The Effect of Sodium Chloride on Beta-Hemolytic Streptococci

Bola Fashola

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1987
THE EFFECT OF SODIUM CHLORIDE ON
BETA-HEMOLYTIC STREPTOCOCCI

A Thesis
Presented to
the Faculty of the Department of Biology
Western Kentucky University
Bowling Green, Kentucky

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Bola Fashola

December, 1987
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THE EFFECT OF SODIUM CHLORIDE ON
BETA-HEMOLYTIC STREPTOCOCCI

Recommended December 7, 1987
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Scott Ford

Approved 1-25-88
(Date)

Dean of the Graduate College
DEDICATION

This thesis is dedicated to my beloved family for their love and their support. Though distant in miles, they are never far from my heart.
ACKNOWLEDGEMENTS

My sincerest appreciation goes to Dr. Larry P. Elliott, my major advisor. A constant source of information and support, he exceeded by far the call of duty. A great part of my success as a graduate student is attributed to his belief in me. I thank him for his guidance and encouragement.

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The drug of choice for the treatment of Streptococcal pharyngitis is penicillin G. However, a common home remedy prescribes the use of salt-water solutions for gargling.

Members of Beta-hemolytic streptococcal groups A, B, and C were isolated from the upper-respiratory tracts of patients diagnosed as having streptococcal pharyngitis. These cultures were obtained from HCA Greenview Hospital (Bowling Green, Kentucky) and used to study the effects of sodium chloride on the isolates.

The minimum inhibitory concentration of sodium chloride was determined for each of eight hospital isolates. Group A streptococci were inhibited at a concentration of 7.2% sodium chloride while Group C streptococci were inhibited at a 7.0% concentration. Group B streptococci were more resistant, and inhibition of growth occurred at 12.0% sodium chloride concentrations.

Scanning electron microscopic studies showed no significant differences in the external structure of cells treated with sodium chloride when compared to non-treated
cells. Despite the lack of changes in the external structure of treated cells, fine structural alterations were observed with transmission electron microscopic studies.

Treatment of the cells with sodium chloride resulted in a condensation of nucleoid deoxyribonucleic acid (DNA) and some loss of ribosomes. These changes were followed by a dissolution of the cytoplasmic cell contents resulting in an intact cell wall with capsule.

Other parameters such as the rate of growth, minimum bactericidal concentrations, DNA content and protein content of cells treated with sodium chloride were examined and compared to control cells.
INTRODUCTION AND LITERATURE REVIEW

The genus *Streptococcus* consists of a large number of biologically diverse bacteria. These organisms belong in the family Streptococcaceae and are gram-positive, cytochrome negative, coccoidal bacteria that usually grow in pairs or chains of various lengths. Most streptococci are facultative anaerobes and non-motile. Some species of *Streptococcus* are primary pathogens for man and animals, while others can produce pathologic processes only under particular conditions (Rotta, 1978). Some are purely saprophytic, forming part of the normal microbial flora of animals including humans.

In spite of the considerable improvements made in the diagnosis, therapy, and prevention of streptococcal infections, streptococcal diseases still present a public health problem which cannot be disregarded. Streptococcal diseases and their effects remain a major health problem world-wide, particularly in developing countries (Quinn, 1982; Wannamaker, 1979).

The identification of streptococci may be achieved by several methods. One of the two most commonly used methods involves testing for group-specific polysaccharide antigens as devised by Rebecca Lancefield (Finegold and Baron, 1986).
The Lancefield method serves as a basis for the differentiation and classification of beta-hemolytic streptococci. The second method, used in conjunction with the first, involves the primary grouping of isolates based on the hemolytic pattern of colonies growing on mammalian blood agar. This method is the most commonly used in clinical laboratories (Finegold and Baron, 1986). Agar containing 5% sheep blood is used most frequently. Most hemolytic streptococci produce a hemolysin that causes clear and complete hemolysis on the blood agar. Organisms that produce this type of hemolysin are said to be beta-hemolytic, and they include pathogenic streptococci such as *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus equisimilis*, and *Streptococcus anginosus* and a number of other species (Coykendall et al., 1987).

An improved understanding of the pattern of interaction of streptococci with the human host has occurred in the last two decades. Until the mid-1960's, there were very few reports of human infections by streptococci other than Group A and the Group D enterococci (Feingold et al., 1966). Beta-hemolytic streptococci are classified into groups A, B, C, D, F, G and a few strains not containing group antigens are occasional etiologic agents of human disease (Finegold and Baron, 1986).

*Streptococci* that contain cell wall antigens of Lancefield group A are known as *Streptococcus pyogenes*. Strains of *S. pyogenes* are usually beta-hemolytic and are
among the most important bacterial pathogens of humans (Shlaes, 1985). Lancefield Group A organisms constitute 48.4% of all beta-hemolytic streptococci isolated from human infections (Pollock and Dahlgreen, 1974). The natural reservoir for *S. pyogenes* are humans (Facklam et al., 1985). The organism, found in the respiratory tract of humans, is transmitted from person to person normally through close contact with asymptomatic carriers (Stamm et al., 1978). Food contaminated with *S. pyogenes* has been known to serve as a vehicle for infection (Facklam et al., 1985). *S. pyogenes* is known to cause impetigo, glomerulonephritis, sinusitis, pyoderma, cellulitis, tonsillitis, bacteremia, erysipelas, osteomyelitis, arthritis, endocarditis, meningitis, lymphadenitis and pharyngitis (Finegold and Baron, 1986; Shlaes, 1985; Facklam et al., 1985).

Extensions of infections from the pharynx into the paranasal sinuses, tonsils, and other parts of the respiratory tract can lead to abscesses, pneumonia, otitis media and other suppurative processes (Finegold and Baron, 1986). Although streptococcal pharyngitis, one of the most commonly acquired streptococcal diseases, is generally thought to be caused by beta-hemolytic streptococci Group A, evidence obtained from various investigations has proven other beta-hemolytic streptococci are causative agents of pharyngitis (Chretien et al., 1979; Benjamin and Perriello, 1976; Ferrier and Blair, 1977; Forrer and Ellner, 1979).

Group B beta-hemolytic streptococci, which comprise
19.9% of all clinically isolated beta-hemolytic streptococci (Pollock and Dahlgreen, 1974), have gained increasing significance as human pathogens over the last two decades (Eickhoff et al., 1964; Yow, 1974). Microorganisms that belong in this group are known causes of meningitis, pneumonia, and severe genitourinary tract infections (Jewes and Jones, 1986; Leland et al., 1978; Tapsall, 1986; Wilkinson, 1978). These organisms are also considered the leading cause of neonatal sepsis in many U.S. hospitals (Shlaes, 1985; Szilagyi et al., 1978; Merritt and Jacobs, 1978) and cause serious infections in immunocompetent patients. The early stage of neonatal sepsis is usually respiratory distress and mortality rates range from 30% to 80% (Jewes et al., 1986). Upper respiratory tract infections due to Group B streptococci have received little attention until recent years. In a study performed by Chretien et al., (1979), the presence of Group B Streptococci in the throats of patients suffering from pharyngitis was determined, and the role of the bacterium as a causative agent for the disease was evaluated. From 1,110 throat cultures obtained, 38 patients (3.4% of the throat cultures) had Group A Streptococci present at the time of clinical pharyngitis. However, 49 patients (4.4% of the throat cultures) were found to carry Group B beta-hemolytic S. agalactiae. The representative organism for Lancefield Group B streptococci in humans is Streptococcus agalactiae. Approximately 11% of all beta-hemolytic streptococci
isolated from humans are classified as Group C (Pollock and Dahlgreen, 1974). These streptococci produce infections similar to those of Group A (Shlaes, 1985; Feingold, 1966). Although considered to be of low pathogenicity, Group C streptococci may cause fatal diseases such as acute endocarditis (Finnegan et al., 1974). Group C includes three streptococcal species. Of these three, Streptococcus equisimilis is isolated most often from human infections and has been implicated as a cause of exudative pharyngitis (Benjamin, 1976; Chretien et al., 1979).

Group F streptococci (S. anginosus) and Group G streptococci, which have no universally accepted species designation, have also been isolated from the pharynx. Pharyngeal carriage of Group G has been reported in 23% of healthy persons and 12% of those with pharyngitis; however, nonsuppurative sequelae associated with Group A has not been reported (Facklam and Carey, 1985).

Streptococcal pharyngitis (strep throat) is one of the most frequently encountered streptococcal diseases, and penicillin G is usually the drug of choice for treatment. Despite appropriate penicillin therapy, a carrier state is believed to develop in 19% to 37% of patients (Facklam and Carey, 1985; Kaplan et al., 1981; Shlaes, 1985). While penicillin is used clinically for treatment, sodium chloride is the indigenous home remedy used world-wide for treatment of streptococcal pharyngitis. The Kaiser Permanente Health Education Department recommends gargling with warm salt
water every two hours (Griffith, 1987). The salt water solution is prepared by dissolving 0.25 teaspoon of sodium chloride in 8 oz of warm water. The solution consists of approximately 0.72% sodium chloride. A survey of students attending Western Kentucky University showed that on the average, one tablespoon of salt was dissolved in an eight ounces of water and used for gargling. This amount constitutes about a 2.4% concentration of sodium chloride. Despite the popularity of the use of sodium chloride as a home remedy, no data on the susceptibility of beta-hemolytic streptococci to sodium chloride are available.

The purposes of this study were: (1) to determine whether beta-hemolytic streptococci Groups A, B, and C are indeed susceptible to sodium chloride; (2) to determine the minimum concentrations of sodium chloride that would result in inhibition; (3) to study the effects of sodium chloride on the growth of streptococci; (4) to evaluate the effects of sodium chloride on the morphology of streptococcal cells of Groups A, B, and C; (5) to evaluate the effects of sodium chloride on the fine structure of these streptococci.
MATERIALS AND METHODS

CULTURE ISOLATES

All clinical strains of beta-hemolytic streptococci were upper-respiratory isolates obtained from HCA Greenview Hospital in Bowling Green, Kentucky. A total of eight strains of streptococci were obtained. Each strain was Gram-stained and tested for catalase production. The organisms, which were Gram-positive cocci and catalase negative, were streaked for isolation on commercially obtained sheep blood agar (Gibco Laboratories, Madison, Wisconsin) and observed for hemolysis. The beta-hemolytic streptococci obtained were serologically grouped using a Streptex latex agglutination kit (Wellcome Reagents Div., Burroughs Wellcome Co., Triangle Park, N.C.). Of the eight strains obtained, three were identified as Streptococcus pyogenes (Group A), three as Streptococcus agalactiae (Group B), and two as Streptococcus equisimilis (Group C).

The cultures were maintained on Tryptic Soy Agar (Difco Laboratories, Detroit Michigan) and Tryptic Soy Broth (Difco Laboratories, Detroit, Michigan) with an incubation period of 18-24 h at 37 C under aerobic conditions. The cultures were transferred bi-weekly and stored at 5 C.
McFARLAND NEPHELOMETER STANDARDS

A set of 10 new, capped 16 X 100 mm test tubes was thoroughly cleaned and rinsed. A sulfuric acid solution was prepared at a concentration of 1.0%, as was a 1.0% aqueous solution of barium chloride. The test tubes were labeled 1 to 10 and designated amounts of the two solutions were added to each test tube as shown in Table 1 to make a total volume of 10 ml per tube (Finegold and Baron, 1986; Hendrickson, 1985). The test tubes were tightly capped, sealed with Parafilm, and refrigerated. The McFarland standards were transferred to room temperature 1-2 h before use.

INOCULA

A standard inoculum for each organism was prepared by inoculating 1 or 2 loopfuls of culture into 10 ml of Tryptic Soy Broth (TSB) which was incubated for 18-24 h at 37 C. These cultures were then adjusted to a turbidity visually comparable to a McFarland standard of 1.0; which corresponds to a concentration of approximately 3x10^8 microorganisms per ml. The cultures were further diluted 1:500 with TSB, resulting in a standard inoculum containing approximately 10^5.
TABLE 1. Preparation of McFarland Nephelometer Standards.

<table>
<thead>
<tr>
<th>Tube Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
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<tr>
<td>Barium Chloride (ml)</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Sulfuric Acid (ml)</td>
<td>9.9</td>
<td>9.8</td>
<td>9.7</td>
<td>9.6</td>
<td>9.5</td>
<td>9.4</td>
<td>9.3</td>
<td>9.2</td>
<td>9.1</td>
<td>9.0</td>
</tr>
<tr>
<td>Cell Density (X10 /ml)</td>
<td>3</td>
<td>6</td>
<td>9</td>
<td>12</td>
<td>15</td>
<td>18</td>
<td>21</td>
<td>24</td>
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to $10^6$ organisms per ml.

MINIMUM INHIBITORY CONCENTRATION

The susceptibilities of Groups A, B, and C streptococci were determined using a broth macrodilution method (Finegold and Baron, 1986; Jones et al., 1985). One ml of the standard inoculum was added to an equal volume of a series of serially diluted concentrations of sodium chloride solutions ranging from 24% to 1.5% (w/v). The sodium chloride solutions were prepared in TSB. One ml of standard inoculum was added to an equal volume of TSB; this served as a growth control (Figure 1). The test tubes of inoculated broth were incubated at 37 C for 18-24 h and checked for turbidity. The lowest concentration of sodium chloride that inhibited growth of the streptococci, determined by a lack of visual turbidity, and the highest concentration at which the streptococci grew suggested a range of the minimum inhibitory concentration (MIC).

Sodium chloride concentrations ranging from the highest concentration of sodium chloride with visible turbidity to the lowest concentration without visible turbidity were prepared in increasing concentrations of 0.2% in an effort to obtain a more exact concentration of inhibition. This procedure was performed in eight replications for each organism.
FIGURE 1. Minimum Inhibitory Concentration Determination.
1 ml of control broth and NaCl concentrations.

1 ml of the standard inoculum (1x10⁶ CFU/ml) was added to each.

INCUBATION (18-24 h) 0.001 ml of control was subcultured for colony count.

The lowest concentration of NaCl with no visible turbidity was designated the MIC.
MINIMUM BACTERICIDAL CONCENTRATION

The minimum bactericidal concentrations (MBC) of sodium chloride were determined by transferring 0.1 ml from test tubes that showed visible turbidity to Tryptic Soy Agar plates. The plates were incubated overnight at 37 C, and the number of colonies growing on each plate was determined. The concentration of sodium chloride that resulted in a 99.9% kill of the microorganisms was designated the minimum bactericidal concentration (Shoenkneckt et al., 1985)(Figure 2).

GROWTH CURVES

The effect of sodium chloride on the growth patterns of \textit{S. pyogenes}, \textit{S. agalactiae}, and \textit{S. equisimilis} was determined by growth curves. Standard inocula of all three groups were prepared by inoculating 5 ml of broth stock cultures into 15 ml of TSB. After overnight incubation at 37 C, the standard inoculum for each organism was diluted with sterile TSB until a 48% transmission value was obtained using a Bausch and Lomb Spectronic 20 spectrophotometer.

A volume of 5 ml of the adjusted \textit{S. pyogenes} inoculum was transferred into 20 ml of TSB, which served as a
FIGURE 2. Minimum Bactericidal Concentration Determination.
0.1 ml was subcultured from MIC tubes that lacked visual turbidity.

INCUBATION (18-24 h)

CFU were determined from concentrations that allowed less than 0.1% of 5x10^5 CFU/ml.
control, and into 20 ml of 7.2% sodium chloride solution prepared in TSB, which served as the experimental. Five ml of the adjusted S. agalactiae inoculum were added to 20 ml of TSB and 20 ml of TSB containing 12.0% sodium chloride. The procedure was repeated using S. equisimilis with a TSB control but a 7.0% sodium chloride solution.

The absorbance of each of the controls and experimental cultures was taken immediately after inoculation. Subsequent absorbance readings of the control and experimental cultures were taken at 1 h intervals for 13 h. Absorbance readings were taken at 620 nm. The absorbance obtained from such readings was plotted on semi-logarithmic paper to derive a growth curve for each of the streptococcal cultures at the various sodium chloride concentrations.

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopic (SEM) studies were performed using the cultures obtained from the minimum inhibitory concentration experiments. The sodium chloride concentrations in TSB that allowed sufficient growth of cells were chosen for SEM procedures.

The broth cultures were collected and centrifuged at 4,000 X g for 10 min. The resulting pellets were washed twice, each time for 15 min, in a 0.2M cacodylate buffer at a pH of 6.5. The washed cells were then fixed in a 1%(v/v)
solution of glutaraldehyde prepared in cacodylate buffer. Fixation was performed overnight at 5°C (Amako and Umeda, 1976). After fixation, the cells were again centrifuged and the pellets washed in cacodylate buffer in two consecutive 15-min periods. The fixed cells were then dehydrated sequentially in 20%, 35%, 50%, 60%, 70%, 80%, and 90% ethanol. The cells were dehydrated at each concentration for 10 min, and finally with two changes of absolute ethanol for 10 min each (Amako and Umeda, 1976).

Glass coverslips were attached to aluminum stubs with Elmer's glue. After drying, the coverslips were trimmed around the edges to obtain approximately the same circumference as the aluminum stubs. The coverslips were each coated with polylysine (Mazia et al., 1975; Weiss, 1984), and the excess polylysine was rinsed off with water. One drop of the cells suspended in 100% ethanol was added to each polylysine-coated coverslip. The thin layer of cells was air dried and coated with a gold-palladium alloy (Au 60%, Pd 40%) using an EMSCOPE SB 250 sputter coater. The coated cells were examined with a Super IIIA ISI Scanning Electron Microscope. Scanning electron micrographs were taken using polaroid type 52 polapan film.
The streptococcal cells used for transmission electron microscopic (TEM) studies were also obtained from the minimum inhibitory concentration experiments. The cells were washed, pelleted, and fixed in glutaraldehyde as described previously for SEM studies. The fixed cells were washed twice in 0.2M cacodylate buffer and post-fixed in 1.0% osmium tetraoxide (osmic acid, OsO₄) for 2 h. The cells were washed twice for 15 min and covered with melted agar tempered to 55-57 °C. The cell-agar mixture was left to solidify and then cut into 2 mm blocks. The cell-agar blocks were dehydrated in a graded series of ethanol prior to infiltration. A volume of Spurr low viscosity embedding plastic was added to the 100% ethanol cell suspension in a 1:3 mixture. After 2 h, an amount of Spurr plastic sufficient to make a 2:2 mixture of plastic and ethanol was added and infiltrated overnight. This was followed by a 3:1 infiltration mixture for 2 h. Finally, the cells were infiltrated in 100% Spurr plastic twice, each time for 2 h. The cells, after infiltration, were embedded in Spurr using Beem gelatin capsules and polymerized at 70 °C for 8 h. The polymerized capsules were trimmed at the tip to the shape of a trapezoid, and ultra-thin sections were prepared using a Sorval ultra-microtome. The sections were collected with 200-mesh copper grids and examined with a Zeiss EM9 S-2 Transmission Electron Microscope.
PROTEIN ASSAY

The media in which the streptococcal cells were cultured were assayed for protein employing the Biorad micro-assay procedure (Biorad Chemical Division, Richmond, California). Control cultures of S. pyogenes and S. equisimilis cells were prepared in TSB, while the experimental cultures were prepared by inoculation of TSB containing 4.5% and 6.5% sodium chloride; experimentals. S. agalactiae controls were prepared by culturing the cells in plain TSB, while the experimentals were prepared by inoculation of TSB containing 7.5% and 10.5% NaCl.

The overnight cultures were diluted with TSB to attain, approximately, a standard optical density for controls and experimentals. The adjusted overnight cultures were vortexed, and 5 ml were removed from each test tube. The removed volumes were then centrifuged three times each at 4,000 X g for 10 min, and the supernatant was retained. The supernatant, now devoid of cells, was assayed for protein using the Biorad micro-assay procedure.

From the supernatant of each concentration of sodium chloride in which the cells were cultured, 4 ml were transferred into clean, oven-dried test tubes. Sterile TSB and TSB containing the various concentrations of sodium chloride were used as reagent blanks from which 4 ml were placed into each clean tube. To 4 ml of the supernatants
and blanks, 1 ml of a Biorad dye reagent concentrate was
added. The mixture was vortexed lightly and allowed to
develop for a period of 45 min. The absorbance of each of
the supernatants was measured at 595 nm versus the reagent
blank of corresponding sodium chloride concentrations. A
standard curve of the absorbance of crystalline bovine
albumin was plotted from which the concentration of protein
in the control and experimental supernatants was determined.

DNA ASSAY

Overnight cultures of streptococcal cells grown in TSB
at various concentrations of sodium chloride and control
cells grown in broth containing no added sodium chloride
were obtained. The turbidity of each of the cultures was
equated by diluting with TSB and measuring the optical
density with a spectrophotometer. From each adjusted
culture, 5 ml were removed and centrifuged at 4,000 X g for
10 min. The supernatants were saved, and the cells were
discarded. Calf thymus DNA (Difco Laboratories, Detroit,
Michigan) was used as a DNA standard.

A diphenylamine reagent was used for detection of DNA
(Herbert et al., 1971). The commercially obtained
diphenylamine (Sigma Chemical Co. St. Louis, Missouri) was
recrystallized (Ault, 1987) with petroleum ether (Figure 3).
The reagent was prepared by dissolving 1.5 g of the
Diphenylamine

Dissolved in hot petroleum ether and filtered by gravity. Activated charcoal added for color absorbance.

Filter containing diphenylamine and soluble impurities.

Insoluble material retained by filter. Discard.

Set aside to cool for crystallization. Crystals collected by suction filtration.

Filtrate containing soluble impurities. Discard.

Crystals of diphenylamine retained by filter. Air dried. Melting point determined.
recrystallized diphenylamine in 100 ml of re-distilled glacial acetic acid and adding 1.5 ml of concentrated sulfuric acid ($H_2SO_4$). The reagent was stored in the dark when not in use.

A measured volume of each sample (2.5 ml) was mixed with 2 ml of the diphenylamine reagent. Standards containing known amounts of DNA, plain TSB, and various concentrations of sodium chloride in TSB were prepared in the same manner as reagent blanks. The test tubes were placed in a boiling water bath for 15 min after which the absorbance was measured at 600 nm with a spectrophotometer standardized against the reagent blanks. The amount of DNA present in each sample was deduced from the values obtained with the DNA standards (Herbert et al., 1971).

STATISTICAL ANALYSIS

Statistical analyses of all data obtained were performed using a randomized complete block design. This design allows for a two-way classification of data into blocks and treatments when sources of variation other than treatment effects are present. The different strains of each species of bacteria were classified into a statistical block; thus, the variability attributable to the difference in strains was measured and excluded from the experimental error (Steel and Torrie, 1980). Also, differences among
treatment means will contain no contribution attributable to variation among strains.

The Duncans Multiple Range test was used for comparison of treatment means and controls and to determine the level of significance of differences among all means.
RESULTS

MINIMUM INHIBITORY CONCENTRATIONS

The minimum inhibitory concentrations of sodium chloride for the streptococcal organisms tested are shown in Table 2. All strains of *S. pyogenes* obtained were inhibited at a total sodium chloride concentration of 7.2%. *S. agalactiae* strains tested showed a variation in the amount of sodium chloride required for inhibition of growth. Two strains of *S. agalactiae* were inhibited at 12.0% while the third strain was inhibited at a 13.0% sodium chloride concentration. Both strains of *S. equisimilis* were inhibited at a concentration of 7.0%.

MINIMUM BACTERICIDAL CONCENTRATIONS

Bactericidal activity was demonstrated by sodium chloride at a concentration of 9.0% for *S. pyogenes* (Table 3). The minimum bactericidal concentration of sodium chloride for *S. equisimilis* was 8.0%. In the case of *S. agalactiae* however, sodium chloride did not demonstrate any
<table>
<thead>
<tr>
<th>Isolates Tested</th>
<th>Strain #</th>
<th>MIC (% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pyogenes</td>
<td>I</td>
<td>7.2</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>II</td>
<td>7.2</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>III</td>
<td>7.2</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>I</td>
<td>12.0</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>II</td>
<td>12.0</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>III</td>
<td>13.0</td>
</tr>
<tr>
<td>Streptococcus equisimilis</td>
<td>I</td>
<td>7.0</td>
</tr>
<tr>
<td>Streptococcus equisimilis</td>
<td>II</td>
<td>7.0</td>
</tr>
</tbody>
</table>
TABLE 3. Minimum Bactericidal Concentrations of Sodium Chloride Tested Against Streptococcal Isolates.

<table>
<thead>
<tr>
<th>Isolates Tested</th>
<th>Strain #</th>
<th>MBC (% NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus pyogenes</td>
<td>I</td>
<td>9.0</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>II</td>
<td>9.0</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>III</td>
<td>9.0</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>I</td>
<td>25.5*</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>II</td>
<td>25.5*</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>III</td>
<td>25.5*</td>
</tr>
<tr>
<td>Streptococcus equisimilis</td>
<td>I</td>
<td>8.0</td>
</tr>
<tr>
<td>Streptococcus equisimilis</td>
<td>II</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Cell viability was observed at this concentration.
bactericidal activity; viable cells were present in as high as 25.5% total sodium chloride concentration.

GROWTH CURVES

The typical growth pattern of *S. pyogenes* (Group A) in TSB is shown in Figure 4. The organism had approximately a 2-h lag phase. There appears to be a temporary decrease in the number of organisms followed by a gradual increase in the rate of growth and multiplication. The lag phase is succeeded by the exponential growth or logarithmic phase during which the organism attains a steady or balanced rate of growth resulting in a rapid increase in cell mass. This phase lasted about 3 h for *S. pyogenes*. The logarithmic phase is followed by the stationary phase during which the reproductive and death rates become equivalent. On examining the experimental growth curve of *S. pyogenes* cells inoculated into TSB containing 7.2% sodium chloride, a different pattern was observed. The various phases observed with the control cells were absent in the experimental cells. These cells appeared to remain in a continuous lag phase (Figure 4).

The growth curve of *S. agalactiae* in TSB (controls) showed a pattern similar to the growth curves of *S. pyogenes* controls except for a shorter lag phase (1 h) (Figure 5). The experimental cells once again showed no apparent growth.
FIGURE 4. Growth curve of *Streptococcus pyogenes* control and experimental cells grown in 7.2% (w/v) NaCl-TSB.
○ Control

▲ Cells grown in 7.2% NaCl

![Graph showing absorbance over time.](graph.png)
There was an initial decline in the number of organisms present followed by a leveling off of activity (Figure 5).

The growth patterns of control and experimental cells of *S. equisimilis* are shown in Figure 6. The results are similar to those obtained with *S. pyogenes* and *S. agalactiae*.

SCANNING ELECTRON MICROSCOPY

Studies conducted with a scanning electron microscope showed no significant differences in the external structure of control and experimental cells (Figs. 7-9). The cells appeared turgid, hence, the spherical morphology of the cells was maintained.

TRANSMISSION ELECTRON MICROSCOPY

Despite the lack of noticeable morphological changes in experimental cells examined with a scanning electron microscope, changes in cells treated with sodium chloride were observed with a transmission electron microscope.

The bulk of the cytoplasm of control cells was filled with densely staining ribosomes in a lighter matrix. The nucleoid lacked any distinct margin and appeared as a region of filamentous and extremely fine material (deoxyribonucleic
FIGURE 5. Growth curve of *Streptococcus agalactiae* control and experimental cells grown in 12.0% (w/v) NaCl-TSB.
○ Control
△ Cells grown in 12.0% NaCl

![Graph showing absorbance over time](image)

Absorbance (620nm) vs. Time (hours)
FIGURE 6. Growth curve of Streptococcus equisimilis control and experimental cells grown 7.0% (w/v) NaCl-TSB.
○ Control
△ Cells grown in 7.0% NaCl

ABSORBANCE (620nm)

TIME (hours)
FIGURE 7. (A) Scanning electron micrograph of *Streptococcus pyogenes* control cells grown in TSB. X15,000. (B) Scanning electron micrograph of *S. pyogenes* experimental cells cultured in 6.5% NaCl-TSB. X10,700.
DARK DOCUMENTS

"May not film well."
FIGURE 8. (A) Scanning electron micrograph of *Streptococcus agalactiae* control cells grown in TSE only. X10,500. (B) Scanning electron micrograph of *S. agalactiae* experimental cells cultured in 11.5% NaCl-TSE. X15,600.
DARK DOCUMENTS

"May Not Film Well."

FIGURE 9. (A) Scanning electron micrograph of *Streptococcus equisimilis* control cells grown in TSB. X10,600. (B) Scanning electron micrograph of *S. equisimilis* experimental cells cultured in 5.5% NaCl-TSB. X15,700.
DARK DOCUMENTS

"May not film well."

acid) lying in the center of the cell (Clawson and Dajani, 1970; Dajani, 1972). Mesosomes were frequently observed in the control cells.

A large number of bacteria from each sample were examined in order to determine the extremes of change for the treated cells. The nucleoid of the treated cells became more prominent with an enlargement of the clear zone and dense aggregation of the DNA (Figures 10-25). The pattern of ribosomal distribution also became altered in the treated cells. Regions of cytoplasm which became devoid of identifiable ribosomes appeared as darkened areas (Figure 24). Mesosomes observed in control cells were not present in treated cells.

One of the most prominent features displayed by treated cells was abortive attempts at division (Figures 11-25). These were in the form of membrane formation at the plane of division. The formed membranes appeared to lack orientation in their extensions. This resulted in the occurrence of several incomplete divisions which led to large, multi-segmented, and misshapen cells. The final stage observed in these cells was a process of dissolution of the entire cytoplasmic contents, causing the occurrence of bacterial ghosts composed of intact cell wall and capsule which lacked any organized internal structure.
FIGURE 10. (A) Transmission electron micrograph of *Streptococcus pyogenes* strain #1 control cells (grown in TSB only). (The nucleoid is central, composed of fine, filamentous DNA. The cytoplasm contains darkly staining ribosomes which give the cells rough appearance. The presence of mesosomes is indicated by arrows.) X42,000. (B) *S. pyogenes* strain #1 experimental cells grown in 6.5% NaCl-TSB. [Condensation of the DNA has occurred, causing an expansion of the clear zone and resulting in greater prominence of the nucleoid region (arrow).] X40,000.
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WELL.
FIGURE 11. (A) Abortive attempts at cell division by S. pyogenes strain #1. X40,000. (B) S. pyogenes strain #1 experimental cells demonstrating internal clearing of the cytoplasmic contents, leading to the occurrence of a bacterial ghost. X40,000.
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[Handwritten text]
FIGURE 12. (A) Control cells of *S. pyogenes* strain #2. (The nucleoid lacks any distinct margins or limiting membranes.) X40,000. (B) Experimental cells of *S. pyogenes* strain #2 (in 6.5% NaCl-TSB). [The cells demonstrate dense aggregation of nucleoid DNA (arrow). X40,000.]
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FIGURE 13. (A) Experimental cells of *S. pyogenes* strain #2 in 6.5% NaCl-TSB showing extensions of membrane in the cytoplasm. X40,000. (B) *S. pyogenes* strain #2 experimental cells (6.5% NaCl-TSB) showing complete dissolution of the cytoplasm, with intact cell wall. X40,000.
Dark

Documents

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Well.
FIGURE 14. (A) Control cells of *S. pyogenes* strain #3. Single, non-viable cell is observed in the death phase. Note that the cells are undergoing different stages of growth. The presence of mesosomes is indicated by arrows.) X40,000. (B) Experimental cells of *S. pyogenes* strain #3 (6.5% NaCl-TSB). [The cytoplasm appears to be pulled from the cell wall as compaction of the cell begins. Condensation of the nucleoid material is apparent (arrow).] X40,000.
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FIGURE 15. (A) *S. pyogenes* strain #3 in 6.5% NaCl-TSB showing erratic extensions of membranes, resulting in unusually large and misshapen cells. X40,000. (B) Experimental cells of *S. pyogenes* strain #3 undergoing dissolution. [Also, note extension of membranes outside a cell in which dissolution of its contents is occurring (arrow).] X40,000.
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FIGURE 16. (A) Streptococcus agalactiae strain #1 control cells grown in TSB. Mesosome (arrow) is apparent, and the nuclear material is fine and filamentous. X100,000. (B) S. agalactiae cells grown in 11.5% NaCl-TSB. (Nucleoid DNA is compacted into dense aggregates.) X40,000.
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FIGURE 17. (A) *S. agalactiae* strain #1 experimental cells (11.5\% NaCl-TSB). (Abortive attempts at cell division are demonstrated by membrane extensions which result in partially segmented cells.) X40,000. (B) Internal clearing of the cytoplasmic contents is observed in experimental cells of *S. agalactiae* strain #1 in 11.5\% NaCl-TSB. X40,000.
Dark Documents

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FIGURE 18. (A) Control cells of S. agalactiae strain #2. (The presence of a mesosome is indicated by an arrow.) X100,000. (B) Experimental cells of S. agalactiae strain #2 grown in 11.5% NaCl-TSB showing compaction of the nucleoplasm. X40,000.
Dark Documents

"May not film well."

WELL.
FIGURE 19. (A) Multi-segmented cells of *S. agalactiae* strain #2 (grown in 11.5% NaCl-TSB) resulting from attempts at cell division. X40,000. (B) Bacterial ghosts of *S. agalactiae* strain #2 cells cultured in 11.5% NaCl-TSB. X40,000.
DARK DOCUMENTS

"May not film well."

WELL.
FIGURE 20. (A) *S. agalactiae* strain #3 control culture. (The nucleoid has no delineating membrane, thus is unrestricted,) X40,000. (B) Experimental cells of *S. agalactiae* strain #3 cultured in 11.5% NaCl-TSB. There appears to be a condensation of the cytoplasm as well as the nucleoid (arrow). X40,000.
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FIGURE 21. (A) Membranous extensions of *S. agalactiae* strain #3 grown in 11.5% NaCl-TSB results in a pinching from an unusually large cell. X40,000. (B) Experimental cells of *S. agalactiae* strain #3 displaying internal clearing. (The cells were cultured in 11.5% NaCl-TSB.) X40,000.
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FIGURE 22. (A) *Streptococcus equisimilis* strain #1 control culture. (The nucleoid material appears to be spread throughout the cell.) X40,000. (B) *S. equisimilis* cells grown in 6.5% NaCl-TSB showing aggregation of the nucleoid DNA. Strain #1. X40,000.
Dark Documents

“May not film well.”
FIGURE 23. (A) Experimental cells of *S. equisimilis* strain #1 (6.5% NaCl-TSB) showing extensions of membranes in attempts at fission. X40,000. (B) *S. equisimilis* experimental culture showing various stages of cell modification due to treatment with sodium chloride. (Note the cell in which dissolution is beginning to occur and the cell in which clearing has occurred.) Strain #1. X40,000.
DARK DOCUMENTS

"May Not Film Well."
FIGURE 24. (A) Control culture of *S. equisimilis* strain #2 showing the unrestricted and filamentous form of the nucleoid material. X40,000. (B) Experimental culture of *S. equisimilis* in 6.5% NaCl-TSB. There is a drastic expansion of the clear zone with condensation of the DNA. [Loss of ribosomes is evidenced by darkened areas (arrows).] Strain #2. X40,000.
DARK DOCUMENTS

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"DARK DOCUMENTS

"May NOT Film WELL."
FIGURE 25. (A) Unsuccessful attempts by *S. equisimilis* cells (strain #2) to divide, result in the formation of membranes that appear to wander about the cytoplasm. (The cells were grown in 6.5% NaCl-TSB.) X70,000. (B) *S. equisimilis* experimental cells undergoing dissolution of the cytoplasmic contents. (The cells (strain #2) were cultured in 6.5% NaCl-TSB.) X95,000.
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S. pyogenes and S. equisimilis were cultured in TSB, TSB with 4.5% NaCl, and TSB containing 6.5% NaCl. S. agalactiae cells were cultured in plain TSB, TSB with 7.5% NaCl, and TSB with 10.5% NaCl.

On examining the amount of protein present in the growth media of the organisms, a specific trend was observed. More protein was detected in the medium of S. pyogenes and S. equisimilis cells grown in 4.5% NaCl-TSB than in the medium of control cells. The same result was obtained with S. agalactiae cells in 7.5% NaCl versus control cells. Examination of the data obtained with S. pyogenes and S. equisimilis cells grown in 6.5% NaCl, indicated less protein detected in the medium than in the 4.5% NaCl broth. However, the protein contents of the 6.5% NaCl medium appeared greater than that of the control in most cases. The same trend was observed with S. agalactiae cells in 10.5% NaCl. There was less protein in the 10.5% NaCl medium than the 7.5% NaCl medium, which had more protein than the control medium. The 10.5% NaCl medium in turn appeared to have a greater protein content than the control (Tables 4-6).

The amount of protein present in the 7.5% NaCl medium of S. agalactiae was significantly greater than the amounts present in the 10.5% NaCl medium and the control medium.
TABLE 4. Protein Content (ug/ml) of S. pyogenes Culture Media Containing Various Concentrations of Sodium Chloride After Removal of the Bacterial Cells.

<table>
<thead>
<tr>
<th>Treatment (% NaCl in TSB)</th>
<th>Strain #</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Control (0.5)</td>
<td></td>
<td>5.22</td>
<td>20.78</td>
<td>9.20</td>
</tr>
<tr>
<td>4.5</td>
<td></td>
<td>0.44</td>
<td>9.56</td>
<td>9.20</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>13.11</td>
<td>13.13</td>
<td>11.86</td>
</tr>
</tbody>
</table>

Each value represents a mean of two trials.

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2.45 n.s.</td>
</tr>
<tr>
<td>Strain</td>
<td>1.66 n.s.</td>
</tr>
</tbody>
</table>

n.s. = non-significant differences.
TABLE 5. Protein Content (ug/ml) of *S. agalactiae* Culture Media Containing Various Concentrations of Sodium Chloride After Removal of the Bacterial Cells.

<table>
<thead>
<tr>
<th>Treatment (% NaCl in TSB)</th>
<th>Strain</th>
<th>Strain</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Control (0.5)</td>
<td>3.56</td>
<td>11.89</td>
<td>3.58</td>
</tr>
<tr>
<td>7.5</td>
<td>1.56</td>
<td>11.86</td>
<td>3.58</td>
</tr>
<tr>
<td>10.5</td>
<td>5.22</td>
<td>11.89</td>
<td>7.11</td>
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</tbody>
</table>

Each value represents a mean of two trials.

**Analysis of Variance**

<table>
<thead>
<tr>
<th>Source</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>42.57**</td>
</tr>
<tr>
<td>Strain</td>
<td>3.17 n.s.</td>
</tr>
</tbody>
</table>

** = highly significant differences.

n.s. = non-significant differences.

**Duncan's Multiple Range Test**

- ug/ml of protein in 7.5% NaCl vs control medium **
- ug/ml of protein in 7.5% NaCl vs 10.5% NaCl medium **
- ug/ml of protein in 10.5% NaCl vs control medium n.s.
TABLE 6. Protein content (ug/ml) of *S. equisimilis* Culture Media Containing Various Concentrations of Sodium Chloride After Removal of the Bacterial Cells.

<table>
<thead>
<tr>
<th>Treatment (% NaCl in TSB)</th>
<th>Strain #</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>5.22</td>
<td>17.56</td>
</tr>
<tr>
<td>4.5</td>
<td></td>
<td>16.89</td>
<td>16.44</td>
</tr>
<tr>
<td>6.5</td>
<td></td>
<td>9.56</td>
<td>11.86</td>
</tr>
</tbody>
</table>

Each value represents a mean of two trials.

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>F-Value</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.94 n.s.</td>
</tr>
<tr>
<td>Strain</td>
<td>1.48 n.s.</td>
</tr>
</tbody>
</table>

n.s. = non-significant differences.
However, the difference in the amount of protein present in the 10.5% NaCl medium was not significantly greater than the amount present in the control medium (Table 5). A randomized complete block design and Duncan's multiple range test were used for statistical analyses (Steel and Torrie, 1980).

Statistical analysis of these data led to the conclusion that the differences were not significant (with the exception of *S. agalactiae*).

DNA ASSAY

No specific trend was observed in terms of the amounts of deoxypyrinucleic acid (DNA) present in the culture media of control and experimental cultures of the streptococci tested. There appeared to be no significant differences in the amount of DNA detected (Tables 7-9).
TABLE 7. DNA Content (ug/ml) of *S. pyogenes* Culture Media Containing Various Concentrations of Sodium Chloride Following Removal of the Bacterial Cells.

<table>
<thead>
<tr>
<th>Treatment (% NaCl in TSB)</th>
<th>Strain 1</th>
<th>Strain 2</th>
<th>Strain 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.5)</td>
<td>17.02</td>
<td>6.60</td>
<td>7.98</td>
</tr>
<tr>
<td>4.5</td>
<td>0.00</td>
<td>5.32</td>
<td>5.32</td>
</tr>
<tr>
<td>6.5</td>
<td>11.17</td>
<td>7.98</td>
<td>8.40</td>
</tr>
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</table>

Each value represents a mean of two trials.

Analysis of Variance

<table>
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<tr>
<th>Source</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>2.16 n.s.</td>
</tr>
<tr>
<td>Strain</td>
<td>0.33 n.s.</td>
</tr>
</tbody>
</table>

n.s. = non-significant differences.
TABLE 8. DNA Content (ug/ml) of *S. agalactiae* Culture Media Containing Various Concentrations of Sodium Chloride Following Removal of the Bacterial Cells.

<table>
<thead>
<tr>
<th>Treatment (% NaCl in TSB)</th>
<th>Strain #</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>Control (0.5%)</td>
<td>17.02</td>
<td>15.43</td>
<td>20.74</td>
</tr>
<tr>
<td>7.5</td>
<td>13.30</td>
<td>17.02</td>
<td>15.96</td>
</tr>
<tr>
<td>10.5</td>
<td>19.15</td>
<td>20.21</td>
<td>29.79</td>
</tr>
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</table>

Each value represents a mean of two trials.

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>5.05 n.s.</td>
</tr>
<tr>
<td>Strain</td>
<td>3.01 n.s.</td>
</tr>
</tbody>
</table>

n.s. = non-significant differences.
TABLE 9. DNA Content (ug/ml) of S. equigimilis Culture Media Containing Various Concentrations of Sodium Chloride, Following Removal of the Bacterial Cells.

<table>
<thead>
<tr>
<th>Treatment (% NaCl in TSB)</th>
<th>Strain # 1</th>
<th>Strain # 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.5)</td>
<td>7.98</td>
<td>30.32</td>
</tr>
<tr>
<td>4.5</td>
<td>14.89</td>
<td>18.08</td>
</tr>
<tr>
<td>6.5</td>
<td>20.74</td>
<td>7.98</td>
</tr>
</tbody>
</table>

Each value represents a mean of two trials.

Analysis of Variance

<table>
<thead>
<tr>
<th>Source</th>
<th>F-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.08 n.s.</td>
</tr>
<tr>
<td>Strain</td>
<td>0.18 n.s.</td>
</tr>
</tbody>
</table>

n.s. = non-significant differences.
DISCUSSION

The data obtained from the minimum inhibitory concentration experiments showed a variation in the susceptibility of the three species of streptococci tested to sodium chloride. *Streptococcus agalactiae* demonstrated a higher degree of resistance to sodium chloride than *Streptococcus pyogenes* and *Streptococcus equisimilis*. All strains of *S. pyogenes* tested were inhibited at a sodium chloride concentration of 7.2% while *S. equisimilis* strains were inhibited at 7.0%. Of the three strains of *S. agalactiae* tested, two were inhibited at a sodium chloride concentration of 12.0%; the third strain was inhibited at a total sodium chloride concentration of 13.0%.

One of the criteria most commonly used to distinguish between enterococcal and non-enterococcal streptococci is the ability of enterococci to grow at a 6.5% NaCl concentration and higher (Facklam, 1981). Since the non-enterococcal streptococci tested were capable of growth in the presence of 6.5% NaCl and higher, perhaps less emphasis should be placed on this criterion as a means of identification. An alternative is to test for hydrolysis of L-pyrrolidonyl-B-naphthylamide (PYR). Group A streptococci...
and enterococci uniquely hydrolyze PYR (Wellstood, 1987); these organisms could then be differentiated by performing a bacitracin test.

From a survey of Western Kentucky University students, it was determined that an average of 2.4% NaCl is dissolved in water used for gargling in the event of the occurrence of sore throat. The Kaiser Permanente Health Education Department (Freemont, California) recommends the use of approximately 0.72% NaCl for gargling (personal communication with Griffith, E., Correspondence Editor, Readers Digest). These percentages fall far below the concentration required for inhibition of the streptococcal organisms that cause strep throat. These findings seem to suggest that the use of such concentrations (0.72% - 2.4%) of sodium chloride might simply have a soothing effect on the infected area. Certainly, concentrations of 7.0% - 13.0% should be used in order for inhibition of growth to occur.

The exposure of streptococci to various concentrations of sodium chloride enabled the determination of the MIC. Essentially no growth was observed when growth curves of the streptococci were determined at the obtained MIC. On examining the growth curves of the three species of *Streptococcus*, the experimental organisms appeared to remain in the lag phase of growth. Perhaps this was a continuous attempt at acclimatization. There was no significant decrease in the absorbance of the cultures suggesting that
while there might be a reduction in number of cells, no lysis occurred as a result of treatment with sodium chloride.

The bactericidal concentration of sodium chloride for _S. pyogenes_ was 9.0%; and 8.0% for _S. equisimilis_. _Streptococcus agalactiae_ cells demonstrated higher resistance to sodium chloride. Although inhibition of growth occurred at 12.0% - 13.0% NaCl concentrations, a bactericidal effect was not obtained when tested with sodium chloride concentrations as high as 25.5%; the organisms remained viable.

Since the experimental streptococcal cells were placed in hypertonic solutions, it was expected that dehydration of the streptococcal cells might occur causing changes in the ultrastructure. Scanning electron microscopic studies were conducted on cells cultured at sodium chloride concentrations slightly lower than the MIC. This was in order to obtain a sufficient volume of cells for SEM procedures. Comparison of electron micrographs (SEM) of experimental and control cultures of the streptococci revealed no significant differences in external morphology. The ability of the streptococci to maintain the integrity of the exterior components reflects the stability of these structures and implies the lack of direct involvement in the action of sodium chloride on the cells (Dajani and Wannamaker, 1969; Dajani et al., 1970)

Since SEM studies failed to reveal significant alterations in the external morphology of the streptococci,
possible alteration of fine structures was sought by transmission electron microscopy. The exposure of these microorganisms to concentrations of sodium chloride slightly lower than the MIC led to a series of events which caused a dissolution of the cytoplasmic contents. The first of the series of structural changes observed was a condensation of the nucleoid DNA into dense aggregates. Next was a loss of ribosomes in scattered regions of the cytoplasm, followed by unsuccessful attempts at cell division. These attempts were exemplified by erratic development and extension of membranes resulting in the occasional occurrence of large, partially segmented, and misshapen cells. Finally, there was a dissolution of the nucleoid and the remainder of the cytoplasmic contents. Such dissolution led to the formation of well preserved bacterial ghosts composed of cell wall and capsule. Mesosomes, observed in control cells, were not apparent in any of the experimental cells.

Matheson et al. (1976) showed that most organisms able to adapt to and grow in environments of high sodium chloride concentrations are unable to achieve complete osmotic balance with their environments. Vreeland et al. (1983) suggested that one mechanism by which microorganisms could survive without osmotic balance would be to control water movement. Perhaps these streptococci possess an osmotic mechanism with which they control or inhibit the loss of cell water in high solute concentrations. Such mechanism might involve a tightening of the cell wall, causing a
decrease in permeability and removing most of the cell-associated water (Vreeland et al. 1984). As suggested of Halomonas elongata in a similar study by Vreeland et al. (1984), the increased ionic content of the surrounding environment might cause an increase in the strength of hydrophobic reactions between macromolecules and as a result, tougher and greater structural support against the stresses imposed by a concentration of solutes in the environment. A compaction of the cytoplasmic contents would be expected to have the effect of structuring the cell wall, further decreasing its permeability thus making the cells less susceptible to sodium chloride-induced dehydraton (Vreeland et al., 1984). The role of ion transport phenomena and metabolic adaptations in streptococcal halotolerance has received scant attention (Rosen, 1986). A physiological accommodation could result from the development of an osmoregulatory mechanism induced by sodium chloride and from the intracellular accumulation of organic compounds such as amino acids, polyhydroxy alcohols, or carbohydrates from available organic nutrients (Rosen, 1986).

The loss of ribosomes in scattered regions of the cytoplasm was indicated by darkening of such areas. This suggests a degradation of ribonucleic acid (RNA) and perhaps, subsequently, a cessation of protein synthesis (Clawson and Dajani, 1970).

There was some variation in the degree of the modification of structures from one cell to another within
modification of structures from one cell to another within the same sample. Such findings suggest that the sodium chloride does not act equally on each organism in a culture due to a dose-response phenomenon. Another possibility might be that the streptococcal cells are susceptible to sodium chloride only at certain stages of development.

No significant differences were observed in the amount of protein present in the culture media of experimental and control cells of _S. pyogenes_ and _S. equisimilis_. In the case of _S. agalactiae_, there was a significantly greater amount of protein in the culture medium of experimental cells at 7.5% NaCl concentration as opposed to the amount detected in the media of control and experimental cells grown at a 10.5% NaCl concentration. This might be a result of leakage, perhaps, before the _S. agalactiae_ cells were able to activate the mechanism with which cell permeability is decreased.

There were no significant differences in the amount of DNA present in growth media of control and experimental cultures of all streptococci tested. Since the nucleoid DNA is condensed into aggregates without any signs of fragmentation, there would be very little chance of leakage prior to degradation of the cell contents.
SUMMARY

Several strains of *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus equisimilis* isolated from the upper-respiratory tracts of patients clinically diagnosed of pharyngitis were obtained. These organisms were cultured in the presence of various concentrations of sodium chloride in an effort to determine its effect.

The MIC of sodium chloride for *S. pyogenes* was 7.2%, 7.0% for *S. equisimilis*, and 12.0% - 13.0% for *S. agalactiae*. Growth curve data obtained using cells cultured at these concentrations correlated with the MIC data. Bactericidal activity was observed at 9.0% for *S. pyogenes* and 8.0% for *S. equisimilis*. No bactericidal activity on *S. agalactiae* was observed at concentrations tested; viable cells remained at 25.5% NaCl.

Scanning electron microscopy showed no differences in the ultrastructure of control and experimental cells, but fine-structural changes were observed with transmission electron microscopy. First in a series of events was an aggregation of nucleoid deoxyribonucleic acid and some loss of ribosomes. Erratic membrane extensions ensued in
unsuccessful attempts at division causing the occurrence of multisegmented cells. Next was a dissolution of the cytoplasmic contents which resulted in empty bacterial ghosts with maintained structural integrity.

The growth medium of the streptococcal cells were tested for possible variation in the amounts of protein present as a result of treatment of the cells with sodium chloride. No significant differences were detected in growth media of control and experimental cells of *S. pyogenes* and *S. equisimilis*. The amount of protein detected in the growth medium of *S. agalactiae* containing 7.5% NaCl was significantly greater than the amount present in control and 10.5% NaCl media.

No significant differences were observed in the amount of DNA present in the growth media of control and experimental cells of all streptococci tested.
LITERATURE CITED


