THE EFFECTS OF NACL ON THE ADENOSINE TRIPHOSPHATASES OF *STREPTOCOCCUS FAECALIS*

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TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	24
	Stock Cultures	24
	Working Cultures	24
	Growth	24
	Batch Cultures	26
	Cell Membrane Preparation	26
	Protein Determination	30
	ATPase Assay	32
	Kinetic Studies	33
	SDS-Polyacrylamide Gel Electrophoresis	34
III.	RESULTS	36
	Growth Characteristics of *Streptococcus faecalis*	36
	ATPase Activity by Fraction of Cell Membrane Preparation	36
	DCCD Inhibition of Membrane-bound ATPase	44
	ATPase Activity	44
	ATPase Activity in the Presence of NaCl	45
	ATPase Activity in the Presence of Trypsin	45
	ATPase Activity in the Presence of NaCl/Trypsin	45
	ATPase Activity in the Presence of EDTA and Citrate	47
	Kinetic Studies	49
	NaCl and Trypsin Interaction as Determined by Electrophoresis	53
IV.	DISCUSSION	57
LITERATURE CITED		75

LIST OF TABLES

Table | Page

I. Enhanced Oil Recovery Potential from Already Discovered Fields (Billions of Barrels) 2

II. Tertiary Oil Recovery 4

III. A Typical Run by MEOR. Isolate HR-66E. Grown in Tryptic Soy Broth w/1% Sucrose (TSBS) at 37C as Measured by Gas Chromatography 10

IV. Comparison of F_1F_0-ATPase Subunits from Various Sources . . 21

V. Volumes (ml) of Components of Biuret Assay 31

VI. ATPase Activity by Fraction from Four Cell Membrane Preparations 41

VII. Effects of NaCl(5%), Trypsin(0.074 mg/ml), and NaCl(5%)/Trypsin(0.074 mg/ml) on F_1 and F_1F_0-ATPases of *S. faecalis* Grown and Harvested in the Absence (A) and Presence (B) of NaCl(5%) 46

VIII. Effects of EDTA (2mM) and Citrate (10mM) on F_1 and F_1F_0-ATPases from Cells Grown and Harvested in the Absence (A) and Presence (B) of NaCl(5%) 48

IX. V_{max} and K_M Values for F_1 and F_1F_0-ATPases from Cells Grown and Harvested in the Absence (A) and Presence (B) of NaCl in the Presence of no NaCl(5%), NaCl(5%), Trypsin(0.074 mg/ml), and NaCl(5%)/Trypsin(0.074 mg/ml) . 50

LIST OF FIGURES

Figure | Page

1. Growth of HR-66E in Tryptic Soy Broth plus 1%(w/v) Sucrose (TSBS) with and without 5%(w/v) NaCl at 37C 9

2. Schematic for the Preparation of Streptococcal Cell Membranes 28

3. Growth of S. *faecalis* in Glucose(1%), Tryptone(1%), Yeast Extract(0.5%) and K_2HPO_4(1%) with and without NaCl(5%) as Measured by Absorbance vs. Time 38

4. Growth of S. *faecalis* in Glucose(1%), Tryptone(1%), Yeast Extract(0.5%), and K_2HPO_4(1%) with and without NaCl(5%) as Measured by Viable Cell Count vs. Time 40

5. Schematic for the Preparation of Streptococcal Cell Membranes 43

6. Electropherogram of Bovine Serum Albumin (BSA) 55

CHAPTER I

INTRODUCTION

In the past ten to fifteen years, attention has been focused on diminishing oil reserves and the high cost of producing those oils. It was soon after the oil embargo of the early seventies that many oil companies began to spend their efforts and monies in the exploration for new reserves and the development of new technologies that would enable them to recover a higher percentage of oil from those reserves already found. With the uncertainty of the foreign market, it became very desirable that the United States become independent of imported oil. One can see results of this effort in the Alaskan oil field becoming operational. This fairly new field has helped in easing United States dependency on foreign oil, but complete independence will not be achieved for some time. To arrive at this goal will necessitate the development of technologies that will enable a higher percentage of recovery from oil reserves already in operation. It has been estimated that some 3,000 to 4,000 billion barrels have been discovered in the world and of these, only 500 billion barrels have been produced (Table I). This leaves some 2,500 to 3,500 billion barrels yet to be produced. Of this enormous amount of oil left, only 36% can be recovered by primary and secondary recovery methods.

Primary recovery is recovery of crude oil that can be produced naturally, i.e., after the well is drilled, oil is forced out by

TABLE I

ENHANCED OIL RECOVERY POTENTIAL FROM ALREADY DISCOVERED FIELDS*
(BILLIONS OF BARRELS)

	World		United States	
Oil originally in place	3000-4000		500-600	
Oil already produced	500	(14%)	150	(27%)
Recoverable by primary & secondary methods	1100-1400	(36%)	180-225	(37%)
		50%		64%
Oil remaining in place		50%		36%

*Taken from Improved Oil Recovery p. 1-17, Dec. 1982 issue of Exxon Background Series prepared by the Public Affairs Department of Exxon Corporation.

elevated pressure in the reservoir, or can be pumped out directly. This is because of such things as gases, dissolved gases, and water in the formation, which force the oil to the surface.

After a period of time these natural forces diminish and a need for a better means of producing the oil arises, thus secondary recovery is used. Secondary recovery is replenishing those forces that have diminished, i.e., injecting water or gas into an injection well and recovering oil from a recovery well. Even after primary and secondary recoveries of oil have diminished, there remains some 50% of the original oil in the formation. An oil company then must make a decision to either stop production on that well or invest an enormous amount of money into what is called tertiary oil recovery (Table II). The high cost of tertiary oil recovery is seen in the relatively low percentage of yield (of the 50% remaining after primary and secondary recoveries, only 8.6% can be recovered by tertiary oil recovery), thus still leaving a large portion of the oil remaining in the formation (Table I).

In recent years investigators have looked elsewhere for a more efficient means of tertiary oil recovery both in expense and yields. One technology being developed is Microbially Enhanced Oil Recovery (MEOR). MEOR is usually considered an _in situ_ process. It involves an injection of microorganisms and nutrients downwell; after a period of time the metabolic products become the components responsible for the release of oil from the formation and serve as the forces that bring the oil to the surface. The types of metabolic products generally considered to be useful in the recovery of oil are acids, gases (CO_2 and H_2), solvents (usually ethanol, n-butanol, or acetone) and surfactants.

TABLE II

TERTIARY OIL RECOVERY*

I. Thermally Enhanced Recovery

   A. Steam Stimulation
   B. Steam Flooding
   C. In Situ Combustion

II. Miscible Recovery

   A. Hydrocarbon Miscible Recovery
   B. Carbon Dioxide Miscible Recovery
   C. Nitrogen Miscible Recovery

III. Chemically Enhanced Recovery

   A. Polymer Flooding
   B. Surfactant Flooding
   C. Alkaline Flooding

*Taken from Improved Oil Recovery p. 1-17 Dec. 1982 issue of Exxon Background Series prepared by the Public Affairs Department of Exxon Corporation.

The concept of using microbes to increase oil recovery began with ZoBell (1947) when he was the first to state that surfactants produced by bacteria could be used in tertiary oil recovery, and also showed that bacteria could lower the surface tension of the growth medium (ZoBell, 1947). He also received a patent for a bacteriological process using *Desulfovibrio* as a means of EOR. However, *Desulfovibrio* produces as one of its metabolic end-products H_2S, which is toxic and responsible for anaerobic corrosion of pipes used in the oil field. The idea of using *Desulfovibrio* in any oil recovery process has long since been abandoned. Many processes have been developed and used, and of these, many have been with an aerobic bacterium with the ability to utilize hydrocarbons for growth. However, other workers in this field have emphasized the desirability of using anaerobic bacteria; most oil reservoirs are quite anaerobic (Clark et al., 1981). These bacteria must also meet the following criteria (Hitzman, 1982) as well:

a) Produce copious amounts of gas which will serve to repressurize the formation, CO_2, the most abundant gas produced by fermentative anaerobes, is also useful in that it will lower the viscosity of the crude when dissolved in it.

b) Produce copious amounts of small molecular weight organic acids. These acids will act upon limestone in such a way as to produce additional CO_2.

c) Produce copious amounts of small molecular weight solvents. Solvents such as alcohols and acetone should render the oil that is trapped in the formation more accessible.

d) Produce copious amounts of low molecular weight nonionic emulsifiers which are capable of solubilizing the oil by forming oil-in-water emulsions (Grula et al., 1982). Surfactants, or compounds capable of lowering the oil-water interfacial tension, may also play a role in the release of oil.

Work in Dr. Grula's lab began in 1980; the goal of the project was isolation and screening of clostridial strains that best met the criteria given above. Over seventy strains were isolated during the course of the work. Since the strains were strictly anaerobic, Gram positive, sporeforming organisms, they were by definition, members of the genus Clostridium. Strains were also screened for the ability to grow, produce metabolic end products, and sporulate in a medium containing inexpensive and readily available nutrients. In particular, the carbon-energy source required a cheap and abundant material; beet molasses appeared to fill the bill the best (Grula and Grula, 1984).

However, these organisms and their ability to grow in an oil reservoir environment are not without problems. For a microorganism to grow in an oil reservoir environment such conditions of increased temperature and pressure, the availability of nutrients, competition with indigenous flora, previous chemical treatment, and salinity must be overcome. Although one can probably isolate bacteria that grow well in any one condition as described above, it is highly improbable that a bacterium would be isolated from an environment that possessed all of the above constraints. Therefore, to overcome such constraints as pressure and temperature, a limit was placed on the depth of the well designated for MEOR. To provide adequate nutrients but keep the process (MEOR) economically feasible, molasses was chosen as the food source, but even different sources of molasses affected the isolates in different ways. To overcome the indigenous flora a large inoculum would be used and hopefully those organisms already downwell would not be able to grow on the food source used. To overcome the problem of previous chemical treatment, those wells that have undergone certain treatments

may not even be considered for MEOR. However, they might be considered if several preflushes were done to remove any compound that might be detrimental to the MEOR isolate(s).

Problems encountered thus far have been relatively simple to overcome in an applied sense and indeed the problem with salinity can be solved in an applied sense as well. In most cases oil reservoirs contain saline concentrations ranging from 1.3% to as high as 15.6% (Clark et al., 1981). To find an isolate with those characteristics deemed beneficial for EOR and at the same time overcome salinity concentrations as high as 15.6% is not likely. But to find isolates that can grow in the presence of 1.3% to 7.5% NaCl can be done, and indeed has been done (Grula et al., 1982).

The clostridial isolates used in MEOR at Oklahoma State University grow well in low to moderate (2.5 to 7.5%) saline concentrations and at the same time maintain the characteristics necessary for EOR. Therefore, in an applied sense, the problems encountered in an oil reservoir environment have been partially solved, taking into consideration the limitations and boundaries mentioned above.

However, to make progress in developing strains, and for the purpose of fundamental understanding, the effect that salt has upon these clostridial isolates becomes an important question. It was interesting to note that some isolates were more resistant to high salt concentrations than others; not only in their growth characteristics, but also in the quantities of their end products. For example, in the presence of 5% NaCl, HR-66E showed a lag period of approximately six hours and at the same time showed a decrease in the amounts of gas, solvent, and acid production (Figure 1 and Table III, respectively, data

Figure 1. Growth of HR-66E in Tryptic Soy Broth plus 1% (w/v) Sucrose (TSBS) with (⊡) and without (⊙) 5% (w/v) NaCl at 37 C

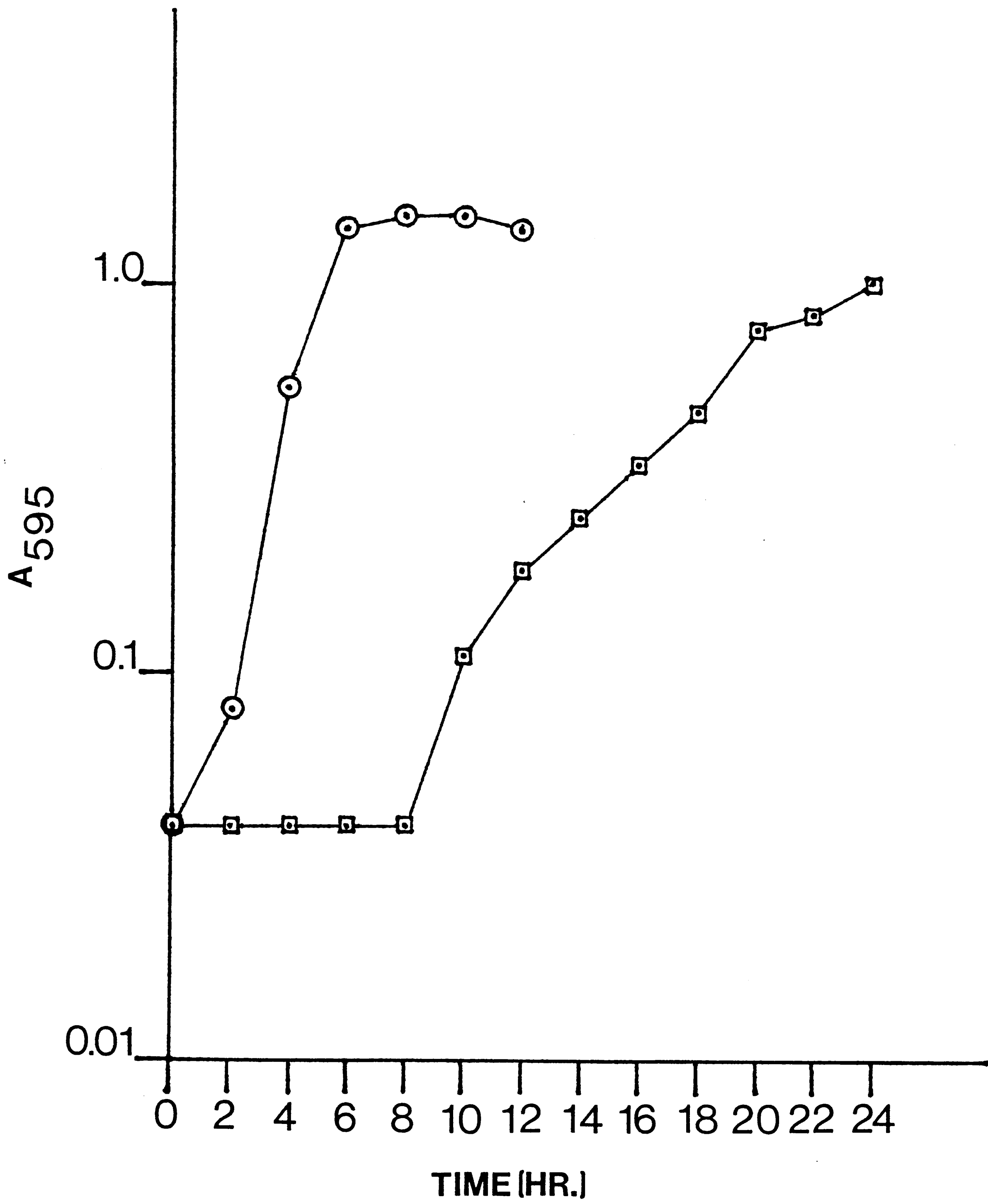

1.0
0.1
0.01
A_{595}
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (HR.)

TABLE III

A TYPICAL RUN BY MEOR ISOLATE, HR-66E GROWN IN TRYPTIC SOY BROTH W/1% SUCROSE (TSBS) @ 37°C AS MEASURED BY GAS CHROMATOGRAPHY*

Acids (24 Hrs.)	TSBS with 0.5% NaCl	TSBS with 5.0% NaCl
Acetic	65,554	trace
Propionic	30,555	trace
Isobutyric	trace	-
Butyric	trace	-

Solvents (24 Hrs.)	TSBS with 0.5% NaCl	TSBS with 5.0% NaCl
Methanol, Formaldehyde**	17,559	trace
Ethanol	110,517	trace
Acetone	357	-
Isopropanol	7,433	-
Propanol	4,552	-
3°-Butanol	5,603	-
2°-Butanol	828	-

Gases (72 Hrs.)	TSBS with 1.0% NaCl	TSBS with 5.0% NaCl
CO_2	591,178	466,543

*Integrated values give in mm^2 (injection volume=0.25 ml).

**Methanol and formaldehyde were indistinguishable by our analysis.

of Table III from personal communication with H. Russell). It should be noted that the lowered quantities of metabolic end products occurred with as nearly as much total growth as in the control. These findings along with earlier work done which suggested that transport of solutes across the membrane was inhibited by the presence of NaCl led to the hypothesis that NaCl is evidently affecting those mechanisms responsible for transport of substrates and possibly the transport of electrolytes across the membrane (Grula and Grula, 1984).

Translocation of substrates and electrolytes has been divided into two categories, primary translocation and secondary translocation (Mitchell, 1967). Those translocations that are energized by a direct chemical reaction involving the making and breaking of a covalent bond are called primary (Mitchell, 1967). Primary translocations are of two types (see review by Thauer et al., 1977). The first is solute modification or group translocation. Group translocation involves a chemical modification of the substrate being transported (Simoni and Postma, 1975). An example, is the phosphotransferase system (PTS) in which glucose goes to glucose-6-phosphate and pyruvate via the energy rich phosphoenolpyruvate. Several facultative and anaerobic bacteria have been found to contain PTS as well as several photosynthetic bacteria. It appears however, that strictly aerobic organisms do not contain a PTS (Boos, 1974).

The second type of primary translocation is called carrier modification. This type involves a chemical alteration of the carrier in such a way that the substrate is transported across the membrane without any alterations of itself (Simoni and Postma, 1975). Examples of this type are the ion-specific ATPases, in particular the

Na^+/K^+-ATPase of animal plasma membranes and the Ca^{2+}-ATPase from sarcoplasmic reticulum (see review by Thauer et al., 1977). Earlier reports indicated that procaryotes do not possess this kind of transport (Thauer, 1977). Now, however, reports indicate they do and will be discussed later in the text.

Secondary translocations are those transports which do not involve an energization by chemical modifications, but rather by physical modifications, namely the chemiosmotic potential (Mitchell, 1967). The driving force responsible for such a transport is the protonmotive force (PMF). The PMF consists of a chemical (ΔpH, interior alkaline) and an electrical (ΔΨ, interior negative) potential difference. In animal cells it consists of a sodium gradient (ΔpNa^+, interior low sodium) and a membrane potential (ΔΨ, interior negative) and it is appropriately called sodium-motive force (Goldner, 1973).

Within secondary translocation, there are three classes. Symport involves the transport of one substrate coupled to the transport of a second substrate at the same time and in the same direction (Mitchell, 1967). Antiport involves the transport of one substrate coupled to the transport of a second substrate at the same time but in opposite directions (Mitchell, 1967). And finally, uniport involves the transport of a single substrate across the membrane without a second substrate being transported (Mitchell, 1967). It is well documented that this protonmotive force is generated in one of two ways depending upon what group of organisms one is talking about (Thauer, 1977; Harold, 1972 and Futai, 1982). For instance, in aerobic and facultative organisms (growing aerobically) the PMF is generated by the redox reactions of the electron transport chain (Thauer, 1977). However, in fermentative anaerobic

bacteria and in facultative organisms in the absence of oxygen, the PMF is generated by an electrogenic H^+-translocating ATPase (Thauer, 1977). Herein, in part, lies the focus of this study.

The ATPase complexes have been studied extensively since Skou showed an ATPase activity in mitochondria (Skou, 1957). In procaryotes, an ATPase was recognized by Abrams in 1960, in *Streptococcus faecalis*. Soon after Abrams discovery, ATPases were found in *Staphylococcus aureus*, *Micrococcus lysodeikticus*, *Agrobacterium tumefaciens*, *Clostridium pasteurianum*, and in *Escherichia coli* (Gross and Coles, 1968; Munoz et al., 1968; Shonukan, 1982; Riebeling et al., 1975 and Evans, 1970). It is now generally accepted that like mitochondria and chloroplast, procaryotes possess ATPases. These ATPases have been shown to be closely associated with the cytoplasmic membrane in procaryotes (Abrams, 1960). These membrane-associated ATPases can be solubilized under numerous conditions depending upon which organism is in question (Abrams, 1976). Subsequently, it was found that N,N'-dicyclohexylcarbodiimide (DCCD) was a strong inhibitor of the membrane-bound ATPase in *S. faecalis* and in *E. coli* whereas the soluble ATPase was unaffected by DCCD (Harold et al., 1969 and Roisin and Kepes, 1973).

It is now recognized and accepted that ATPases consist of two main portions, one hydrophobic and the other hydrophilic. The hydrophobic portion apparently is a transmembrane protein embedded in the membrane, and serves as a channel for protons and also a kind of specialized receptor (see reviews by Futai and Kanazawa, 1983 and Munoz, 1982). The hydrophilic portion is an extrinsic protein and is the catalytic portion of the ATPase complex. It also serves as a regulator of proton flow

through the hydrophobic portion (Futai and Kanazawa, 1983 and Munoz, 1982). The hydrophobic portion is conventionally called the F_0 portion while the hydrophilic portion is called the F_1 portion after the terminology of Racker (Racker, 1967). The DCCD-sensitive ATPase requires the F_1 portion to be attached to the F_0 portion; therefore the membrane-associated activity constitutes the F_1F_0-ATPase and the soluble activity constitutes the F_1 portion exclusively as described earlier.

Since the H^+-translocating ATPase of strict anaerobes is responsible for the generation of PMF and PMF, in most cases, is the driving force in the transport of substrates across the membrane, perhaps in the presence of salt the ATPase is directly inhibited by the NaCl in such a manner as to inhibit transport and consequently inhibit growth.

The study of clostridial ATPases began with this salt phenomenon in mind. However, due to some unexpected results encountered both in the isolation of membranes and in the ATPase activity, an alternative was taken.

It was decided after much deliberation and the awareness of time that a second model for the elucidation of the effects of salt on ATPase must be chosen.

Several organisms were viewed for their availability, ease to work with, and the amount of work done on the organism. It was found that most studies of the function of bacterial ATPases have been carried out in E. coli and S. faecalis (Abrams, 1976).

S. faecalis is a homolactic fermentative organism without a cytochrome system which normally relies on anaerobic fermentation of sugars for all of its energy needs (Abrams, 1976). Grown on glucose,

there is no evidence that it has any capacity to carry out oxidative phosphorylation even though it has an abbreviated respiratory chain that terminates at the flavin level (Faust and Vandemark, 1970). Therefore, unlike a respiring organism like *E. coli*, its ATPase functions as a hydrolytic enzyme and not a synthetase. Whether the ATPase functions as a H^+-translocating ATPase and generates a PMF, or as a specific ion (Ca^{2+}, K^+, Na^+) pump, it still remains that the ATPase of *S. faecalis* is very similar in function to the ATPase of the strict anaerobe, *Clostridium* (Heefner, 1982 and Thauer, 1977). Therefore *S. faecalis* was chosen because of its similarities to *Clostridium* and perhaps through this study the outcome would prove useful in studying the same effects on clostridial isolates used in MEOR.

Streptococcus faecalis is a homofermentative organism with lactic acid being the primary product when grown on glucose. *S. faecalis* is a Gram-positive coccus occurring in pairs or short chains. It is generally non-motile and rarely pigmented. Gamma(γ) hemolysis is seen on blood agar and gelatin is not hydrolyzed. It possesses no catalase activity and grows well in media containing 6.5% NaCl. Arginine can be used as a source of energy. Its Lancefield grouping is D (Deibel and Seely, 1974). The effects of NaCl on procaryotes have been studied extensively. These studies however, have been in the area of transport across the membrane of intact cells and vesicles. Such studies have been done using *Streptococcus faecalis* (Harold et al., 1970 and Harold and Papineau, 1972 I and II), *Escherichia coli* (Reenstra et al., 1980), *Bacillus alcalophilus* (Mandel et al., 1980), *Azotobacter vinelandii* (Bhattacharyya, 1978), *Halobacterium halobium* (Luisi, 1980), *Salmonella typhimurium* (Niiya, 1980), and in a marine bacterium, *Alteromonas*

haloplanktis (Niven, 1978). All of the studies above have been to elucidate the mechanisms involved in Na^+ transport and in most cases the mechanism responsible for the transport of Na^+ is a Na^+/H^+ antiporter. This, of course, is a secondary translocation powered by PMF as stated earlier. However, in the past few years evidence has surfaced that some (S. faecalis and E. coli and possibly A. vinelandii) procaryotes possess primary cationic pumps as in the eucaryotes (Bhattacharyya and Barnes, 1976 and the review by Heefner, 1982).

In transport studies involving S. faecalis, Heefner and Harold were able to show that glycolyzing cells were able to expel Na^+ against a concentration gradient of 100-fold (Heefner and Harold, 1980). This extrusion was carried out at an alkaline pH in buffer containing excess K^+ and even occurred in the presence of DCCD and other reagents that dissipate the PMF (Heefner and Harold, 1980). However, at acid pH, or in the presence of low K^+, sodium extrusion requires both ATP and the PMF (Heefner and Harold, 1980). In a subsequent paper Heefner and coworkers were able to show a similar phenomenon using everted vesicles of S. faecalis (Heefner et al., 1980). Again the mechanism of $^{22}Na^+$ movement (this time an accumulation) was dependent on the pH and ionic environment (Heefner et al., 1980). Heefner and Harold again in 1982 were able to show using inverted membrane vesicles prepared in the presence of protease inhibitors, an ATP-driven sodium transport system (Heefner and Harold, 1982). Once again, they showed that these vesicles were able to accumulate $^{22}Na^+$ even in the presence of reagents that dissipate the PMF as long as ATP was provided (Heefner and Harold, 1982). In addition, they were able to show the presence of a sodium-stimulated ATPase distinct from the H^+-translocating ATPase (Heefner and Harold,

1982). On the other hand, vesicles prepared without the presence of protease inhibitors accumulated $^{22}Na^+$ via the secondary Na^+/H^+ antiport system (Heefner and Harold, 1982). In that same paper, Heefner and Harold postulated, at least in S. faecalis, the presence of the Na^+/H^+ antiport system is due to proteolytic digestion of the sodium pump. In the same bacterium, Kinoshita and coworkers were able to show, both in a mutant defective in the generation of PMF and in the wild-type strain, a Na^+ stimulated ATPase (Kinoshita et al., 1984). The wild-type strain required a high Na^+ (120mM) and carbonylcyanide-m-chlorophenylhydrazone (CCCP) (20mM) in the medium, whereas, the mutant did not (Kinoshita et al., 1984). However, Na^+-stimulated ATPase activity was increased as the Na^+ content of the growth medium increased (Kinoshita et al., 1984). The increase in enzyme activity corresponded to the increase in transport activity for Na^+ in both whole cells and membrane vesicles (Kinoshita et al., 1984).

Other ATP-energized cationic transport systems have been found in S. faecalis as well. For instance, a ATP-linked Ca^{2+} transport system was found using whole cells and membrane vesicles (Kobayashi et al., 1978). Calcium accumulation was not inhibited by either proton conductors or by DCCD (Kobayashi et al., 1978). The presence of ATP and Mg^{2+} was required for $^{45}Ca^{2+}/Ca^{2+}$ exchange as well (Kobayashi et al., 1978). In 1980, Bakker and Harold showed the presence of a ATP-linked K^+ transport system in S. faecalis that also required a PMF (Bakker and Harold, 1980). However, Kobayashi was able to show that K^+ accumulation occurred in a mutant defective in the generation of PMF and that it had characteristics similar to the ATP-linked transport system already described above (Kobayashi, 1982). Hugentobler and coworkers have

isolated and purified a K^+-ATPase from S. faecalis (Hugentobler et al., 1983). This K^+-ATPase is quite similar to the (Na^+-K^+) -ATPase found in eucaryotic cells in that its molecular weight is 78,000 daltons and it is inhibited by micromolar concentrations of vanadate (Hugentobler et al., 1983).

E. coli apparently is the only other bacterium in which an ATP-linked cationic transport system has been described. In this system, K^+ is accumulated by four kinetically and genetically distinct transport systems (Rhoads and Epstein, 1977; Epstein et al., 1978 and Wieczorek and Altendorf, 1979).

In addition to the above studies conducted with membrane vesicles and everted vesicles, investigations have been performed using isolated membrane-bound and solubilized forms of the ATPase enzymes. In 1968, Gross and Coles showed a stimulation of a Mg^{2+}-dependent ATPase activity of Staphylococcus aureus at a NaCl concentration of 60mM (Gross and Coles, 1968). In that same year Munoz and co-workers showed no stimulatory effect of NaCl on the membrane-bound, Ca^{2+}-activated ATPase of Micrococcus lysodeikticus (Munoz et al., 1968). However, the following year an inhibitory effect of some 30% to 40% was seen in the solublized form at a concentration of 100 mM (Munoz et al., 1969).

The Mg^{2+}-ATPase of Escherichia coli showed a 30% increase in activity in the presence of 140 mM NaCl, but the soluble ATPase showed a 60% loss in activity when sodium acetate was used at a concentration below 100 mM (Halkenocheid and Bonting, 1969 and Evans, 1970). It was interesting to note that Evans was able to show an anionic effect as well. In concentrations above 100 mM, with maximum inhibitory effect at 184 mM, the following anions (used as the sodium salt) showed a

decreasing order of inhibition with NO_3^- > I^- > Cl^- > CH_3COO^-, but as mentioned, concentrations below 100 mM the Na^+ cation inhibition masked that of the anions (Evans, 1970). The moderately halophilic bacterium, Vibrio parahaemolyticus showed a significant amount of increase in activity, with the optimum NaCl effect between 1.2 and 1.6 M (Hayashi and Uchida, 1965). The membrane-bound ATPase of Azotobacter vinelandii showed no stimulation by NaCl (Jurtshuk and McEntire, 1975). A NaCl concentration of 100 mM had no inhibitory or stimulatory effect on the membrane-bound Mg^{2+}-ATPase of the strict anaerobe, Clostridium pasteurianum (Riebeling and Jungermann, 1976).

However, in our model system, Streptococcus faecalis, some activation occurred with Na^+, but only with concentrations above 50mM. For example, the solubilized Mg^{2+}-ATPase was stimulated by 40% in the presence of 200 mM NaCl (Abrams, 1965). This effect was also seen with KCl (200mM) and with KCl (100mM) + NaCl (100mM) (Abrams, 1965).

It appears, after a thorough investigation of the literature, that the direct effect of NaCl on bacterial ATPase depends on whether the enzyme is membrane bound (F_1-F_0) or soluble (F_1 portion only) (Gross and Coles, 1968; Munoz et al., 1968; Munoz et al., 1969; Halkenocheid and Bonting, 1969; Evans, 1970; Hayashi and Uchida, 1965; Jurtshuk and McEntire, 1975; Riebling and Jungermann, 1976 and Abrams, 1965). In most of the organisms reviewed, the effect of NaCl depends upon the relationship of the enzyme to the membrane.

The F_1F_0-ATPase or membrane-bound enzyme shows either no effect or a stimulatory effect while the F_1 subunit or the solubilized enzyme is inhibited by NaCl (Munoz et al., 1968; Hafkenocheid and Bonting, 1969; Riebeling and Jungermann, 1976; Munoz, 1969 and Evans, 1970).

Since the F_1F_0-ATPase or membrane-bound form is the functional form in the intact microorganism, NaCl in low concentrations should not inhibit the growth, clostridial uptake or metabolism of the microorganism. However, as stated earlier, growth, uptake, and metabolism were inhibited in the presence of NaCl. These results, with respect to the findings in the literature, could indicate one of two things. One, the NaCl could be affecting other functions of the cell other than those dealing with transport; or two, the NaCl inhibition is dependent upon the concentration of NaCl used. This second assumption is based on the fact that the NaCl concentration used in our experiments was at least four times that of those used in the literature.

However, in the case of *S. faecalis*, the solubilized form showed a 40% stimulation in the presence of NaCl (Abrams, 1965). Although this difference warrants an explanation, the literature has not expounded upon this difference. The author is not clear as to why a stimulation was observed instead of an inhibition as was the case of other microorganisms mentioned earlier. It is interesting to note, however, that this difference is not the first encountered when dealing with ATPases of *S. faecalis*. For instance, structurally the F_1 and F_0 subunits of *S. faecalis* are almost identical to those of the respiring organisms and organelles but yet their function, *in vivo*, is as a phosphohydrolase and not as a synthetase (Table IV). In addition, when the F_1F_0-ATPase of *S. faecalis* is compared to the F_1F_0-ATPase of *C. pasteurianum* their functions (i.e., phosphohydrolysis) are quite similar. However, the structure of the clostridial F_1F_0-ATPase is unlike that of the streptococci F_1F_0-ATPase or any others mentioned in Table IV. It consists of only four subunits, a, c, d_a, and f_a, with

TABLE IV

COMPARISON OF F_1-F_0-ATPase SUBUNITS FROM VARIOUS SOURCES**

	F_1 (daltons)					F_0 (daltons)		
Source	α	β	γ	δ	ε	I	II	III
S. faecalis	55,000	50,000	35,000	20,000	10,000	27,000	15,000	6,000
E. coli	56,000	52,000	32,000	22,000	11,500	28,000	19,000	8,500
PS3	56,000	53,000	32,000	15,500	11,000	-	13,500	5,400
Mitochondria	59,000	56,000	36,000	15,000	12,000	19,000	11,000	8,000
Chloroplasts	59,000	56,000	37,000	17,500	13,000	-	15,500	7,500

*Taken from D. L. Heefner in Molecular and Cellular Biochemistry (1982), Vol. 44, p. 86.

**The mitochondria were obtained from *Neurospora crassa* and the chloroplasts from spinach leaves. Molecular weights were estimated by sodium dodecyl sulfate electrophoresis.

molecular weights of 65,500, 57,500, 43,000, and 15,000 daltons, respectively (Clark et al., 1979).

The subunits a, c, and d_a constitute the F_1 portion and the single polypeptide f_a, constitutes the F_0 portion (Clark et al., 1979). It appears that S. faecalis has the function of the old i.e., a phosphohydrolase and not a synthetase, and the structure of the new i.e., resembles other bacteria and eucaryotes in subunit size and number. Some investigators have gone as far as to speculate that streptococci evolved from aerobic, not anaerobic, bacteria to explain this paradox, and indeed in some strains of S. faecalis, when grown aerobically in the presence of heme, produce an abbreviated respiratory chain (Whittenburg, 1964 and Pritchar and Wimpenny, 1978). This speculation, albeit broad in its application, should not be dismissed but should await further research. However, it would be quite easy to explain the stimulation of the F_1 subunit by NaCl as just another characteristic possessed by the F_1F_0-ATPase of S. faecalis. This, too, like the postulate presented above, must await further research.

In order to better understand the effects of NaCl upon ATPases of S. faecalis, this study was conducted in the following manner. First, membranes from cells grown and harvested without NaCl present were isolated. These membrane preparations were assayed for both membrane-bound and soluble ATPase activities with and without the presence of NaCl. Trypsin was also incorporated into these assays to see if an increase in activity could be observed. To further the understanding of the effect of NaCl, K_M and V_{max} values were determined to see if the effect dealt with the affinity of the enzyme for the substrate or the rate of reaction or both.

Second, membranes from cells grown and harvested with NaCl present were isolated. These membrane preparations were assayed as above and their K_M and V_{max} values were determined also.

In response to the data obtained in the above experiments, a third set of experiments was done. This third set of experiments involved the use of citrate and ethylenediaminetetraacetic acid (EDTA) as a means of characterizing the ATPases from cells grown and harvested without NaCl and from cells grown and harvested with NaCl.

It is the intention of this thesis and the hope of the author that these data will contribute to the overall understanding of the effect of NaCl upon the ATPase of *S. faecalis*.

CHAPTER II

MATERIALS AND METHODS

Stock Cultures

Streptococcus faecalis was obtained from the stock cultures of the Department of Botany and Microbiology at Oklahoma State University. It is a Gram positive coccus occurring in pairs and short chains. It grows in media containing 6.5% NaCl and shows a gamma (γ) reaction on blood agar and it possesses no catalase activity.

S. faecalis was maintained on agar (Difco) slants containing GTYP, which is the following: 1%(w/v) glucose (Fisher), 1%(w/v) tryptone (Difco), 0.5%(w/v) yeast extract (Scott) and 1%(w/v) K_2HPO_4 (Fisher). These stocks were transferred on a weekly basis and allowed to grow at 37 C for twelve hours at which time they would be stored at 4 C until used.

Working Cultures

Five ml of GTYP broth was inoculated from an agar slant and allowed to grow for six hours at 37 C. This preparation was used as an inoculum for batch cultures, and for growth curve determinations.

Growth

Growth of *S. faecalis* was determined by two ways. First, growth was measured by absorbance spectrophotometrically with a Bausch and Lomb

Spectronic 100 spectrophotometer at a wavelength of 595nm. Inoculation was done by pipetting 0.1 ml of a six hour culture into a triplicate set of test tubes containing 5 ml of GTYP broth. A second set containing GTYP with 5%(w/v) NaCl (Fisher) was also inoculated. An initial optical density was measured, and then tubes were placed in a 37 C incubator (air) and allowed to grow. At intervals of two hours, readings were taken and values were averaged and recorded as the optical density at that particular time interval. Readings were taken in this fashion until the twelfth hour at which time the experiment was terminated. Growth was also monitored by viable cell count. Inoculation was done by pipetting 0.1 ml of a six hour culture into a triplicate set of test tubes containing 5 ml of GTYP broth and a triplicate set of test tubes containing 5 ml of GTYP broth with NaCl (5%). At the same time, 0.1 ml aliquots were pipetted into sterile 9.9 ml water blanks serially to the appropriate dilutions. One ml aliquots from each tube from three different dilutions were then pipetted into triplicate sets of sterile petri plates. Melted agar was poured into the plates and swirled to allow an even distribution of cells. Plates were allowed to solidify and then were incubated at 37 C for 24 hours at which time colonies were counted using a American Optical colony counter. This process was repeated at two hour intervals through the twelfth hour. To assure uniformity and accuracy in counting colonies, the 24 hour incubation prior to counting colonies began at each two hour interval and only plates showing 30-300 colonies were counted.

Batch Cultures

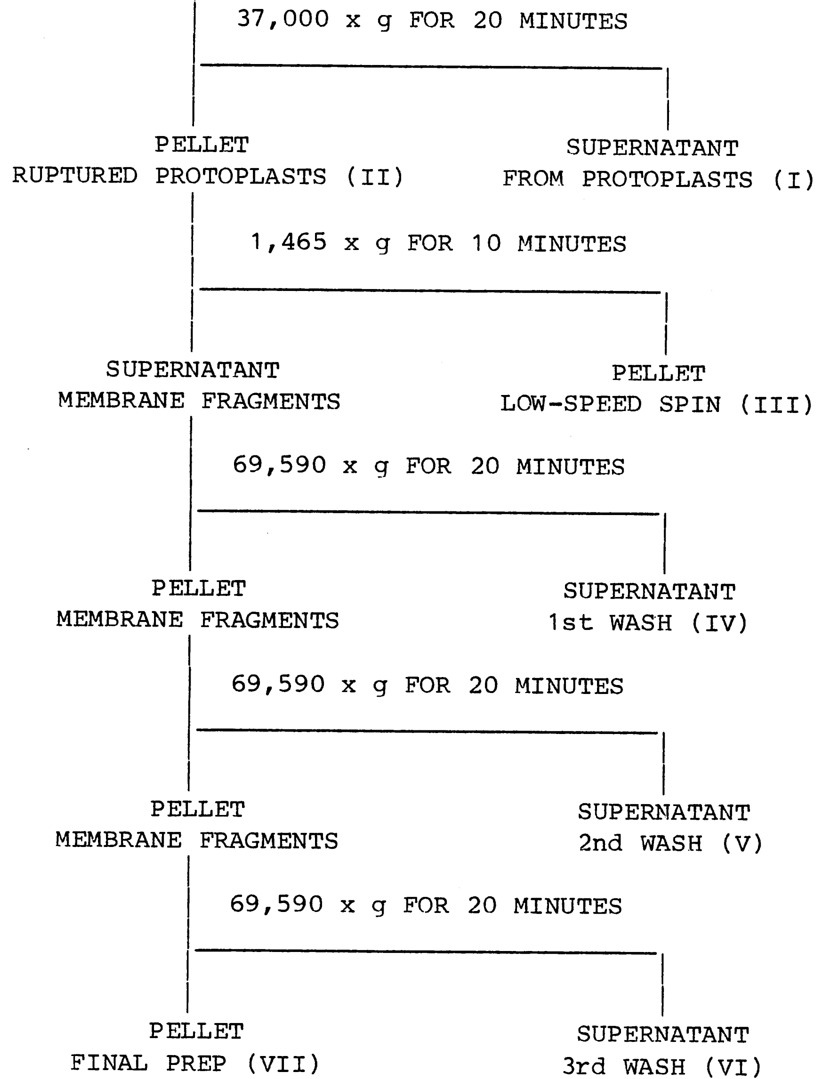
A 2 ml aliquot of a six hour culture was used to inoculate a one liter flask containing 800 ml of GTYP broth with or without NaCl (5%). Cells were allowed to grow approximately 18 hr. at 37 C with constant swirling by means of a magnetic stirrer. These cells, when harvested, were used in making cell membrane preparations for the purpose of ATPase assays.

Cell Membrane Preparation

The cell membrane preparation used in these experiments is a modification of the one used by Abrams (1965) with _S. faecalis_. It excludes the LiCl washes and terminates prior to the washes devoid of $MgCl_2$. A schematic of the preparation steps is seen in Figure 2. Each product has been designated with a Roman numeral to coincide with that of Table III in Chapter III.

800 ml of GTYP was inoculated with two ml of a six hour culture and allowed to grow for 18 hr. at 37 C with constant swirling. Cells were then harvested with a Sorvall model RC-2B centrifuge with a GSA rotor at 5860 x g for 20 minutes. Cell pellets were then resuspended with ice-cold distilled water and centrifuged again at 5860 x g for 20 minutes. Two additional washes were performed and centrifuged in the same manner. After the third wash, cell pellets were resuspended in 238 ml of 0.5 M glycylglycine (Sigma) and 2 mM $MgCl_2$ (Baker) at a pH of 7.2. Lysozyme (Sigma) was then added at a concentration of 0.2 mg/ml to the cells and allowed to incubate for 60 minutes in a waterbath at 37 C. A second dose of lysozyme (0.1 mg/ml) was added for an additional 60 minutes at 37 C. Protoplast formation was complete after the second

Figure 2. Schematic for the Preparation of Streptococcal Cell Membranes


PROTOPLASTS AFTER LYSOZYME TREATMENT
37,000 x g FOR 20 MINUTES
PELLET
RUPTURED PROTOPLASTS (II)
SUPERNATANT
FROM PROTOPLASTS (I)
1,465 x g FOR 10 MINUTES
SUPERNATANT
MEMBRANE FRAGMENTS
PELLET
LOW-SPEED SPIN (III)
69,590 x g FOR 20 MINUTES
PELLET
MEMBRANE FRAGMENTS
SUPERNATANT
1st WASH (IV)
69,590 x g FOR 20 MINUTES
PELLET
MEMBRANE FRAGMENTS
SUPERNATANT
2nd WASH (V)
69,590 x g FOR 20 MINUTES
PELLET
FINAL PREP (VII)
SUPERNATANT
3rd WASH (VI)

lysozyme treatment as revealed by phase contrast microscopy. Protoplasts were then harvested by centrifugation in a Sorvall model RC-2B centrifuge in a SS-34 rotor at 37,000 x g for 20 minutes. Protoplasts were then resuspended in 20 ml of 1 mM $MgCl_2$ and homogenized with a Bellco teflon/glass homogenizer in the presence of DNAse (Sigma) at a concentration of 2mg/ml for ten minutes at 37 C. To remove insoluble portions of the preparation, a low-speed spin was incorporated using the Sorvall model RC-2B with a SS-34 rotor at 1465 x g for ten minutes. The pellet was discarded and the supernatant was centrifuged using a Spinco model L centrifuge with a type 40 rotor at 69,590 x g for 20 minutes. The pellet was resuspended in 1 mM $MgCl_2$ and centrifuged again at 69,590 x g for 20 minutes. A 33 mM solution of Tris-hydroxymethylaminomethane (TRIS) (Sigma) buffer containing 2 mM $MgCl_2$ pH 7.5 (1 N HCl was used to pH buffer to 7.5) was used to resuspend the pellet. Centrifugation was accomplished as before at 69,590 x g for 20 minutes. The pellet was again resuspended in 33 mM TRIS buffer containing 2 mM $MgCl_2$ pH 7.5 (1 N HCl was used to adjust buffer to pH 7.5). Samples of the suspension were taken and protein content was determined. An appropriate dilution by buffer was done to give a final protein concentration of 5 mg/ml as a working stock.

Cell membranes prepared in this manner will be referred to as membrane preparations from cells grown and harvested in the absence of NaCl. When membrane preparations from cells grown and harvested in the presence of NaCl were desired, the above procedure was used except all steps were supplemented with 5% (w/v) NaCl including growth medium and dilution buffer used in making the working stock. This preparation

(supplemented with NaCl) will be referred to as membrane preparations from cells grown and harvested in the presence of NaCl.

ATPase assays were usually run immediately after preparation. If assays were performed on the following day the preparation was kept at 4 C and not frozen. It was found that freezing preparations caused a substantial loss in ATPase activity which is in agreement with Schnebli and Abrams (Schnebli and Abrams, 1970).

Protein Determination

Protein concentration was determined by biuret reaction according to a modification of the method of Yonetani (Yonetani, 1961). A standard curve was done using bovine serum albumin (BSA) and protein concentration for each cell membrane preparation was determined using the following reagents:

Biuret reagent (2x)

(A)	$CuSO_4 \cdot 5H_2O$	4.5 grams
(B)	$KNaC_2H_4O_6 \cdot 4H_2O$	18.0 grams
(C)	NaOH	90.0 grams

Each of the above, A, B, and C, were dissolved in 375 ml of distilled water. Solutions B and C were then added together and mixed. Solution A was then added to the NaOH/tartrate solution with constant stirring. The biuret reagent was then diluted to a total volume of 1500 ml. If at anytime a white precipitate was formed, the solution was discarded.

The biuret assay was conducted using the volumes shown in the following Table V.

TABLE V

VOLUMES (ml) OF COMPONENTS OF BIURET ASSAY

Tube No.	BSA[1]	Sample	H_2O_2[2]	K-DOC[3]	Buffer[4]	H_2O	Total
1	-	-	.05	.05	0.1	2.8	3.0
2	0.2	-	.05	.05	0.1	2.6	3.0
3	0.2	-	.05	.05	0.1	2.6	3.0
4	-	0.1	.05	.05	-	2.8	3.0
5	-	0.1	.05	.05	-	2.8	3.0

1. Bovine serum albumin (BSA) was made at a concentration of 40 mg/ml.

2. H_2O_2 was used at a concentration of 30% (concentrated).

3. Deoxycholate (K-DOC) was made at a concentration of 10% (w/v) in 0.5 N KOH at a pH of 8.0.

4. The buffer used was 33 mM TRIS with 2 mM $MgCl_2$ at a pH of 7.5 (1 N HCl was used to adjust the buffer to pH 7.5).

The water, H_2O_2 and K-DOC were added together and mixed by vortexing. To these solutions the standards and samples were added followed by vortexing. Two ml of 2x biuret reagent was then added to each tube followed by vortexing. Tubes were then transferred to a 37 C waterbath for 10 minutes. Standards and samples were then read by a Bausch and Lomb Spectronic 100 spectrophotometer at a wavelength of 540 nm.

ATPase Assay

Prior to the ATPase assay, the working stock (5 mg/ml) was diluted 1/5 in 10 mM Tricine, pH 8.5 containing one of the following: A) no additives, B) NaCl 5% (w/v), C) citrate 10 mM (trisodium salt, Sigma), or D) ethylenediaminetetraacetic acid (EDTA) (2 mM) disodium salt, Sigma). Since citrate and EDTA were used as sodium salts, sodium controls of equal concentration (on a molar basis) were used to account for the effect of sodium.

These solutions, containing 1 mg/ml of the membrane preparation, were allowed to incubate for 15 minutes at 37 C. From these, a 0.1 ml aliquot was used for the ATPase assay.

Experiments involving trypsin (Sigma) at a final concentration of 0.074 mg/ml were conducted in similar fashion except trypsin digestion was terminated at the end of 15 minutes by the addition of trypsin inhibitor (Sigma) at a final concentration of 0.37 mg/ml.

The release of inorganic phosphate (P_i) from ATP was measured according to the method of Adolfsen and Moudrianakis (Adolfsen and Moudrianakis, 1971). 0.1 ml samples containing 100 µg of protein were assayed in a total volume of 1 ml containing 15 mM ATP (Disodium salt,

Sigma), 15 mM $MgCl_2$, carbonylcyanide-m-chlorophenylhydrazone (CCCP) at a concentration of 2.5 x 10^{-6}M and 10 mM Tricine (Sigma) at a pH of 8.5 (1 N NaOH was used to adjust the buffer to pH 8.5). N, N'-dicyclohexylcarbodiimide (DCCD) (Sigma) at a concentration of 2.4 x 10^{-4}M was used with a 15 minute incubation prior to initiation of assay when determination of the membrane-bound ATPase activity was desired. These values, which represent that portion of the total activity which is DCCD-sensitive, were subtracted from the total activity to get a value that represents the membrane-bound ATPase activity. Since CCCP and DCCD were dissolved in 100% ethanol, an ethanol control of equal concentration (0.5%) was used to assure no interference from ethanol.

The assay was started by the addition of ATP and allowed to proceed for ten minutes at 37 C. The reaction was terminated by adding 1 ml of a 10%(w/v) solution of trichloroacetic acid(TCA) (Fisher). P_i was assayed by the addition of 2 ml of 0.5%(w/v) ammonium molybdate (Baker) and 2.5%(w/v) $FeSO_4 \cdot 7H_2O$ (Sigma) in 0.5 N H_2SO_4 (Fisher). Ammonium molybdate and $FeSO_4$ were made up in 0.5 N H_2SO_4 daily at a concentration of 1%(w/v) and 5%(w/v) respectively. Prior to assay, equal volumes of both were mixed and used as described. This mixture was allowed to react for ten minutes at 37 C and then P_i was determined by measuring absorbance at 650 nm, using a Gilford spectrophotometer.

Kinetic Studies

Kinetic studies were conducted as in ATPase assay except a range of 1.67 x 10^{-4}M to 1.25 x 10^{-2}M of ATP was used. The K_M and V_{max} values were computed by a Michaelis-Menten computer program; where 1/v and 1/s values were fitted by a least squares linear regression technique.

Polyacrylamide Gel Electrophoresis (PAGE)

Twelve 1 ml samples were prepared using 10 mM Tricine pH 8.5 as a buffer and contained the following:

1. Bovine serum albumin (BSA) at a concentration of 1 mg/ml.
2. BSA (1 mg/ml) and trypsin at a concentration of 0.67 mg/ml.
3. BSA (1 mg/ml) trypsin (0.67 mg/ml) and NaCl at a concentration of 5.0%(w/v).
4. BSA (1 mg/ml) and NaCl (5.0%)(w/v).
5. trypsin (0.67 mg/ml).
6. trypsin (0.67 mg/ml) and NaCl (5.0%)(w/v).
7. BSA (1 mg/ml), trypsin (0.67 mg/ml) and NaCl at a concentration of .01%(w/v).
8. BSA (1 mg/ml), trypsin (0.67 mg/ml) and NaCl at a concentration of 0.1%(w/v).
9. BSA (1 mg/ml), trypsin (0.67 mg/ml) and NaCl at a concentration of 0.5%(w/v).
10. BSA (1 mg/ml), trypsin (0.67 mg/ml) and NaCl at a concentration of 2.5%(w/v).
11. BSA (1 mg/ml), trypsin (0.67 mg/ml) and NaCl at a concentration of 5.0%(w/v).
12. BSA (1 mg/ml) and trypsin (0.67 mg/ml).

Samples were allowed to react for 15 minutes at 37 C at which time the experiment was terminated. Termination was carried out by the addition of trypsin inhibitor, distilled water or an appropriate NaCl solution. These terminators were used in order to achieve uniformity of the solutions with respect to NaCl and protein concentrations prior to transferring to gel. Samples were then diluted 1 to 50 in a total volume of 0.5 ml with a sample buffer containing the following

Sample Buffer

Distilled Water	4.0 ml
0.5 M TRIS-Cl, pH 6.8	1.0 ml
Glycerol	0.8 ml
10%(w/v) Sodium Dodecyl Sulfate (SDS)	1.6 ml
2-Mercaptoethanol	0.4 ml
0.5%(w/v) Bromophenol Blue	0.2 ml

These samples were then placed in a waterbath and boiled for approximately 4 minutes. Samples were cooled and 100μl aliquots of each were layered on a 10% polyacrylamide slab gel prepared according to a procedure furnished by BioRad Laboratories.

Electrophoresis was then performed using a BioRad Laboratories polyacrylamide gel apparatus model Protean 16 cm in a Glycine-TRIS base buffer (pH 8.4) using a current of 30 mA for approximately 3.5 hours or until the tracking dye (bromophenol blue) was 1 cm from the bottom.

CHAPTER III

RESULTS

Growth Characteristics of *Streptococcus faecalis*

Growth Characteristics of *S. faecalis* are shown in Figures 3 and 4. Figure 3 shows that growth of *S. faecalis* in the absence of NaCl begins without a lag phase and reaches a stationary phase within four hours of inoculation. However, growth of *S. faecalis* in the presence of NaCl shows some lag within the first two hours (as compared to the first two hours of growth of *S. faecalis* in the absence of NaCl) and takes twice as long to reach stationary phase. As shown in Figure 4, the viable cell number of *S. faecalis*, in the presence of NaCl, is reduced by 32% within the first two hours. In addition the doubling time over the next six hours is increased from 32 minutes to 53 minutes.

ATPase Activity by Fraction of Cell Membrane Preparation

Table VI shows the ATPase activity by fraction from four cell membrane preparations. Figure 5 shows where these fractions were taken and assayed for ATPase activity. The low-speed spin pellet (III) gave the highest specific activity (specific activity is defined as nmoles of P_i per minute per milligram of protein), but accounted for only 7.9% of the yield. The 1st wash supernatant (IV) gave the highest percentage of yield, but had the lowest specific activity. The final preparation

Figure 3. Growth of *S. faecalis* in Glucose (1%), Tryptone (1%), Yeast Extract (0.5%) and K_2HPO_4 (1%) with (⊡) and without (⊙) NaCl(5%) as measured by Absorbance vs. TIME

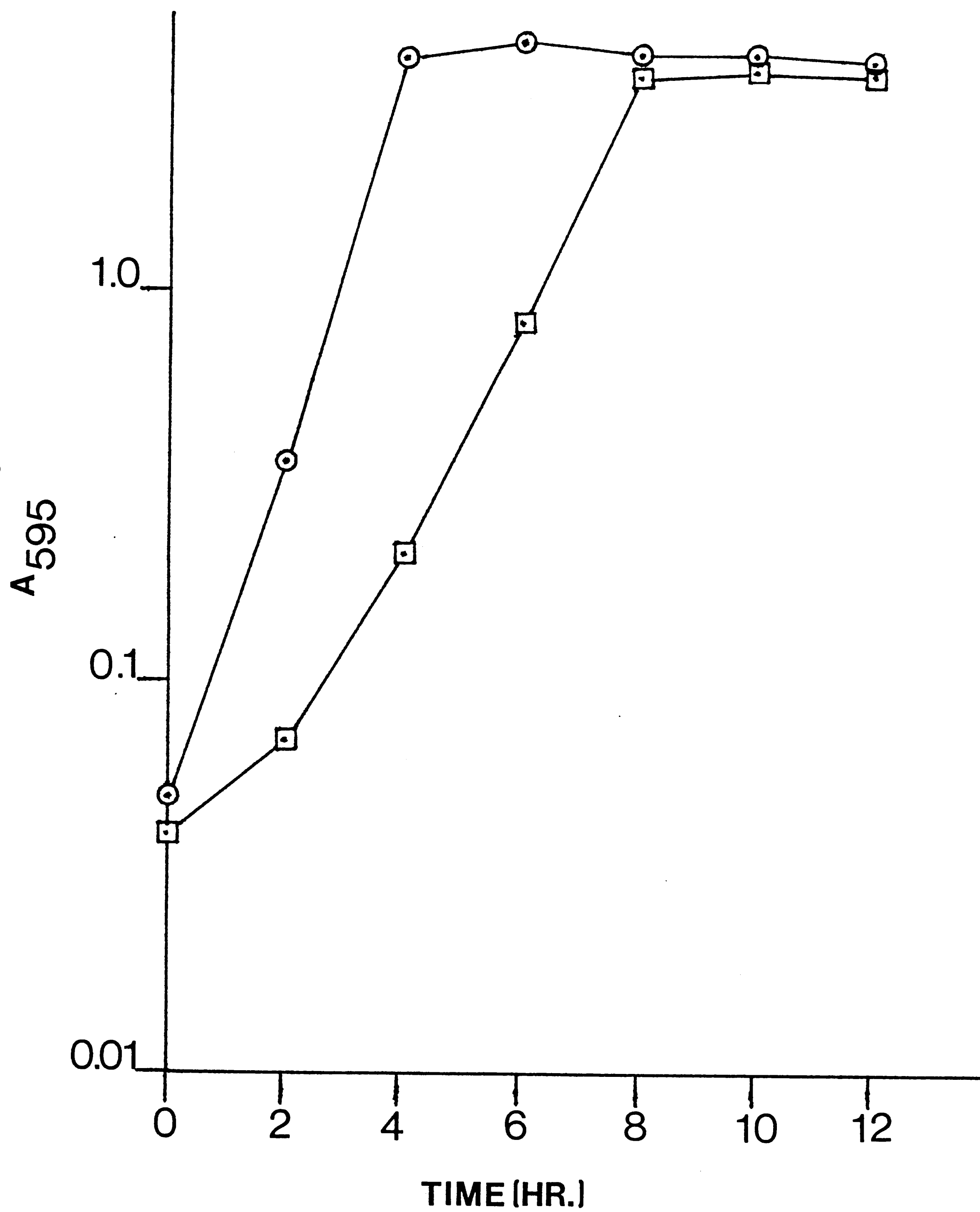

1.0
0.1
0.01
A595
0
2
4
6
8
10
12
TIME (HR.)

Figure 4. Growth of *S. faecalis* in Glucose (1%), Tryptone (1%), Yeast Extract (0.5%), and K_2HPO_4 (1%) with (⊡) and without (⊙) NaCl (5%) as measured by viable cell count vs. TIME

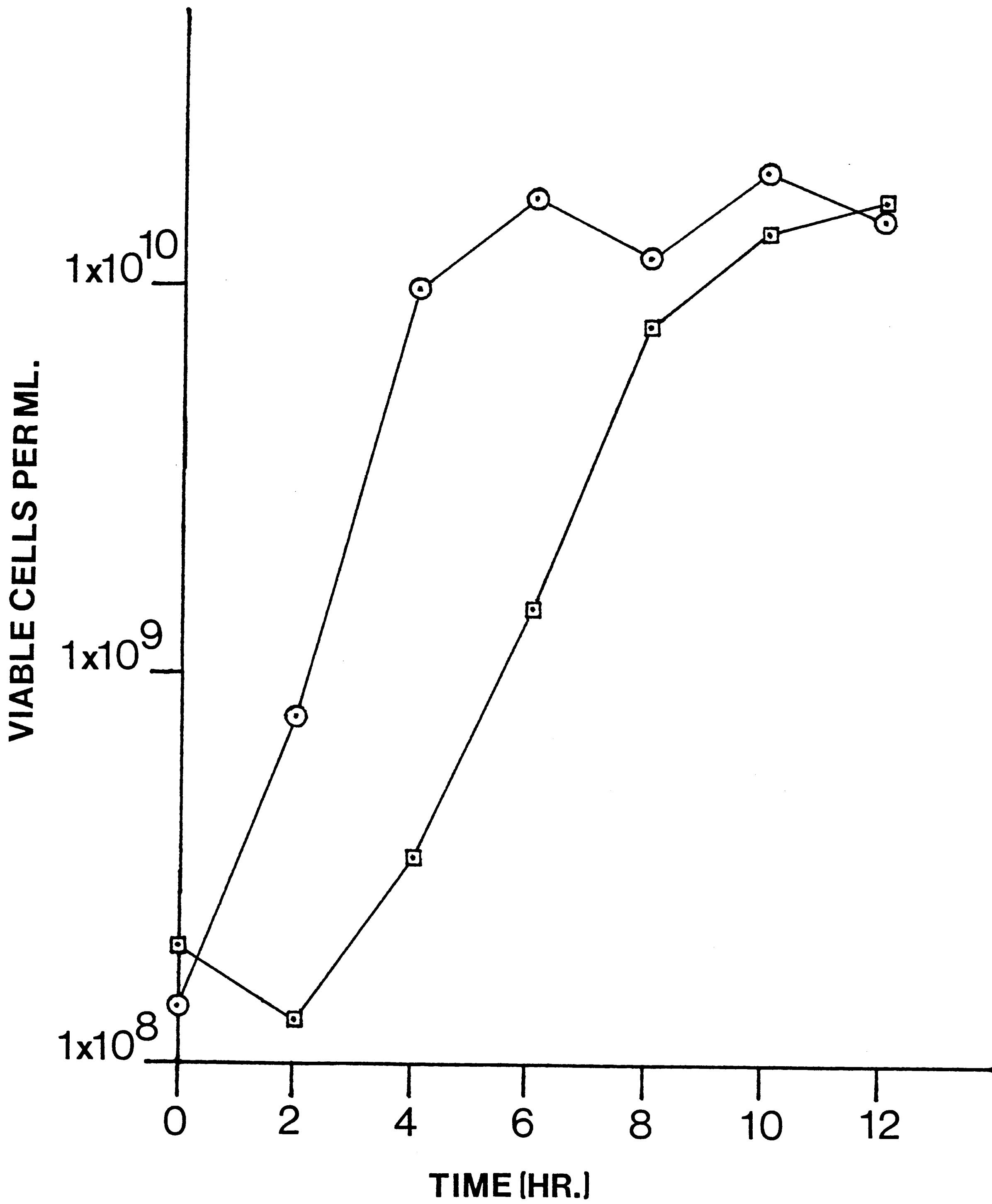

1x10^{10}
1x10^{9}
1x10^{8}
VIABLE CELLS PER ML.
0
2
4
6
8
10
12
TIME (HR.)

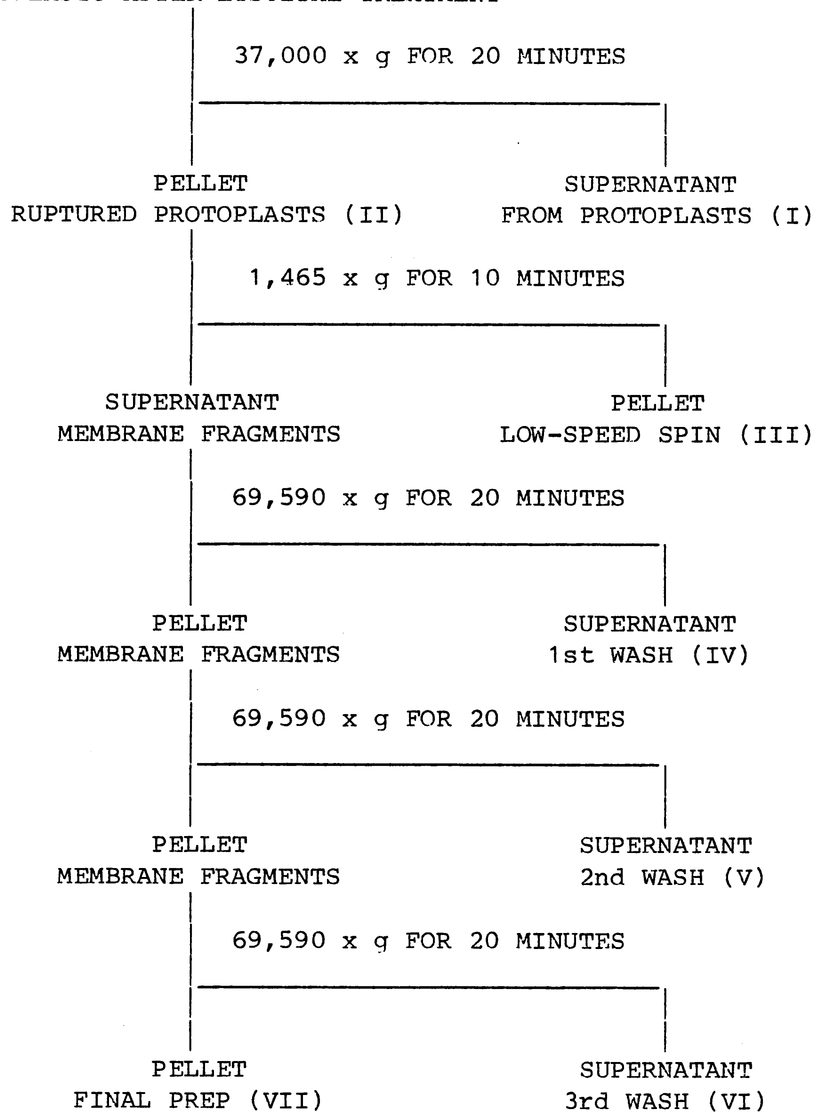
TABLE VI

ATPase ACTIVITY BY FRACTION FROM FOUR CELL MEMBRANE PREPARATIONS*

Fraction	Volume ml.	Protein mg./ml.	Activity nmoles P_i / min.	Specific Activity nmoles P_i / min. • mg.	Total Activity nmoles P_i / min.	Recovery %
Protoplast Supernatant (I)	236 ±4	7.8 ±1.2	0	0	0	0
Ruptured Protoplast (II)	27 ±6	6.9 ±1.8	115 ±12	152 ±43	29,164 ±11,128	100.0
Low-Speed Spin Pellet (III)	5 ±0	1.7 ±0.2	46 ± 6	283 ±37	2,278 ±314	7.9
1st Wash Supernatant (IV)	26 ±1	4.0 ±0.4	46 ±17	105 ±56	12,268 ±189	42.1
2nd Wash Supernatant (V)	15 ±0.6	1.3 ±0.2	30 ± 6	220 ±18	3,702 ±605	12.6
3rd Wash Supernatant (VI)	8 ±0.1	0	7.5 ± 0.8	0	0	0
Final Preparation (VII)	5 ±1	8.4 ±2.0	203 ±59	212 ±41	8,678 ±258	29.8
						92.4

*Values are expressed in nmoles of P_i $min.^{-1}$ $mg.^{-1}$ and represent four preparations with their respective standard deviations.

Figure 5. Schematic for the Preparation of Streptococcal Cell Membranes


PROTOPLASTS AFTER LYSOZYME TREATMENT
37,000 x g FOR 20 MINUTES
PELLET
RUPTURED PROTOPLASTS (II)
SUPERNATANT
FROM PROTOPLASTS (I)
1,465 x g FOR 10 MINUTES
SUPERNATANT
MEMBRANE FRAGMENTS
PELLET
LOW-SPEED SPIN (III)
69,590 x g FOR 20 MINUTES
PELLET
MEMBRANE FRAGMENTS
SUPERNATANT
1st WASH (IV)
69,590 x g FOR 20 MINUTES
PELLET
MEMBRANE FRAGMENTS
SUPERNATANT
2nd WASH (V)
69,590 x g FOR 20 MINUTES
PELLET
FINAL PREP (VII)
SUPERNATANT
3rd WASH (VI)

contained 29.8% of the total activity with a specific activity of 212 nmoles of P_i x min.$^{-1}$ mg.$^{-1}$.

DCCD Inhibition of Membrane-bound ATPase

DCCD is known to be a potent inhibitor of membrane-bound ATPase and was used in these experiments to determine what percentage of the total activity was sensitive to DCCD (Harold et al., 1969). It was found that 2.4 x 10^{-4}M DCCD for 15 minutes inhibited 19% of the total activity. No substantial increase in inhibition was seen when using concentrations of DCCD ranging from 1.0 x 10^{-5}M to 1.0 x 10^{-3}M at incubation periods of 10, 15, 20 and 30 minutes.

ATPase Activity

Membrane preparations from cells grown and harvested without NaCl present showed an activity of 43 $\pm$17 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ for the membrane-bound ATPase; whereas, the soluble ATPase gave a value of 189 $\pm$23 nmoles of P_i x min.$^{-1}$ x mg.$^{-1}$ (Table VII).

On the other hand, membrane preparations from cells grown and harvested with NaCl present showed 59 $\pm$16 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ and 350 $\pm$53 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ for the membrane-bound and soluble ATPase, respectively (Table VII). It can be seen that cell membrane preparations from cells grown and harvested with NaCl increase in both their membrane-bound ATPase and soluble ATPase activities by 37% and 85%, respectively.

ATPase Activity in the Presence of NaCl

When membrane preparations from cells grown and harvested without NaCl were assayed in the presence of NaCl, an increase in membrane-bound ATPase activity of 98% from 43 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 85 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when compared to the same when assayed in the absence of NaCl (Table VII). In contrast, the soluble ATPase was inhibited by 36% from 189 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 120 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when assayed in the presence of NaCl (Table VII). When membrane preparations from NaCl grown and harvested cells are assayed in the absence of NaCl a decrease in activity by 20% from 59 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 47 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ is seen by the membrane-bound ATPase; whereas, the soluble ATPase exhibits a 42% increase in activity from 350 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 492 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ (Table VII).

ATPase Activity in the Presence of Trypsin

No appreciable amount of gain or loss from trypsin activity is seen with either membrane-bound or soluble ATPases from cells grown and harvested without NaCl (Table VII). However, the membrane preparation from cells grown and harvested in NaCl show a substantial amount of inhibition in the presence of trypsin (Table VII). The membrane-bound ATPase appears to be more sensitive to the action of trypsin than does the soluble ATPase with inhibitions of 71% and 22%, respectively (Table VII).

ATPase Activity in the Presence of NaCl/Trypsin

An increase in activity of 184% from 43 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 122 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ was shown by the membrane-bound ATPase

TABLE VII

EFFECTS OF NaCl (5%), TRYPSIN (0.074 mg/ml), AND NaCl 5%/TRYPSIN (0.074 mg/ml) ON F_1 AND F_1F_0-ATPases OF S. Faecalis GROWN AND HARVESTED IN THE ABSENCE (A) AND PRESENCE (B) OF NaCl (5%)

(A)

	F_1-ATPase	F_1F_0-ATPase
Control*	189 ± 23***	43 ± 17
NaCl	120 ± 25	85 ± 17
Trypsin	188 ± 21	46 ± 20
NaCl/Trypsin	38 ± 10	122 ± 21

(B)

	F_1-ATPase	F_1F_0-ATPase
Control**	350 ± 53	59 ± 16
(-)NaCl	492 ± 66	47 ± 18
Trypsin	274 ± 53	17 ± 9
NaCl/Trypsin	336 ± 46	203 ± 22

*Control represents activity in the absence of NaCl.

**Control represents activity in the presence of NaCl.

***Values are expressed in nmoles of P_i $min.^{-1}$ $mg.^{-1}$ and represent eight measurement involving three different preparations with their respective standard deviations.

from membrane preparations from cells grown and harvested in the absence of NaCl when assayed in the presence of NaCl/Trypsin (Table VII). A similar increase of 244% from 59 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 203 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ was also shown by the membrane-bound ATPase from membrane preparations from cells grown and harvested in the presence of NaCl when assayed in NaCl/Trypsin (Table VII). In contrast, the soluble ATPase activity from membrane preparations from cells grown and harvested in the absence of NaCl showed an inhibition of 80% from 189 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 38 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when assayed in the presence of NaCl/Trypsin (Table VII). However, the soluble ATPase from membrane preparations from cells grown and harvested in the presence of NaCl showed only a slight inhibition of 4% from 350 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 336 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when assayed in the presence of NaCl/Trypsin (Table VII).

ATPase Activities in the Presence of EDTA and Citrate

EDTA and citrate had an inhibitory effect on the membrane-bound ATPase of membrane preparations of cells grown and harvested in the absence of NaCl (Table VIII). In the presence of EDTA, a decrease of 32% from 43 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 29 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ was seen and a decrease of 46% from 43 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 23 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ was seen in the presence of citrate (Table VIII). EDTA and citrate had a similar inhibitory effect on the membrane-bound ATPase of membrane preparations of cells grown and harvested in the presence of NaCl as well (Table VIII). A decrease in activity of 58% from 59 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 25 nmoles of

TABLE VIII

EFFECTS OF EDTA (2mM) AND CITRATE (10mM) ON MEMBRANE-BOUND AND SOLUBLE ATPases OF *S. faecalis* GROWN AND HARVESTED IN THE ABSENCE (A) AND PRESENCE (B) OF NaCl (5%)

	(A)	
	F_1-ATPase	F_1F_0-ATPase
Control*	189 ± 23***	43 ± 17
EDTA	356 ± 10	29 ± 17
Citrate	232 ± 6	23 ± 11
	(B)	
	F_1-ATPase	F_1F_0-ATPase
Control**	350 ± 53	59 ± 16
EDTA	64 ± 34	25 ± 11
Citrate	Nil	21 ± 7

*Control represents activity in the absence of NaCl.

**Control represents activity in the presence of NaCl.

***Values are expressed in nmoles of P_i min.$^{-1}$ mg.$^{-1}$ and represent eight measurement involving three different preparations with their respective standard deviations.

P_i min.$^{-1}$ mg.$^{-1}$ was seen when assayed in the presence of EDTA (Table VIII). In the presence of citrate a decrease in activity of 64% from 59 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 21 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ was seen as well (Table VIII).

The soluble ATPase of membrane preparations of cells grown and harvested without NaCl show an increase of 88% from 189 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 356 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ for EDTA and 23% from 189 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 232 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ for citrate (Table VIII). On the other hand, the soluble ATPase of membrane preparations of cells grown and harvested with NaCl showed a decrease in activity (Table VIII). In the presence of EDTA, an 82% decrease from 350 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 64 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ was seen and in the presence of citrate activity is completely inhibited (Table VIII).

Kinetic Studies

Table IX shows the results of the kinetic studies done on the ATPases from membrane preparations from cells grown and harvested without NaCl. In the presence of NaCl, the K_M value for soluble ATPase is increased by 27% from 3.0 mM to 3.8 mM; and the V_{max} value is increased by 26% from 96 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 121 nmoles of P_i min.$^{-1}$ mg.$^{-1}$. The membrane-bound ATPase K_M and V_{max} values are decreased. K_M is lowered by 10% from .76 mM to .68 mM and the V_{max} is lowered by 18% from 56 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 46 nmoles of P_i min.$^{-1}$ mg.$^{-1}$.

In the presence of trypsin, no values were recorded due to large inconsistencies between assays within a single preparation, as well as between preparations.

TABLE IX

V_{max} AND K_M VALUES FOR F_1 AND F_1F_0-ATPases OF CELLS GROWN AND HARVESTED IN THE ABSENCE (A) AND PRESENCE (B) OF NaCl IN THE PRESENCE OF NO NaCl (5%), NaCl (5%), TRYPSIN (0.074 mg/ml), AND NaCl (5%)/TRYPSIN (0.074mg/ml)

	(A)					(B)			
	F_1-ATPase		F_1F_0-ATPase			F_1-ATPase		F_1F_0-ATPase	
Treatment	V_{max}	K_M	V_{max}	K_M	Treatment	V_{max}	K_M	V_{max}	K_M
Control*	96±19***	3.0±1.7	56±18	.76±.09	Control**	289± 46	.41±.13	52± 28	.68±.33
NaCl	121±23	3.8±1.7	46±20	.68±.08	(-)NaCl	627±175	3.1±.98	32± 15	1.3±.80
Trypsin	-	-	-	-	Trypsin	155± 79	.92±.40	367±113	5.2±3.1
NaCl/Trypsin	37±10	3.7±.09	98±14	.33±.18	NaCl/Trypsin	373± 65	.81±.24	221± 35	.72±.41

*Control represents activity in the absence of NaCl.

**Control represents activity in the presence of NaCl.

***V_{max} expressed as nmoles of P_i $min.^{-1}$ $mg.^{-1}$ and K_M expressed in mM. Values represent four measurements involving two different preparations with their respective standard deviations.

The NaCl/trypsin treatment affects the soluble ATPase from membrane preparations from cells grown and harvested in the absence of NaCl in such a way as to decrease its V_{max} by 61% from 96 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 37 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ and increase its K_M by 23% from 3.0mM to 3.7mM. However, its K_M value of 3.7mM is quite similar to the K_M value observed in the presence of NaCl alone.

The membrane-bound ATPase from membrane preparations from cells grown and harvested in the absence of NaCl and assayed in the presence of NaCl and trypsin shows an increase in V_{max} by 75% from 56 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 98 nmoles of P_i min.$^{-1}$ mg.$^{-1}$, while its K_M is decreased by 56% from .76mM to .33 mM.

Table IX also shows the V_{max} and K_M values determined for the ATPases from membrane preparations from cells grown and harvested in the presence of NaCl. The control, in this case, would be with NaCl present. The K_M value for the soluble ATPase is decreased by some 86% from 3.0mM to .41mM when compared to the control of the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl (Table IX). However, the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl appears to have little or no change in its V_{max} and K_M values when compared to the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl (Table IX). In addition, the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl when assayed in the presence of NaCl shows an identical K_M value of .68mM and a similar V_{max} value of 46 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when compared to the K_M and V_{max} values of the membrane-bound ATPase of membrane preparations from cells grown and

harvested in the presence of NaCl and assayed in the presence of NaCl (Table IX).

When the soluble ATPase from membrane preparations from cells grown and harvested with NaCl are assayed in the absence of NaCl, the K_M reverts back to that of the soluble ATPase from membrane preparations from cells grown and harvested without NaCl (Table IX). The V_{max} however, is changed drastically exhibiting an increase of 553% from 96 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 627 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ when compared to the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl (Table IX). It also exhibits an increase of 117% from 289 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 627 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ over its own control (Table IX). The K_M is also increased by 656% from .41mM to 3.1mM when the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl and assayed in the presence of NaCl is compared to the same when assayed in the absence of NaCl (Table IX).

The soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl when assayed with trypsin shows a decrease in V_{max} of 46% from 289 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 155 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ and an increase in K_M of 124% from .41mM to .92mM (Table IX). The membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl shows an increase in V_{max} of 606% from 52 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 367 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ and an increase in K_M of 665% from .68mM to 5.2mM (Table IX).

In the presence of trypsin and NaCl, the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl

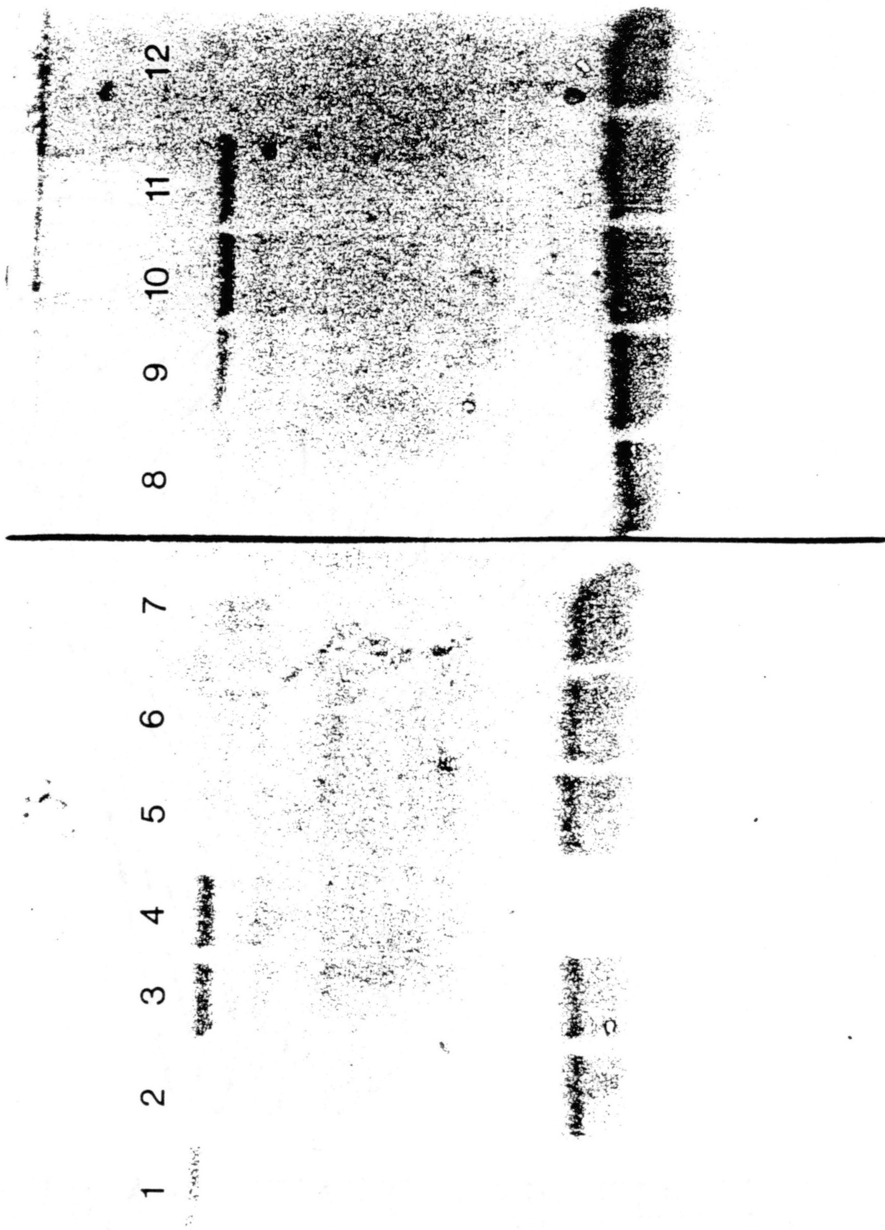
shows only a slight increase in V_{max} of 29% from 289 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 373 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ and a large increase in K_M of 98% from .41mM to .81mM (Table IX). The membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl and assayed in the presence of trypsin and NaCl, exhibits a K_M increase of only 6% from .68mM to .72mM but exhibits a substantial increase in V_{max} of 325% from 52 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 221 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ (Table IX).

NaCl and Trypsin Interaction as Determined by Electrophoresis

Determination of the effect of NaCl on trypsin activity was done by using sodium dodecyl sufate polyacrylamide gel electrophoresis (SDS-PAGE). Figure 6 shows the results of the SDS-PAGE with the following conclusions. Lane 1 shows the migratory pattern of bovine serum albumin (BSA) as being one distinct band occurring near the top of the gel (Figure 6). Its pattern is unaffected by NaCl (5%) as seen in lane 4 (Figure 6). The migratory pattern of trypsin is seen in lane 5 as being a smeared band occuring near the bottom of the gel (Figure 6). Its pattern, like BSA, is unaffected by NaCl as evident in lane 6 (Figure 6). The action of trypsin digestion on BSA can be seen in lane 2 with the disappearance of the BSA band that was seen in lane 1 (Figure 6). The effect of NaCl on the digestive action of trypsin on BSA is clearly seen when lane 3 is observed (Figure 6). The appearance of the BSA band in the presence of trypsin and NaCl indicate that the trypsin digestion of BSA is inhibited in the presence of NaCl. This inhibition is dependent upon the concentration of NaCl as evident by the gradual

Figure 6. Electropherogram of Bovine Serum Albumin (BSA), Trypsin, and NaCl in the following situations:

Lane 1 Contains BSA (1 mg/ml).
Lane 2 Contains BSA (1 mg/ml) and trypsin (0.67 mg/ml).
Lane 3 Contains BSA (1 mg/ml), trypsin (0.67 mg/ml) and NaCl (5%).
Lane 4 Contains BSA (1 mg/ml) and NaCl (5%).
Lane 5 Contains trypsin (0.67 mg/ml).
Lane 6 Contains trypsin (0.67 mg/ml) and NaCl (5%).
Lane 7 Contains BSA (1 mg/ml), trypsin (0.67 mg/ml), and NaCl (.01%).
Lane 8 Contains BSA (1 mg/ml), trypsin (0.67 mg/ml), and NaCl (.1%).
Lane 9 Contains BSA (1 mg/ml), trypsin (0.67 mg/ml), and NaCl (.5%).
Lane 10 Contains BSA (1 mg/ml), trypsin (0.67 mg/ml), and NaCl (2.5%).
Lane 11 Contains BSA (1 mg/ml), trypsin (0.67 mg/ml), and NaCl (5.0%).
Land 12 Contains BSA (1 mg/ml) and trypsin (0.67 mg/ml).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darkening of the BSA band seen in lanes 9 through 11 (Figure 6). It appears the minimal NaCl concentration that is effective in the inhibition of trypsin digestion on BSA occurs at 0.5%.

CHAPTER IV

DISCUSSION

NaCl at a concentration of 5% (0.86M) lowers the growth rate of *S. faecalis* as shown by an increase of doubling time from 32 minutes to 53 minutes (an increase of 66%). The viable cell count indicates a 32% loss in cell number from 1.96×10^8 to 1.33×10^8 cfu/ml within the first two hours when cells are inoculated into a growth medium containing NaCl (5%). It appears that NaCl serves as a selecting agent against that portion of a population that is incapable of growing in 5% NaCl. However, the remaining 68% of the population has been altered in some way as evidenced by the increase in doubling time.

Although there is a detrimental effect on growth of *S. faecalis* in the presence of NaCl, there appears to be no precise quantitative relationship between the extended doubling time and the effects of NaCl upon the ATPase of *S. faecalis*. One would hope that such a relationship would exist; it is tempting to speculate that the growth inhibitory property of NaCl stems from its inhibition of ATPase.

Earlier work with my strain of *S. faecalis* revealed a low ATPase activity. Abrams and coworkers reported a specific activity of 600 nmoles of P_i $min.^{-1}mg.$ $protein^{-1}$ for the membrane-bound ATPase of *S. faecalis* (ATCC 9790). When the ATPase was solubilized, the specific activity rose to 10 μmoles of P_i $min.^{-1}mg.$ $protein^{-1}$ (a 16-fold increase in specific activity upon release from the membrane)(Abrams,

1965). When these values from *S. faecalis* (ATCC 9790) are compared to those of my strain of *S. faecalis* a 13-fold decrease from 600 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 43 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ is seen in the specific activity of the membrane-bound ATPase. Likewise, a 52-fold decrease from 10 μmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 0.189 μmoles of P_i min.$^{-1}$ mg.$^{-1}$ in specific activity is seen when the solubilized ATPase of *S. faecalis* (ATCC 9790) is compared to the solubilized ATPase of my strain of *S. faecalis*. In addition to the above finding, the membrane-bound ATPase activity of my strain of *S. faecalis* represents only 19% of the total ATPase activity. This too, is different from *S. faecalis* (ATCC 9790). Harold and Baarda demonstrated in 1969, in the presence of DCCD (1×10^{-5}M) for 10 minutes, that maximal inhibition was 78.6 $\pm$5.4% for 44 measurements involving five different preparations (Harold and Baarda, 1969). Since then, it has been well documented that *S. faecalis* membrane-bound ATPase is highly sensitive to DCCD (Abrams and Baron, 1967; Abrams and Baron, 1970; Abrams et al., 1972).

The small but consistent ATPase activity accompanied by a low percentage of membrane-bound ATPase inhibited by DCCD led to the following possibilities: 1) a large percentage of the total ATPase activity was being lost in the preparatory steps of membrane isolation, 2) the time of exposure in the presence of DCCD and/or the concentration of DCCD is not sufficient to inhibit the majority of the F_1F_0-ATPases, 3) the ATPase enzymes are in a cryptic form which requires a trypsin digestion to stimulate the enzyme's activity, or 4) this strain of *S. faecalis* is a mutant possessing a DCCD-resistant membrane-bound ATPase.

First, ATPase assays were conducted on each fraction of the membrane preparation. These assays revealed no substantial loss in ATPase activity, i.e., loss of activity on the order of μmoles of P_i min.$^{-1}$ mg.$^{-1}$ (See Table VI and Figure 5, Chapter III) at any one step in the preparation. These fractions were also assayed for membrane-bound ATPase activity by using DCCD. The results indicate no substantial loss, again on the order of umoles of P_i min.$^{-1}$ mg.$^{-1}$, of membrane-bound ATPases in any of the seven fractions assayed. Secondly, experiments were conducted with varying concentrations of DCCD at different periods of time in an attempt to show a larger percentage of membrane-bound ATPase activities. This too, as indicated on page 44 of Chapter III, failed to increase the percentage of membrane-bound ATPase activity. Thirdly, a trypsin treatment was incorporated in an attempt to increase total ATPase activity, both soluble and membrane-bound ATPase activity, as in the case of Micrococcus lysodeikticus, Mycobacterium phlei and Escherichia coli. These bacteria were shown to possess a latent ATPase and in the presence of trypsin, ATPase activity was either observed for the first time or increased substantially (Carreira et al., 1976; Ritz and Brodie, 1977 and Carreira et al., 1973). However, Harold reported that S. faecalis does not require a trypsin treatment for activation of its ATPase (see review by Harold, 1972).

Trypsin treatment of the strain of S. faecalis in these studies had differing effects depending upon the source of ATPase. For example, trypsin had no effect on ATPases of membrane preparations from cells grown and harvested in the absence of NaCl and assayed in the presence of trypsin. On the other hand, ATPases of membrane preparations from

cells grown and harvested in the presence of NaCl and assayed in the presence of trypsin showed an inhibition. The effect of trypsin will be discussed later in detail. Nevertheless, trypsin did not stimulate ATPase activity in either case as originally thought.

Fourthly, the possibility that my strain of S. faecalis is a mutant possessing an ATPase resistant to DCCD is quite conceivable. Abrams and coworkers in 1972 were first to show a DCCD-resistant mutant of S. faecalis (Abrams et al., 1972). Their mutant, SF-dcc-8, required an increase in DCCD concentration of approximately 100-fold to produce a 50% inhibition as compared to the parental strain (Abrams et al., 1972). However, the specific activity of the membrane-bound ATPase in both the parent and mutant strains was the same. There was no substantial loss in membrane-bound ATPases specific activity to accompany the resistance to DCCD in mutant SF-dcc-8. Although the strain of S. faecalis in this study apparently has a membrane-bound ATPase that is resistant to DCCD, its low specific activity is still left unexplained.

Failure to enhance ATPase activity and/or increase the percentage of membrane-bound ATPase activity has led the author to conclude that these characteristics, of low total activity and a population of membrane-bound ATPases that are resistant to DCCD, are inherent to this particular strain of S. faecalis. This could mean that in this strain of S. faecalis the H^+-translocating membrane-bound ATPase (DCCD sensitive) represents only a small portion (19%) of the total population of membrane-bound ATPases. It could also mean that in this strain 80% of the H^+-translocating membrane-bound ATPases has lost its sensitivity to DCCD. These possibilities will be addressed later in the text.

This particular strain of S. faecalis also possesses some other unique characteristics as well. As indicated in Chapter III, the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl assayed in the presence of NaCl, is stimulated by 98%. This NaCl stimulation has been seen only one other time in this species. Kinoshita and coworkers have shown, using membrane vesicles of a mutant of S. faecalis defective in the generation of protonmotive force, an increase in activity of 12% when grown on low Na^{+}(5-10mM) medium (Kinoshita et al., 1984). The specific activity of 89.7 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when assayed in the presence of low Na^{+} is quite comparable to the value shown by the strain used here.

The soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl activity which is three times higher than the membrane-bound ATPase activity from the same type of preparation, shows an inhibition (36%) in the presence of NaCl. This contradicts earlier work by Abrams, who showed a 40% stimulation in the presence of NaCl (200mM) (Abrams, 1965). This difference cannot be explained unless one considers the possibility of a second isozyme of ATPase.

Furthermore, when the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl is assayed in the presence of NaCl and trypsin, a decrease in activity of 80% from 189 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 38 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ occurs. This decrease in activity indicates a possible synergism between NaCl and trypsin, i.e., NaCl is acting upon the soluble ATPase in such a way as to render the soluble ATPase accessible to trypsin digestion. This synergistic effect by NaCl and trypsin is also seen when the

membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl are assayed in the presence of NaCl and trypsin. However, the effect is stimulatory instead of inhibitory. Although NaCl alone stimulates the membrane-bound ATPase 98% from 43 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 85 nmoles of P_i $min.^{-1}$ $mg.^{-1}$, it still enhances the action of trypsin in such a way as to increase the membrane-bound ATPase activity an additional 44% from 85 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 122 nmoles of P_i $min.^{-1}$ $mg.^{-1}$.

In addition to the above findings, SDS-PAGE electropherogram shows a second effect that must be considered (Figure 6, Chapter III). This electropherogram indicates that NaCl inhibits trypsin digestion on bovine serum albumin (BSA). This inhibition would appear to be a salt (NaCl) denaturation of trypsin, thus rendering the enzyme inactive in its digestion of BSA. However, in light of the findings when ATPases are assayed in the presence of NaCl and trypsin, there would appear a second mode of inhibition (inhibition of trypsin on BSA). Perhaps the salt (NaCl) is denaturing the substrate (BSA) instead of the enzyme (trypsin) to the point where trypsin is unable to digest BSA. If this is the case, then perhaps the ATPase in the presence of NaCl is affected in such a way as to be accessible to the action of trypsin. Further research is needed in this area to better understand the relationship between NaCl and trypsin.

So far, these findings, i.e., the mode of action of NaCl, trypsin, and NaCl/trypsin on both the F_1 and F_1F_0-ATPases of membrane preparations from cells grown and harvested in the absence of NaCl, indicate the significance of the relationship of F_1 to the F_0 and, more importantly, the relationship to the membrane. It appears that as long as

the F_1 (catalytic) portion is membrane bound, NaCl stimulates the activity, but detached F_1 is susceptible to inhibition by NaCl. The increase in activity of the membrane-bound ATPase in the presence of NaCl/trypsin gives the indication of a regulatory component associated with both the F_0 and the F_1 portions of the ATPase. To date, most, if not all, regulatory components of ATPase have been associated with the F_1 exclusively (Carreira et al., 1976; Ritz and Brodie, 1977 and Dreyfus and Satie, 1984).

Several other characteristics of this strain of _S. faecalis_ were observed. The membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl has a K_M value of 0.76mM similar to the proton-translocating ATPase of _S. faecalis_ (ATCC 9790). Its value of 0.76mM is within the range of 0.7 - 0.9mM as recorded by Abrams (Abrams and Smith, 1971). The soluble ATPase of this strain of _S. faecalis_ showed a K_M value of 3.0mM which is in agreement with Schnebli and Abrams when in 1970 reported a K_M of 2.5mM for the soluble ATPase (Schnebli and Abrams, 1970). These data indicate that the ATPase of this strain of _S. faecalis_ reported here may be a proton-translocating ATPase as in the case of _S. faecalis_ (ATCC 9790) (Abrams and Smith, 1971 and Schnebli and Abrams, 1970).

In this study, experiments were also done to determine the effect of NaCl, trypsin, and NaCl plus trypsin combined on K_M amd V_{max} of soluble and membrane ATPases of membrane preparations from cells grown and harvested in the absence of NaCl. It must be pointed out that there are discrepancies between the data obtained from K_M and V_{max} experiments and the data obtained from the initial specific activity experiments (compare Table VII to Table IX, Chapter III). For example, the observed

velocities of Table VII indicate a decrease in activity for the F_1-ATPase and an increase in activity for the F_1F_0-ATPase of membrane preparations from cells grown and harvested in the absence of NaCl and assayed in the presence of NaCl. However, the calculated values for V_{max} indicate just the opposite, i.e., the increase in activity is seen for the F_1-ATPase (see Table IX, Chapter III). In addition, several of the observed velocities of Table VII exceed the corresponding theoretical V_{max} values that were calculated and are shown in Table IX. A possible explanation for these discrepancies could be that the methods employed in determining the V_{max} values did not use substrate (ATP) concentrations high enough to establish saturation of the enzymes (ATPases), thus determining the V_{max}. In Chapter II, substrate (ATP) concentrations as high as 12.5mM were used in determining V_{max}; this concentration is 4 times higher than the K_M (3.0mM) of S. faecalis (ATCC 9790) (Schnebli and Abrams, 1970). These concentrations of substrate used are quite sufficient for the determination of V_{max}. Nevertheless, a discussion of these data will be done in hopes of understanding these phenomena.

The results in Chapter III indicate that the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl assayed in the presence of NaCl and NaCl plus trypsin in combination decreases the affinity of the ATPase for the substrate (MgATP). The rate (V_{max}) of the reaction is increased in the presence of NaCl and decreased in the presence of NaCl plus trypsin. The decrease in rate (V_{max}) in the presence of NaCl plus trypsin is in agreement with the decrease in specific activity determined earlier (compare Table VII to Table IX, Chapter III). Again, it appears as a

synergistic effect by NaCl and trypsin on the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl. On the other hand, the increase in V_{max} of the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl and assayed in the presence of NaCl does not agree with the decrease in specific activity as stated earlier (compare Table VII to Table IX, Chapter III). It is unfortunate that kinetic data could not be obtained for the ATPases of membrane preparations from cells grown and harvested in the absence of NaCl and assayed in the presence of trypsin alone to shed further light on the subject. However, the specific activities shown in Table VII, Chapter III, would indicate there would be no change in K_M or V_{max}.

The affinity of the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl for the substrate in the presence of NaCl is increased and at the same time the rate (V_{max}) is decreased.

Trypsin in the presence of NaCl may remove or inhibit some inhibitor protein in or around the active site of the membrane-bound ATPase. Trypsin not only increases the affinity, but increases the rate of reaction of the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl as well. This coincides nicely with data reported in Table VII, Chapter III. However, the effect only occurs when the F_1-ATPase is bound to the membrane and NaCl is present in the assay.

To further elucidate the effect of NaCl, a second set of experiments was conducted with ATPases of membrane preparations from cells grown and harvested with NaCl. This set of experiments would

indicate if NaCl in the growth medium affected the ATPase. As noted in Chapter III, S. faecalis grows well in NaCl (5%) and perhaps its ability to grow in NaCl can be attributed to an adaptive ability by the ATPase. The membrane-bound ATPase of membrane preparations of cells grown and harvested in the presence of NaCl activity was surprisingly increased by only 37% from 43 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 59 nmoles of P_i min.$^{-1}$ mg.$^{-1}$. This at first glance would appear unusual, especially in light of the 85% increase from 189 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 350 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ in activity of the soluble ATPase. However, once again the role and function of the membrane must be considered. The membrane-bound ATPase of membrane preparations of cells grown and harvested in the presence of NaCl seems to be altered, but when assayed without NaCl, it behaves as the membrane-bound ATPase of membrane preparations from cells grown and harvested without NaCl. In addition, its K_M value of .68mM in NaCl is identical to the K_M value of the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl when assayed in the presence of NaCl. On these grounds, it appears that the membrane-bound ATPase is unaltered when cells are grown and harvested with NaCl.

However, the large K_M value for the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl and assayed in the absence of NaCl indicates that the membrane-bound ATPase is altered. Perhaps the decrease in affinity is responsible for the decrease in activity. But, the author feels this is unlikely due to only a 20% loss in activity from 59 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 47 nmoles of P_i min.$^{-1}$ mg.$^{-1}$. The increase in K_M seems to be undefined as of now.

Although the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence or absence of NaCl appears to be the same enzyme, the trypsin treatment indicates they are not. The membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl is unaffected by trypsin while the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl is inhibited 71% in the presence of trypsin. This evidence indicates an alteration in the conformation of the F_1F_0-ATPase. It would appear that the alteration in the conformation only occurs when the F_1-ATPase is bound to the membrane. This is evident by the decrease in activity of only 22% from 350 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ to 274 nmoles of P_i min.$^{-1}$ mg.$^{-1}$ when soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl is assayed in the presence of trypsin. However, Table IX shows that the K_M value of the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl and assayed in the presence of trypsin is increased from 0.41mM to 0.92mM, indicating a decrease in affinity. Therefore, the effect of trypsin on soluble ATPase is not completely ruled out.

From these data discussed above, it appears that this strain of *S. faecalis*, when grown in a growth medium containing 5% NaCl, produces a second isozyme of ATPase.

Further comparison of trypsin treatment cannot be done because of the failure of obtaining kinetic data on the ATPases of membrane preparations from cells grown and harvested in the absence of NaCl. It can be pointed out that kinetic information from ATPase assays of membrane preparations from cells grown and harvested in the presence of

NaCl were obtained and as stated the K_M and V_{max} values obtained for the soluble ATPase give validity to the inhibitory action of trypsin on both the soluble and membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl.

The decrease in V_{max} and the increase in K_M agrees with the specific activity data of Table VII in that trypsin inhibits the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl. However, the large V_{max} value for the membrane-bound ATPase indicates a large stimulation of 606% in the presence of trypsin. This does not agree with the 71% inhibition by trypsin as seen in Table VII, nor does it coincide with the decrease in affinity when assayed in the presence of trypsin. This, too, warrants further investigation before a conclusion can be drawn.

It appears that the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl is quite similar to the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl, with the exception of the decrease in activity when the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl is assayed in trypsin. In the presence of NaCl/trypsin, the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl shows an increase in activity from 59 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ to 203 nmoles of P_i $min.^{-1}$ $mg.^{-1}$, a 244% increase. This increase is similar to the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl of 184%. However, the K_M value of the membrane-bound ATPase of membrane preparations from cells grown and harvested in the presence of NaCl does not decrease as in the case of

the membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl, but increases slightly from 0.68mM to 0.72mM.

On the other hand, the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl appears to be quite different from the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl. The soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl showed an 86% increase in affinity and an 85% increase in rate when compared to the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl. These increases represent a significant change in the soluble ATPase when NaCl is present in the growth and harvest medium.

Further evidence is seen in the NaCl/trypsin treatment. Remembering the presence of NaCl/trypsin inhibits the soluble ATPase of membrane preparations from cells grown and harvested in the absence of NaCl, the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl shows no effect. However, the K_M value of 0.81mM of the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl when assayed in NaCl/trypsin indicates the affinity is decreased. This decrease in affinity does not correspond to the increase in rate. This discrepancy is unaccounted for at this time.

It is interesting to note that when this soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl is assayed in the absence of NaCl its rate of reaction increases by 40%. This increase was thought to be unusual in that an ATPase adapted to

NaCl should not function as well when NaCl is not present, and indeed the increase in K_M is proof that its affinity is decreased. However, the increase in rate still remains a question.

Due to the differences encountered with ATPases from cells grown and harvested in the presence and absence of NaCl, a third set of experiments were conducted. These experiments involved the use of citrate and EDTA. Adolfsen and Moudrianakis showed in Alcaligenes faecalis that in the presence of citrate and EDTA the activity of the ATPases was inhibited (Adolfsen and Moudrianakis, 1973). Therefore, these compounds were used to see if the ATPases from cells grown and harvested in the presence and absence of NaCl responded differently. The membrane-bound ATPase of membrane preparations from cells grown and harvested in the absence of NaCl shows an inhibition by both EDTA (32%) and citrate (46%). The same response is seen in the membrane-bound ATPases of membrane preparations from cells grown and harvested in the presence of NaCl; in the presence of EDTA an inhibition of 58% and in the presence of citrate an inhibition of 64% is seen (Table IX). This would indicate similarities between the two membrane-bound ATPases of membrane preparations with the membrane-bound ATPases from cells grown and harvested in the presence of NaCl being slightly more sensitive to EDTA and citrate.

The soluble ATPase shows different responses. In the case of cells grown and harvested in the absence of NaCl, the soluble ATPase activity of membrane preparations is increased 88% and 23% respectively for EDTA and citrate. On the other hand, the soluble ATPase of membrane preparations from cells grown and harvested in the presence of NaCl

shows an inhibition of 82% in the presence of EDTA and an inhibition of 100% in the presence of citrate.

The following conclusions have been made in light of the data presented. First, and foremost, the extremely low activity and apparent insensitivity of ATPase activity to DCCD leads one to believe that the ATPase encountered in this strain of *S. faecalis* is not the membrane-bound proton translocating ATPase seen in other strains of *S. faecalis*. In addition, its ability to be stimulated by NaCl in the membrane-bound form is a contradiction to the published data for proton-translocating ATPases (Schnebli and Abrams, 1970).

It appears after extensive review of the literature that the ATPase activity seen in this strain of *S. faecalis* favors the recently discovered Na^+-ATPase proposed by Kinoshita (Kinoshita et al., 1984). The Na^+-ATPase described by Kinoshita and coworkers exhibits a low activity of 47.1 nmoles of P_i $min.^{-1}$ $mg.^{-1}$ and a resistance to DCCD by the wild-type strain. However, this activity is seen only when the wild-type is grown on a high-Na^+(120mM) and in the presence of the proton conductor CCCP (20μM) (Kinoshita et al., 1984). In addition, a mutant from the wild-type strain when grown on high-Na^+ medium showed a large stimulation when assayed with NaCl (Kinoshita et al., 1984).

It would seem from the above that this strain of *S. faecalis* used in these studies may possess a Na^+-ATPase that has a high activity without the necessity of DCCD, CCCP and high-Na^+ content being present in the medium. In addition, this would also indicate that this strain of *S. faecalis* used in these studies contains a very low percentage of DCCD sensitive H^+-translocating ATPases.

Furthermore, the evidence presented also indicates that in the presence of high NaCl (0.86M) a second isozyme of ATPases appear. This second ATPase shows several differences in the soluble form (F_1) such as an increase in activity in the presence of NaCl and an increase in affinity for the substrate (MgATP). In addition, its sensitivity to trypsin, EDTA, and citrate is characteristic only of the F_1-ATPase from cells grown and harvested in the presence of NaCl. On the other hand, the membrane-bound ATPase from both preparations appears to be the same with the exception to its sensitivity to trypsin and its decrease in affinity when grown and harvested in the presence of NaCl.

The activities of the ATPases in the presence of NaCl/trypsin seem to follow the same assumption as well. For instance, the soluble ATPase of cells grown and harvested in the absence of NaCl shows a large decrease in activity; whereas, the soluble ATPase of cells grown and harvested in the presence of NaCl shows no change when assayed in the presence of NaCl/trypsin. This would indicate a second isozyme of ATPases.

On the other hand, the membrane-bound ATPase of both preparations indicate a stimulation in the presence of NaCl/trypsin which demonstrates no change in ATPases grown in the presence or absence of NaCl.

In conclusion, the above only indicates the possible inducement of a second isozyme of ATPase. It must be pointed out that this change only occurs in the F_1 portion of the ATPase. Since ATPases of bacterial cells do not function _in vivo_, apart from the membrane, it seems that the presence of NaCl does not induce an alteration in the F_1F_0-ATPase in my strain of _S. faecalis_. However, if one considers the hypothesis that this strain of _S. faecalis_ possesses two different types of ATPases (one

sensitive to DCCD and one resistant to DCCD), then the change induced by NaCl is explanable. If the hypothesis is true, what these data would represent are two different membrane-bound ATPase activities, not the F_1 and F_1F_0 activities from the same ATPase. Then the change induced by NaCl would indicate that one membrane-bound ATPase (DCCD-resistant) was altered and the other membrane-bound ATPase (DCCD-sensitive) unaltered when the bacterium was grown and harvested in the presence of NaCl. This hypothesis has support when one considers that the cell membrane isolation procedure employed for this work was designed exclusively to harvest membranes with a high percentage of ATPases bound to the membrane. Precautions throughout this work were taken to insure that dissociation of the F_1 did not occur. However, there is no proof that the F_1 stayed associated to the membrane. I can only speculate that F_1 stayed bound on the design of the cell membrane isolation procedure employed.

It must be reiterated that before a clearcut explanation to these phenomena can be made, further research must be done.

Finally, will this work aid in understanding the effect of NaCl on clostridial isolates used in microbial enhanced oil recovery (MEOR)? We have seen the effect of NaCl on the ATPase(s) of _S. faecalis_ to be one of alteration when _S. faecalis_ is grown and harvested in the presence of NaCl. This alteration has been hypothesized to occur either at the F_1 portion of the ATPase or if there are two isozymes of ATPase the alteration affects one isozyme and not the other.

It is not certain if NaCl would affect the ATPase of _Clostridia_ in the same manner as it does in the ATPase of _S. faecalis_. For instance, _S. faecalis_ is known to grow in at least 6.5% NaCl which indicates it

possesses the genetic machinery to overcome any severe detrimental effect NaCl (6.5%) might have. In fact, 6.5% NaCl is used as a diagnostic tool in the identification of *S. faecalis* (Deibel and Seely, 1974). In contrast, *Clostridia* are not characterized as being able to grow in NaCl although isolates have been found that do grow in NaCl. In addition, *S. faecalis* differs in structure when compared to *Clostridia* (see Table IV, Introduction) and since the effect of NaCl on ATPase occurs at the structural level, it is not certain if the effect would be the same.

One thing is certain: before the effect of NaCl on the ATPase of *Clostridia* can be studied, it must be determined if the *Clostridium* in question has one or more type of ATPases.

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VITA 2

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