945	Frazier & West hoff (1978)
Pseudomonas fluorescens	0.97 (NaCl)	Gould & Measures (1977)
Salmonella (16 spp.)	0.945	Christian & Scott (1953)
Leuconostoc citrovorum	0.975 (NaCl)	Christian & Waltho (1961)
Salmonella oranienburg	0.95-0.97	Christian (1955)
halotolerant:		
Staphylococcus aureus (14 spp.)	0.86-0.88	Scott (1953)
Micrococcus (3 spp.)	0.83 (NaCl) 0.92 (glyerol)	Marshall *et al.*, (1971)
Staphylococcus albus	0.885 (NaCl)	Marshall *et al.*, (1971)
Halomonas elongata	0.83 (NaCl)	Vreeland & Martin (1980)
slight halophiles:		
Vibrio metchnikovii	0.96-0.995 (NaCl) 0.95 (NaCl) 0.97 (glycerol)	Scott (1957) Marshall *et al.* (1971)
moderate halophiles:		
Vibrio costicola	0.86-0.98 (NaCl)	Kushner (1978)
Paracoccus halodenitrificans	0.86-0.98 (NaCl)	Kushner (1978)

TABLE 1 CONT'D

Limiting water activities (a_w) for microbial growth

Organism	a_w lower limit	References
extreme halophiles:		
Halobacteria, Halococci	0.75-0.88 (NaCl)	Kushner (1978)
Halobacterium	0.75	Gould & Measures (1977)
FUNGI		
non-xerophilic:		
Aspergillus flavus	0.902	Pitt & Christian (1968)
Aspergillus niger	0.843	Pitt & Christian (1968)
Rhizopus spp.	0.94	Scott (1957)
xerophilic:		
Xeromyces bisporus	0.605	Scott (1957), Pitt & Christian (1968)
YEAST		
non-osmophilic:		
Candida pseudotropicalis	0.931 (NaCl)	Battley & Bartlett (1966)
Saccharomyces cerevisiae	0.94 (NaCl) 0.92 (glucose) 0.90 (salt)	Onishi (1957)
osmophilic:		
Saccharomyces rouxii	0.86 (NaCl) 0.857 (sucrose) 0.845 (glucose)	Onishi (1957)
Saccharomyces rouxii	0.60	Brown (1976) Gould & Measures (1977)

TABLE 2

Salt Response of Different Microorganisms

Category	Reaction	Examples
Non-halophile	Grows best in media containing less than 0.2 M salt	Most normal eubacteria and most freshwater microorganisms
Halotolerant	Non-halophile which can tolerate salt. If the growth range extends above 2.5 M salt, it may be considered extremely halotolerant.	*Staphylococcus epidermidis* Solute-tolerant yeasts, fungi and algae. *Halomonas*
Slight-halophile	Grows best in media containing 0.2-0.5 M salt	Many marine microorganisms
Moderate-halophile	Grows best in media containing 0.5-2.5 M salt. Organisms able to grow in less than 0.1 M salt are considered facultative halophiles	*Vibrio costicola*, *Paracoccus halodenitrificans* *Pseudomonas* sp.
Borderline extreme halophiles	Grows best in media containing 1.5-4.0 M salt	*Ectothiorhodospira halophila* *Actinopolyspora halophila* *Halobacterium volcanii*, *H. mediterranei*
Extreme halophiles	Grows best in media containing 2.5-5.2 M (saturated) salt	*Halobacterium salinarium* *Halococcus morrhuae*

(From Kushner 1984; In Press)

Non halophilic organisms consist of most fresh water and normal eubacteria; halotolerant organisms are considered non-halophiles which can tolerate salt. Slight halophiles, as the name implies, have a slight salt requirement for optimal growth and thus many marine organisms belong to this grouping.

Moderate halophiles have been classified as those organisms able to grow in the range of about 3 to 20% NaCl (0.5 to 3.5M). It has been shown that a number of marine bacteria are capable of growing in up to 20% and even 30% NaCl (Forsyth et al., 1971); thus, many marine bacteria, which normally grow in the 3% NaCl found in the oceans, could be considered moderate halophiles.

The inhibitory effect of NaCl or upper NaCl limit for the halophiles may be due to the NaCl itself or to the effect the NaCl has on water activity (Kushner, 1978). Most non-halophilic bacteria cannot grow below an a_w value of 0.90 (corresponding to 2.83M NaCl) whereas the halotolerant bacteria can grow at an a_w of 0.87 (3.7M NaCl) and some moderately halophilic bacteria can grow at an a_w of 0.86 (3.9M NaCl) (Table 1). Extreme halophiles can grow in salt saturated solutions (5.15M NaCl) at a_w values near 0.75. Thus non-halophilic to halophilic organisms can be roughly grouped according to their limiting a_w values.

The definition of the lower salt concentration for growth of several strains of salt tolerant and halophilic bacteria is incomplete unless the growth temperature is also considered. Several investigators have observed that the minimal NaCl requirement for growth of the salt

tolerant moderate and extremely halophilic bacteria is increased by raising the temperature of incubation (Gibbons and Payne, 1961; Goldman et al., 1961; Ingram, 1957; Ishida et al., 1974; Novitsky and Kushner, 1975; Tesone et al., 1981). For example the lowest salinity which will support growth of the moderate halophile Vibrio costicola at 30°C is 0.5M NaCl. At 20°C this organism can grow down to 0.2M NaCl (Kushner, 1978). Other moderate halophiles should be re-examined in this respect to determine if this temperature-salinity effect is a general phenomenon.

2. Vibrio costicola

Vibrio costicola is a gram negative moderately halophilic, aerobic, curved rod. This organism was originally isolated from pork ribs curing in salt brine (Smith, 1938). V. costicola can grow over a NaCl range of 0.2-3.5M (Shindler et al., 1977) and it has been previously shown that growth at higher NaCl concentrations was not due to a selection of more resistant cells, but rather a result of individual adaptation (Forsyth and Kushner, 1970).

A knowledge of how intracellular ionic content varies with the environment is required to understand how growth at different salt concentrations affects cellular components. For V. costicola the internal ionic composition varies when the cells are grown at different salt concentrations and the sum of internal Na^+ and K^+ ions is at least as high as that of the environment (Table 3). Only in the medium containing 2.0M NaCl was the total Na^+ plus K^+ concentration found to be slightly lower than the external NaCl concentration.

So far five cytoplasmic enzymes from V. costicola have been examined from cells grown in 1.0M NaCl (Table 4). The aspartate transcarbamylase has a salt optimum at 2.0M NaCl. In contrast the threonine deaminase, glycerol dehydrogenase, NADP-specific malic enzyme and pyruvate kinase have a salt optimum at 0.1-0.5M and further NaCl in the reaction mixture is inhibitory. The opposite is true for most enzymes from extreme halophiles which require high NaCl for stability and activity (Kushner, 1978, 1984: In press). Yet what is striking about V. costicola's enzymes is that they are strongly inhibited by NaCl levels well below measured cell-associated Na+ and K+ concentrations (Table 3). Even in vitro protein synthesis (Wydro et al., 1977) was found to be optimal at 0.2M NaCl and non functional at the higher concentrations thought to be inside the organism itself. Since the cells are viable and in fact grow optimally in 1.0M NaCl, it was suggested that the intracellular NaCl must somehow be separated from these salt sensitive structures (Wydro et al., 1977). Recent experiments have now clarified, to some extent, this mystery (Kamekura and Kushner, ASM Annual Meeting, 1984). Cell free chloride was measured by direct ashing of cell pellets. NaCl or KCl do not evaporate upon direct ashing whereas chloride bound to the cell envelope or amino acids does (Cotelove 1964). The intracellular chloride concentration in the form of salts was found to be approximately 0.02M and 0.98M respectively in 1.0 and 3.0M NaCl grown cells (Kamekura and Kushner, in preparation). However, when cell pellets were first heated in the presence of NaOH, chloride bound to the envelope or amino acids is converted to NaCl. Upon ashing the total cell, associated chloride in 1.0 and 3.0M NaCl-grown cells, was approximately 0.22 and 1.3M

TABLE 3

Effect of medium NaCl concentration on cell-associated cations in *Vibrio costicola*

Ion concentration (M)				
in medium		cell-associated		References@
Na+	K+	Na+	K+	
1.0	0.004	0.684	0.221	Christian & Waltho (1962)
0.6	0.008	0.505	0.524	Shindler et al. (1977)
1.0	0.008	0.584	0.661	
1.6	0.008	1.09	0.594	
2.0	0.008	0.898	0.567	

@ The difference between the K+ concentration found by the two laboratories may be due to the fact that Christian and Waltho (1962) measured ions in stationary-phase cells grown in complex media and Shindler et al. (1977) used exponential cells grown on defined media which were re-aerated after centrifuging.

TABLE 4

Effect of Salt on Cytoplasmically Located Enzymes from *Vibrio costicola*

Enzymes	NaCl Concentration of Growth Media (M)	Salt Activity Range (M)	Optimal Salt Activity (M)	Reference
Glycerol dehydrogenase (Cell free extracts)	0.7	0-3.0 (NaCl)	0.5 (NaCl)	Baxter & Gibbons, 1954
	2.6	0-3.0 (NaCl)	0.5 (NaCl)	
NADP-oxidoreductase (Purified)	1.0	0-0.9 (KCl or NH_4Cl)	0.1 (KCl or NH_4Cl)	Salvarrey & Cazzulo, 1980
Aspartate transcarbamylase (Cell free extracts)	1.0	1.0-4.5 (NaCl)	2.0 (NaCl)	Shindler, 1976
Threonine deaminase (Cell free extracts)	0.6	0-3.0 (NaCl)	0.1 (NaCl)	Shindler, 1976
	1.0	0-3.0 (NaCl)	0.1 (NaCl)	
	2.2	0-3.0 (NaCl)	0.1 (NaCl)	
Pyruvate kinase (purified)	1.0	0-1.0 (KCl or NaCl)	0.125 (KCl or NaCl)	DeMedicus & Rossignol, 1977

respectively. Whatever the form of Cl^- ions, these results show that their intracellular concentration is much lower than their extracellular one.

Thus a significant proportion of intracellular Cl^- in V. costicola is not found in the form of NaCl but rather bound to other compounds which are as yet unknown. Therefore, in such a bound state it is likely that the Cl^- is no longer available to interact with NaCl or more appropriately Cl^- sensitive structures. Secondly Cl^- rather than Na^+ is responsible for inhibition of in vitro protein synthesis since the Na^+ - salts such as Na-acetate, Na-glutamate and Na-aspartate enhance in vitro protein synthesis in the presence of NaCl (Kamekura and Kushner, in preparation).

In the few cases examined, the salt response of enzyme activity in moderate halophiles does not seem to change regardless of the salt concentration in which the cells were grown (Kushner, 1978). This is true for the threonine deaminase and glycerol dehydrogenase (Table 4). Similarly ribosomes of V. costicola maintain the same sedimentation coefficient and the protein synthesizing system was not affected regardless of the salt concentration of the growth media (Wydro et al., 1975, 1977).

Of the membrane components so far examined in V. costicola, the adenosine triphosphatase, membrane bound 5'-nucleotidase and respiratory enzyme system of intact cells show optimal activity in the presence of 0.2-1.0M NaCl, and further NaCl in the reaction mixture is inhibitory (Table 5). However the $NADH_2$ oxidase was activated by salt with an optimum of 0.5M (Table 5) and seems to be fairly resistant to

further salt increases in the reaction mixture.

A recent finding showed the transport of α-aminoisobutyric acid across the membrane in V. costicola can be induced to become less salt sensitive in non growing cells by allowing incubation of these cells in a higher NaCl solution (Kushner et al., 1983). However, before elucidation of the mechanisms of salt resistance can be understood, it is necessary to examine the effect of NaCl on the physiology of the membrane itself.

Much of the past research on V. costicola and other moderate halophiles has been concentrated on salt response of cytoplasmic enzymes and other cytosolic components. However, it is likely that the cellular membrane will give a better understanding of the adaptational processes, first because the membrane is the first barrier between the cell and its environment; secondly, the membrane regulates the exchange of solutes between the inside and outside of the cell; and lastly, the membrane contains many enzymes vital to cell survival (e.g. respiratory chain).

3. Bacterial Membrane Composition

The cell envelope of gram-negative bacteria consists of an inner cytoplasmic membrane and an outer membrane separated by a thin layer of peptidoglycan (Fig. 1). Basically, each membrane is composed of a phospholipid bilayer plus associated protein; however the outer membrane also contains lipopolysaccharide on the external surface. The outer membrane contains many fewer polypeptide species than are present in the cytoplasmic membrane. Approximately 20 species of polypeptide have been identified on SDS-polyacrylamide gels in the outer membrane of E. coli

TABLE 5

Effect of Salt on Cytoplasmically Located Enzymes from *Vibrio costicola*

Enzymes	NaCl Concentration of Growth Media (M)	Salt Activity Range (M)	Optimal Salt Acitivity (M)	Reference
$NaDH_2$ oxidase (membrane bound)	1.0	0-1.0 (NaCl)	0.5 (NaCl)	Unemoto et al., 1977
5'-nucleotidase (purified)	1.0	0-3.0 (NaCl or KCl)	2.0-3.0 (NaCl or KCl)	Bengis-Garber & Kushner 1981
(membrane bound)	1.0	0-3.0 (NaCl or KCl)	0.2-0.4 (NaCl or KCl)	
Adenosine triphosphatase	1.0	0-3.0 (NaCl or KCl)	0.5 (NaCl or KCl)	Higa & Cazzulo 1978
Respiratory enzyme system	0.5	0-5.-4.0 (NaCl)	0.5 (NaCl)	Kushner, et al., 1983
	1.0	1.0-4.0 (NaCl or KCl)	1.0 (NaCl or KCl)	

and Salmonella spp. (Wright and Tipper, 1979). In most gram negative organisms, between one and four major proteins make up 70% or more of the mass of protein in the outer membrane (Schnaitman, 1970). The outer membrane is relatively simple in terms of protein composition compared to the cytoplasmic membrane so that it is easily purified. It forms the interface between the cell and its external environment and thus contitutes a physical and functional barrier which controls access of solutes to the cytoplasmic membrane.

3. A) Effect of Salt and Other Factors on Outer Membrane Protein Compostion.

The effect of increasing the external salinity has been examined for E. coli K-12 which has four major outer membrane proteins designated a, b, c, and d in order of decreasing molecular weight. The presence of higher NaCl or KCl in the media (0.3M) caused the relative amount of protein c to increase with a roughly equal decrease observed in protein b (Hasegawa et al., 1976; van Alpen and Lugtenberg, 1977). The function of both these salt-regulated proteins has now been elucidated. Lugtenhaus (1977) showed that mutants lacking either protein b or c alone had unchanged ability to take up various metabolites such as thymidine, methionine, glucose or leucine. But in a mutant lacking both proteins incorporation was inhibited, suggesting that these proteins function as passive diffusion pores (porins) in the outer membrane. In very high concentrations of these solutes some were taken up in the double mutants, suggesting the presence of other secondary pores in the outer membrane.

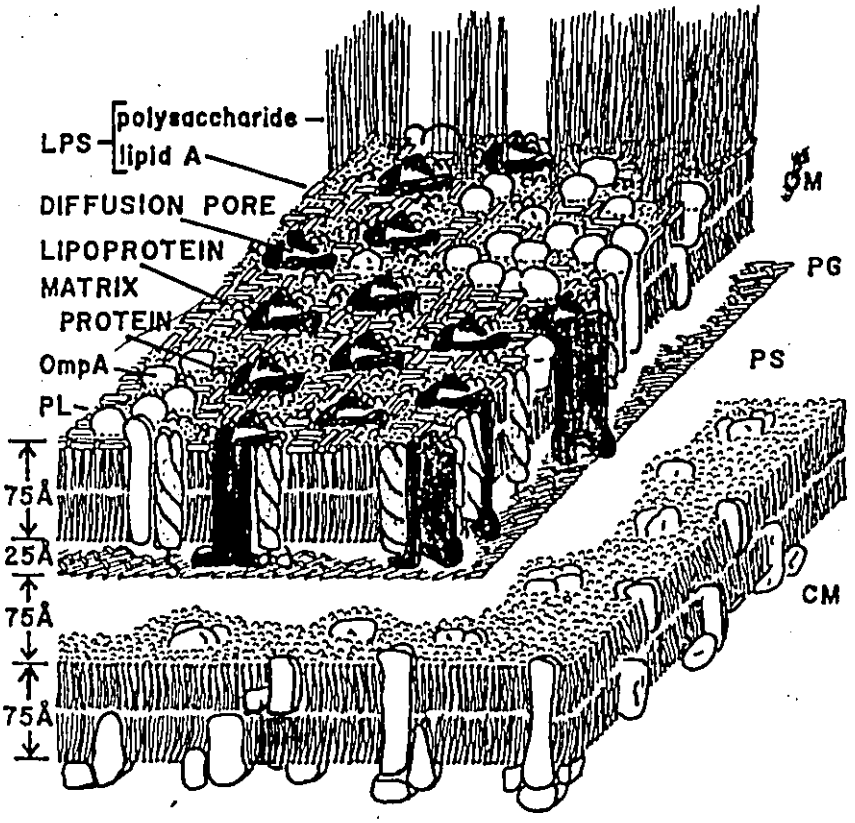


Figure 1 Schematic Representation Illustrating the Possible Molecular Architecture of the E. coli Cell Envelope

Abbreviations used are:PL, phospholipid;OM, outer membrane; LPS, lipopolysaccharide; PG, peptidoglycan; PS, periplasmic space; OMP A, outer membrane protein; and CM, cytoplasmic membrane.

(From DiRienzo et al. 1978)

E. coli K-12 has two porin proteins which have slightly different permeability properties (Nikaido, 1979). For example mutants less permeable to Cu^{2+}, chloramphenicol or adenosine 5'-monophosphate usually lack protein b (Lutkenhaus, 1977; Chai and Foulds, 1978; van Alpen et al., 1978). Thus the salt-repressible porin (protein b) appears to be most effective in the diffusion of a number of solutes and could form the basic all-purpose channels. But the question remains why this porin is repressed by high ionic strength. Nikaido (1979) suggested that when enteric bacteria are excreted with feces, the salt concentration of the environment would increase as a result of the drying out of the feces. This might act as a signal of an environmental change to the bacteria so that they shut down the most effective pores and survive in a state of minimal metabolic activity or with minimal exchange of material with the outside.

NaCl-regulated changes in major protein species of the outer membrane have also been observed for the moderate halophile Pseudomonas halosaccharolytica (Hiramatsu et al., 1980 a, b); however, whether they are porins or not has not yet been investigated.

Increasing the external concentration of several carbohydrates and dextrans in the medium caused the same effect as increasing external salinity in the growth media for E. coli K-12. Protein c increased and protein b decreased (Kawaji et al., 1979; van Alpen and Lugtenberg, 1977). Therefore the external osmolarity seems to be the controlling factor.

Schnaitman (1974) found that as E. coli entered stationary phase, one of the major proteins declined whereas the relative amount of the other was elevated. Similarly differences were observed for Vibrio cholerae. Cells in early exponential phase showed elevated levels of some protein bands compared to cells in mid-exponential or stationary phase (Kelley and Parker, 1981).

Under conditions of iron starvation, Vibrio cholerae (Sigel and Payne, 1982), Azotobacter vinelandii (Page and von Tigerstrom, 1982), E. coli and other Enterobacteriaceae (Wright and Tipper, 1979) over-produce certain outer membrane proteins which appear to be involved in uptake of ferric chelates.

In E. coli cerulenin specifically blocks the activity of the 3-oxoacyl-(acyl-carrier-protein) synthetase thus inhibiting fatty acid synthesis. Bocquet-Pages et al. (1981) noted a decrease in both porin species, b and c and an inhibition of 5'-AMP uptake in the presence of cerulenin. Similarly in a E. coli auxotroph defective in sn-glycerol-3-phosphate acyltransferase, phospholipid synthesis stops immediately upon glycerol deprivation. Again both porin proteins decreased in relative amounts. Thus inhibition of lipid synthesis might inactivate preexisting pores or perhaps interefere with pore synthesis or assembly.

B) Effect of Salt on Phospholipid Composition

Lipids in a biomembrane must fulfill two requirements. First they must form a stable bilayer together with membrane proteins and, second, they must be in a sufficiently fluid state under physiological conditions.

However, both of these conditions can be affected not only by membrane composition but also by ionic strength, temperature, and pH (Melchior, 1982). Prokaryotes exist in environments subject to changes in these factors and their lipid composition in many instances changes in response to the environment.

Kates et al. (1961) first noted that NaCl concentration of the media could affect lipid composition. They noted that although the total amount of lipid remained constant in Micrococcus halodentrificans (at present renamed Paracoccus halodentrificans), the content of total phosphorus increased and the unsaponifiable matter decreased as the salinity of the medium was raised.

Reports on the moderate halophile Pseudomonas halosaccharolytica indicate a gradual increase in phosphatidylglycerol and a concomitant decrease in phophatidylethanolamine with increasing NaCl concentrations in the medium (Hiramatsu et al., 1980 a, b; Ohno et al., 1976 b; Ohno et al., 1979). The same results were obtained in a moderately halophilic Paracoccus sp. (Hiramatsu et al., 1980 a). It should be pointed out that for the above organisms the total amount of negatively charged phospholipids increased with increasing NaCl. In contrast, in the halotolerant gram-positive Staphylococcus aureus (Kanemasa et al., 1972) and Staphylococcus epidermidis (Komaratat and Kates, 1975) a decrease in phosphatidylglycerol and increase in which also resulted in an overall increase in cardiolipin was observed with increasing salt concentration in the media which also resulted in on overall increase in the negatively-charged phospholipids. Thus Hiramatsu et al. (1980 a) suggested that the changes in relative amounts of individual phospholipids in response to different salt concentrations in the media differ from gram positive to gram negative bacteria. However not all bacteria follow these same trends. For the slight halophile

Planococcus citreus the changes in phospholipid composition may be due to growth rate rather than salt concentration. As the NaCl content of the media is raised from 0.5% to 3% (optimal growth salinity), the relative amount of phosphatidylglycerol and cardiolipin doubles, and a further increase to 10% caused a 50% decrease. The opposite changes were observed for phosphatidylethanolamine (Thirkell and Summerfield, 1977). Thus phosphatidylglycerol and cardiolipin were at a maximal concentration when grown at the highest growth rate whereas phosphatidylethanolamine was found to be at its lowest concentration.

The fatty acids have also been shown to be regulated by environmental NaCl concentration. In S aureus (Kanemasa et al., 1972) and S. epidermidis (Komaratat and kates, 1975) the proportion of branched chain fatty acids increases with increasing NaCl in the media. Increasing NaCl causes a significant increase in cyclopropanoic acids of P. halosaccharolytica, (Hiramatsu et al., 1980 a; Hyono et al., 1980; Ohno et al., 1975, 1979). Peleg and Tietz (1971) noted that an unidentified moderately halophilic bacterium contained remarkably high contents of cyclopropane acids when cultured on media containing 2 and 4M NaCl; however, no significant changes in individual fatty acid proportions were observed when bacteria were grown at such high NaCl concentrations.

In the non-halophile E. coli, high salt (0.6M) concentrations induced cyclopropane fatty acid enrichment and a concomitant decrease of unsaturated fatty acids for cells at stationary phase (McGarrity and Armstrong, 1975). Thus, the above observations suggest high NaCl induces cyclopropane synthetase. The melting point of cyclopropanoic fatty acids lies between those of unsaturated and saturated fatty acids. Therefore regulation of cyclopropanization of unsaturated fatty acids by NaCl may contribute to maintain suitable membrane integrity in a salty environment.

c) Effect of other factors on Phospholipid Composition

It is quite possible that the minimum growth temperature of microorganisms could be directly determined by the lower boundary of the gel to liquid-crystalline phase transition. Generally, the gel state will not support normal membrane function (Steim et al., 1969; Overath et al., 1970). It has been suggested that the upper temperature limit of growth may be determined by the point above the transition phase where increased thermal motion of acyl chains causes the cell membrane to become leaky or unstable so that low molecular weight solutes can no longer be retained (McElhaney, 1976). Furthermore, the physical state of the lipids could influence the proper functioning of enzymes or other membrane bound proteins required for transport which are critical to cell survival (Singer and Nicholson, 1972).

Bacteria have evolved a mechanism which enables the type of fatty acids in the membrane to change in response to altered environmental temperatures (Bhakoo and Herbert, 1979; Cronan, 1975; Kates and Hagen, 1964; Marr and Ingraham; McElhaney, 1976; Ray et al., 1971; Taneia et al., 1979). Generally the proportion of unsaturated fatty acids of the

phospholipids increases as the temperature is lowered. Inversely, as the temperature is raised, the proportion of saturated fatty acids increase. Thus bacteria are capable of controlling to a certain extent the transition temperature of their membranes as a function of growth temperature in order to maintain their normal physiological cell processess.

Prokaryotes not only possess a short-term adaptation mechanism to changes in thermal environments as described above but also there seems to be long-term evolutionary adaptation as well. Psychrophiles are characterized by high proportions of unsaturated or shorter fatty acids, whereas thermophiles possess a high content of longer saturated and predominately isobranched acids (Langworthy, 1982; McElhaney, 1976). In general, lipids from organisms which have evolved to grow at low temperatures are enriched in fatty acids which have relatively low melting points, whereas those adapted to growth at high temperatures are rich in fatty acids having higher melting points.

In E. coli it has been shown that as cells pass from the exponential phase to stationary phase of growth, the proportion of phosphatidylglycerol decreases whereas cardiolipin increases (Cronan, 1969; Cronan and Vagelos, 1972; DeSiervo, 1969; Randle et al., 1969). Similar changes have been observed in Planococcus citreus (Komura et al., 1975) and Pseudomonas halosaccharolytica (Ohno et al., 1976). and an unidentified moderate halophile (Stern and Tietz, 1973). A stoichometric loss of phophatidylglycerol and concomitant increase in cardiolipin is understandable because phosphatidylglycerol is the precursor of

cardiolipin (Cronan and Vagelos, 1972; Randle et al., 1969).

Increased proportions of cyclopropane fatty acids with a corresponding diminuation of monounsaturated acids in stationary phase cultures have been found for E. coli, (Cronan, 1968; Sijher et al., 1980), P. halosaccharolytica (Ohno et al., 1976; 1979) and Serratia marcescens (Kates et al., 1963). The biochemical basis for these changes is the transfer of a methylene group from S-adenosylmethionine across the double bond of the monounsaturated acid. However, the rate of methylation is dependant on growth phase and is much higher as cells enter stationary phase (Kates, 1964). The significance of this conversion in cells at stationary phase may be to protect the unsaturated acids from roxidation while altering membrane properties in the least amount possible (Kates et al., 1963).

Not all bacteria examined have been found to alter their phospholipid composition as a function of growth phase. The phospholipid and fatty acid composition remained constant over the growth cycle for Bordetella pertussis (Kawai and Moribayashi, 1982) and no significant changes were observed in the composition of individual phospholipids for S. aureus in mid-exponential or stationary phases of growth (Kanemasa et al., 1972).

4) Osmoregulation of Intracellular Solutes in Response to Environmental Salinity.

The environmental NaCl concentration has been shown to alter envelope components of bacteria but the possibility of adaptation of cytoplasmically located components must also be considered.

The cell water of an organism is an important part of the cell since it contains dissolved solutes and other internal components which undergo intracellular interactions. Such solutes and their interactions have control over many important functions such as enzymes activity as well as other physiological changes. Thus, it is quite obvious that changes in concentrations of dissolved solutes within the cell may drastically affect cell function as well as cell survival.

Almost any solute dissolved in the cell water can act as an osmoregulator. An osmoregulator can be defined as any solute which will increase in concentration within the cell simultaneously as the cell is exposed to increasing external concentrations of a different solute and vice versa. Osmoregulation is the formation and removal (via destruction or excretion) of a particular solutes(s) which in turn will alleviate osmotic tensions and dehydration. How osmoregulation occurs and exactly what solutes are involved is a particularly interesting question when considering halophilic organisms. This is because such organisms can grow in differing salt concentrations and must accordingly be able to adjust their internal solute concentrations in order to prevent cell lysis.

Ben-Amotz and Avron (1973) showed that the halophilic alga Dunaliella parva, contained large amounts of intracellular glycerol

which would increase or decrease when cells were grown in higher or lower NaCl concentrations respectively. Boriwitzka and Brown (1974) showed that the algae, Dunaliella tertiolecta and D. virdis contained glycerol in amounts which varied directly with the NaCl concentration of the growth media. They assumed that the reason D. virdis can tolerate higher salt concentrations than D. tertiolecta is that it produces more glycerol. They also noted that glucose 6-phosphate dehydrogenase and a NADP specific glycerol dehydrogenase were inhibited by high salt concentration whereas high glycerol concentrations allowed the enzymes to function normally. Thus it seems that not only does glycerol act as an osmoregulator but also as a compatible solute, a solute that allows enzymes to function at high concentrations (i.e., which does not inhibit enzyme activity) and seems to protect the enzymes from inhibition or inactivation by other inhibitory substances if present (Brown, 1976). Glycerol seems to act as compatible solute in halophilic algae as do polyols in yeasts (Brown, 1974). Betaine may be an important compatible solute in some halophilic species (Galinski and Truper, 1982; Rafaeli-Eshkol and Avi-Dor, 1968) as well as non halophiles such as members of the Enterobacteriaceae (Le Rudulier and Bouillard, 1983); polyamines in E. coli (Munro et al., 1972) were suggested to act as osmotic balancers.

The extreme halophiles, on the other hand, accumulate K+ ions under growth conditions of higher salinity (Christian and Waltho, 1961, 1962; Ginzburg, 1970; Yancey et al., 1982). For example intracellular K+ concentrations of approximately 7 molal have been recorded in species of Halobacterium growing in a saturated NaCl medium (5.1M) (Lanyi, 1974).

Since the proteins of the halobacteria are enriched in aspartyl, glutamyl and weakly hydrophobic residues, Lanyi (1974) suggested that such amino acid substitutions enable halophile proteins to have the proper conformational and functional states at high KCl concentrations.

Several reports underline the potential importance of amino acids in osmoregulation. In some bacteria a significant proportion of one to three amino acids (glutamate, proline, alpha-aminobutyric acid, and or glutamine) is accumulated in direct response to increasing external salinity. Glutamate seems to be generally accumulated by gram negative organisms and proline by gram positive organisms (Table 6). However, as shown in Table 6, some gram negative organisms in addition also accumulate proline and similarly some gram positive species accumulate glutamate. Even in halophyte plants, proline seemed to be involved in osmostic adjustment as medium salinity was changed (Flowers and Hall, 1978; Stewart and Lee, 1974).

Accumulation of glutamic acid requires the cell to accumulate an accompanying cation in most cases in order to retain electrical neutrality. The disadvantages of K+ accumulation, however, are similar to those of the inhibitory effect of NaCl on enzymatic activity. Thus gram positive organisms seemed to have evolved a mechanism to convert glutamate to proline which is not charged at neutral pH and this does not require the presence of a neutralizing cation (Measures, 1975).

TABLE 6

Amino acids showing major increase in the free cytoplasmic pool during growth in the presence of NaCl

Organism	Increased Amino Acids	References
Gram Negative:		
Halomonas elongata	glutamate, glutamine alanine	Vreeland et al. (1983)
Vibrio alginolyticus	glutamate, proline	Unemoto & Hayashi (1972)
Rhizobium japonicum	glutamate	Yelton et al. (1983)
Rhizobium sp.	glutamate	Yap & Lim (1983)
Beneckea harveyi	glutamate	Mackemson & Hastings (1979)
Salmonelli typhimurium	glutamate, glutamine	Csonka (1981)
Aerobacter aerogenes	glutamate	Tempest & Meers (1970)
Erwinia carotovora	glutamate	Tempest & Meers (1970)
Escherichia coli	glutamate, GABA, proline	A
Pseudomonas fluorescens	glutamate	Tempest & Meers (1970)
Klebsiella aerogenes	glutamate, proline	A
Pseudomonas aeruginosa	glutamate	A
Vibrio parahaemolyticus	glutamate	A
Salmonella oranienbrug	glutamate, proline	A
Gram Positive:		
Staphylococcus aureus	glutamine, proline	Anderson & Witter (1982)
S. aureus	proline	Koujima *et al.* (1978)
Bacillus subtilis	proline	Tempest & Meers (1970); A
Streptococcus faecalis	GABA, proline	A
Sarcina lutea	proline	A

TABLE 6 CON'T

Amino acids showing major increase in the free cytoplasmic pool during growth in the presence of NaCl

Organism	Increased Amino Acids	References
Bacillus cereus	proline	A
Micrococcus lysodeikticus	proline	A
Clostridium sporogenes	glutamate, GABA, proline	A
Lactobacillus plantarum	glutamate, proline	A
Bacillus megaterium	glutamate, proline	A
Serratia marcescens	glutamate, proline	A

(A = Measures, 1975; GABA = alpha-aminobutyric acid)

5) Goals of Thesis

The goal of this thesis was to investigate the adaptation to external salt concentration in the medium of the moderate halophile, Vibrio costicola. The cell membranes are the first barrier between the external environment and the cell. Therefore, the first site of NaCl regulation would likely be at the membrane level. Thus, we investigated some individual components of the membranes, i.e. protein and lipid composition and certain membrane-bound enzyme activities as a function of environmental salt concentration.

Also examined was the effect of salt on certain intracellular solutes which possibly could be involved in adaptation via osmoregulation.

CHAPTER 2

MATERIALS AND METHODS

1. Media and Growth Conditions

Vibrio costicola NRC 37001 was maintained on agar slants consisting of 1% proteose peptone (Difco), 1% tryptone (Difco) (1% PPT), 1.5% agar (Difco) and 1M NaCl, pH 7.6. One day prior to inoculation of liquid PPT media with 0.2, 0.5, 1.0, 2.0 or 3.0 M NaCl pH 7.6, cells were transfered from slants to PPT agar plates containing NaCl equal to the concentration of the liquid media. Cells were grown with vigorous shaking (110 rpm) at 30 °C, except for those in the medium containing 0.2M NaCl, which were shaken at 20 °C; in this NaCl concentration the cells will not grow at 30 °C. Growth was monitored by measuring absorbance at 660nm on a Bausch and Lomb Spectronic 20 spectrophotometer.

Semi-synthetic media consisted of 0.5% glucose, 0.5% casamino acids, 0.001% yeast extract, minerals (0.312% K_2HPO_4, 0.028% KH_2PO_4, 0.01% $MgSO_4.7H_2O$, 0.2% $(NH_4)_2SO_4$) according to Forsyth and Kushner (1970) and the various concentrations of NaCl as detailed above. Cells were harvested at the late exponential phase of growth (O.D. at 500 nm; 0.8-1.2) and washed in the medium above, using half the volume of the growth media.

Escherichia coli RR1 was grown in a medium consisting of 1% tryptone, 0.5% yeast extract, 1% NaCl and 0.1M phosphate buffer pH 7.2.

Vibrio parahaemolyticus LCDC 82-289, Vibrio metchnkovii LCDC SB574, and Vibrio alginolyticus LCDC SB272 were transfered from blood agar

plates (prepared from blood-agar base pH 7.3 (Difco) containing 5% defibrinated sheep blood) to trypticase soy broth (Difco). The cells were washed in 0.5M NaCl, 4mM $MgCl_2$ and 0.1M phosphate buffer pH 7.0.

An unidentified moderate halophile HX, NRC 41227 was also grown in 1M NaCl PPT at 30°C.

2. Total Membranes Prepared by Pressure Bomb Treatment for Enzymes Assays

Cells of V. costicola were washed twice in 10% sucrose, 2mM $MgCl_2$ and 50 mM Tris-hydrochloride (pH 8) containing NaCl equal to the concentration used in the growth media. Membranes were prepared according to the method of Bengis-Garber and Kushner (1981) as follows. Washed cells (100g wet weight) were hand homogenized in a final volume of 300 ml of 50 mM Tris-HCl (pH 8), 10% glycerol and NaCl equal to one half the concentration of the growth media. Lysozyme and disodium ethylene diamine tetraacetate were also added to a final concentration of 0.25 mg/ml and 10mM respectively and the mixture was stirred gently for 20 minutes at room temperature. Next the suspension was placed in a cell disruption bomb (Parr Instrument Co., Moline, III.) and subjected to argon pressure (1500 psi) for 10 minutes. The pressure was slowly released and the pressure treatment was repeated twice more. The broken cell suspension was centrifuged at 100,000 g for 90 minutes. The pellets (membranes) were homogenized in 50 mM tris-HCl (pH8), 10% glycerol and one half the NaCl of the growth media. The membranes were pelleted a final time by centrifugation at 100,000 g for 60 minutes. The membrane preparation was resuspended in the same buffer and stored at -70°C. Purify of membrane fragments were verified by electron microscopy (not shown).

3. Sonicated Whole Cell Preparations for Enzyme Assays

Sonicated material was prepared by suspending washed whole cells at the ratio of 1 g per 7 ml of 10% glycerol, 50mM tris-HCl (pH8), 2mM $MgCl_2$, NaCl equal to one half that of the growth medium, and then sonicated for 2 minutes at 55,000 cycles per second (Branson sonicator).

4. Sonicated Membrane Preparations for Enzymes Assays

Sonicated membranes were prepared by exactly the same method as described above for "Total Membranes Prepared by Pressure Bomb for Enzyme Assays" except that two 30 second sonication pulses replaced the pressure bomb treatment.

5. Protein Assay

Protein content was measured by the method of Lowry et al. (1951) with slight modification. Tris buffer and sucrose were removed by extracting with acetone:methanol (5:2 v/v) as described by Bengis-Garber and Kushner (1981) prior to the Lowry assay. Protein in cell material was solubilized in 1% sodium dodecyl sulfate as a substitute for NaOH. Bovine serum albumin was used as the standard. The standard curve was the same for protein dissolved in water or sodium dodecyl sulfate.

6. Enzyme Assays

The adenosine triphosphatase (ATPase) and 5'-nucleotidase can be selectively assayed by their different requirements for Mg^{2+} as previously described by Bengis-Garber and Kushner (1981). Briefly, the

reaction mixture for the ATPase contained in a final volume of .1 ml, 2mM $MgCl_2$, 50mM tris-HCl (pH 8), NaCl in a range 0-3.0M and 30 to 100 μg of protein. The reaction was initiated by addition of 4mM ATP, ADP or AMP and the mixture incubated for 5 to 15 minutes at 30°C. The reaction was terminated by the addition of 0.1 ml 60% trichloroacetic acid. Reaction tubes were immediately placed on ice and the protein was pelleted at 8500g (swinging bucket Beckman J-6 centrifuge). The supernants were then transferred to clean tubes and inorganic phosphorus measured colorimetrically as follows (Taussky and Shorr, 1953). Two and a half g $FeSO_4 \cdot 7H_2O$ in 30 ml H_2O was mixed with 5 ml 10% ammonium molybdate in 1ON H_2SO_4. Of this ferrous sulfate solution 0.66 ml was added to each of the supernatants and incubated 10 minutes at room temperature. The optical density was read at 640 nm. KH_2PO_4 was used as standard.

The assay for the 5'-nucleotidase was the same as above, except that 20 mM $MgCl_2$ was used in the reaction mixture (Bengis-Garber and Kushner, 1981).

7. Anti-5'-nucleotidase Preparation

Antibody against purified 5'nucleotidase, produced in a rabbit as described by Nelson et al. (1973), and Dr. Bengis-Garber and Kushner, (1982), was kindly supplied by Dr. C. Bengis-Garber.

8. Inhibition of ATP and AMP Hydrolysis by Antibody against 5'nucleotidase

Whole cells or envelope preparations were suspended in the reaction

mixture, antibodies were added, and incubated for 10 minutes at room temperature. The reaction was then started with addition of substrate.

The above experiment was also done on membranes or whole cells which had first been solubilized with 2% triton X-100 (2mg protein/ml) for 30 minutes on ice followed by a 15 minute incubation at room temperature. Antibodies were then added as described above. After the ferrous sulfate solution as previously described was added, 25 μl of 1% SDS was added to triton treated cells only, in order to solubilize any cell material which might interfere with the colorimetric Pi analysis.

As a control whole cells or membrane fractions were also incubated for 10 minutes with non-immune serum.

9. Broad Range Isoelectric Focusing

An LKB isoelectric focusing apparatus (Sweden) was used. The anode electrode solution used was 1M H_3PO_4 and 1M NaOH for the cathode. Approximately 75 μg of purified 5'-nucleotidase was applied and the ampholine gel was run at 1500 V for 1.5 hours at 10°C. The pH gradient was determined by placing a surface pH electode along several points between the anode and cathode. The 5'-nucleotidase isolated as described by Bengis-Garber and Kushner (1982) was kindly supplied by Dr. Bengis-Garber.

10. Electron Microscopy

Cells were fixed in 2% formaldehyde-2% glutaraldehyde in 1M NaCl and 50mM tris buffer (pH 7.6) for 2 hours. The cells were pelleted and resuspended in buffer overnight, then incubated in ice with 1% osmium tetroxide in NaCl for 1 hour. The material was pelleted and resuspended in 50% acetone (3 min.), then 70% acetone (3 min.), 80% acetone (3 min.), 90% acetone (3 min.), 95% acetone (5 min.), 100% acetone (20 min.) and

finally 100% acetone for a further 20 min. The cells were then immediately infiltrated using Spurrs (1969) resin. Micrographs of thin sections were taken using a AEI-EM or Phillips-EM.

11. Separation of Outer Membrane from Cytoplasmic Membranes

The procedure of Osborn et al. (1972) was used with some modifications. Spheroplasts were prepared by gently mixing 2M NaCl-grown washed cells (3g wet weight) suspended in a final volume of 9 ml of 50mM tris-HCl (pH 7.6), 10% glycerol, 1M NaCl, 10mM EDTA for 20 minutes at room temperature. The spheroplasts were lysed by sonic oscillation for 2 minutes (Branson sonicator). Whole cells were removed by centrifugation (5356xg for 20 minutes) and the supernatant was centrifuged at 100,000g for 90 minutes. The membrane pellets were washed in 1M NaCl, 50mM tris-HCl (pH 8) and 10% glycerol and pelleted by centrifugation at 100,000g for 60 minutes. Homogenization of the pelleted membrane to a final volume of 1 ml was carried out using the same buffer as above with the addition of a final concentration of 18% sucrose.

An isopycnic sucrose density gradient was prepared by layering in a stepwise fashion 2.1 ml of each 50, 45, 40, 35 and 30% cold sucrose solutions (w/w) in a 9/16"dia. x 3½" polyallomer tube. All of the sucrose solutions contained 5mM EDTA and 50mM tris-HCl, pH 7.6. The total membrane preparation (1.0 ml) was layered on the surface of the gradient and spun at 38,000 g for 16 hours. The bottom of the tubes were punctured with a coarse needle (18 gauge bore size) and 8-drop fractions collected. Fractions were stored at -20°C.

Prior to loading some of the total membrane preparations on the sucrose gradient, inner membranes were selectively solubilized with a final concentration of 0.5% sodium-lauryl sarcosinate for 20 minutes at room temperature as described by Filip et al. (1973).

12. Radiolabeling of Outer and Inner Membranes

A) Radioactive Labelling the Inner Surface of the Outer Membrane

V. costicola was grown in 1% PPT containing 2M NaCl and 4μCi/ml ^{3}H-diaminopimelic acid (DAP) for 16 hours. DAP is a component unique to the peptidoglycan layer of gram negative bacteria. Matrix proteins protruding from the inner surface of the outer membrane next to the peptidoglycan are non-covalently attached to both the peptidoglycan and to lipoproteins when solubilized in 2% SDS at temperatures below 70°C (Rosenbusch, 1974). Thus, after polyacrylamide gel electrophoresis and autoradiography, those proteins which are radioactively labeled can be designated as outer membrane matrix proteins or lipoprotein which protrude from the inner surface of the outer membrane.

B) Determination of Outer Membrane Fraction by Labeling the Peptidoglycan

V. costicola was grown in 500 ml PPT containing 2M NaCl and 0.5 μCi/ml ^{3}H-diaminopimelic acid for 16 hours. The distribution of the radioactivity after sucrose gradient centrifugation of membranes was measured by the modified procedure of Yamato et al. (1975).

C) Determination of Protein-Containing Fractions of Separated Membranes

Cells were grown in PPT containing 2M NaCl and 15 μCi ^{14}C-leucine/100ml media to stationary phase. Processing of these cells was as described above for ^{3}H-DAP labeled cells.

D) Labeling of Surface Proteins with ^{125}I-Iodosulfanilic Acid

The procedure for radioactive surface protein labeling was described by Williams and Jirousek (1979). V. costicola was grown in 2M NaCl PPT and washed twice in 0.1 M K-phosphate buffer pH 7.5 containing 2M NaCl and 2 mM $MgCl_2$. The cells were suspended to a final concentration of 8.7mg protein/ml and 50 μl of suspended cells were mixed with 7 μl of diazotized iodosulfanilic acid (^{125}I) of specific activity 1500 μCi/umole. Samples of such reaction mixtures were incubated at 30 °C, 20 °C or 4 °C for 15 minutes. The reaction was terminated by centrifuging down the cells (5000rpm), discarding the supernatant and resuspending the cells in phosphate buffer as above containing 1% bovine serum albumin. The cells were washed 3 times with same buffer, which removes any loosely bound radioactivity from the cell surface. To detect which surface proteins were labeled, the whole cell proteins were separated by

polyacrylamide gel electrophoresis and the radioactivity was detected by autoradiography, by the methods described below.

13. Liquid Scintillation Counting of Sucrose Gradient Fractions

Labeled protein contained within individual sucrose gradient fractions was precipitated with 50 µl 60% ice cold trichloroacetic acid. Filtration of each fraction was carried out through 0.22 µm millipore filters. Each test tube in which gradient fractions had been stored was washed twice with 5 ml cold 5% TCA, and the washing were also filtered through the same filter. Each filter was dried with a gentle stream of warm air and radioactivity was counted for 10 minutes after adding 10 ml Scintiverse I to a vial containing the filter.

14. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The detergent sodium dodecyl sulfate (SDS) dissociates proteins into their constituent polypeptide chains. Polyacrylamide gel electrophoresis in the presence of SDS separates the polypeptide chains based on their molecular weights. The procedure of Weber and Osborn (1975) was used in the preparation of the polyacrylamide gels.

Protein bands were detected by staining with coomassie brilliant blue and glycoproteins by the periodic acid-Schiff technique (Fairbanks et al., 1971).

15. Autoradiography

After the polyacrylamide gel has been destained, the gel was dried beneath sheets of Whatman 3MM filter paper at about 80°C under vacuum on

a Bio-Rad drier for 2 hours. The surface of the dried gel was covered with Saran wrap with the edges of the plastic wrap folded and taped down on the back of the filter paper. In total darkness Kodak X-Omat X-ray film (XAR-2) was placed over the gel and the system sealed in double intensifing screen (or calcium tungstate medical X-ray screen which acts as a solid state scintilation). Exposure was carried out at -70°C for 2 days with ^{125}I-labeled material and 6 days for ^{3}H-labeled material. The X-ray film was developed for 4 minutes, washed in water for 30 seconds and fixed for 4-5 minutes using Kodak RP X-Omat chemicals.

Autoradiography was performed using gels labeled with either ^{125}I or ^{3}H. However, due to the low emission energy of ^{3}H, this isotope cannot be detected by conventional autoradiography. In this case, it is necessary to impregnate the gel with a scintillator, thus instead of the β-particles reacting directly with the film, they cause the scintillant to produce multiple photons which in turn activates the film (Bonner and Laskey, 1974; Chamberlain, 1979). Therefore, prior to drying, the ^{3}H-labeled gels were impregnated with a scintillator (Enlightning rapid autoradiography enhancer) for 30 minutes.

16. 2-Keto-3-deoxyoctonate(KDO) Analysis

Membrane fractions precipitated with 5 ml cold 10% trichloroacetic acid were collected by centrifugation at 4°C for 10 min. at 20,000 g and washed twice by centrifugation with 5 ml distilled H_2O to remove residual sucrose. The precipitate was suspended in 0.7 ml of 0.02N H_2SO_4 and hydrolyzed at 100°C for 20 min. to liberate KDO from lipopolysaccharide. The colorimetric assay of KDO was as described by

Weissbach and Hurwitz (1959); 0.2 ml of hydrolyzed sample was added to 0.25 ml of 0.25N HIO_4 in 0.125N H_2SO_4 for 20 min. at room temperature. Next 0.5 ml 2% $NaAsO_2$ in 0.5N HCl was added and after 2 minutes, 2 ml of 0.3% thiobarbituric acid (pH 2) was mixed into the reaction mixture. The mixture was heated to 100°C for 10 min. After cooling the optical density was read at 548 mm.

17. Endotoxin Detection Assay

Freeze dried limulus lysate (Pyrotell) was reconstituted with 5 ml depyrogenized free water. Small aliquots (0.1 ml) were distributed in depyrogenized tubes and stored at -20°C until use. Reaction was initiated by addition of 0.1 ml of endotoxin solution to each tube and then incubated at 37°C for 1 hour. A positive reaction is the formation of a firm gel that does not slip out of the tube upon inverting. The limulus lysate was tested with Escherichia coli endotoxin and found to have a sensitivity of 0.5 ng/ml.

18. Free Amino Acid Extraction from Whole Cells

Cells of V. costicola grown in PPT with 0.5, 1.0, 2.0 or 3.0M NaCl were washed in 0.1M phosphate buffer (pH 7.6), 2mM $MgCl_2$, and NaCl as above. The pellet was resuspended to a known volume in the same washing buffer and a small aliquot was removed to determine protein concentration. The remainder was centrifugated (7000 rpm) in an acid-washed 30 ml corex tube. The supernatant was discarded and the sides of the Corex tube were rinsed with distilled water and dried using a Kimwipe being careful not to wet or touch the pellet.

The extraction of the pellet was carried out by resuspension in 5 ml ice cold 5% trichloroacetic acid as described by Unemoto and Hayashi (1979) for 30 minutes. The cell fragments were removed by centrifugation (10,000 g for 15 minutes) and the supernatant was transfered to a clean tube from which the TCA was removed by extraction with 4 ml ether 4 times. Finally the amino acids extracts were filtered through 0.25 l millipore filters and stored frozen until analysis.

19. Amino Acid Analysis

Amino acids prepared as just described were chromatographically indentified and quantified by injection into a Durham automatic amino acid analyzer by Dr. M. Yaguchi at the National Research Council, Ottawa, Ont.

20. Hydrolysis of Proteins Into Free Amino Acids

Both membrane preparations and isolated 5'-nucleotidase (See Bengis-Garber and Kushner, 1981) contained tris buffer which was removed by ice-cold (5:2) acetone:methanol extraction as described by Bengis:Garber and Kushner (1981). The hydrolysis of each preparation was then carried out as outlined by Moore and Stein (1963) with some minor modifications. In a volume of 1 ml., a final concentration of 1.5 mg protein and 6N HCl were placed in 16 x 125 mm heavy walled Pyrex tubes. A section of the tube a few centimeters below the top was constricted by flaming to approximately 1mm diameter, the tube's content's frozen in an ethanol bath and the tube was evacuated below a pressure of 50 microns. To completely degas the solution, the tube was twice more thawed and frozen

during continuous pumping down. Finally, the constricted top was sealed by flaming before the vacuum was removed.

Hydrolysis was performed by incubating the sealed tubes at 110°C for 48 hours. After cooling the seals were broken and HCl was removed by a water pump until dryness. The sample was resuspended in 0.5 ml distilled water and the drying procedure repeated until the pH was near neutral.

For membrane preparations, lipids were removed from the hydrolyzed solution by adding 1 part $CHCl_3$ and 1 part MeOH to 1 part hydrolysate, and the lower phase was removed with a Pasteur pipette.

21. Thin Layer Chromatography of Amino Acids

From stock solutions (8.3 mg/ml) of individual amino acids 1 µl was spotted on Brinkmam SIL-G25 thin layer chromatography (TLC) plates which had been previously activated for 2 hours at 110°C. Separation was achieved with the solvent system 1-butanol-acetic acid-water (3:1:1 v/v). Amino acids were visualized by spraying with 0.3% ninhydrin in acetone and then heating at 110°C.

To stablize colored spots, the plates were sprayed with a solution of 1 ml saturated aqueous copper nitrate, 0.2 ml 10% nitric acid and 100 ml 96% ethanol (Randerath, 1971). The plate was then placed in an air-tight enclosure with a beaker of concentrated ammonia for a few minutes.

22. Glycerol Assays

A) The concentration of glycerol from cell extracts were measured by a method from M. Avron (1973). A 0.2 ml sample was added to 1 ml

periodate reagent (65 mg $NaIO_4$ dissolved in 100 ml 6% acetic acid containing 7.7g ammonium acetate. This was mixed well and left for 5 min. at room temperature. Next 2.5 ml acetylacetone reagent (1ml acetyl acetone + 99 ml isopropanol) was added and then incubated at 37°C for 25 minutes, cooled to room temperature and the absorbances read at 410 nm. Glycerol was used as the standard.

B) A second procedure for glycerol determination (Ben-Amotz and Avron, 1973) was carried out as follows. One ml of sample was deproteinized by addition of 30% trichloroacetic acid and the protein pelleted by centrifugation for 10 min. at 4° at 2000 g. Of this deproteinized solution 0.5 ml was added to a tube containing 1.5 ml distilled H_2O. Next 0.1 ml 10N H_2SO_4 and 0.5 ml 0.1M sodium periodate were mixed and allowed to react at room temperature for 5 min. After the incubation period 0.1 to 0.5 ml was pipetted into a new tube followed by addition of 10 ml 6.3 mM chromotropic acid reagent (1g 1, 8-dihydroxynaphthalene filtered through Whatman #1 paper. Sulphuric acid solution was prepared by adding 300 ml concentrated H_2SO_4 to 1560 ml H_2O. The cooled sulphuric acid solution was added to the dissolved chromotropic acid to a final volume of 500 ml. The reagent was stored in a dark bottle and made fresh every 2-3 weeks). The mixture was boiled for 30 minutes, cooled and the optical densities read at 570 nm. Glycerol was used as the standard.

23. Lipid extraction

Isolated membranes of V. costicola grown at 0.2, 1.0, 2.0 and 3.0 M NaCl were suspended in 0.2, 0.5, 1.0, and 1.5M NaCl, respectively,

containing 10% glycerol, 50mM Tris-HCl (pH8) and 2mM $MgCl_2$. Glycerol protected the nucleotidases being studied (Bengis-Garber and Kushner 1981, 1982). Whole cells were suspended in the same amount of NaCl as used in the growth media. Lipid extraction from membranes and whole cells was carried out by the method of Bligh and Dyer (1959) as modified by Kates (1972).

To avoid lipid degradation by lipase action during extraction, in some experiments cells were extracted by boiling hot isopropanol (Kates 1972): 1.5 g wet cell weight was mixed with 15 ml hot isopropanol and then centrifuged. The supernatant was removed and the pellet extracted twice more with 10 ml hot isopropanol and a final time with 10 ml 1:1 $CHCl_3$-isopropanol. All the supernatants were pooled and brought to dryness on a rotary evaporator. The residue was immediately resuspended in methanol-chloroform (1:1) and converted to two phases by addition of 0.9 volumes of water (Kates, 1972).

24. Lipid analysis and identification.

Dry weights were determined on a portion of each total lipid extract. A portion of the lipids was applied to Brinkmam SIL-G25 or non commercial silica gel-G or H (0.25-0.5 mm) thin layer chromatography (TLC) plates which had previously been activated for 2 hrs. at 110°C. Separation was achieved in the solvent system chloroform-methanol-acetic acid-water (85:15:10:3.5 v/v). Lipids were visualized by spraying with 40% H_2SO_4 and charring, or by iodine vapor. Phospholipids were visualized by the molybdate spray reagent (Vaskovsky and Kostetsky, 1968), glycolipids by the α-napthol stain (Kates 1972) and amino lipids

by spraying with 0.25% ninhydrin in acetone. Identification was also achieved by cochromatography with phospholipid standards. Phospholipids were separated from neutral lipids on a silicic acid column as described by Kates (1972). Individual phospholipids were analyzed quantitatively by scraping isolated lipid bands (as well as suitable blank areas) from TLC plates and measuring lipid phosphorus by the modified method of Bartlett (1959) (Kates 1972). For fatty acid, analysis, lipids were extracted from the silica gel three times by the Bligh and Dyer (1959) procedure (Kates 1972), and subjected to methanolysis (Kates 1972) with addition of heptadecanoic acid (25 μg per 100 μg lipids) as internal standard. Absolute values were caluclated by comparison to internal standard peak heights. The fatty acid methyl esters obtained were analyzed by gas liquid chromatography (Pye Unicam) on a column (4mm x 2m) of 5% SP 2330 at 185 ^{0}C. Fatty acid standards used for identification were 14:0, 16:0, 16:1, 18:0, and 18:1.

25. Source of Materials

Materials were purchased from: Nucleotides, Triton X-100, Tris and lysozyme, Sigma Chemical Company. Proteose peptone and tryptone: Difco laboratories, Detroit, Mich. Silica gel (SIL G-25) plates for thin layer chromatography: Brinkman Instruments Canada Ltd. Phospholipid standards (Cardiolipin, Phophatidylglycerol, phosphatidylethanolamine and phosphatidylcholine): Serdary Research Laboratories, London, Ont. heptadecanoic acid and chromotropic acid sodium salt: Eastman Organic Chemicals, Rochester, N.Y.

Low molecular weight protein standards (40,000-100,000 daltons), acrylamide, N,N'-methylenebisacrylamide, N, N, N'N'-tetramethylethylenediamine (TEMED) and ammonium persulfate: Bio rad laboratories, Richmond, California. Molecular weight standards (14,300-85,800 daltons) for SDS gel electrophoresis (product #44223 2U): BDH Chemicals Ltd., Broom Road England. ^{3}H-diaminopimelic acid: Amersham Radiochemicals, Oakville, Ont. ^{14}C-leucine, ^{3}H-leucine, ^{125}Iodosulfofanilic acid labeling kit and Enlightning rapid autoradiography enhancer: New England Nuclear, Boston, Mass. Kodak X-Omat X-ray film (XAR-2): Eastman Kodak Co., Rochester, N.Y. Scintiverse I: Fisher Scientific. Limulus lysate: Pyrotell Assoc. of Cape Cod Inc., Woods Hole M.A. Escherichia coli endotoxin standard: Mallinckrodt Inc., St. Louis, Mo. Ampholine PAG plates: LKB-Produkter AB, Sweden. Fatty acid standards (14:0, 16:0, 16:1, 18:0 and 18:1): Supelco Corporation.

All solvents were distilled before use for phospholipid analysis. All other chemicals were of analytical grade.

Note: Most individual experiments as reported in Results section were repeated at least twice.

CHAPTER 3

RESULTS

1. Growth and Morphological Characteristics of Vibrio costicola

Vibrio costicola are short curved rods, of dimension approximately 0.5 to 2.0 μm (Buchanan and Gibbons, 1974). The cells grow singly or in chains. At salt concentrations near the upper extreme permitting growth (3.0M NaCl), the cells were slightly thinner in diameter, but much longer than cells grown at the optimal NaCl concentration (1.0M NaCl). Swollen cells, often found at the end of a chain were also observed, as previously reported (Shindler, 1976).

Internal structure was elucidated by thin sections (Fig. 2 and 3). The cytoplasmic region consists of electron opaque ribosomes surrounding a centrally disposed nucleoid seen as one or more irregularly shaped bodies of low electron density which are simply areas of densely packed DNA. Cells grown in 0.2, 0.5, 1.0, and 2.0M NaCl PPT contained spherical organelles or vesicles of unknown function. Some bacteria have been found to possess storage or reserve products of similar morphology in their cytoplasm (Holt and Beveridgef, 1982). In other bacteria gas vacuoles have been reported (Walsby, 1972), but there is no reason to think V. costicola contains gas vacuoles.

Typical growth curves for V. costicola in 0.5, 1.0, 2.0 and 3.0M PPT at 30°C are shown in Figure 4 (0.5 1 of media in 2.8 1 fernbach flasks at 62 rpm).

Figure 2 Electron Micrograph of Vibrio costicola Cells Grown in 1.0M NaCl PPT

This section stained with 2% uranyl acetate and 6% lead citrate. Magnification is 55,645X. The cell wall (cw), cytoplasmic membrane (cm) and nucleoplasm of the bacteria are clearly defined. Lighter staining organelles or vesicles (v) are found in the cytoplasm.


cw
cm

Figure 3 Electron Micrograph of Vibrio costicola Whole Cells Grown in 0.5M Media.

Thin sections were stained with 2% uranyl acetate and 6% lead citrate. Magnification is 44,000X. Spherical vesicles (v) were found in the cytoplasm (c) and external to the cell. The nucleoplasm (n), cell wall (cw) and cell membrane (cm) are all clearly defined.

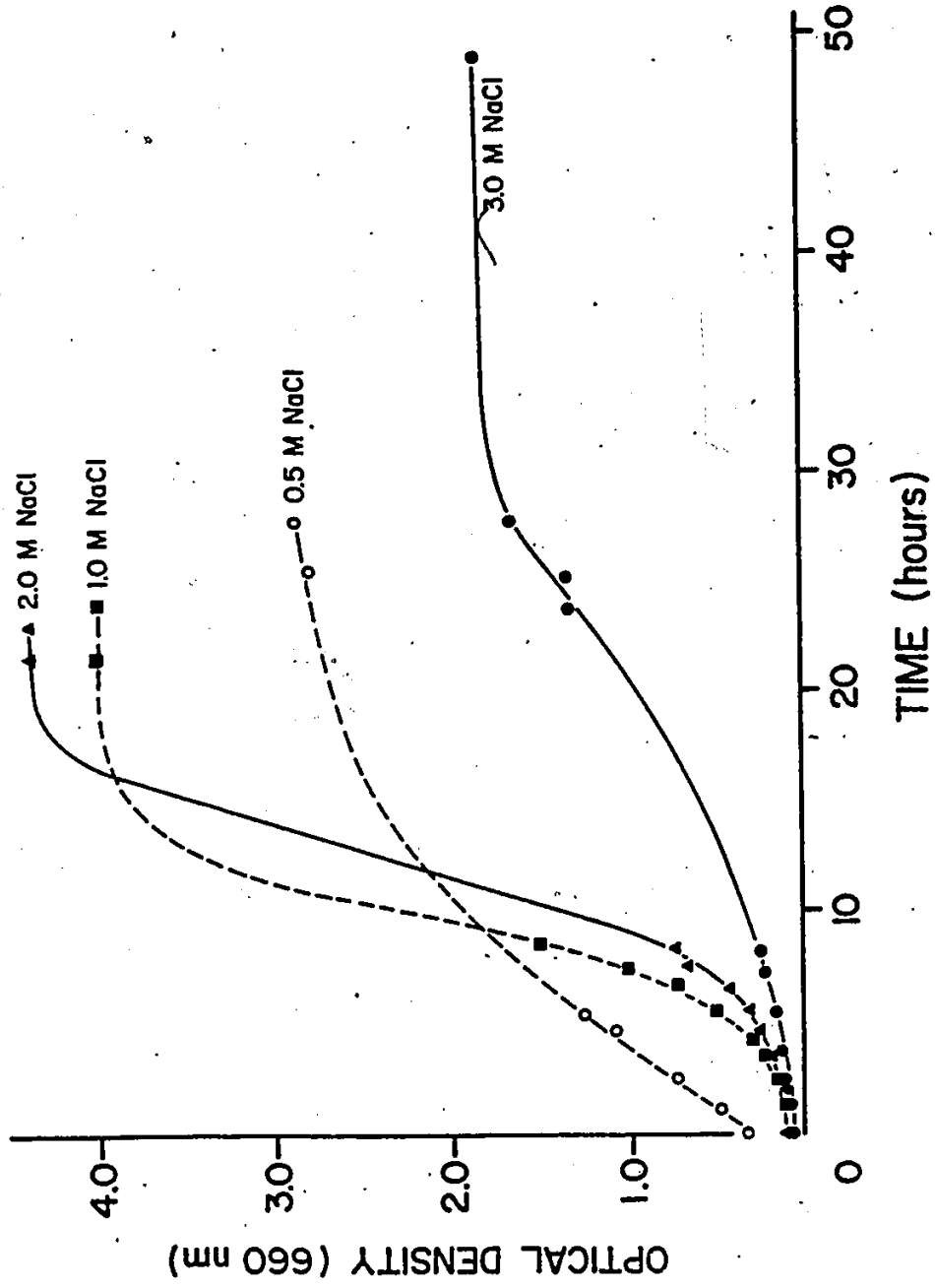


Figure 4 Typical Growth Curves of Vibrio costicola Grown in 0.5, 1.0, 2.0, and 3.0M NaCl PPT.

2. Changes in Lipid Components in Cultures Grown in the Presence of Different NaCl Concentrations

A) Phospholipid Composition of V. costicola Grown in Media Containing Different NaCl Concentration

Lipids from whole cells grown at various salinities on complex media were separated by thin layer chromatography (TLC) (Fig. 5). At all concentrations the major lipids were phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin (CL). Lesser amounts of lyso-PE, neutral lipid, glycolipid and two other phospholipids, called lipid X and lipid Y, tentatively identified by comparison of Rf values from elsewhere (Senff et al., 1976) as lyso-Cl and lyso-PG, respectively, were also present. Increases in the relative amounts of PG and decreases in PE as a function of NaCl concentration were evident from the analyses shown in Table 7. Quantitatively, the proportion of PG almost doubled whereas that of PE decreased by about half when the salinity of the media was increased from 0.5 to 3.0M NaCl (Table 7). The proportions of lysophospholipids (lipid X, lipid Y and lyso-PE did not show any significant differences in cells grown in the range of 0.5 to 3.0M NaCl. Usually, little difference in the proportions of the major phospholipids PG and PE occurred between 0.5 and 1.0M NaCl grown cells.

These results show that there were greater proportions of negatively charged phospholipids in the cells grown at the higher NaCl concentrations (Table 7, Fig. 6).

Figure 5 Thin layer chromatograph of lipids extracted from whole cells of Vibrio costicola grown in 0.5M, 1.0M, 2.0M and 3M NaCl.

Lipids were extracted from whole cells of V. costicola grown on 1% proteose peptone tryptone plus NaCl at the indicated molarities; 15 µg lipid of each of the 4 extracts was spotted on a Brinkmann G-25 plate which was developed in one dimension in the solvent system chloroform:methanol:acetic acid:water(85:15:10:3.5). All lipids were visualized by charring with 40% H_2SO_4. Identification was made separately by comparison of Rf values to standard phospholipids and by staining with α-naphtol for sugars (Kates 1975), ninhydrin for amino groups and ammonium molybdate (Vaskovsky and Kostetsky, 1968) for phosphate.

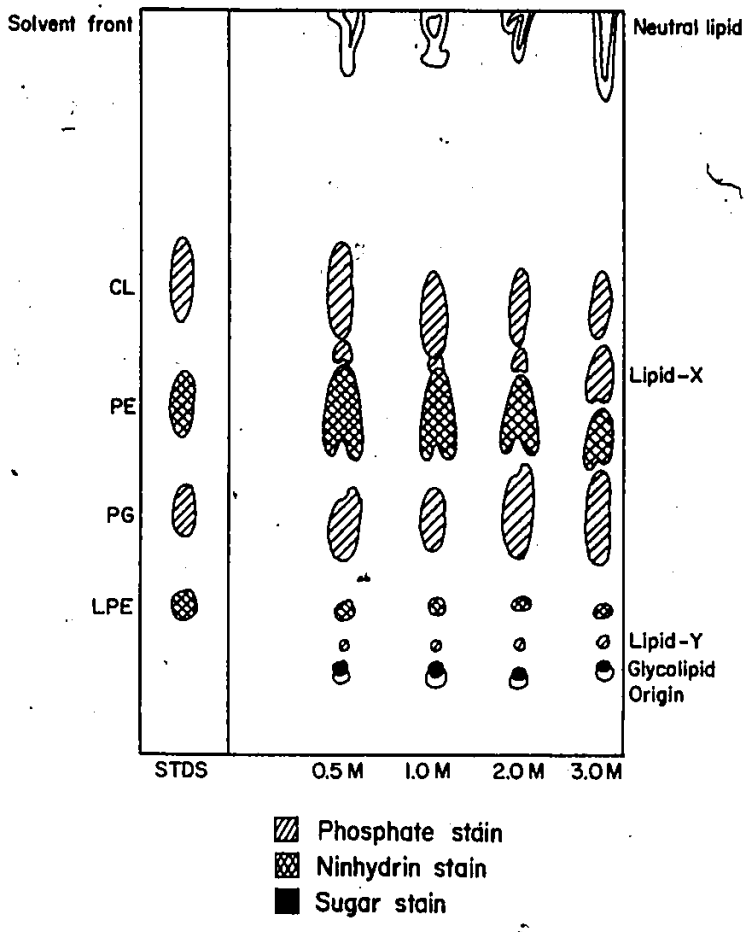

Solvent front
Neutral lipid
CL
PE
PG
LPE
Lipid-X
Lipid-Y
Glycolipid
Origin
STDS
0.5 M
1.0 M
2.0 M
3.0 M
Phosphate stain
Ninhydrin stain
Sugar stain

TABLE 7

Polar Lipid Composition (Mole %) of *Vibrio costicola* Grown in the Presence of Different Salt Concentrations

Lipid Component	R_f	NaCl concentrations of media (molarity) 0.5	1.0	2.0	3.0
Lipid Y[b]	0.05	5.1±1.1	4.1±0.7	4.4±0.1	6.4±2.5
Lyso phosphatidyl ethanolamine	0.11	5.8±2.0	5.4±1.6	5.5±2.3	6.8±1.5
Phosphatidyl glycerol	0.25	22.9±2.9	26.5±3.3	34.1±3.9	39.7±1.7
Phosphatidyl ethanolamine	0.42	55.2±1.8	54.0±2.5	47.3±4.3	33.5±4.0
Lipid X[b]	0.49	3.9±1.4	3.2±0.9	3.4±1.6	5.6±1.7
Cardiolipin	0.59	7.9±1.7	6.6±0.7	5.3±2.0	8.0±0.7
Moles negative polar lipid / Moles neutral polar lipid		0.65	0.68	0.89	1.48
Growth Rate generation time (hr.)		1.6	1.6	3.1	7.8
Incubation time (hr.)		4.8±0.5	4.8±1.0	6.0±1.0	24.0±1.5

Note: Mole % data were calculated from percent total lipid phosphorus values assuming cardiolipin and lysocardiolipin contains two phosphate groups per molecule and one phosphate per molecule for the other phospholipids. Values are expressed as means ± standard error of the mean (Dean and Dixon 1951, Bailey 1959) of 4 batches (n=10) for 0.5M NaCl, 4 batches (n=11) for 1M NaCl, 2 batches (n=7) for 2M NaCl and 3 batches (n=7) for 3M NaCl; n indicates the total number of analyses for all batches.

[b]Lipid Y, tentatively lyso-PG; Lipid X, tentatively lyso-CL

[c]cells were harvested at the late exponential phase.

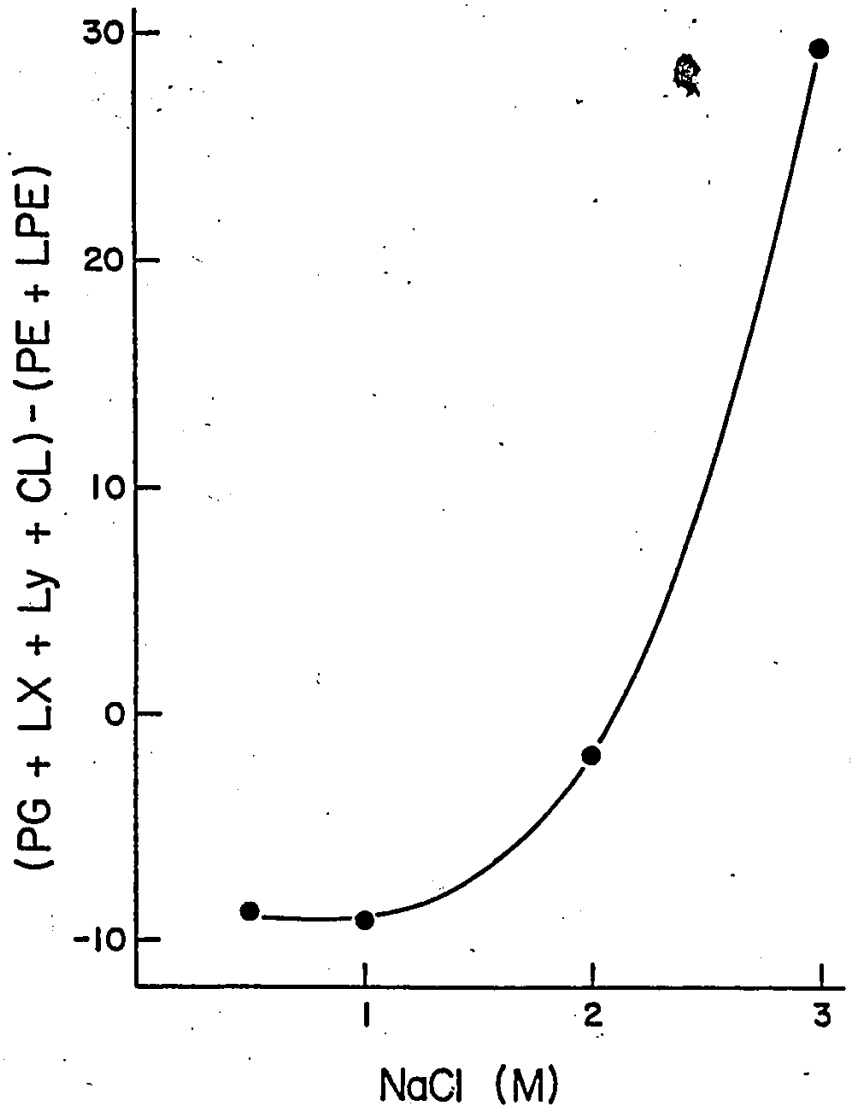


Figure 6 Effect of NaCl concentration in the growth media on the proportion of negatively charged phospholipids to neutral lipids.

The ordinate shows percentages of the total negatively charged phospholipids minus the neutral phospholipids.

Although only a single set of membranes were analysed, their lipids also showed higher PG and lower PE when the NaCl in the growth media was varied from 0.2 to 3.0M (Table 8).

To determine whether the lipid changes observed were affected by the organic composition of the growth medium, V. costicola was grown on semi-synthetic media containing various salt concentrations. The relative amounts of phospholipid components showed the same changes with differences in salinity (Table 9) as those found for cells grown in complex media (Table 7).

Bacterial lipases, which degrade lipids, may well be stimulated by cell lysis. It was important to consider the possibility that phosphatidase or lipase action might have taken place during lipid extraction. Therefore, in some experiments, portions of cell suspension were extracted with hot isopropanol (Kates, 1972) which should immediately denature any lipolytic enzymes stimulated by cell lysis, and a second portion extracted by the standard Bligh and Dyer (1959) procedure. Each method produced identical proportions of phospholipids from 0.5 and 1.0M cultures (Table 10). Thus, the results obtained by the Bligh and Dyer (1959) technique of extraction probably represents the actual lipid composition of the cells at the time of harvesting without any change due to the action of lipolytic enzymes.

TABLE 8

Polar Lipid Composition (Mole %) of Vibrio costicola Membranes Prepared from Cells Grown in Complex Medium in the Presence of Different Salt Concentrations

	NaCl concentrations of media (molarity)			
Lipid Component	0.2	1.0	2.0	3.0
Lipid Y	-	1.1	1.6	2.9
Lyso phosphatidyl ethanolamine	14.0	10.3	14.2	3.9
Phosphatidyl glycerol	19.5	23.1	25.1	43.5
Phosphatidyl ethanolamine	59.4	60.5	46.9	37.3
Lipid X	0.5	0.5	3.5	7.4
Cardiolipin	6.5	4.5	8.1	4.9

N o t e : Values are given as moles percent of total lipid phosphorus (See Table 7); n=1.

TABLE 9

Polar Lipid Composition (Mole %) of Vibrio costicola Whole Cells in Medium of Forsyth and Kushner (1970) Supplemented with 0.5% Casamino Acids and 10 µg Yeast Extract Containing Different Salt Concentrations

	NaCl concentrations of media (molarity)			
Lipid Component	0.5	1.0	2.0	3.0
Lipid Y	-	-	0.3±0.1	1.4±0.1
Lyso phosphatidyl ethanolamine	0.3±0.1	1.1±0.1	0.2±0.1	2.5±0.2
Phosphatidyl glycerol	8.2±1.4	21.2±4.2	33.6±1.9	35.8±0.2
Phosphatidyl ethanolamine	89.2±0.1	72.9±9.0	59.5±2.8	52.8±5.9
Lipid X	-	2.7±1.8	1.9±0.9	0.5±0.3
Cardiolipin	2.3±0.9	1.9±0.4	4.6±5.5	1.0±1.0

Note: n=2.

TABLE 10

Comparison of the Polar Lipid Composition of Vibrio costicola by Bligh and Dyer (1959) Versus the Hot-Isopropanol Extraction Procedure (Kates, 1972).

Lipid Component	NaCl concentration of growth media 0.5M Isopropanol extracted	0.5M Bligh/ Dyer	1.0M Isopropanol extracted	1.0M Bligh/ Dyer
Lipid Y	4.5±0.4	3.6±1.0	3.6±0.6	4.2±1.3
Lyso-phosphatidyl ethanolamine	4.2±1.4	4.1±0.2	5.3±0.7	4.2±0.6
Phosphatidyl glycerol	25.3±3.9	25.3±1.2	27.9±1.4	28.7±1.5
Phosphatidyl ethanolamine	55.6±6.6	57.6±0.1	53.0±0.9	53.0±2.3
Lipid X	3.0±0.7	3.8±0.6	3.3±0.8	3.5±0.7
Cardiolipin	7.5±0.9	5.7±0.1	6.8±0.1	6.5±0.7
@	n=2	n=2	n=2	n=4

@ n indicates the total number of analyses for each lipid extract.

The above results show that the proportions of the individual phospholipids PG and PE change with changes in salinity. However, the phospholipid content of the total lipids (about 80%), as well as the cellular phospholipid on a cell protein basis did not change significantly with increasing salt concentration (Table 11).

B. Effect of Growth Phase in Phospholipid Composition

To determine whether other factors, such as phase of growth, also influenced lipid composition, cells from the same pre-inoculum were grown in PPT containing 1.0M NaCl. No significant changes in the proportions of any of the phospholipids were found at the early exponential, late exponential and stationary phases of growth (Table 12).

C. Fatty Acid Composition of V. costicola Harvested in Media Containing Various Concentrations of NaCl

No significant changes in the total fatty acid composition as a function of growth phase in 1.0M NaCl were observed (Table 18). The fatty acid composition found was similar to that reported for other Vibrio species (Oliver and Colwell 1973b), with 16:0, 16:1, and 18:1 as the major fatty acid species (Fig. 7).

However, differences in fatty acid composition were observed when V. costicola was grown at different NaCl concentrations. For whole cells grown in complex media (Table 14), the proportion of palmitoleic (16:1) acid did not change with increased NaCl concentration while the percentage of palmitic (16:0) acid was lowest at the optimal salinity of

TABLE 11

Content of Phospholipids in Cells Grown at Different NaCl Concentrations

NaCl in Growth medium (M)	µg lipid P / mg protein	% lipid P / total lipids	*% phospho-lipids in total lipids
0.5	5.7±0.3	3.5±0.4	78.9±9.0
1.0	5.7±0.6	3.8±0.01	86.7±0.2
2.0	3.9±1.1	3.3±0.6	78.2±14.0
3.0	3.7±1.3	3.3±0.5	77.8±11.8
@n=	5	5	5
@b=	3	3	3

Lipids were extracted from cells and weighed after drying under N_2 and then in vacuo. Aliquotes were hydrolyzed and analysed for phosphate content. Results are expressed as means ± standard error of the mean.

* % phospholipids=%P $\frac{\text{average mol. wt. of phospholipid}}{31}$

(Average mol. wt. calculated by using fatty acid mol. wts. in Table 14).

@ b indicates the number of batches extracted at a particular NaCl concentration.

n indicates the total number of analyses for all batches.

TABLE 12

Phospholipid Composition of Vibrio costicola Cells at Harvested Different Phases of Growth in 1M NaCl

Lipid	Phases of Growth		
	EARLY EXPONENTIAL	LATE EXPONENTIAL	STATIONARY
Lipid Y	3.7±3.0	5.4±2.5	6.3±3.9
Lyso phosphatidyl ethanolamine	5.9±3.5	5.6±1.3	6.3±1.3
Phosphatidyl glycerol	22.6±0.2	25.6±0.9	24.0±2.0
Phosphatidyl ethanolamine	56.9±0.8	51.2±7.1	51.6±9.6
Lipid X	3.9±0.3	4.7±0.1	3.7±0
Cardiolipin	6.7±1.3	7.5±2.3	8.2±2.4
Growth Rate generation time (hr.)	1.4	4.2	16.4
Incubation time (hr.)	2.3±0.3	6.0±7.0	24.0±0.0

Note: Values are given as moles percent of total lipid phosphorus (See Table 1) and expressed as means ± mean deviations. Two independent batches of cells were extracted.

TABLE 13

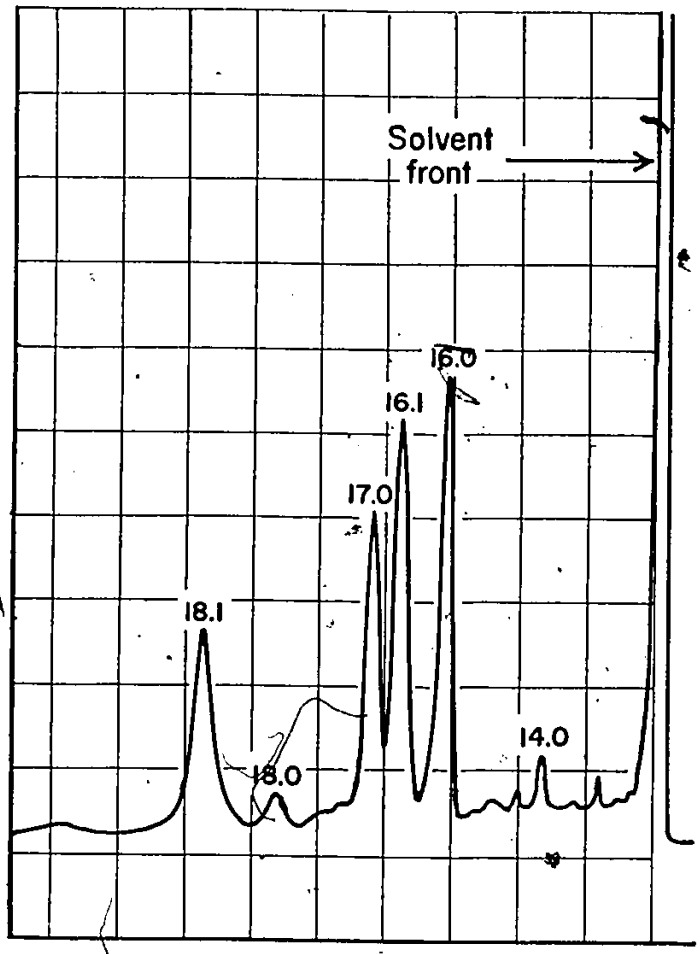
Percent Fatty Acid Composition of 1M NaCl Grown Vibrio costicola Cells Harvested at Different Phases of Growth

Growth Phase	%Fatty Acid								
	12:0	12:1	14:0	15:0	15:1	16:0	16:1	18:0	18:1
Early Exponental	1.4±0.8	0.5±0.9	1.0±0.6	0.5±0.4	0.1±0.1	29.7±0.1	37.0±7.3	2.0±2.2	27.6±6.7
Late Exponental	1.0±0.2	0.5±0.5	3.2±0.9	0.7±0.7	-	29.6±3.7	30.6±4.2	3.5±1.6	27.4±5.3
Stationary	0.7±0.7	1.2±0.6	2.7±2.6	1.6±0.7	-	30.0±3.6	29.1±10.9	3.9±1.3	30.2±3.9

Mean ± SE of 3 independent experiments.

Figure 7 Gas chromatogram of total fatty acids from 1M grown cells of Vibrio costicola in the late exponential phase of growth.

An aliquote of total lipid extract (10 μg dry weight) was methanolyzed with 25 μg heptadecanoic acid (17:0) as the internal standard (see experimental procedures). The concentrated sample was injected at 250°C int. an SP 2330 column wlith the hydrogen flows at 14 16/in^2, oxygen at 1316/in^2 and the carrier gas at 1516/iη^2.


Solvent front
16.0
16.1
17.0
18.1
18.0
14.0

1.0M and higher at 0.5M, 2.0M and 3.0M NaCl. Stearic (18:0) acid, which was present in low concentrations, did not show any significant change with NaCl concentration. In summary, the changes in proportions of total saturated fatty acids reflect largely those of 16:0 acid while the changes in proportions of the mono-unsaturated fatty acids reflect largely those of the 18:1 and 16:1 acid (Table 14).

Table 14

Fatty acid composition of *Vibrio costicola* grown at different salt concentrations

Fatty Acid[a]	NaCl Concentration of Growth Media			
	0.5M	1.0M	2.0M	3.0M
14:0	1.6±0.8	0.5±0.0	0.8±0.3	1.4±0.6
15:1	0.7±0.7	1.3±0.0	0.4±0.4	0.5±0.5
16:0	32.5±3.2	18.9±0.0	24.9±2.8	29.0±0.8
16:1	32.5±2.2	35.5±0.0	38.7±3.2	31.2±6.1
17:1	1.1±0.4	3.6±0.0	1.2±0.1	1.4±0.3
18:0	2.3±1.1	2.7±0.0	3.0±0.5	6.2±3.6
18:1	26.9±1.1	37.9±0.0	31.3±0.5	30.0±1.0
20:?	2.4±2.0	-	-	1.3±1.3
Total saturated	36.4±4.3	22.1±0.0	28.7±3.3	36.6±4.4
Total monounsaturated	61.2±4.4	77.9±0.0	71.6±4.0	63.1±7.6

Note: Data are averages of 2-4 replicate analyses of a single batch at each NaCl concentration.

[a]Traces of 12:0, 12:1, 14:1, 15:0, and 17:0 were also present (less than 0.5%).

3. Effect of NaCl Concentration of Media on the Membrane-Bound Protein Composition

Not only does the lipid composition in membranes have the capability of changing with changing salinity but also proportions of different proteins have been found to change in the moderate halophile Pseudomonas halosaccharolytica (Hiramatsu et al., 1980b). The same is true for V. costicola. Figure 8 shows that a membrane protein of approximate molecular weight 58,000 increases dramatically with increasing salinity. Table 15 shows the computed amount of this protein relative to the total protein. The protein increases over 10 fold relative to the total when cells are grown in the highest salinity (3.0M NaCl) compared to the lowest (0.2M NaCl). The NaCl regulated protein (58K), designated protein X is not present in the cytoplasmic fractions of V. costicola. A second gel was run in order to determine if the expression of this protein or any other protein varied due to growth phase (Fig. 9). However, no differences were observed in exponential versus stationary cells grown in 0.5 or 1.0M NaCl.

In order to determine if increased NaCl concentration would induce further production of protein X from cells grown in 0.5M NaCl PPT, the cells were transferred to fresh media containing 2.0M NaCl PPT. After a 8.5 hour period of growth, the optical density increased from 0.09 to only 0.165, during which time the relative amount of this protein did not change (Fig. 10). However, samples at 24 and 48 hours after inoculation where the optical density had increased to over 1.5 showed that protein X now became the major protein (Fig. 10).

Figure 8 SDS-polyacrylamide gel electrophoresis of membranes from Vibrio costicola grown at various salinities.

Electrophoresis on 20% acrylamide gels were performed according to Weber and Osborn (1975) and samples were run in duplicate. Molecular weights were calculated from standards used on other gels. (See Figure 20)

Cells were harvested from late exponential phase of growth.

(n=4)

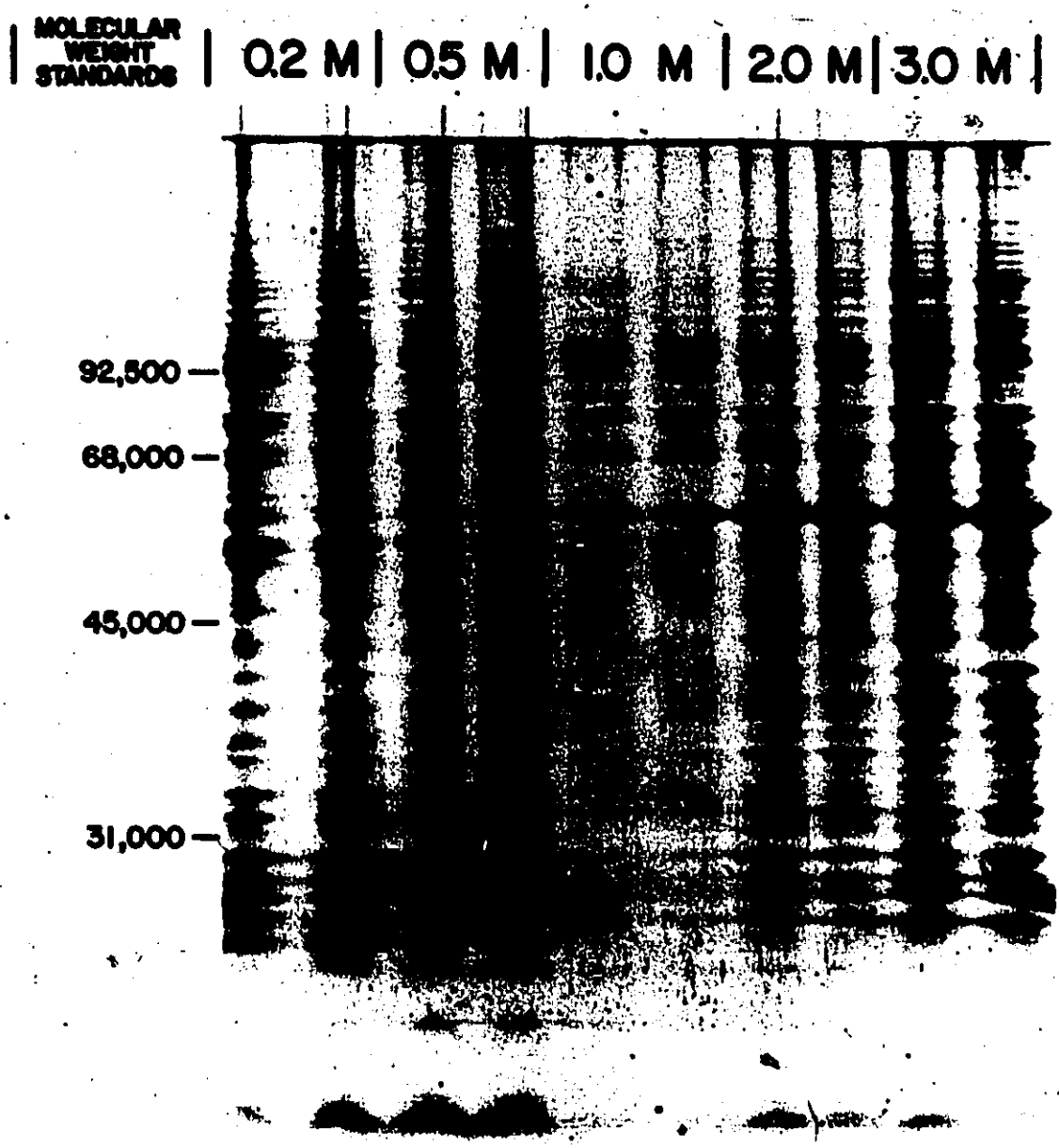

MOLECULAR WEIGHT STANDARDS
0.2 M
0.5 M
1.0 M
2.0 M
3.0 M
92,500
68,000
45,000
31,000

TABLE 15

Relative Amounts of Protein X (58K protein) as Computed from a Density Scan of Gel from Figure 8.

NaCl Concentration of of Growth Media	$\frac{\text{Area of Protein X}}{\text{Area of Total protein}}$ x 100
0.2M	4.1
0.5M	5.0
1.0M	15.9
2.0M	39.6
3.0M	45-55

The density scan was done on a negative film of the gel in figure 8; OD was read at 540 nm.

Figure 9. SDS-polyacrylamide gel electrophoresis solubilized whole cells of Vibrio costicola grown at different NaCl concentrations on PPT.

Electrophoresis on 15% acrylamide gel in the presence of SDS was preformed according to Weber and Osborn (1975). Protein (20-35 μg) was applied on the gel and stained with coomassie brilliant blue.

LANE A: Cells grown in 0.5M NaCl PPT harvested at stationary phase; LANE B: Cells grown in 0.5M NaCl PPT harvested at exponential phase; LANE C: Cells grown in 1.0M NaCl harvested at exponential phase; LANE D: Cells grown in 1.0M NaCl harvested at stationary phase.

Note: n=2

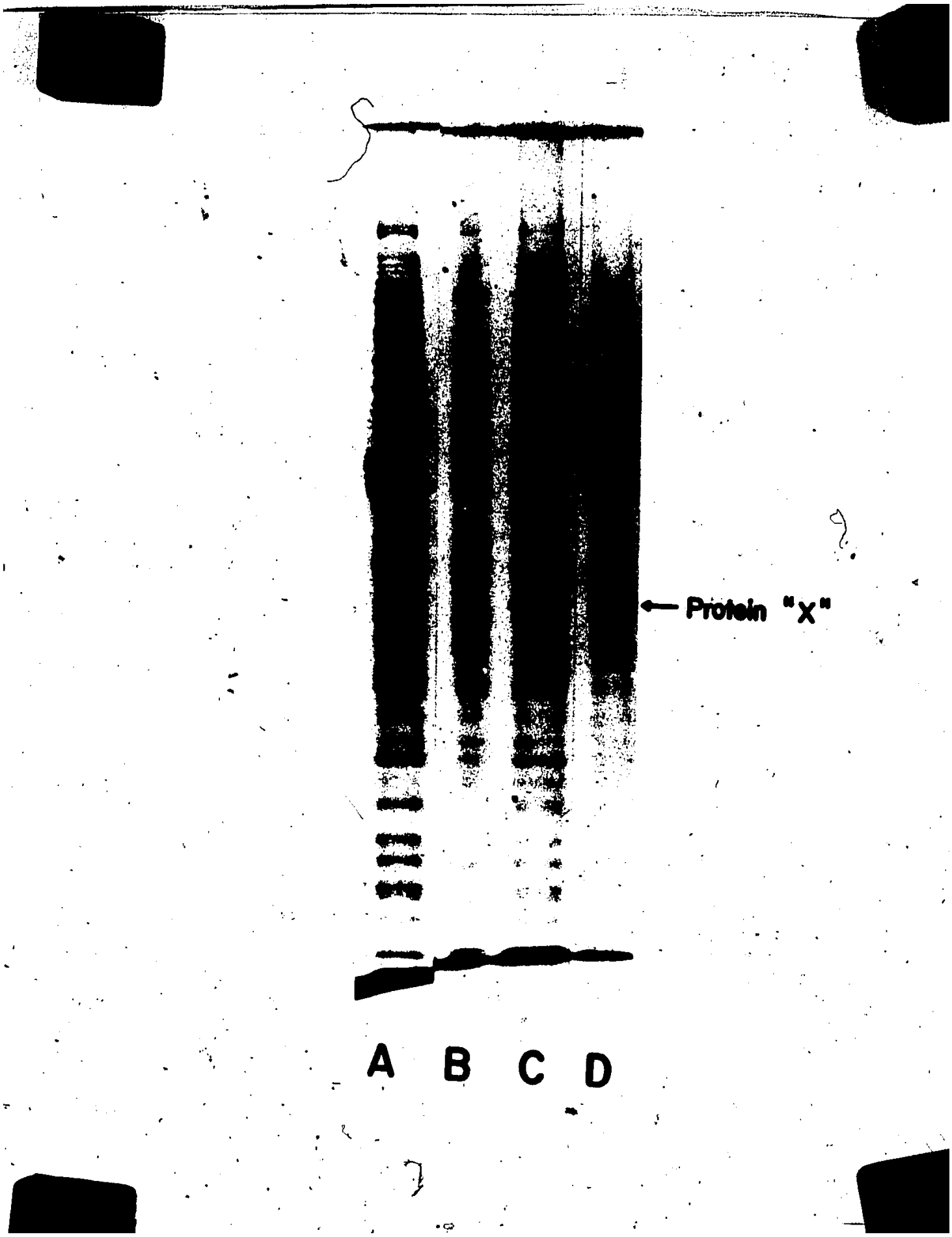
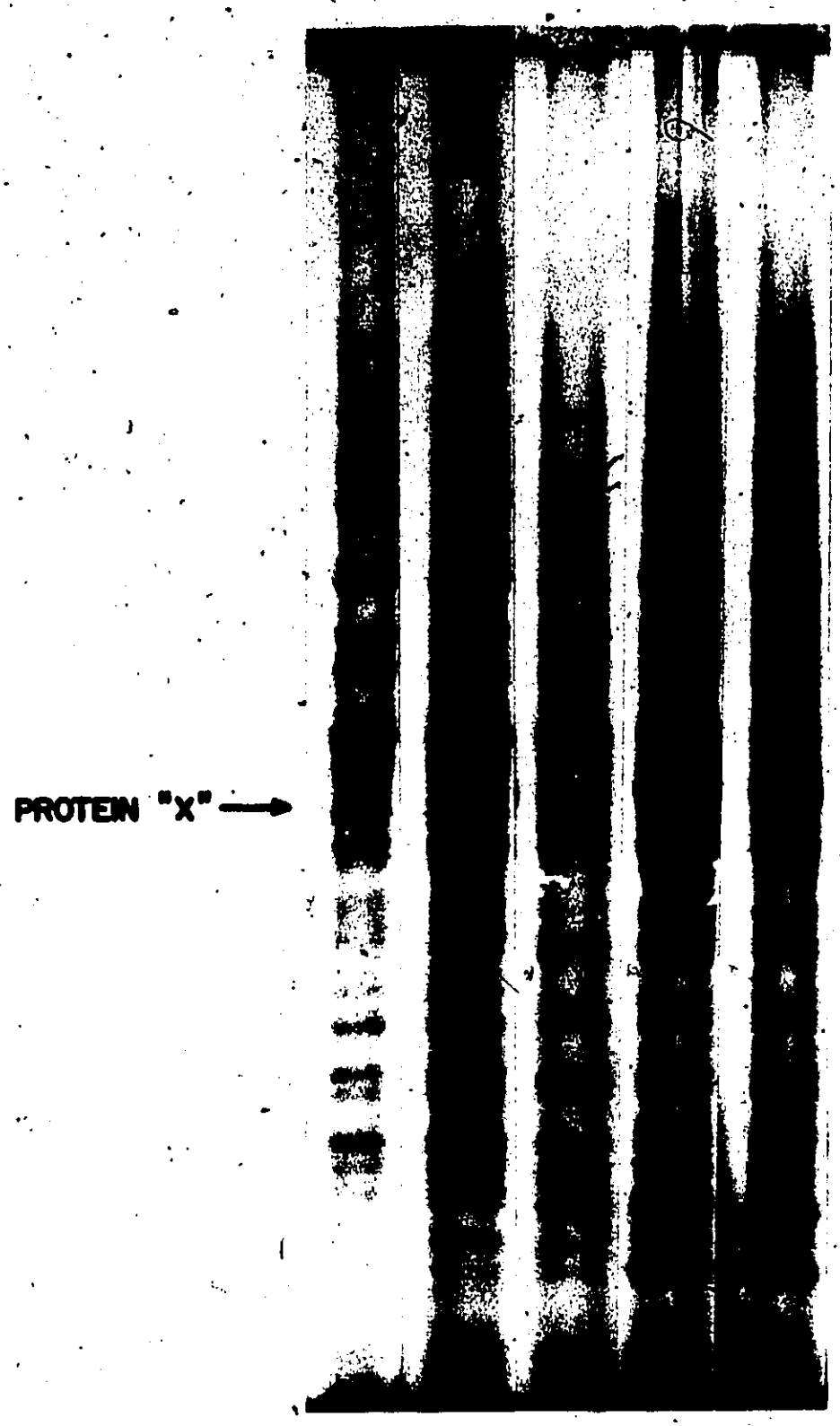

← Protein "X"
A B C D

Figure 10 SDS-polyacrylamide gel electrophoresis pattern of solubilized whole cells transfered from 0.5M NaCl PPT to 2.0M NaCl PPT. Electrophoresis was performed on a 15% SDS acrylamide gel. LANE A: Preculture of cells grown in 0.5M NaCl PPT; LANE B: Cells transfered from 0.5M NaCl PPT preculture to fresh 2.0M NaCl PPT and harvested after 2 hours; LANE C: Transfered cells harvested after 8.5 hours; LANE D: Transfered cells harvested after 24 hours; LANE E: Transfered cells harvested after 48 hours.


PROTEIN "X"
A
B
C
D
E

4. Localization of 58K Protein (Protein X) as an Outer Membrane Protein

To localize protein X, total membranes from cells labeled with ^{14}C-leucine were separated on a sucrose gradient into outer and inner membrane (Fig. 11). Outer membrane, being most dense due to lipopolysaccharide association (Yamato et al. 1975) was found in fractions 4-10, inner membrane in fractions 16-22 and some protein which was solubilized during centrifugation through the sucrose gradient on the top of the gradient (Fig. 11). However, the outer membrane can be better purified using sodium-lauryl sarcosinate (sarkosyl) which preferentially solubilizes inner membrane in Escherichia coli (Filip et al. 1973). Figure 12 shows that much of the inner membrane fraction for V. costicola was solubilized by sarcosyl treatment. SDS gel electrophoresis of pooled fractions #5-10 corresponding to the outer membrane fraction showed that protein X was the major protein with approximately 13 other minor protein bands present (Fig. 13). Protein X also appeared in fractions 28-30. This suggests that some of the outer membrane was also solubilized by sarkosyl treatment or by the absence of salts in the gradient.

Diaminopimelic acid (DAP) (a constituent of the peptidoglycan) is covalently attached to the outer membrane of E. coli (Braun and Rehn, 1969). Although lipoprotein undoubtedly plays a major roles in this attachment, certain major outer membrane porins have also been shown to be peptidoglycan associated (Wright and Tipper, 1979; Osborn and Wu, 1980; Nikaido and Nakae, 1979) Yamato et al. (1975) have previously shown that over twice the amount of DAP remains associated with outer membrane fractions than with the inner membranes of E. coli. Therefore, DAP could be used as marker in order to verify that the fractions being analyzed were truely outer membrane.

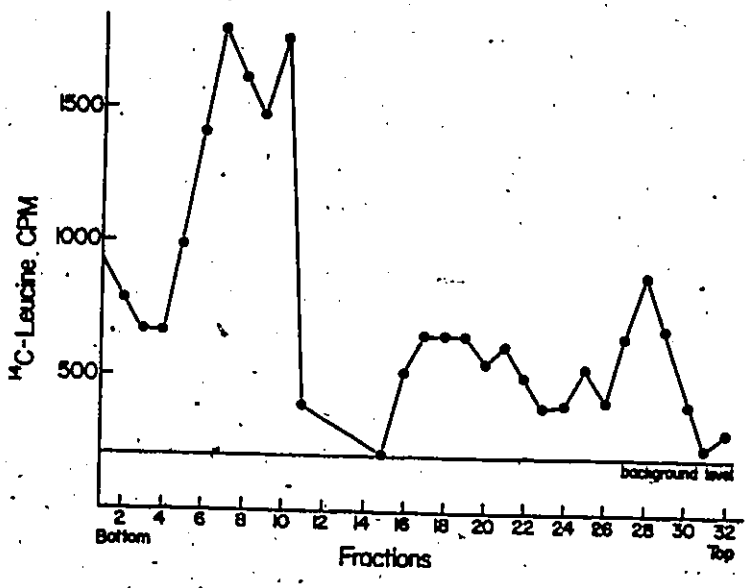


Figure 11 Sucrose density gradient fractionation of ^{14}C-leucine labelled membranes from 2.0M NaCl grown cells.

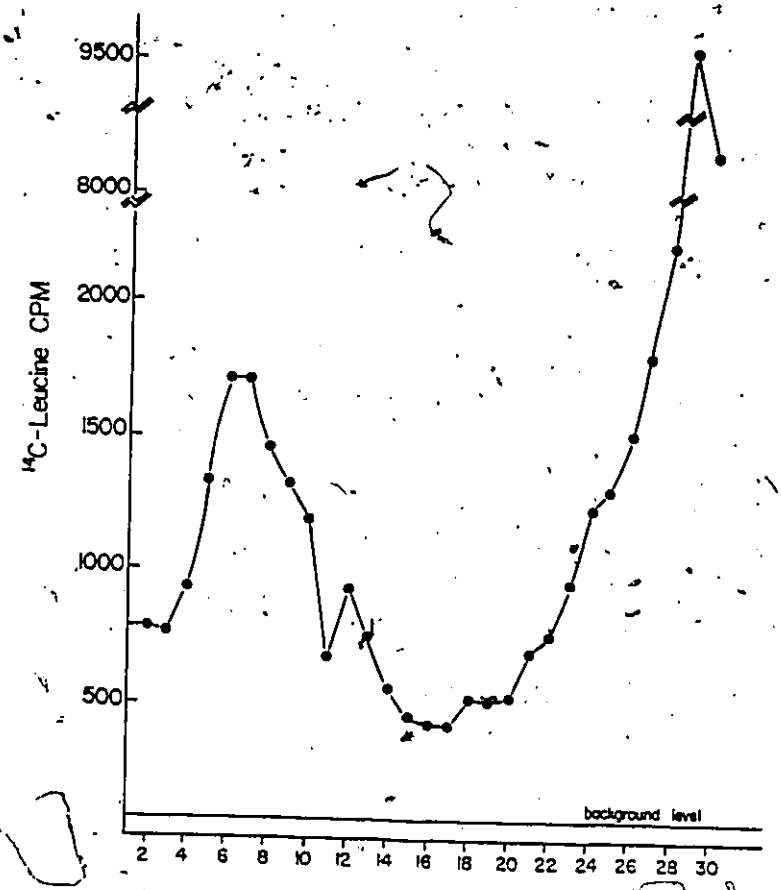
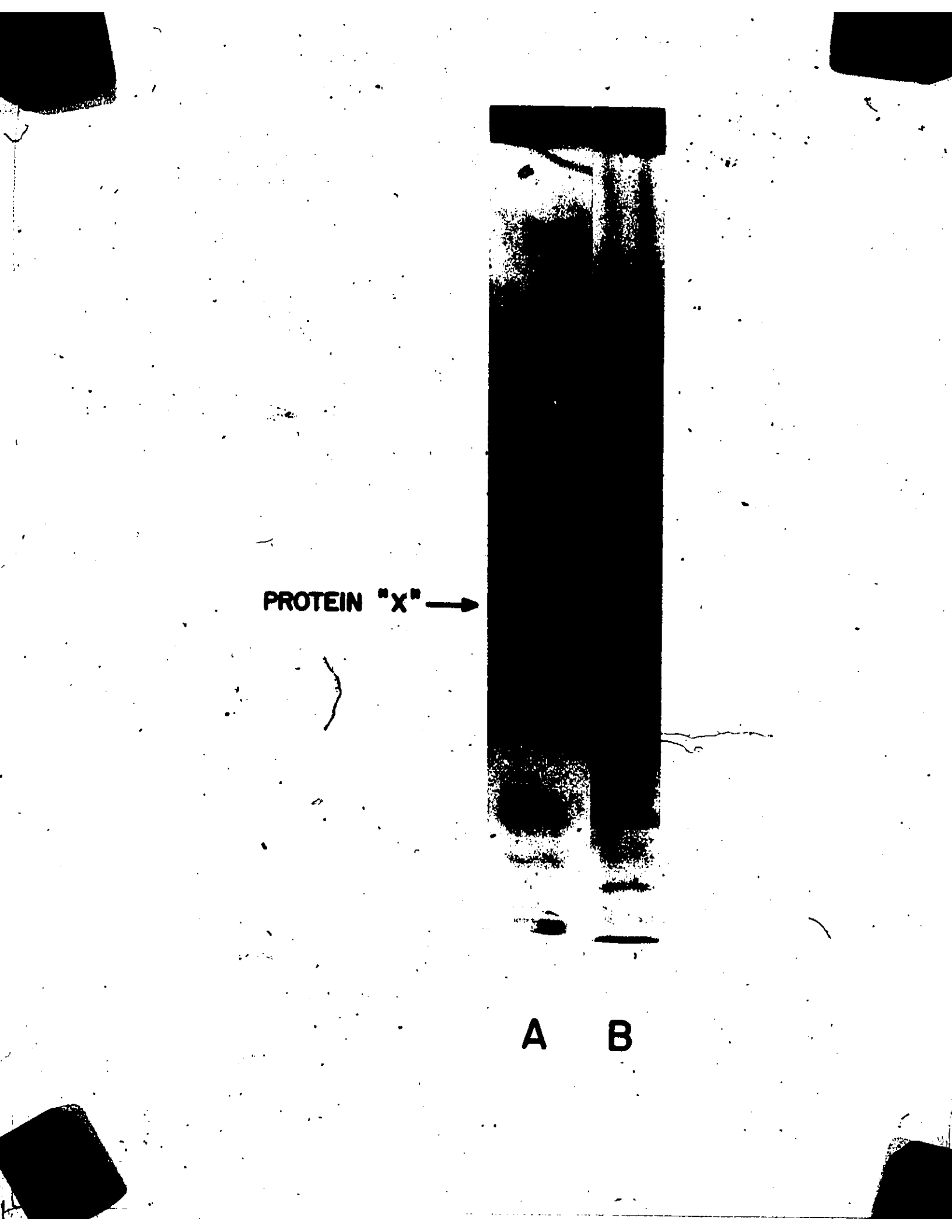


Figure 12 Sucrose density gradient fractionation of ^{14}C-leucine labelled membranes from 2.0M NaCl grown cells which had been treated with sarkosyl.

Figure 13 SDS-polyacrylamide gel electrophoresis (15% gel) of pooled fractions from 2.0M NaCl grown cells solubilized with Sarkosyl and then fractionated by sucrose density gradient.

LANE A: Pooled fraction #5-10 which contain outer membrane (See Fig. 12); LANE B: Pooled fractions #28-30 which contain solubilized protein from both inner and outer membrane.

NOTE: n=1


PROTEIN "X"
A
B

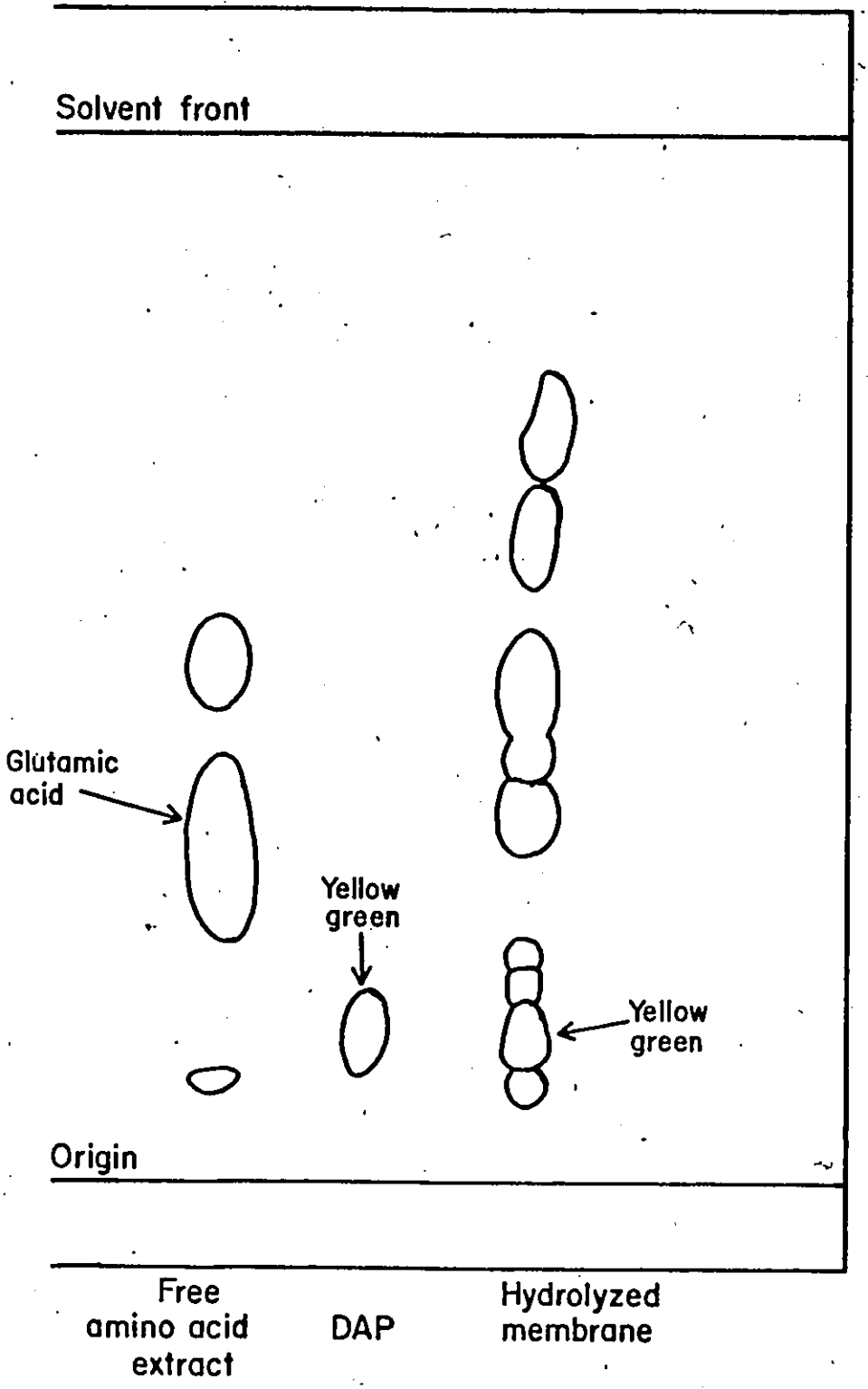
Using thin layer chromatography (Fig. 14), it was verified that DAP was a component of V. costicola total membranes. Therefore, cells were grown in the presence of ^{3}H-DAP. Figure 15 shows that most of the radioactivity was associated with the outer membrane fraction. Upon sarkosyl digestion, however, some DAP was found in the inner membrane fraction, but the outer membrane fraction contained twice the amount (Fig. 16).

Attempts were made to use 2-keto-3-deoxy-octonate (KDO), a component unique to the outer membrane of gram negative bacteria to estimate the purity of our membrane preparation. However, KDO could not be clearly demonstrated in any of the outer membrane fractions nor in whole cells of V. costicola. This was not due to a failure to detect KDO when present. However, membranes from Halophile HX had a very high KDO content (0.202 umol/mg protein) as well as membranes from E. coli (0.72 umol/mg protein). In comparison to V. costicola membranes, Halophile HX contained 119 time more KDO and E. coli 42 times more.

Razuidden and Kawasaki (1976) reported that Vibrio cholerae was the only gram negative bacterium analyzed thus far which lacked KDO. Since we also found only trace levels in V. costicola. I thought it possible that the absence of KDO is a characteristic of Vibrio species in general. For this reason a few other Vibrio species were screened for KDO. Membranes from Vibrio alginolyticus, Vibrio metchnkovii and Vibrio parahemolyticus all gave zero readings for KDO where 1 mg protein or less was analyzed. Analysis of larger protein concentrations of whole cells from V. costicola, V. metchnikovii or V. alginolyticus did not give absorbance readings greater than 0.09. To verify whether these hydrolyzed cells actually contained KDO, the spectrum was compared

Figure 14 Thin layer chromatography of cell free amino acid extracts and hydrolyzed membrane from Vibrio costicola.

Free amino acid extracts and hydrolyzed membrane from cells grown in 2.0M NaCl PPT were prepared as described in Materials and Methods. Membrane protein (33.8 mg) was hydrolyzed, dried and resuspended in 0.5 ml distilled H_2O. Five microliters of hydrolyzed membrane preparation, 10 µl of free amino acid extract and 1 µl of 0.1M diaminopilelic acid (DAP) were spotted on the TLC plate. The plate was developed in one dimension with 1-butanol-acetic acid-water (3:1:1 v/v). Amino acids were visualized by spraying with ninhydrin.


Solvent front
Glutamic acid
Yellow green
Yellow green
Origin
Free amino acid extract
DAP
Hydrolyzed membrane

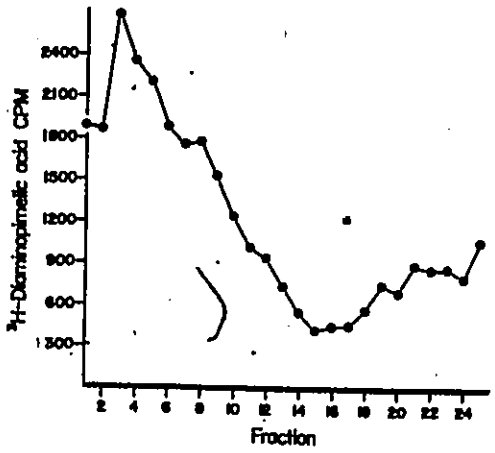


Figure 15 Sucrose density gradient fractionation of ^{3}H-DAP labelled sonicated membranes from 1.0M NaCl grown cells.

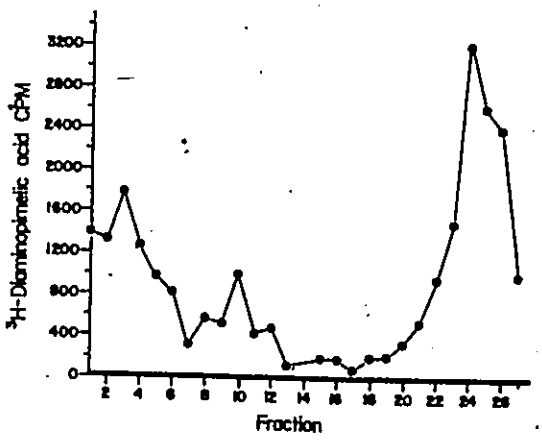
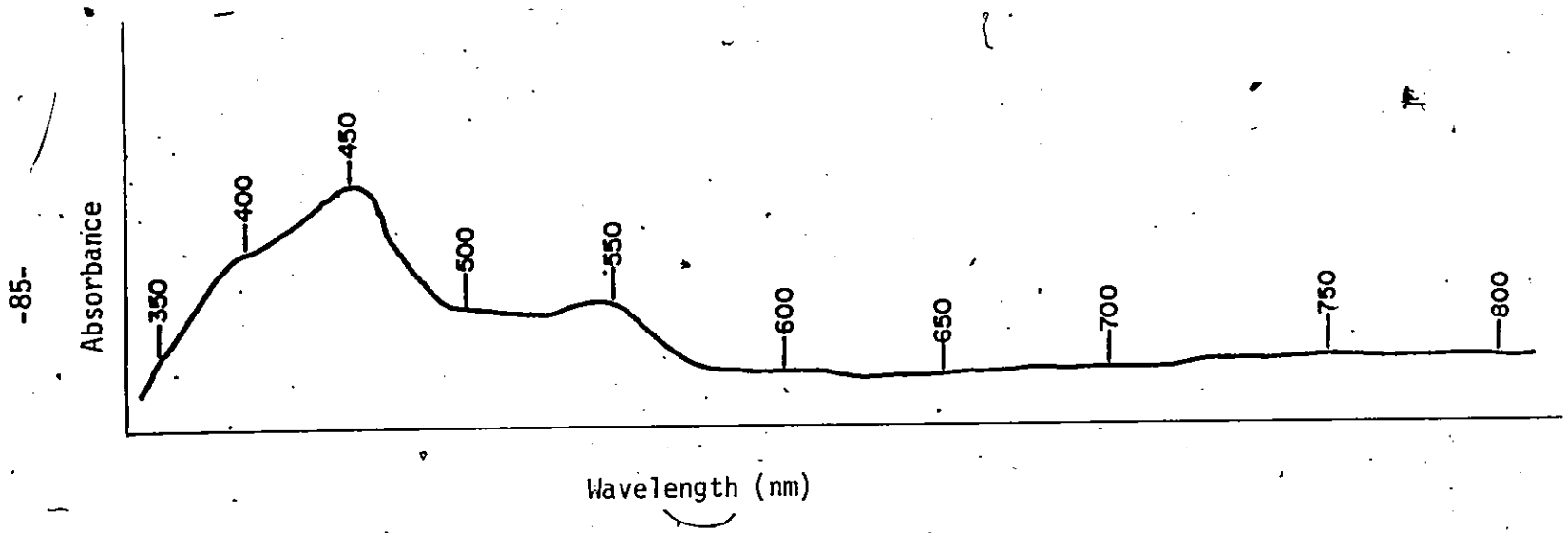


Figure 16 Sucrose density gradient fractionation of ^{3}H-DAP labelled sonicated membranes from 1.0M NaCl grown cells which had been treated with sarkosyl.

Figure 17 Optical Density Spectrum of KDO Analysis of Vibrio costicola Whole Cells. (Pure KDO produces a peak at 550 nm)



to the spectrum of pure KDO. Figure 17 shows two detected compounds, KDO at 550nm and an unknown component at 450nm. Thus, the estimated amount of KDO present in the 550nm peak for the above organisms was almost negligible, less than 0.0002 μmole/mg protein. When this work was being done, I become aware of a report by Hisatune et al., (1982) who reported that virtually all members of the family Vibrionaceae lack KDO.

As a final test for membrane purity, pooled sucrose gradient fractions were tested for the presence of endotoxin which constitutes part of the outer membrane. The assay using limulus lysate has a sensitivity of 0.05 ng/ml endotoxin. Pooled fractions 5-10 from figure 12 formed a firm gel indicating a positive reaction. Fractions 16-20 and 23-25 formed a very soft gel indicating a negative reaction. Thus, pooled fractions 5-10 contained outer membrane whereas fractions 16-20 and 23-25 did not.

To further characterize the NaCl-regulated outer membrane protein (protein X), gels were stained with periodic Schiff reagent to detect glycoproteins. Figure 18 (lane A) shows that 4 glycoproteins were detected from total membrane protein separated by SDS gel electrophoresis. A carbohydrate staining band was detected at the position of the 58K protein suggesting that protein X was a glycoprotein.

5. Determination if Protein X is an Integral Transmembrane Protein

Porins (matrix proteins) are the major proteins found in outer

membrane and they are also known to be bound to lipopolysaccharide (Rosenbusch et al., 1979; Yu and Mizushima; 1977). The fact that protein X is the major outer membrane protein in V. costicola and it is associated with carbohydrate suggests that this protein may well be a porin (Osborn and Wu, 1980). Porins are proteins existing as trimers which form pores that span the entire membrane. In order to obtain further evidence that the 58K protein was a porin, it was necessary to determine if the protein did in fact span the entire outer membrane. Thus, whole cells of V. costicola were labeled with ^{125}I-iodosulfanilic acid which labels only those proteins protruding outside the outer membrane towards the external surface. These cells were run on SDS gels and then subjected to autoradiography to detect which proteins were labeled. It was found that the protein X did indeed label (Fig. 19). Next, to determine if this protein protruded out on the inner surface of the membrane, cells were grown in the presence of ^{3}H-diaminopimelic acid. Via autoradiography it was also shown that the 58K protein was labeled (Fig. 20). Thus, from the above data we suggest that this NaCl regulated protein may well be a porin.

6. Amino Acid Composition of Membrane Isolated from Cells Grown in Different NaCl Concentrations

Since the protein proportions of the membrane changes as a function of NaCl concentration in the growth media, it seemed possible that the total charge of the membrane itself might also change. To investigate this, the amino acid composition was analyzed from hydrolyzed total membrane preparations of cells grown at different salinities. The only significant change observed from Table 16 was an increase in the

COLOURED PICTURES
Images en couleur

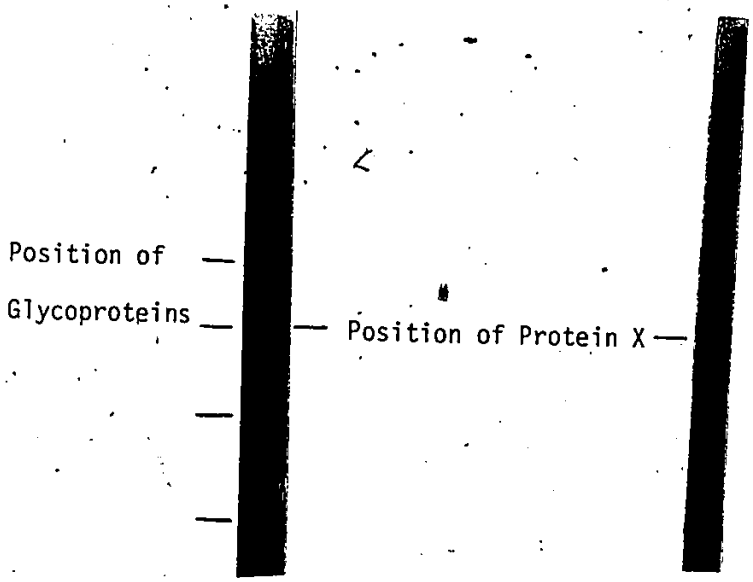


Figure 18 Glycoprotein staining following SDS polyacrylamide gel electrophoresis (15% acrylamide gel).

Lane A: Solubilized total envelope preparation from 2.0M NaCl grown cells stained with periodic acid schiff reagent for detection of glycoproteins; Lane B: Cells as above stained with Coomassie brilliant blue.

Note: n=1

proportion of glycine (an uncharged amino acid) with increasing NaCl concentration of the growth media. However, since the ratio of aspartic acid to asparagine and glutamic acid to glutamine is not known here, no conclusions can be made about the actual acidity of the membrane.

Figure 19 a) SDS Gel Electrophoresis of Cells Grown in 2.0M NaCl PPT which have been surface labeled with ^{125}I-iodosulfanilic acid (12% acrylamide gel) LANE A: 1 ml of a 8.7 mg/ml protein suspension of intact cells were incubated with 40 μg ^{125}I-diazotized salts at 20°C. Reaction was terminated by washing cells in buffer containing bovine serum albumin: LANE B: 50 μl of above intact cell suspension was incubated with 1 10^{-4}M (7 μl) ^{125}I-diazotized salt: LANE C: outer membrane Pooled fractions #5-11, see figure 12). Arrows designate positions of radiolabeled proteins.

Figure 19 b) Autoradiograph Detecting ^{125}I-iodosulfanilic acid labeled proteins in LANES A and B from Figure 19 a.

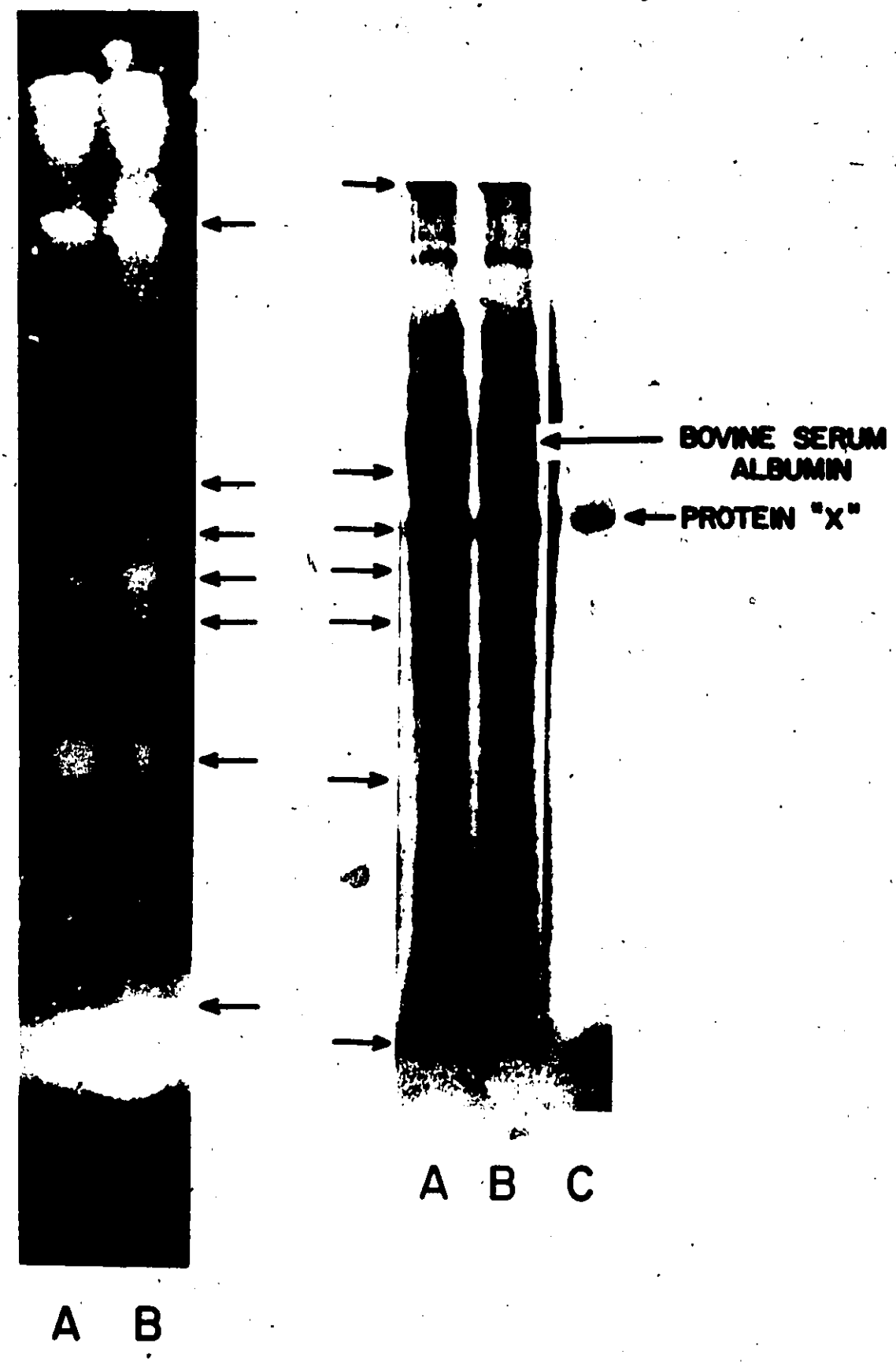
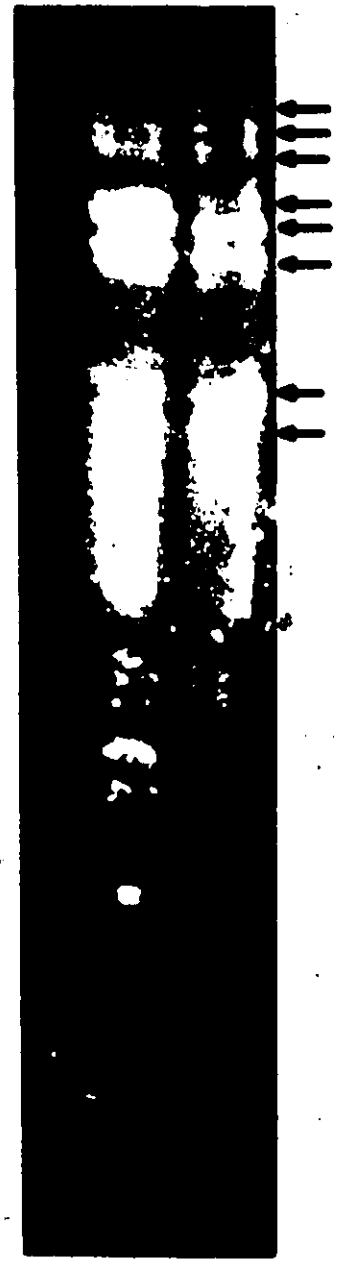

BOVINE SERUM ALBUMIN
PROTEIN "X"
A B
A B C

Figure 20 a) SDS Gel Electrophoresis of Cells Grown in 2.0M NaCl PPT containing ^{3}H-diaminopimelic Acid (12% acrylamide gel)

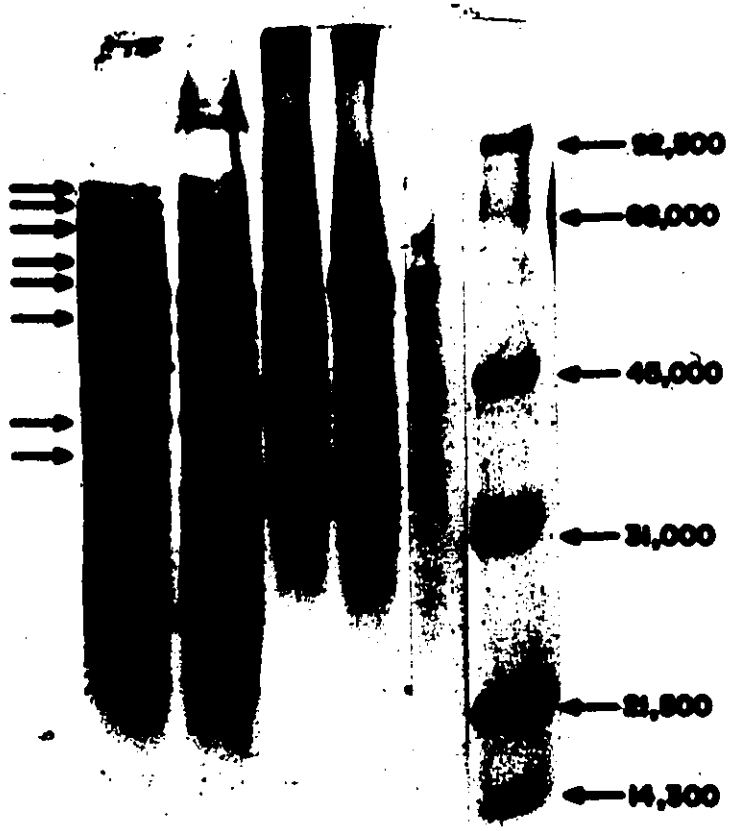
LANE A: Washed whole cells were solubilized in 2% SDS, 10mM tris (pH 7.3), 0.7M mercaptoethanol and 10% glycerol for 30 minutes at 60°C prior to leading on the gel; LANE B: Washed whole cells were solubilized in the same solution above for 5 minutes at 100°C; LANE C; Unlabeled membranes prepared from 0.5M NaCl PPT grown cells; LANE D: Unlabeled membranes prepared from 2.0M NaCl PPT grown cells; LANE E: Outer membrane (Pooled fractions #5-11, see figure 12).

LANE C, D and E were used as reference markers for position of NaCl regulated protein.

Figure 20 b) Autoradiograph detecting ^{3}H-DAP labeled proteins in LANES A and B from Figure 20a.



A B



A B C D E Molecular weight standards

TABLE 16

Mole percent Amino Acids in Membranes Derived from Various Salinities

AMINO ACID	0.2M	0.5M	1.0M	2.0M	3.0M
?	-	0.07	0.05	0.06	-
CYSTEIC ACID	0.72	0.16	0.15	0.18	0.16
ASPARTIC ACID + ASN.	10.32	10.34	10.90	11.66	10.94
THREONINE	4.16	4.45	4.45	3.93	3.88
SERINE	2.97	3.10	3.27	2.84	2.32
GLUTAMIC ACID + GLUTAMINE	9.56	10.66	9.95	10.26	10.02
PROLINE	4.38	4.10	3.85	3.22	3.09
GLYCINE	9.78	9.69	10.18	14.92	17.71
ALANINE	10.54	10.89	10.98	10.56	10.18
VALINE	8.10	8.24	8.29	8.16	8.13
?	0.03	0.10	0.14	0.11	0.11
METHIONINE	1.09	0.76	1.22	0.68	0.54
?	0.17	0.18	0.07	0.16	0.16
ISOLEUCINE	5.70	5.97	5.60	5.23	5.14
LEUCINE	9.91	10.47	9.23	8.63	8.05
TYROSINE	2.10	2.27	1.88	2.76	2.32
PHENYLALANINE	4.31	4.42	3.71	3.78	3.40
?	0.08	0.35	0.30	0.50	0.29
HISTIDINE	2.34	2.13	1.94	1.52	1.75
ORNITHINE	0.72	0.65	0.64	0.27	0.49
LYSINE	6.52	6.36	7.03	6.28	7.13
?	1.26	1.36	0.74	0.27	0.11
TRYPTOPHAN	0.07	-	0.75	0.75	0.96
ARGININE	4.72	3.04	3.07	3.28	3.12

7. Effect of NaCl on Adenosine Triphosphatase Activitiy from Vibrio costicola Grown at Various Salinities

Bengis-Garber and Kushner (1981) established that V. costicola contains two membrane-bound enzymes capable of hydrolyzing adenosine 5'-triphosphate, namely, the 5'-nucleotidase and the DCCD sensitive ATPase, which could be selectively assayed by their different Mg^{2+} requirements.

Initial results indicated the F_0F_1 ATPase activity of Vibrio costicola membranes to be very resistant to NaCl when the cells were grown at higher molarities but the enzyme from cells grown at the lower molarities was NaCl sensitive (Table 17a). However, other 2.0 and 3.0M NaCl membrane preparations were less salt tolerant. The specific activity of the ATPase was measured to see if these inconsistencies were due to changes in enzyme activity related to the growth phase. In 1M and 2M NaCl containing cultures cells harvested from the mid-exponential phase of growth expressed the highest specific activity relative to cells harvested at early exponential or stationary phases of growth (Table 17b and c). However, cells at the early exponential growth phase seem to be slightly more NaCl resistant than cells harvested at mid-exponential and stationary phase. These results could suggest that two ATPases are formed, one in early exponential phase, which is relatively salt-resistant, and another in mid-exponential and later phases that is salt-sensitive.

It was though possible that the method of preparation and subsequent handling of membrane material caused changes in enzyme activity and salt response. Membranes were prepared by pressure bomb and frozen. An aliquot of this preparation was analyzed simultaneously with a second

aliquot which had a further sonication treatment for 2 minutes. Table 17d shows that sonication did not affect ATPase activity of the membranes.

Freshly prepared pressure-bombed membranes versus the same membranes later frozen were compared to determine whether or not freezing of the previously prepared vesicles had affected the results. Table 17e and f showed that for 2.0 and 3.0M grown vesicles, freezing caused no changes. For 1.0M grown cells (Table 17g) there was either no change due to freezing or some increase of specific activity (Table 17h). Although Bengis-Garber detected no change on freezing in ATPase activity of membrane preparations from 1.0M grown cells (personal communication.), other preparations of fresh versus frozen pressure bombed material gave activation, no change or some slight repression of specific activity.

Sonicated membranes were also analyzed for differences in ATPase activity caused by freezing. Membranes from 0.5M, 1.0M, 2.0M and 3M grown cells all showed increased specific activity after freezing (Table 17i). It is likely that sonication may produce vesicles which break upon freezing and thawing. This, in turn could render more of the enzyme accessible to substrate in the reaction mixture.

8. Effect of NaCl on 5'-nucleotidase Activity from V. costicola Grown at Various Salinities

The experiments shown above for the F_0F_1 ATPase have also been done for the 5'-nucleotidase. It was found that the 5'-nucleotidase had a NaCl optimum at approximately 0.5M for 0.5, 1.0, 2.0 and 3.0M membranes

TABLE 17

Activity at ATPase from Vibrio costicola Grown at Different Concentrations of NaCl.

	NaCl Concentration of Growth Media (M)	Conditions of Preparation	Maximum Activity $\frac{\mu mole\ Pi}{mg\ protein \times min.}$	% Maximal Activation in NaCl (M) 0	0.2	05	1	2	3
a	0.5	Pressure bombed	0.72	39	100	93	63	28	7
	1.0	Membranes	0.61	33	100	96	82	25	16
	2.0		0.58	40	69	86	100	86	85
	3.0		0.42	23	66	93	100	98	100
b.		Pressure bombed membranes from cells harvested at:							
	1.0	- Stationary growth phase	1.19	33	97	99	76	46	25
	1.0	- Mid-exponential growth phase	2.05	29	100	98	83	54	29
	1.0	- Early exponential growth phase	0.5	40	100	96	70	50	50
c		Pressure bombed membranes from cells harvested at:							
	2.0	- Stationary growth phase	0.79	22	76	100	95	78	70
	2.0	- Mid-exponential growth phase	1.25	8	50	100	92	78	71
	2.0	- Early exponential growth phase	0.36	28	100	100	100	83	83
d		Pressure bombed membranes from cells harvested at:							
	2.0	- Exponential phase	2.3	10	52	100	99	87	74
	2.0	- Same membranes with a further sonication treatment	2.3	9	50	83	100	87	74
e		Pressure bombed membranes							
	2.0	- Fresh	1.90	14	82	100	93	74	24
	2.0	- Frozen once	1.95	15	85	100	91	72	46

TABLE 17 CON'T
Activity at ATPase from Vibrio costicola Grown at Different Concentrations of NaCl.

	NaCl Concentration of Growth Media (M)	Conditions of Preparation	Maximum Activity µmole Pi/ mg protein x min.	% Maximal Activation in NaCl (M) 0	0.2	05	1	2	3
f		Pressure bombed membranes							
	3.0	- Fresh	1.90	14	95	100	85	58	26
	3.0	- Frozen once	2.08	24	89	100	87	62	34
g		Pressure bombed Membranes:							
	1.0	- Fresh	0.75	9	91	100	64	43	32
	1.0	- Frozen once	0.775	25	77	100	77	58	45
h		Pressure bombed membranes assayed:							
	1.0	- Fresh	1.13	18	75	100	97	47	32
	1.0	- Frozen once	1.56	32	91	100	98	66	50
i		Menbranes prepared by sonication assayed:							
	0.5	- Fresh	1.05	38	90	100	85	59	38
	0.5	- Frozen once	1.6	22	80	100	73	54	39
	1.0	- Fresh	1.5	13	65	100	96	73	69
	1.0	- Frozen once	1.78	16	89	100	98	79	67
	2.0	- Fresh	0.95	27	76	100	84	53	53
	2.0	- Frozen once	1.77	17	85	92	100	86	70
	3.0	- Fresh	0.98	23	73	100	81	73	46
	3.0	- Frozen once	1.88	30	80	93	100	66	56

and that activity decreased only slightly with increasing NaCl in the reaction mixture (Table 18a). Thus this enzyme seemed to be fairly resistant to high NaCl concentrations. Also there is no change in salt response of the enzyme after growth at different NaCl concentration.

Sonicated frozen membranes from 0.5, 1.0, 2.0 and 3.0M grown cells always showed an increase in specific activity compared to freshly assayed membranes (Table 18b).

Growth phase also affected the specific activity of the 5'-nucleotidase. In media containing 1.0M and 2.0M NaCl cells harvested from the mid-exponential phase had the highest activity, stationary phase cells had less, and cells from the early exponential phase had the least amount (Table 19c and d).

Lastly, activation of 5'-nucleotidase by NaCl was similar for sonicated whole cell material and pressure bombed prepared membranes with AMP and ATP as the substrate (Table 18a, e, f and g).

In summary, both ATPase and 5'-nucleotidase seem able to function over a wide range of NaCl concentrations. There is no clear evidence for changes in salt response of either enzyme due to growth at different NaCl concentrations perhaps because of the complexity of the system studied.

A specific antibody against the 5'-nucleotidase of V. costicola was used as a control to monitor if the enzyme activity measured was truly the 5'-nucleotidase. Table 19 shows that the antisera strongly inhibited the enzyme from cells treated with triton X-100 which caused disruption and degradation of the cell membrane. There was also some inhibition in untreated (intact) cells; however, the effect in triton treated cells was

TABLE 18

Activity at 5'-nucleotidase from Vibrio costicola at Different Concentrations of NaCl.

	NaCl Concentration of Growth Media (M)	Conditions of Preparation	Maximum Activity μmole Pi / mg protein x min.	% Maximal Activation in NaCl (M) 0	0.2	05	1	2	3
a	0.2	Pressure bombed	1.4	67	67	100	89	82	71
	0.5	Membranes (late exponential	2.1	66	90	100	90	76	66
	1.0	growth phase)	3.1	71	90	100	96	90	80
	2.0		2.9	76	86	93	100	93	79
	3.0		1.4	57	86	100	100	99	97
b		Membranes prepared by sonication assayed:							
	0.5	- Fresh	2.2	59	77	100	68	63	45
	0.5	- Frozen once	4.9	63	93	100	100	81	61
	1.0	- Fresh	9.7	59	87	100	95	76	66
	1.0	- Frozen once	11.0	58	79	100	100	74	65
	2.0	- Fresh	8.0	53	100	95	86	78	69
	2.0	- Frozen once	8.6	55	78	100	98	86	63
	3.0	- Fresh	4.5	71	84	96	100	82	82
	3.0	- Frozen once	6.1	59	87	97	100	77	77
c		Pressure bombed membranes from cells harvested at:							
	1.0	- Stationary growth phase	3.9	66	100	100	92	74	58
	1.0	- Mid-exponential growth phase	9.1	72	100	98	92	81	71
	1.0	- Early exponential growth phase	1.7	59	94	100	97	80	74
d		Pressure bombed membranes from cells harvested at:							
	2.0	- Stationary growth phase	4.75	80	100	99	92	53	40
	2.0	- Mid-exponential growth phase	7.7	52	97	97	92	79	65
	2.0	- Early exponential growth phase	1.9	63	89	100	97	76	61

TABLE 18 CONT'D

Activity at 5'-nucleotidase from Vibrio costicola at Different Concentrations of NaCl.

	NaCl Concentration of Growth Media (M)	Conditions of Preparation	Maximum Activity μmole Pi mg protein x min.	% Maximal Activation in NaCl (M) 0	0.2	05	1	2	3
e	0.2	Pressure bombed	0.98	41	51	89	100	94	86
	0.5	Membranes (AMP as substrate)	1.64	45	72	72	97	73	87
	1.0		2.3	37	68	70	100	99	95
	2.0		3.04	39	75	88	100	86	68
	3.0		2.80	11	25	39	50	57	100
f		Sonicated fresh whole cell culture							
	0.2		0.52	69	96	100	92	62	48
	0.5		1.33	61	90	100	83	69	69
	1.0		0.84	39	56	100	99	53	49
	2.0		1.40	59	84	95	100	80	72
	3.0		0.82	61	90	100	100	76	68
g		Sonicated fresh whole cell culture (AMP as substrate)							
	0.2		0.38	17	33	59	80	91	100
	0.5		1.04	18	35	54	78	94	100
	1.0		0.66	27	54	72	84	96	100
	2.0		1.12	25	46	65	82	92	100
	3.0		0.59	24	54	68	81	95	100

* NOTE: ATP was used as substrate unless otherwise indicated.

much larger. Similar results were obtained by Bengis-Garber and Kushner (1982), who found that the anti-5'-nucleotidase did not interfere with the 5'-nucleotidase as well, unless the cells were first solubilized. Thus these results suggest that the antigenic site is membrane bound since the binding site of the antibody is less accessible to whole cells or membrane preparations.

Further characterization was performed on the 5'-nucleotidase isolated from cells grown in 1.0M NaCl. Isoelectric focusing showed the enzyme to have pI of 5.8. Examination of amino acid composition (Table 20) a composition similar to that of amino acids of total membranes (Table 16).

TABLE 19

Inhibition of 5'-nucleotidase of Vibrio costicola by Anti-5'-nucleotidase
(μmole Pi released mg protein^{-1} min.$^{-1}$)

		NaCl concentration of growth media (molarity)				
		0.2	0.5	1.0	2.0	3.0
Intact Cells	No serum	0.31	0.66	0.75	1.16	1.33
	Control serum	0.34	0.79	0.75	1.27	1.45
	*Antisera (10μl)	0.21	0.47	0.61	0.77	1.18
	*Antisera (20μl)	0.20	0.40	0.71	0.74	1.11
2% Triton Treated Cells	No serum	0.32	0.81	1.09	1.15	1.38
	Control serum	0.32	0.84	0.95	1.15	1.51
	*Antiserum (10μl)	0.11	0.84	0.95	0.32	0.46
	*Antiserum (20μl)	0.10	0.18	0.19	0.29	0.35

Note: All of the above assays were measured in 0.5M NaCl and 20mM $MgCl_2$; ATP was the substrate.

*Antisera contained 20 mg/ml protein.

TABLE 20

Amino Acid Content from the Purified 5'-nucleotidase of Vibrio costicola

Amino Acid	Mole %
Unidentified	0.3
Unidentified	1.7
Unidentified	0.4
Aspartate	11.5
Threonine	4.4
Serine	3.4
Glutamine + Glutamic acid	12.1
Proline	4.9
Glycine	10.1
Alanine	10.7
Cysteine	0.5
Valine	7.9
Methionine	0.6
Isoleucine	5.3
Leucine	9.2
Tyrosine	1.4
Phenylalanine	1.0
Histidine	2.7
Unidentified	1.2
Lysine	6.7
Unidentified	0.7
Arginine	3.4

Note: The 5'-nucleotidase was purified from cells grown in media containing 1.0M NaCl. The enzyme was kindly supplied by Bengis-Garber and Kushner (1982).

9. Analysis of Internal Solutes as Potential Osmoregulators

A) Analysis for the Presence of Glycerol as a Cytoplasmic Component

Ben-Amotz and Avron (1973) showed that when the halophilic green alga, Dunaliella parva was grown in high NaCl concentrations, the cells produced large amounts of glycerol. Borwitzka and Brown (1974) found that glycerol protected certain cytoplasmic enzymes from NaCl inhibition.

Virtually no glycerol was detected in V. costicola as assayed by either the method of Avron (1973) or that of Ben-Amotz and Avron (1974). The highest amount of glycerol detected in the cytoplasmic fraction of V. costicola was only 0.0018 mmole/g cell protein in contrast to Dunaliellia species that has 12-34 mmole glycerol/g protein (Borwitzka and Brown, 1974).

B) Regulation of Intracellular Amino Acid Composition by External Salinity

Amino acids, especially glutamic acid and proline, have been shown to be accumulated by non-halophilic bacteria in response to increased environmental NaCl (Measures, 1975;Tempest and Meers, 1970).

An adaptation experiment was performed whereby V. costicola grown in PPT with 0.5M NaCl was transfered to PPT media containing 2.0M NaCl and grown to stationary phase. The intracellular amino acid

concentration of a portion of the 0.5M NaCl preculture prior to transfer was compared to the 2.0M NaCl transfered culture. The mole % of glutamine and glutamic acid increased with elevated external NaCl whereas glycine and alanine decreased (Table 21). The position of the glutamine peak also corresponds to the position of threonine, asperigine and serine. However upon acid hydrolysis of samples all of the material from this peak dissappeared and moved to the glutamic acid position. Therefore this peak contained only glutamine. From Table 21, both the total negatively charged free amino acids and the total amino acid content seems to increase as the NaCl concentration of the media is raised.

TABLE 21

Amino Acid Composition of Vibrio costicola before and after increasing the External Salinity of the Growth Medium from 0.5M to 2.0M NaCl

Amino Acid	nmoles amino acid / mg protein		mole %	
	0.5M NaCl	2.0M NaCl	0.5M NaCl	2.0M NaCl
Cystic acid	11.4	48.5	3.9	2.5
Unidentified	8.4	-	2.9	-
Unidentified	8.2	91.5	2.8	4.7
Unidentified	5.8	11.0	1.9	0.6
Aspartate	8.7	97.8	2.9	5.1
Glutamine	3.6	379.0	1.2	19.6
Glutamic acid	60.4	811.8	20.4	42.1
Glycine	69.1	322.1	23.4	16.7
Alanine	78.8	153.7	26.7	8.0
Methionine	1.1	9.6	0.4	0.5
Tyrosine	1.6	6.3	0.5	0.3
Phenylalanine	4.8	32.7	1.6	1.7
Unidentified	6.6	-	2.2	-
Histidine	7.8	34.9	2.6	1.8
Ornithine	2.9	12.1	1.0	0.6
Lysine	16.3	73.2	5.5	3.8
Total	295.5	2084.2		
Molal concentration of total intracellular amino acids	0.168	0.457		

CHAPTER IV

DISCUSSION

Kates et al. (1966) reported that lipids extracted from Vibrio costicola grown in 1.0M NaCl contain PG and PE as major phospholipids as well as CL and a ninhydrin-positive component, first thought to be an amino acyl glycerol as minor components. In the present studies the major phospholipids of V. costicola grown in 1.0M NaCl were also found to be PE, PG and CL as is common in gram negative bacteria (Oliver and Colwell, 1973a), together with minor amounts of the ninhydrin-positive component which we consider to be lyso-PE on the basis of its positive ninhydrin stain and its TLC mobility (Senff et al., 1976). In addition, minor amounts of two phospholipids, lipid X and lipid Y, tentatively identified as lyso-CL and lyso-PG, respectively, and one glycolipid were also present. Oliver and Colwell (1973a) noted that several Vibrio species contained similar phospholipids within the range: PE 60-80%, PG 15-30%, CL 20% and lyso-PE 1-10%. Values for V. costicola fall well within these ranges.

Gram negative marine bacteria have been divided into two general taxomonic groups, the fermentative strains which include Aeromonas, Beneckea and Vibrio (Bauman et al., 1971) and nonfermentative strains. DeSiervo and Reynolds (1975) noted the absence of cardiolipin in most of the nonfermentative isolates. However, the phospholipid composition of the fermentative marine bacteria was also similar to other Vibrio species (Oliver and Colwell, 1973a).

Growth in media of different salinities led to differences in the proportions of individual phospholipids. With increasing salinity, PG increased and PE decreased in V. costicola. Similar trends with increasing NaCl in the growth media have been observed in a moderately halophilic Paracoccus (Hiramatsu et al., 1980b), Vibrio alginolyticus (Ohno et al., 1976b) and Pseudomonas halosaccharolytica (Ohno et al., 1976a, 1979). The gram positive organisms, Staphylococcus aureus (Kanemasa et al., 1972), and Staphylococcus epidermidis (Komaratat and Kates, 1975) showed an apparent opposite effect, i.e., PG decreased and Cl increased with increasing salinity. However, it should be mentioned that there is no PE in these staphylococcus species.

One question that arises is whether the changes observed in phospholipid composition are due to the NaCl concentration of the medium, or to the NaCl regulated grwoth rate of this organism. In complex media where the generation time of cells grown in 0.5M and 1.0M NaCl is the same, very little variation in phospholipid proportions occurs. Conversely significant changes are observed between cells extracted from complex media containing 1.0, 2.0, and 3.0M NaCl where the generation times are markedly different, suggesting that indeed growth rate is the factor controlling the proportions of phospholipids. However, other evidence would argue against this. Although growth rate certainly does change with the NaCl concentration (Table 7), growth rate also changes greatly during the growth cycle (Table 12), but lipid composition was approximately the same in each phase of growth. Such constant composition over the growth cycle has also been

reported for other bacteria (Kanemasa et al., 1972; Komura et al., 1975; Ohno et al., 1979). Secondly, cells grown on semi-synthetic media containing 2.0M NaCl had nearly the same generation time (1.8 h) as those grown on complex media in 0.5M NaCl (1.6h), but quite different phospholipid compositions, characteristic of the low and high-salt grown cells in complex media, respectively. Thus, growth rate alone seems to have little effect on lipid composition.

The major fatty acid species in V. costicola were 16:0, 16:1 and 18:1 as previously found in this organism (Kates et al., 1966). The proportions of fatty acids compare closely to those of other Vibrio species (Oliver and Colwell, 1973b).

In the non-halophile Escherichia coli, the only change observed due increasing NaCl was an increase in cyclopropane fatty acids (McGarrity and Armstrong, 1975). In V. costicola total monounsaturated and total C:18 fatty acids are highest at the optimal salinity for growth (1.0M NaCl) but then decrease in 0.5, 2.0 and 3.0M NaCl grown cells. Total C:16 fatty acids show the opposite effect. Little change in fatty acid composition was observed at different stages of growth in 1.0M NaCl (Table 13). Thus, once again NaCl concentrations rather than growth rate seems responsible for these changes.

The results show a substantial increase in the negatively charged lipids of V. costicola when cells were grown in media containing higher concentrations of NaCl. Kogut and Russell (1984) showed that in adaptation experiments where NaCl is directly added to growing cultures, the

negatively charged lipid groups also increase in V. costicola. A high content of negatively charged membrane phospholipids is found in other halophilic bacteria (Hancock and Kates, 1973; Kates et al., 1963, 1966; Peleg and Tietz, 1971; Stern and Tietz, 1973), particularly extreme halophiles (Evans et al., 1980).

Increase in negative charge of the envelope may partly explain an increased salt requirement (Kushner, 1978). When halobacteria are exposed to sufficiently dilute salt solutions they lyse. Brown (1963), and Kushner and Bayley (1963) first pointed out that salt dependence of envelopes might be due to negatively charged groups on the membrane. The negative charges would cause lysis or dispersion if they were not effectively neutralized by cations. It is less clear why such changes would be advantageous to cells that grow at higher salt concentrations but don't require such high concentrations. Thirkell and Summerfield (1977) and Kanemasa et al. (1972) suggested that negatively charged phospholipids may be advantageous to halophiles since negatively charged phospholipids have been shown to increase membrane permeability to cations (Haest et al., 1972; Hopper et al., 1970; Paphadjopoulos, 1971). In addition, divalent cations might stiffen negative charges (Brown, 1963) and this might be advantageous for the cells. For example, Mg^{+2} and Ca^{+2} ions may form salt bridges between neighboring free negative charges. Monovalent cations (Na^{+}), may produce a similar effect at high NaCl concentrations by forming and oriented positively charged atmosphere along the membrane surface (Brown, 1964).

It was recently found that salt tolerance of the active transport in V. costicola of an amino acid analog changes with the NaCl concentration at which the cells are grown (Kushner et al., 1983). It was suggested that this might be due to changes in membrane composition. The fact that such changes evidently occur, permits this possibility to be examined more closely.

The external salt concentration must somehow effect the underlying enzymatic mechanisms, which in turn controls the changes in lipid composition. Since the major effect of high salinity is increased PG with a concomitant decrease in PE, it is tempting to suggest that activity of one or more enzymes at the metabolic branch point leading to these two phospholipids are altered, or perhaps enzymes involved in breakdown or turnover of these lipids are responsible. In any case the next logical step would be to determine effect of NaCl on enzymes involved phospholipid synthesis and catabolism.

Not only do the lipids in membranes have the capability of changing in response to external salinity but also the membrane protein proportions from V. costicola have been found to be regulated by environmental salinity. From total membranes isolated from cells of V. costicola grown at different NaCl concentrations, one protein (designated) Protein X of MW 58,000 increased dramatically from 4 to over 55% of the total membrane protein as the NaCl concentration of the media was increased from 0.2 to 3.0M respectively.

So far, there has been very little published on the effect of environmental NaCl on membrane proteins in other bacteria. NaCl-regulated changes in the major protein species of the outer membrane have been observed for the moderate halophile Pseudomonas halosaccharolytica (Hiramatsu et al., 1980a, 1980b). Increasing the NaCl concentration from 0.85 to 2.0M, resulted in a decrease of a major 43,000 MW protein and an increase of a submajor 50,000 MW protein, whereas a decrease of 50,000 MW protein and appearance of 41,000 MW protein occurred with changing the NaCl concentration from 2.0M to 3.0M in the medium. For the non-halophile, E. coli K-12, the external salinity has also been shown to affect two of the four major outer membrane proteins, designated a, b, c, and d in order of decreasing molecular weight. The presence of higher NaCl or KCl (300mM) in the media caused the relative amounts of protein c to increase with an almost equal decrease in protein b (Hasegawa et al., 1976; van Alpen and Lugtenberg, 1977). For E. coli K-12 increasing the concentration of several carbohydrates and dextrans in the medium caused the same effect as increasing the external salinity in the growth media. Protein b decreased and protein c increased (Kawaji et al., 1979; van Alpen and Lugtenberg, 1977), thus, the external osmolarity seems to be the controlling factor.

For both E. coli and P. halosaccharolytica, the NaCl regulated changes were observed for proteins localized in the outer membrane. In the present work the outer membrane from V. costicola grown in 2.0M NaCl was purified to determine if Protein X was also a component of the outer membrane. The outer membrane was separated from inner membrane

by the former's higher density, presumably due to its higher carbohydrate content in the form of lipopolysaccharide, and possibly a higher ratio of proteins than are present in the inner membrane (Nikaido and Nakae, 1979; Wright and Tipper, 1979). Approximately 15 protein bands were identified by SDS-polyacrylamide gel electrophoresis in the outer membrane of V. costicola which approximates the number of polypeptides in the outer membranes of other bacteria (Raetz, 1978; Wright and Tipper, 1979). The NaCl-regulated Protein X was found to be the major protein component of the outer membrane. It was necessary to determine whether the changes in protein proportions of V. costicola were influenced by other factors such as growth phase. For example as E. coli entered stationary phase one of the major outer membrane proteins increased whereas the relative amount of another decreased (Schnaitman, 1974). Similarly for Vibrio cholerae, cells in early exponential phase showed elevated levels of some proteins compared to cells in mid-exponential or stationary phase (Kelley and Parker, 1981). However, for V. costicola no differences were detected between cells in exponential growth phase and those at the stationary phase of growth.

The question now remains, what could be the possible functional role of this NaCl-regulated outer membrane protein? It has been well established that the cytoplasmic membrane of other bacteria contains a large variety of enzymatic activities including succinic and lactic dehydrogenases, enzymes involved in phospholipid and lipopolysaccharide biosynthesis, ATPase and various cytochromes. In contrast, the outer membrane is almost devoid of enzymatic activity, with the one exception

being phospholipase A_1 (Raetz, 1978; Wright and Tipper, 1979).

In general, the outer membrane proteins of E. coli have been categorized into three groupings. Braun and Rehn (1969) first reported the existence of a lipoprotein in the outer membrane which is covalently linked to the peptidoglycan. Inouye et al. (1972) and Hirashima et al. (1973) reported that the Braun lipoprotein is present in two forms in E. coli; one third of the lipoprotein is peptidoglycan bound while two thirds exists free in the outer membrane. Anchorage of the outer membrane to the peptidoglycan is crucial for maintaining the integrity of the outer membrane since mutants lacking lipoprotein grow and divide normally, but have an increased sensitivity to cationic dyes and detergents and also leak periplasmic enzymes (Hirota et al., 1977).

The second grouping consists of the minor proteins many of which, in addition to the major proteins (third grouping) have been identified as receptors for phages and colicins. Others have been shown to act as specific receptors involved in the transport of certain solutes (vitamin B_{12}, maltose, maltodextrans, ferric iron and nucleotides) across the outer membrane which are too large to penetrate by passive diffusion (DiRienzo et al., 1978; Nikaido and Nakae, 1979; Osborn and Wu, 1980; Wright and Tipper, 1979).

The final classification entails the major proteins of which most gram-negative organisms have between one and four and which make up 70% or more of the total mass of protein in the outer membrane (Schnaitman, 1970). Generally these proteins show a set of distinctive common properties. First they are in tight, but noncovalent association

with peptidoglycan when solubilized in 2% SDS at temperatures below 70°C. They are released from the peptidoglycan when the temperature is increased to 100°C or by adding 0.5M NaCl (Rosenbusch, 1974) which suggests that these proteins are held to the peptidoglycan by ionic interactions. Secondly, these proteins are generally strongly enough bound to lipopolysaccharide to withstand purification procedures unless these are carried out at high ionic strength. For proteins c and b of E. coli 3 moles lipopolysaccharide is bound per mole polypeptide (Rosenbusch et al., 1979; Yu and Mizushima, 1977). These proteins, unless heat denatured, remain mostly oligomeric. Lastly, they produce transmembrane diffusion pores when added to a phospholipid-lipopolysaccharide mixture (Nakae, 1976a,b) and therefore are called porins. Porins form general hydrophilic pores, ie. fixed aqueous channels that allow passive nonspecific diffusion of solutes across the membrane. These pore proteins do not appear to interact directly with permeable solutes with any specific affinity, and selectivity is primarily determined by the hydrated radius of the solute relative to that of the pore. In Salmonella typhimurium sucrose (MW 342) and raffinose (MW 504) penetrate the cell, stachyose (MW 666) penetrates less well, and verbacose (MW 828) not at all (Decad and Nikaido, 1976). In general, solutes above a certain size class, which varies from 600-1000 MW depending on the type of solute, do not penetrate the outer membrane.

Evidence now suggests that the two major salt-regulated proteins (b and c) of E. coli previously discussed are porins. Lutkenhaus (1977) showed that mutation causing either protein b or c to disappear did not

affect the cell's ability to take up methionine or glucose. However, mutants lacking both proteins showed inhibited incorporation. Further evidence has come from cryptic mutants of E. coli K-12 where the 5'-nucleotidase which is situated in the periplasmic space and produced in normal amounts, retains full activity in cell extracts. The loss of activity is presumed to be caused by the loss of a major outer membrane protein (most likely a porin) which causes the outer membrane to become less permeable to the enzyme's substrates.

The fact that the NaCl-regulated protein of V. costicola was the only major outer membrane protein present in cells grown in 2.0M NaCl suggested that this protein may in fact be a porin. In order to provide further evidence for this assumption, a SDS-polyacrylamide gel was stained with a reagent specific for carbohydrate detection. A carbohydrate staining band was detected at the position of the 58K protein suggesting Protein X was a glycoprotein. This corroborates data from S. typhimurium and E. coli where the major outer membrane proteins (porins) are lipopolysaccharide associated (Osborn and Wu, 1980).

Electron micrographs of negative-stained porin protein-peptidoglycan complex from E. coli show that the porin protein molecules are arranged as a hexagonal lattice layer characterized by a triplet readily penetrated by stain. It was suggested that these indentations represent aqueous pore channels formed by trimeric proteins (Steven et al., 1977).

Analyses by sedimentation equilibrium have yielded trimer molecular weights of several proteins of E. coli and S. typhimurium which are known to be functional pore proteins (Nakae et al., 1979; Tokunaga et al., 1979; Yu and Mizushima, 1977). Furthermore, in freeze fracture electron

microscopy, the outer fracture face of the outer membrane is studded with intramembranous particles whose density is closely correlated with the amounts of the major transmembrane proteins in E. coli. NaCl has no effect on the density of these pores (Veikleij et al., 1977) which can be explained by the fact that NaCl causes an opposite and roughly equal change in relative amounts of proteins b and c (Hasegawa et al., 1976; van Alpen and Lugtenberg, 1977) as discussed above.

The two porin proteins (b and c) of E. coli K-12 have slightly different permeability properties (Nikaido, 1979). For example, mutants less permeable to Cu^{2+}, chloramphenicol or adenosine 5'-monophosphate usually lack protein b (Chai and Foulds, 1978; Lutkenhaus, 1977; van Alpen et al., 1978) but not c, suggesting that porin b produces a more efficient pore for these solutes. Since the salt-repressible porin protein b appears to be effective in the diffusion of a number of solutes (Lukenhaus, 1977; Nikaido et al., 1977; van Alpen et al., 1978), it could form the basic, all-purpose channels. However the question remained why this porin (protein b) would be repressed by high ionic strength. Nikaido (1979) suggested that when enteric bacteria are excreted with feces, the drying out of the feces would result in an increase in the salt concentration of the environment. This could act as a signal to inform the bacteria of a change in the environment so that they repress the most effective pores and survive in a state of minimal metabolic activity and thus with minimal exchange of material from the outside.

Since porins are transmembrane proteins, it was next decided to determine whether or not Protein X of V. costicola was also a transmembrane protein. Exposure of Protein X on the outer surface of the outer membrane was shown by the use of a nonpermeable labeling agent, ^{125}I-iodosulfanilic acid which labels only those proteins protruding outside the outer membrane towards the external surface. To determine if this protein protruded out of the inner surface of the outer membrane, the protein was examined for possible association to peptidoglycan. It has been suggested that the D-diaminopimelic acid residue of the peptidoglycan plays an important role in binding to the major proteins (DiRienzo et al., 1978). Membranes prepared from cells of V. costicola grown in the presence of ^{3}H-diaminopimelic or whole cells reacted with ^{125}I-iodosulfanilic acid were solubilized and separated on SDS-polyacrylamide gels. Autoradiography revealed that the 58K protein was bound to the diaminopimelic acid and ^{125}I-iodosulfanilic acid. Thus, not only does this confirm that Protein X is transmembrane but also that it is peptidoglycan-associated which is one of the properties of porins.

Although evidence thus far tends to suggest Protein X may be a porin, this evidence presented here is far from conclusive. For example the major proteins d and a of E. coli outer membranes are not porins (Osborn and Wu, 1980), however, it has recently been established that protein d is a transmembrane protein. It is exposed at the external face of the membrane because it acts as a receptor for phages (Henning et al., 1976; van Alpen et al., 1977). The observation that the protein is directly cross-linked to the peptidoglycan (Endermann et al., 1978,

Palva and Randall, 1976) and that cleavage of the protein by trypsin or pronase occurs only when the inner surface of the membrane is made accessible to the protease (Henning et al., 1978; Reithmeier and Bragg, 1977) showed that protein d was also exposed at the periplasmic face.

Thus, whether Protein X of V. costicola is a porin or a protein analogous to protein d of E. coli is yet to be established. Logically further classification of the functional properties of Protein X would entail purification of this protein and reconstitution with a phospholipid-lipopolysaccharide mixture. It could then be determined whether Protein X conferred the ability of non specific solute diffusion into vesicles and this could provide the ultimate proof as to its possible role as a pore protein in the outer membrane of V. costicola.

In considering NaCl adaptations at the membrane level, the effect of NaCl on both the membrane bound ATPase and 5'-nucleotidase were examined for V. costicola. Both of these enzymes have previously been purified from membranes of V. costicola grown in media containing 1.0M NaCl (Bengis-Garber and Kushner, 1981). Bengis-Garber and Kushner (1981) showed that the 5'-nucleotidase was active over a fairly wide range of salt concentrations for 1.0M NaCl grown cells. However, to investigate adaptational changes, the enzyme activity was analyzed for cells grown at different salinities. The 5'-nucleotidase was very resistant to high NaCl concentrations (3.0M) and furthermore no differences in the salt response of the enzyme from cells grown in the presence of various concentrations of NaCl was detected. There have only been a few reports dealing with the NaCl response of ATP-hydrolyzing enzymes presumed to be 5'-nucleotidases

due to the high concentration of Mg^{2+} (20-100mM) required for maximal acitivity in bacteria. The 5'-nucleotidase of Vibrio parahaemolyticus (Hayashi and Uchida, 1965), a marine Pseudomonas B-16 (Drapeau and MacLeod, 1963), Vibrio alginolyticus (Hayashi et al., 1970), and a unidentified gram-negative marine bacteria MB3 (Thompson et al., 1969) were all stimulated by NaCl to an optimum (0.2-0.75M) and generally the enzymes were resistant to further increases of NaCl in the reaction mixture. Thus, the salt-responses of the 5'-nucleotidases of these slight halophiles were similar to that found for V. costicola.

On the other hand the F_0F_1 ATPase of V. costicola in some experiments seemed to be very salt-sensitive when the cells were grown at low NaCl concentrations and salt-resistant at high concentrations of NaCl. However, the above observations for V. costicola were not always consistent, possibly due to various changing parameters in the conditions of preparation. For example, the salt reponse and or specific activity changed for cells harvested at different phases of growth, for cell material assayed fresh versus frozen, for membranes produced by different methods such as by sonication versus pressure bomb and possibly by the NaCl concentration of the growth media. Most of these variable parameters also influence activity and salt response of the 5'-nucleotidase.

Thus, for both of these membrane bound enzymes, the complexity of the system does not allow a clear understanding of the effect of the external NaCl concentration of the growth media upon enzyme activity. It has been previously shown that certain membrane-bound enzyme activities could be controlled by the membrane phospholipid composition (Bragg and

Hou, 1976; Coleman, 1973; Jinks et al., 1975; Sandermann, 1975). Since it is now known that individual membrane phospholipids of V. costicola change in response to environmental salinity, the lipid composition may be another parameter not before considered which also influences ATPase and or 5'-nucleotidase activities of V. costicola.

So far, the effect of NaCl on membrane components of V. costicola have been discussed. However, the effect of external salinity on cytosolic components is of equal interest. Interest was particularly aroused when Wydro et al. (1977) noted that in vitro protein synthesis was optimally activated in the presence of 0.2M salt (NaCl, KCl or NH_4Cl) and that at concentrations above 0.5M no activity was observed. Yet,what is striking about this information is that in vitro protein synthesis and certain other cytosolic enzymes (Shindler, 1976) are strongly inhibited by NaCl or KCl levels well below those assumed to be intracellular from Na^+ and K^+ measurements (Shindler et al., 1977). It was once thought that most of the Na^+ and K^+ ions must be somehow separated form these salt sensitive structures. Possible explanations were considered:

1. the bulk of the salts may be associated with the cell envelope
2. the salts may be enclosed or trapped intracellularly within a organelle
3. compatible solutes or osmoregulators may accumulate with increasing salinity and possibly protect salt sensitive structures from inhibition.

Hypothesis 1 was tested indirectly by considering that if the bulk of salts are indeed associated with the membrane, then one would expect that membrane bound enzymes be salt dependent or at least very salt tolerant. However, as previously discussed, the F_0F_1 ATPase was found to be NaCl sensitive depending upon the conditions of preparation for analysis.

In V. costicola, spherical organelles or vesicles have been observed. Thus,it may also be possible that the intracellular salts may be trapped or associated with these structures. In other bacteria spherical shaped gas vacuoles have been reported (Walsby, 1972) and also storage or reserve products in the cytoplasm of similar morphology to those observed in V. costicola have been reported (Holt and Beveridge, 1982). None of these is known to accumulate specific ions.

The third possibility suggests that perhaps V. costicola may produce a compatible solute(s) which would protect the protein synthesizing machinery and other cytosolic salt-sensitive enzymes from inactivation. The existence of the compatible solute, glycerol, has been well documented in species of the halophilic alga, Dunaliellia. Borwitzka and Brown (1974) noted that these alga would accumulate large quantities of glycerol in direct response to increasing NaCl concentrations in the growth media and thus act as an osmoregulator. The glycerol was also shown to act as a compatible solute since certain salt-sensitive

enzymes (glucose-6-phosphate dehydrogenase and a NADP specific glycerol dehydrogenase) were inhibited by high levels of NaCl; however, in the presence of the high NaCl and high glycerol concentrations as found in the cytoplasm, the enzymes could function normally (Borwitzka and Brown, 1974). Thus, the possibility of glycerol as a compatible solute in V. costicola was examined. However, virtually no intracellular glycerol was detected for cells grown in media containing NaCl in the range of 0.2 to 3.0M.

Recently it has been shown that Cl^- rather than Na^+ was responsible for protein synthesis inhibition and that the intracellular Cl^- concentration is very low (<0.1M). Furthermore glutamate, aspartate and other anions have been shown to partially counteract the toxic effect of Cl^- ions. In vitro protein synthesis can take place in 0.6M or higher Na-glutamate (Kamekura and Kushner, in preparation). Thus it may be significant that in V. costicola glutamine and glutamic acid are among the major intracellular amino acids which increase significantly as the external NaCl concentration is raised. It is also possible that the cell associated cations are partly neutralized by the negatively charged glutamic acid and glutamine; however, this remains to be examined.

In summary external NaCl concentration greatly influences individual components both at the membrane and cytosolic levels. Thus, V. costicola has been shown to possess an adaptational behaviour to environmental salinity. The work in this thesis has

characterized certain NaCl induced changes which may be essential for understanding its adaptational mechanisms in the future.

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