

# Research on Ion Transport Across Microbial Membranes

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Contract No. 14-01-0001-636

UNITED STATES DEPARTMENT OF THE INTERIOR • Stewart L. Udall, Secretary  
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## FOREWORD

This is one of a continuing series of reports designed to present accounts of progress in saline water conversion and the economics of its application. Such data are expected to contribute to the long-range development of economical processes applicable to low-cost demineralization of sea and other saline water.

Except for minor editing, the data herein are as contained in a report submitted by the contractor. The data and conclusions given in the report are essentially those of the contractor and are not necessarily endorsed by the Department of the Interior.

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## ACKNOWLEDGEMENTS

The authors wish to express appreciation to Dr. C. R. Bovell, University of California at Riverside, and Dr. H. P. Silverman, TRW Systems Group, for their guidance during the course of this work. Appreciation is also expressed to J. B. Dittman, A. V. Bocksruker, and M. Porter who rendered invaluable technical assistance.

The present report is the final report of a 2-year study of ion transport across microbial membranes supported by the Office of Saline Water. The objectives of the program are to:

- 1) Elucidate the mechanism by which the bacteria Serratia marcescens and S. marinorubra actively transport cations across their cell membranes
- 2) Determine the reason why these two bacteria, which appear to be similar metabolically but differ in their tolerance to salt transport cations at markedly different rates
- 3) Provide information as to the manner in which the bacteria derive and couple energy for the active transport of cations
- 4) Gain insight into the nature of the carrier system involved in cation transport.

Comparisons of growth characteristics, cation content, and rate of cation transport have been made under a variety of conditions with the two microorganisms.

The transport of cations was determined by observing the changes in the amount of light scattered by resting cell suspensions in which the ionic strength of the external solution was adjusted so as to be hypertonic to the interior of the cell. With the proper physical environment and in the presence of compounds capable of being transported into the cells, deplasmolysis and the resultant decrease in scattered light were observed. This approach was supplemented by the quantitative determination of selected intracellular cations by atomic absorption spectrometry in cells prior to and following the addition of hypertonic and hypotonic salt solutions.

It was found that the choice of growth medium and the method of washing and preparing the resting cells played important roles in determining the amount of intracellular salts remaining within the cells, and consequently in the cells' behavior as observed by the light scattering technique. Both microorganisms tend to accumulate  $K^+$  during growth and to exclude  $Na^+$  against concentration gradients. The amount of  $K^+$  accumulated within the cells is increased, particularly

in S. marinorubra, as the ionic strength is raised in the medium in which the organisms are grown. The same tendency to accumulate  $K^+$  and exclude  $Na^+$  prevails when the cells are properly harvested and washed and subsequently resuspended in hypertonic salt solutions. The amount of  $Mg^{++}$  found in logarithmic phase cells was greater than that found in stationary cells of either microorganism, regardless of growth medium. The concentration of  $Mg^{++}$  in S. marcescens was found to increase significantly when this organism was transferred from low-salt glycerol to 3% NaCl-glycerol medium.

Satisfactory retention of accumulated materials can be achieved during the preparation of these cells by washing them with approximately isotonic salt solutions of either monovalent or divalent cations. Best results, in terms of respiration rates, light scattering behavior, and salt retention, were consistently obtained using isotonic solutions of  $MgCl_2$  as the washing medium. Washing in redistilled water, with the associated osmotic shock, appeared generally to decrease cell respiration and the retention of intracellular cations. The degree of shock is increased as the concentration of intracellular salts increases, and can lead to the lysis of the cells.

Potassium transport occurs at considerably faster rates in resting cells of S. marcescens than in S. marinorubra. Smaller amounts of stored glycogen and lower respiration rates in the latter microorganism suggest that the decreased available energy reserves are responsible for the slower rates of ion transport. If an exogenous energy source such as glucose is added to the resting suspension of S. marinorubra, the rate of  $K^+$  transport is markedly increased, as is the rate of respiration.

Maximum rates of  $K^+$  transport occur in S. marcescens at pH 5.5 and this does not appear to be an exchange of extracellular  $K^+$  for intracellular  $Na^+$  or  $H^+$ . Kinetic studies have shown that at pH 5.5 in the presence of either sodium phosphate or sodium bicarbonate buffer, half maximal velocity of  $K^+$  transport occurred at a  $1.6 \times 10^{-3} M$  concentration of KCl.

The process of  $K^+$  transport is inhibited by a variety of metabolic inhibitors including both glycolytic and respiratory inhibitors, as well as by anaerobiosis. Transport of this cation is, however, insensitive to relatively high concentrations of NaF, a potent inhibitor of glycolysis in higher forms. Although possible reactions of inhibitory compounds with the cell envelope or the transport enzymes themselves cannot be eliminated, these results exclusive of the anomalous behavior of the cells toward NaF, indicate that the process of  $K^+$  accumulation depends upon some aspect of electron transport through the respiratory chain. Furthermore, in these resting cell suspensions, the required energy must ultimately be derived from catabolism of stored glycogen.

## 1. INTRODUCTION

The utilization of membrane processes such as electrodialysis and reverse osmosis for desalination of sea water and the search by the Office of Saline Water for more efficient synthetic membranes for this purpose have made the study of biological mechanisms of ion transport increasingly important. It is expected that useful information derived from a study of biological membrane phenomena will allow the development of more efficient synthetic membranes capable of operating for prolonged periods at low temperatures and pressures.

Bacteria readily lend themselves to a study of ion transport because of their relative morphological simplicity. One of the difficulties in interpreting results of measurements of ion movements in tissues and higher cells is that there are many internal membranes. But in bacteria there is no endoplasmic reticulum, no mitochondria, no nuclear membrane, and the plasma membrane is the only osmotic barrier for small molecular weight solutes. Because of their minute size, there is a large ratio of area to volume, and although the plasma membrane is only about 10 m $\mu$  thick, it represents some 10% of the dry weight of the organism.

Bacteria of one kind or another are capable of growth at salt concentrations ranging from essentially zero to that of concentrated brine. Considerable attention has been given to a study of individual bacterial species having various degrees of salt tolerance or actual salt requirements, ranging from freshwater or terrestrial forms to extreme halophiles. All bacteria accumulate K<sup>+</sup> and exclude Na<sup>+</sup> against concentration gradients, and thus possess an intracellular ionic composition that is generally different from that of their environment. Because of these characteristics, bacteria make excellent models for the study of ion transport phenomena.

Serratia marcescens, together with S. marinorubra, a closely related form which many taxonomists feel should be classified as a variant of S. marcescens,<sup>(5)</sup> offers a unique opportunity for a comparative study of ion transport across bacterial membranes. Differences

stemming from salt tolerance or salt requirement can be studied without introducing problems associated with broad species peculiarities and grossly different metabolic pathways.

At the outset of our work in this field, most of the effort was directed toward developing experimental techniques for using the better understood S. marcescens. These studies were later extended to include the lesser known S. marinorubra. We are now at a point where some comparisons can be drawn between these two bacteria.

## 2. MATERIALS AND METHODS

### 2.1 BACTERIA

The bacteria employed in this study were Serratia marcescens HY and Serratia marinorubra S10 obtained from Dr. W. Belser, University of California at Riverside. S. marcescens was maintained on glycerol agar slants by weekly subculture. Following overnight growth at 30°C, the stock cultures were refrigerated at 4°C and served for subsequent inoculation of experimental and subculturing media. S. marinorubra was maintained by a similar growth and subculturing procedure on glycerol agar slants to which 3% NaCl had been added.

### 2.2 MEDIA

The regular glycerol medium consisted of

Glycerol	20 g
Yeast extract	1 g
Peptone	2 g
Distilled water	1000 ml

The final pH was adjusted to 6.8 to 7.0 by the addition of NaOH.

The 3% NaCl-glycerol medium was prepared by adding 3% NaCl to the above medium prior to adjustment of the pH. Solid media, for maintenance of cultures, were prepared by adding 1% agar to the respective liquid media described above.

### 2.3 CULTURE CONDITIONS

Cultures were grown with shaking at 30°C in either a reciprocal or gyratory shaker. Growth was determined by measuring optical density of the culture at 660 m $\mu$  using a Beckman DB spectrophotometer or a Bausch and Lomb Spectronic 20 spectrophotometer.

### 2.4 PREPARATION OF CELLS FOR LIGHT SCATTERING STUDIES

Cells were harvested by centrifugation at 4°C, washed twice, and resuspended in the desired solutions. The cell suspension was filtered through a double thickness of Whatman No. 1 filter paper and adjusted to

a final optical density of approximately 0.3 at 660 m $\mu$ . In initial experiments, distilled water was used to wash the cells, and 0.05 M tris-(hydroxymethyl) aminomethane (Tris) chloride at pH 6.8 was used for resuspension. Cell suspensions were maintained under aerobic conditions throughout the experimental procedures.

## 2.5 LIGHT SCATTERING MEASUREMENTS

Light scattering measurements were performed in a Model 2000-D Brice-Phoenix Universal Light Scattering Photometer (Phoenix Precision Instrument Co., Philadelphia, Pennsylvania), using the 546 m $\mu$  emission maximum of a mercury arc lamp with the photocell placed at 90 degrees relative to the plane of incident light. Results were recorded on a Sargent Model SRL recorder. Three ml aliquots of appropriate cell suspensions in a Brice-Phoenix 10 x 10 mm (ID) light scattering cuvette were placed in the instrument; a base line was established, after which the desired additions were made with a micro-syringe.

## 2.6 PREPARATIONS OF SAMPLES FOR ATOMIC ABSORPTION ANALYSIS

From 30 to 40 ml aliquots of cell culture contained in polypropylene centrifuge tubes were harvested and washed as described above. The solutions used for washing and resuspending were varied to determine their effect on the retention and recovery of intracellular salts. After the desired treatment and required washing to remove adhering salts that would interfere with the analysis, the cells were centrifuged and the pellet resuspended in 9 ml of either 0.05 M tris (pH 6.8) or redistilled water. One ml concentrated HNO<sub>3</sub> was added to aid in disrupting the cells and to effect the release of intracellular cations. The suspensions were then heated in a boiling water bath for 5 to 10 minutes, after which debris was removed by centrifugation and the supernatant decanted into clean polystyrene tubes for subsequent analysis. Samples were analyzed for Na<sup>+</sup>, K<sup>+</sup>, and Mg<sup>++</sup> on a Model 290 Perkin Elmer atomic absorption spectrometer and results were expressed as milligrams of the particular cation per 100 mg (dry weight) of cells as estimated from the standard curve shown in Figure 1. The standard curve was prepared using cells of S. marcescens and S. marinorubra in the logarithmic growth phase. The cells were washed twice in water and dessicated in a vacuum at 50°C.

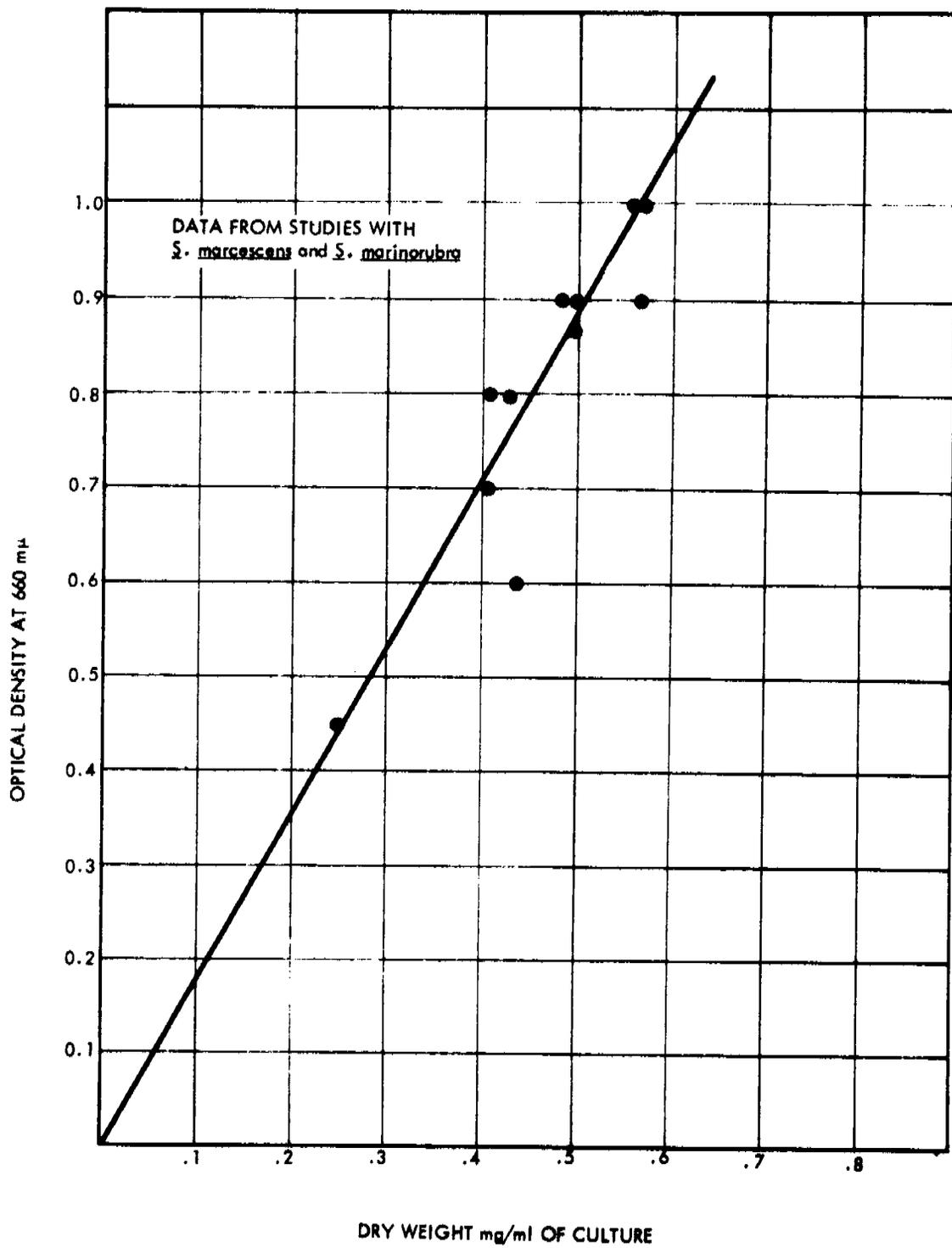


Figure 1. Standard Curve For Estimating Dry Weight of Cells

A rough approximation of the intracellular concentrations of these cations in terms of moles of cation per 1000 grams intracellular water can be made using the following assumptions and factors:

Assume: 1) The dry weight/wet weight ratio = 0.228.

2) The extracellular space = 0.20 ml/gram wet weight of a packed pellet.

These values for E. coli were reported by Schultz, et al. (13)

With the assumption that these figures also apply to S. marcescens and S. marinorubra, known to be similar in size and shape to E. coli, the intracellular molar concentrations of the respective cations can be approximated by multiplying the result expressed in milligrams of cation per 100 milligrams dry weight of cells by the following factors:

$K^+$  -- X 102

$Na^+$  -- X 173

$Mg^{++}$  -- X 164

## 2.7 RESPIRATION MEASUREMENTS

Cells were harvested at the appropriate phase of growth and washed twice with either redistilled water or 0.1M  $MgCl_2$ . The cells were resuspended in 0.05 M tris-HCl, pH 6.8, or other desired solution, and the optical density adjusted to approximately 5.0 at 660 m $\mu$ . Oxygen consumption by 10 ml of cell suspension was measured directly with a Beckman oxygen macroelectrode and Model 160 physiological gas analyzer. Cell suspensions were aerated as required by means of a fritted glass sparger.

## 2.8 GLYCOGEN DETERMINATIONS

Glycogen was determined on aliquots of cells washed twice in distilled water in a modification of a method described by Kerr. (8) The cells were hydrolyzed in boiling 20% KOH. Ethanol was added to precipitate the glycogen and the precipitate was washed once in absolute ethanol. The glycogen was hydrolyzed in 1N HCl at 100°C and the resultant glucose determined with the aid of Glucostat reagent (Worthington Biochemical

Corp.). Results were expressed in terms of milligrams of glycogen per gram of dry cells.

## 2.9 pH MEASUREMENTS

A Corning Model 10 pH meter with expanded scale was used to measure pH.

### 3. RESULTS

#### 3.1 COMPARISON OF GROWTH AND CULTURAL CHARACTERISTICS

The standard 2% glycerol medium chosen for the routine growth of S. marcescens is a common bacteriological medium which has been shown to allow rapid growth of this microorganism. Growth of the organism at 30°C with proper aeration is characterized by the formation of large amounts of pigment as the cells approach the stationary phase of growth. Pigment formation is dependent upon a number of physical parameters, including:

- 1) Temperature, with maximum pigment formation occurring at 30°C
- 2) pH between 6.5 and 7.0
- 3) Sufficient aeration
- 4) Age of culture, with pigment being formed only as the cells approach the stationary phase of growth.

Comparative growth rates of S. marcescens and S. marinorubra in glycerol media reported by Goldner<sup>(6)</sup> showed that the latter microorganism displayed a longer lag period and somewhat slower rate of growth than the former. The final yield of cells was generally considerably less in cultures of S. marinorubra and although some pigmentation was found on slant cultures, no pigment was formed in liquid cultures. The acidic pH of the medium during and following growth of S. marinorubra more than likely is responsible for inhibition of pigment formation.

Although both microorganisms are capable of growth on glycerol, it is evident that the products of metabolism differ either qualitatively and/or quantitatively. It is quite possible that there are some differences in the activity of alternate pathways of metabolism of glycerol which account for the differences observed in cultural characteristics. The final pH of cultures of S. marinorubra was consistently between 4 and 5, while that in cultures of S. marcescens approached these low values only when grown with insufficient aeration or in the presence of 3% NaCl. In either of these conditions, S. marcescens also failed to produce pigment.

The final yield of cells of S. marinorubra after a 24-hour period was found to remain rather constant with increasing concentrations of NaCl, up to 5-6%, in the glycerol medium. Above 5-6% NaCl, the final yields were appreciably limited. Maximum growth rates occurred in 3% NaCl. The growth of S. marcescens, on the other hand, was adversely affected by any addition of NaCl to the glycerol medium, and no growth was found in those media containing 5-6% NaCl.

The effect of history of the inoculum on the rate of growth in a variety of media is shown in Figures 2 and 3. The pH values shown in these figures represent the final pH observed in the respective cultures. Transfer of either microorganism from its normal maintenance medium to one in which the ionic strength was radically different resulted in a long lag period. It was possible that during this lag period there was a process of selection for that fraction of the population capable of either adapting or already capable of thriving under the newly imposed conditions. As depicted for S. marinorubra in Figure 2, there was a lag associated with the transfer from a salt-containing medium to a regular glycerol medium. Subsequent transfer of the glycerol (low salt) adapted cells to fresh low salt glycerol medium did not result in such an extended lag period. It is obvious from the growth curve in Figure 2 that the rate of growth of S. marinorubra in low salt media, with or without prior adaptation to the low salt conditions, did not approach that found in 3% NaCl-glycerol media. There is apparently no strict requirement for either  $\text{Na}^+$  or  $\text{Cl}^-$  as shown by the fact that comparable growth curves for S. marinorubra were observed in media in which the NaCl was replaced by KCl,  $\text{Na}_2\text{SO}_4$  or  $\text{MgSO}_4$  of approximately equivalent ionic strength. (The growth curve is not shown for growth in  $\text{MgSO}_4$ -glycerol.) No attempt was made to determine the optimum concentration of KCl,  $\text{MgSO}_4$ , or  $\text{Na}_2\text{SO}_4$ , but the fact that rapid growth could occur in the presence of high concentrations of either  $\text{K}^+$  or  $\text{Mg}^{++}$  as well as  $\text{Na}^+$  indicates that S. marinorubra does not have a strict requirement for  $\text{Na}^+$ , but has a simple requirement for increased osmotic pressure. Since it is generally agreed that true marine bacteria have a specific requirement for  $\text{Na}^+$ ,<sup>(11)</sup> it is apparent that S. marinorubra is not a true marine bacterium, but rather a salt-tolerant terrestrial organism.

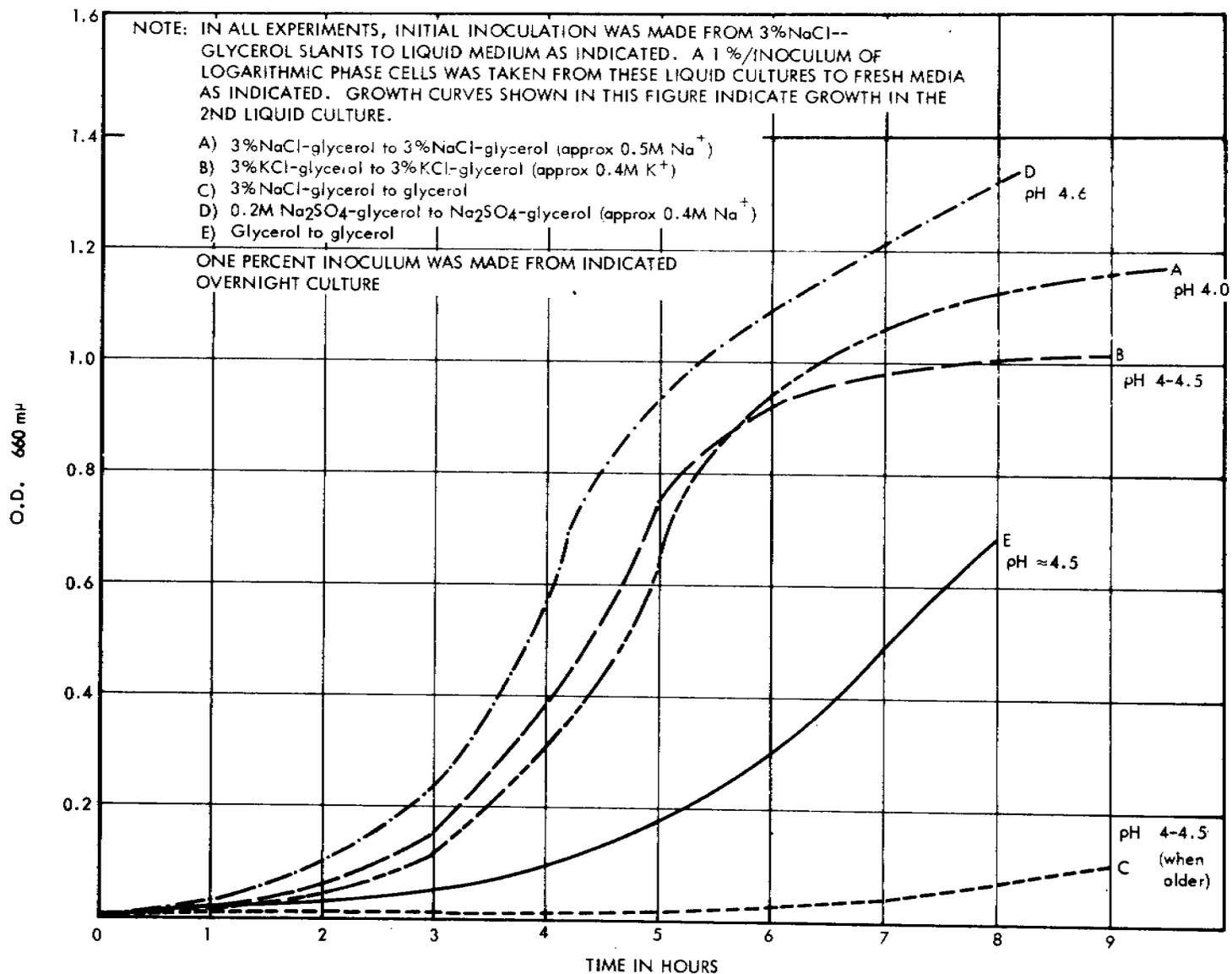


Figure 2. Growth of S. marinorubra

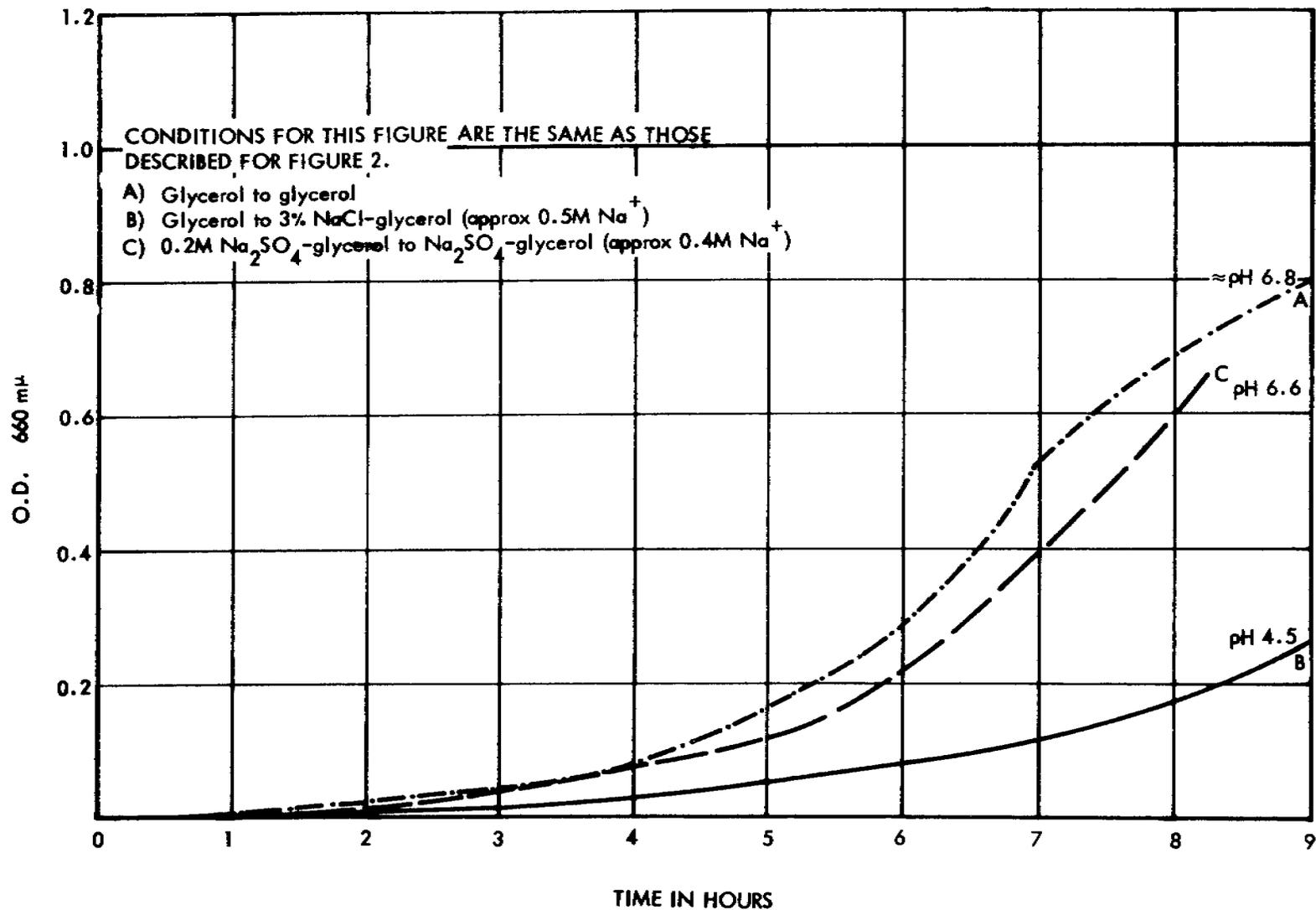


Figure 3. Growth of S. marcescens

Growth of S. marcescens in 3% NaCl was rather slow (Figure 3), resulting in a final pH of approximately 5. Under these conditions, no pigment was produced. Substitution of Na<sub>2</sub>SO<sub>4</sub> for NaCl resulted in a growth curve comparable to that obtained in regular glycerol medium, with a final pH of approximately 6.6 and evidence of pigment formation as the cells entered the stationary phase of growth. The accumulation of acidic end products which occurred when S. marcescens was grown in 3% NaCl-glycerol did not occur in media containing Na<sub>2</sub>SO<sub>4</sub> or in simple glycerol media. Further studies are required to determine what role Cl<sup>-</sup> may play in altering metabolism so as to encourage the production of acidic compounds. It would appear, then, that the decreased rate of growth in 3% NaCl may be partially explained on the basis of low pH resulting from the acidic metabolic products.

### 3.2 INTRACELLULAR GLYCOGEN CONTENT

During early light scattering studies, it was found that resting cells of glycerol-grown S. marcescens deplasmolyzed rapidly in the presence of 0.08M KCl in 0.05M tris buffer at pH 6.8, while S. marcescens grown in 1% glucose or glycerol-grown S. marinorubra deplasmolyzed at a much slower rate. On the assumption that the process of K<sup>+</sup> accumulation even in the direction of concentration gradient is dependent upon an energy source, and ultimately upon endogenous reserves<sup>(9, 10)</sup>, the differences in the rate of deplasmolysis described above could reflect differences in the amount of stored glycogen in the two organisms. Differences in glycogen content were also believed to account for variations in rates of deplasmolysis between cells of S. marcescens grown under different conditions or at different phases of their growth.

The rate of utilization of intracellular glycogen and some measure of the glycogen content of bacterial cells can be obtained by measuring rates of respiration of resting cells. Measurement of the rate of respiration glucose-grown S. marcescens showed that these cells utilized oxygen only one-third as fast as glycerol-grown cells, indicative of lower glycogen content in the glucose-grown cells.

Glycogen assays of cells grown under varying conditions as presented in Table 1 show that in the early logarithmic phase with cells grown in glycerol, there is approximately eight times as much glycogen in S. marcescens as in S. marinorubra. This would account for the increased rates of deplasmolysis and respiration in the former organism. However, the data also indicates that low glycogen stores are not responsible for the decreased rate of deplasmolysis and respiration in the glucose-grown S. marcescens, and one must look for other explanations for the reduced rates of respiration and cation transport in glucose-grown S. marcescens. A possible explanation for the lowered deplasmolysis and respiration rates is that the rate of glycogen utilization is retarded in the glucose-grown cells. When S. marcescens is grown anaerobically in a glucose medium, rates of deplasmolysis are significantly higher than in aerobically grown cells. While this may still indicate increased stores of glycogen in the anaerobically grown cells, it is more likely that it reflects a difference in the rate at which the glycogen is utilized. These differences in the rate of glycogen utilization and ion transport may be caused by a number of factors, including 1) pH of the medium, 2) variations in intracellular cation content, or 3) damage to the cells during preparation procedure as a result of osmotic shock. (7)

In summary, it appears that variations in glycogen content could account for some of the observed differences in deplasmolysis and respiration rates, e.g., the low rates found in logarithmic S. marinorubra are in agreement with its low glycogen stores. However, other factors also could affect the cells' ability to utilize glycogen stores, such as final pH of the medium, shock associated with preparation procedures, age of culture, etc., and must also be considered.

### 3.3 RESPIRATION RATES

The measurement of rates of oxygen utilization by resting cell suspensions was chosen as one means of detecting utilization of intracellular stores of glycogen. The uptake of oxygen is indicative of normal functioning of the typical electron transport chain, but would not detect any fermentative activity where oxygen does not serve as terminal electron acceptor. The respiratory activity seemed to be most interesting to the present study, since it was known from earlier studies that deplasmolysis

Table 1. Glycogen Content of S. marcescens,  
and S. marinorubra

<u>Organism and Medium</u>	<u>OD at Harvest</u>	<u>Approx. Growth Phase</u>	<u>Glycogen Content Mg/Gram Dry Weight</u>
<u>S. marinorubra</u> glycerol	0.18	Early Log	1.1
<u>S. marcescens</u> glycerol	0.20	Early Log	8.3
	1.25	Intermediate Log	14.4
	1.40	Intermediate Log	23.9
	1.60	Intermediate Log	22.2
	2.40	Late Log	30.0
<u>S. marcescens</u> glucose	0.15	Early Log	16.2
	0.40	Early Log	10.9
	0.74	Early Log	12.0

rates were greatest under aerobic conditions and apparently somewhat dependent upon aerobic respiration. The results of a study to determine the rates of respiration in both micro-organisms at different phases of growth and under a variety of conditions are shown in Table 2. Except for the  $MgCl_2$  washes, chosen for better retention of intracellular salts and less osmotic shock in the case of salt-grown S. marinorubra, all cells were washed twice in redistilled water. Logarithmic phase cells were harvested at optical densities near 0.6, while stationary cells were harvested at 30 hours after inoculation with optical densities near 3.0 and 2.0 for S. marcescens and S. marinorubra, respectively.

It was found that under comparable conditions, the rates of endogenous respiration of cultures of S. marinorubra were generally considerably lower than those observed for S. marcescens. As stated earlier, this

Table 2. Respiration Rates in S. marcescens and S. marinorubra<sup>a</sup>

Conditions	<u>S. marcescens</u>		<u>S. marinorubra</u>			
	<u>Early Log</u>	<u>Stationary</u>	<u>Early Log</u>	<u>Stationary</u>		
<u>Glycerol - Grown Cells:</u>						
Endogenous Respiration	16	16-20	6 to 8	6 to 8		
+Glucose 1.67 mM	100-120	100-120	100	100		
+0.8m NaCl						
Endogenous	0	40-100	0	0		
+0.8m KCl						
Endogenous	0	40-100	0	0		
<u>3% NaCl - Grown Cells:</u>			<u>(H<sub>2</sub>O)(MgCl<sub>2</sub>)(H<sub>2</sub>O)(MgCl<sub>2</sub>)</u>			
Endogenous			20	29	3-4	18
+Glucose 1.67mM			44	116	17	38
+Formate 1.67mM			300	228	160	144
+0.8m NaCl						0
+0.8m KCl						0

a) Total volume in each case = 10ml. Results are expressed in terms of mm O<sub>2</sub> utilized per minute.

fact is in agreement with lower glycogen stores in the former micro-organism. However, the oxygen measurement described here does not necessarily indicate the complete metabolic (or specifically, catabolic) activity of either micro-organism. Equivalent rates of oxygen utilization were noted between logarithmic and stationary cells of either type. Age has no apparent effect on the ability to respire, although it is known from glycogen determinations that stationary cells of S. marcescens contain significantly greater glycogen content than logarithmic phase cells. This may mean that regardless of age, the cells have a limited capacity for

utilization of oxygen, or limited rates of glycogen catabolism. Or, it may mean that the relative activities of respiratory and fermentative pathways differ between cells at different ages.

The addition of glucose to cell suspensions resulted in rates of oxygen utilization essentially equal for the two organisms at both the logarithmic and stationary phases. This indicates that the ability to utilize glucose, and the subsequent uptake of oxygen is not the rate limiting factor in endogenous respiration. From this, it would appear that the rate limiting step in endogenous respiration is the initial conversion of glycogen to glucose, and/or limiting amounts of glycogen in the first place. From these studies one can conclude that limited amounts of glycogen coupled with possible limited rates of glycogen catabolism may be responsible for lower rates of respiration and the energy-dependent accumulation of cations in S. marinorubra.

The peculiar stimulation of oxygen utilization in stationary S. marcescens subjected to high salt (0.8M) concentrations was not observed with logarithmic phase cells or by either growth phase of S. marinorubra. This high salt concentration had previously been used in light scattering studies with stationary cells in an attempt to induce plasmolysis. The stimulation of oxygen utilization in the present study requires further work to allow testing of hypotheses for possible causes. The presence of elevated concentrations of salts may act in these stationary cells to uncouple oxidative phosphorylation, or to stimulate those enzymes responsible for glycogen degradation and oxygen utilization.

If the salt-grown cells of S. marinorubra were washed with 0.1 M  $MgCl_2$ , found to favor intracellular  $K^+$  retention in such cells, they appeared to have greater endogenous respiration rates than comparable cells washed in water. Utilization of exogenous glucose also appeared to be faster in the  $MgCl_2$ -washed cells. The disparity between rates of oxygen utilization in logarithmic and stationary salt-grown S. marinorubra, both with and without added glucose, and the slow glucose utilization in comparison with formate respiration are worthy of note. It has been shown that formate rapidly equilibrates across the membranes of E. coli,<sup>(2, 3)</sup> while transport of glucose requires energy and is enzymatically governed. The permeability of S. marinorubra is undoubtedly

greater for formate than for glucose, but as the cells pass from the logarithmic phase to the stationary phase of growth, the permeability is generally lowered.

### 3.4 INTRACELLULAR SALT CONTENT

The study of ion transport across microbial membranes requires a knowledge of the intracellular cation concentrations. Light scattering techniques allow a convenient qualitative analysis of transport of materials across the cell membranes of plasmolyzed cells, but this technique must be coupled with quantitative techniques to give some idea of the initial concentrations of cations within the cells and the magnitude of concentration changes when cells are suspended in hypertonic salt solutions. The accurate determination of cation concentrations in bacterial cells is a difficult problem. Techniques must be developed for the separation of intact bacteria with their contents to distinguish between intracellular and extracellular salts. This may involve: 1) replacing the surrounding medium with some acceptable wash solution and subsequently releasing the contents of the cells by chemical means, or 2) by applying correction factors for the quantities of extracellular salts trapped in the extracellular space of harvested cells and subsequent determination of salts present in the packed pellet of cells after centrifugation or 3) by simply comparing the cation content of freshly prepared media with that of spent media assuming that the difference between these values must be contained within the cells. Consequently, a number of methods for estimating intracellular cation content of bacterial cells have been conceived although each of the established methods has its advantages and limitations. When an attempt is made to correct for cations trapped between packed cells, it becomes apparent that simply subtracting the quantity of salt found in a volume of supernatant growth medium equal to the trapped space between cells may not yield accurate results. It is difficult to include corrections for accumulation of cations in the immediate vicinity of the cell envelopes where interactions with charged groups on the cell surface are strong. Such interactions would undoubtedly cause the removal of cations from the bathing fluid and the concentrations of such trapped ions may well exceed the average concentration found in the remaining supernatant medium. If one attempts to remove these ions not

strongly adhering to the cell wall by specific washing solutions, one is faced with the possibility of osmotic shock, damaging the cells' envelopes thus allowing leakage or leaching of ions from the cells' interiors.

For our own purposes in the determination of intracellular cation concentrations in S. marcescens and S. marinorubra, it was desirable to obtain results on cells washed in water, similar to those routinely used for light scattering studies so that correlations could be made between light scattering and intracellular salt content data. Cells were also examined under a variety of washing conditions which may possibly favor greater retention of intracellular salts in order to accurately determine the salt content of cells under various growth conditions.

For the sake of comparing old and young cells, studies included analyses of logarithmic phase (OD approximately 0.8) and stationary phase (OD approximately 2.0 to 2.5) cells. All experimental procedures were carried out in clean polypropylene tubes to minimize contamination, and all washing solutions were stored in sodium- and potassium-free polypropylene vessels. Following the washing and other various experimental treatments, the cells were resuspended in 9 ml of either redistilled water or tris-HCl buffer, 0.05M pH 6.8. No differences in recovered cations were apparent in either case. One ml concentrated HNO<sub>3</sub> was added and the cells were held in a boiling water bath for 5 to 10 minutes. This procedure allowed recovery of all the K<sup>+</sup>, Na<sup>+</sup>, and Mg<sup>++</sup> as determined by comparison with amounts found to be missing from the spent medium inasmuch as complete digestion of the cells in concentrated nitric acid gave no greater recovery of these cations.

#### 3.4.1 Potassium Content

The results of the determination of intracellular K<sup>+</sup> by atomic absorption spectrometry are shown in Table 3. Most of the data reported represents averages of several determinations made under identical conditions. Of the various washing solutions employed in this study, it was found that 0.1M MgCl<sub>2</sub> generally allowed greatest recovery of intracellular K<sup>+</sup>. This washing procedure was recommended for K<sup>+</sup> retention in cells of Aerobacter aerogenes by Tempest, et al.<sup>(16)</sup> Control studies made on fresh and spent media insured that in S. marcescens essentially all of the

Table 3. Effect of Various Washing Solutions on Intracellular Potassium Content of *S. marcescens* and *S. marinorubra*\*

Organism and Growth Phase	Medium	1X Tris	2X Tris	2X H <sub>2</sub> O	2X MgCl <sub>2</sub> 0.1M	2X MgCl <sub>2</sub> 0.5M	2X NaCl 0.85%	2X Na-PO <sub>4</sub> 0.05M	2X Na-PO <sub>4</sub> 0.05M	2X Na <sub>2</sub> SO <sub>4</sub> 0.1M	2X Na <sub>2</sub> SO <sub>4</sub> 0.1M	2X H <sub>2</sub> O 2X	1X MgCl <sub>2</sub> 0.1M
									2X MgCl <sub>2</sub> 0.1M		2X MgCl <sub>2</sub> 0.1M	2X MgCl <sub>2</sub> 0.1M	2X NaCl 0.85%
<i>S. marcescens</i> 1	a	0.55	--	0.78	0.92	0.18	1.06	--	--	--	--	--	--
<i>S. marinorubra</i> 1	a	0.74	--	0.46	0.88	--	1.05	--	--	--	--	--	--
<i>S. marcescens</i> s	a	0.66	--	0.54	1.03	0.322	--	--	--	--	--	--	-- O. D. 2.7
		--	0.22	0.15	0.31	0.056	0.31	--	--	--	--	--	0.34 O. D. 4.0
<i>S. marinorubra</i> s	a	1.03	0.55	0.50	0.60	0.50	0.77	--	--	--	--	--	0.75
<i>S. marcescens</i> 1	b	--	0.71	0.39	1.12	--	1.19	--	--	--	--	--	--
<i>S. marinorubra</i> 1	b	0.30	0.22	0.10	1.7	2.07	1.7	0.64	0.66	1.8	1.3	0.016	--
<i>S. marcescens</i> s	b	--	0.88	0.53	1.2	2.3	1.2	--	--	--	--	--	1.4
<i>S. marinorubra</i> s	b	--	0.12	0.15	1.17	2.14	1.3	--	--	--	--	--	1.3

\* Results are expressed in terms of milligrams K<sup>+</sup> per 100 mg cells (dry weight).

a. low salt-glycerol media

b. 3% NaCl - glycerol media

1 - logarithmic phase

s - stationary phase

Tris wash was 0.05M at pH 6.8.

Sodium phosphate wash was buffered at pH 6.8.

intracellular  $K^+$  missing from the spent media was recovered by the procedure. The same was true for glycerol-grown S. marinorubra. In 3% NaCl-glycerol grown S. marinorubra, however, recovery was not as good with 0.1M  $MgCl_2$  as with 0.5M  $MgCl_2$ . Furthermore, even these figures do not account for all the  $K^+$  found to be missing from spent medium. Physiological saline (0.85% NaCl) and 0.1M  $Na_2SO_4$  were also effective in reducing the amount of  $K^+$  lost to the washing solution. It would thus appear that ionic strength of these washing solutions and not any specific ion is responsible for intracellular  $K^+$  retention.

Although 0.5M  $MgCl_2$  was generally more effective in preventing loss of  $K^+$  in 3% NaCl-glycerol grown cells, in the case of low salt-glycerol grown cells its use resulted in severe losses of  $K^+$ . It is apparent that in the latter case the washing solution is significantly hypertonic to the interior of the cell. The hypertonicity of the washing solution may actually cause rupture of the cell envelope and the release of intracellular materials. In 3% NaCl-grown cells, the intracellular  $K^+$  concentration (as discussed below) is much higher and 0.5M  $MgCl_2$  does not present a great osmotic shock to the plasma membrane. It should be mentioned, though it is not included in the tabulated data, that growth of either micro-organism in media containing KCl or  $Na_2SO_4$  instead of the usual 3% NaCl resulted in similar accumulation of large amounts of intracellular  $K^+$ .

Potassium content in log phase glycerol-grown cells of S. marcescens is roughly the same as that in S. marinorubra and represents approximately a 100-fold concentration of this cation above the concentration in the medium. The concentration of  $K^+$  in the external medium was approximately  $1 \times 10^{-3}$  M. Growth in 3% NaCl media causes an increase in the concentration of intracellular  $K^+$  of both organisms, the increase in S. marinorubra being the greater, so that the intracellular  $K^+$  concentration approximates the concentration of extracellular  $Na^+$ , resulting in an approximate isotonic condition across the plasma membranes. In 3% NaCl-grown cells (or cells grown in comparable concentrations of  $Na_2SO_4$ ) this would mean at least a 250-fold concentration of  $K^+$  over that found in the medium.

The potassium content of either organism does not vary significantly between the logarithmic and stationary phase of growth until the cells are well into the stationary phase (as shown with S. marcescens in Table 3) at which time the potassium content drops abruptly. There is no associated increase in the sodium content of the cells.

Logarithmic phase cells of glycerol-grown S. marcescens appear to be most resistant to loss of intracellular potassium during the washing procedure with distilled water. Only 15% of the intracellular  $K^+$  was lost to the distilled water washes. S. marinorubra, on the other hand, lost almost 50% of its  $K^+$ , when grown and treated under similar conditions. Stationary cells and cells grown in 3% NaCl all showed greater losses of potassium. In salt-grown cells, S. marcescens lost some 50% of its  $K^+$  to the wash water, while S. marinorubra was found to lose up to 90% of its  $K^+$ . This loss was not prevented by inclusion of metabolic inhibitors such as  $HgCl_2$  and metachloro-ccp. It must be remembered that in the salt-grown cells the intracellular  $K^+$  content is very high, and washing with distilled water presents a great osmotic shock to the cells.

Thus, S. marcescens retains its intracellular salts better than does S. marinorubra. It may also be seen from these results that washing with 0.05M tris generally tends to remove intracellular  $K^+$  from cells of both types. When cells are resuspended in tris buffer, even after washing with  $MgCl_2$  to avoid initial losses of intracellular salts, there is a tendency for the cells to lose  $K^+$ . The impact of this loss of  $K^+$  in the presence of tris buffer is apparent in light scattering studies conducted in the usual manner. Due to this loss, plasmolysis of the cells occurs in salt solutions of lower ionic strength when suspended in tris than when suspended in  $MgCl_2$  which tends to cause retention of intracellular salts.

#### 3.4.2 Sodium Content

From the data on  $Na^+$  concentrations shown in Table 4 and derived from atomic absorption techniques, it is obvious that normal cells under all conditions of growth contain very small amounts of  $Na^+$  relative to  $K^+$ . The intracellular concentration roughly approaches that in the external

Table 4. Effect of Various Washing Solutions on Intracellular Sodium Content of *S. marcescens* and *S. marinorubra*\*

Organism and Growth Phase	Medium	1X	2X	2X H <sub>2</sub> O	2X	2X	2X	2X	2X
		Tris 0.05M pH 6.8	Tris 0.05M pH 6.8		MgCl <sub>2</sub> 0.1M	MgCl <sub>2</sub> 0.5M	H <sub>2</sub> O 2X MgCl <sub>2</sub> 0.1M	Na-PO <sub>4</sub> 0.05M 2X MgCl <sub>2</sub> 0.1M	Na-PO <sub>4</sub> 0.1M 2X MgCl <sub>2</sub> 0.1M
<i>S. marcescens</i> 1	a	0.003	--	0.032	0.037	--	--	--	--
<i>S. marinorubra</i> 1	a	0.005	--	0.016	0.016	--	--	--	--
<i>S. marcescens</i> s	a	0.004	0.002	0.112	0.005	0.002	--	--	--
<i>S. marinorubra</i> s	a	0.002	0.008	0.004	0.002	0.003	--	--	--
<i>S. marcescens</i> i	b	0.044	--	0.405	0.034	--	--	--	--
<i>S. marinorubra</i> i	b	0.089	0.016	0.476	0.014	0.02	0.095	0.155	0.096
<i>S. marcescens</i> s	b	--	0.016	0.423	0.013	0.007	--	--	--
<i>S. marinorubra</i> s	b	--	0.003	0.21	0.005	0.007	--	--	--

\* Results are expressed in terms of milligrams Na<sup>+</sup> per 100 mg cells (dry weight)

- a. Regular glycerol media.
- b. 3% NaCl-glycerol media.

Sodium phosphate wash was pH 6.8.

medium (2.44 mM) in young cells grown in glycerol medium. The sodium content does not increase during growth in 3% NaCl-glycerol ( $\text{Na}^+$  concentration = 510 mM). However, results do indicate that S. marcescens may contain somewhat higher intracellular concentrations of  $\text{Na}^+$  than S. marinorubra.

Water washing does not affect the intracellular sodium content of logarithmic phase cells of low salt glycerol-grown S. marcescens or S. marinorubra. The same is true of stationary cells of S. marinorubra grown in glycerol. However, in all other instances, i. e., in salt-grown cells of both organisms and in stationary low salt glycerol-grown S. marcescens, washing with water results in the appearance of rather large increases in intracellular concentrations of  $\text{Na}^+$ , although this is still much less than the  $\text{Na}^+$  concentration of the growth media. It is therefore likely that the most probable source of this  $\text{Na}^+$  is residual  $\text{Na}^+$  trapped between the cells upon harvesting from the growth media. This accumulation of  $\text{Na}^+$  occurred simultaneously with the severe osmotic shock imposed by the water wash and a resultant loss of intracellular  $\text{K}^+$ . The accumulation of  $\text{Na}^+$  did not occur in the absence of these factors, and the amount of  $\text{Na}^+$  taken up amounted to 15 to 50% of the lost potassium. Once associated with the cells, the  $\text{Na}^+$  was not removed by subsequent water washes, but its removal was effected by washing in tris or  $\text{MgCl}_2$ . Or, if the sodium-rich cells were suspended in 0.08M KCl and 0.05M tris at pH 6.8, the  $\text{Na}^+$  was removed and significant amounts of  $\text{K}^+$  were taken up. The addition of glucose to such suspensions of salt-grown S. marinorubra allowed somewhat greater amounts of  $\text{K}^+$  to be accumulated, but the final intracellular  $\text{K}^+$  concentration, (or at least  $\text{K}^+$  associated with the cells) never approached the concentrations found in cells removed from the original medium and washed in  $\text{MgCl}_2$ .

It is apparent from the results discussed above and by visual observation that the cells involved were not intact, or at least a large proportion of the cells were actually lysed. Such a condition could well account for these results. Elevated intracellular  $\text{K}^+$  concentrations, as a result of growth in the presence of high salt concentrations, or decreased stability of the cell envelope as in the case of stationary S. marcescens grown in glycerol medium (as indicated by loss of  $\text{Mg}^{++}$ , to be discussed later), are necessary predisposing factors for the accumulation of  $\text{Na}^+$  and the

simultaneous loss of  $K^+$ . Lysis of such cells by washing in distilled water would disrupt the cell envelope and expose the internal structures and compounds to the washing medium. Competition between the  $K^+$  originally inside the cells and the residual  $Na^+$  from the growth medium still present in the washing solution probably occurs at the numerous charged sites on the cellular debris, and the higher concentrations of  $Na^+$  result in displacement of the  $K^+$ . The  $Na^+$  bound by electrostatic forces to the cellular debris could not be removed by subsequent washing with distilled water, but would be easily displaced by resuspending the debris in the tris,  $MgCl_2$ , or  $KCl$  as discussed above.

### 3.4.3 Magnesium Content

It is generally agreed that divalent cations play an important role in maintaining the integrity of the cell membrane and probably regulate permeability due to their ability to chelate with acidic or negatively charged groups associated with the membrane. Light scattering data indicated that salts of such divalent cations caused cells of both S. marcescens and S. marinorubra to plasmolyze and to remain in that condition with no apparent uptake of the cations. <sup>(6)</sup> These facts, together with the observed ability of washing solutions of  $MgCl_2$  to favor retention of intracellular  $K^+$  prompted a study to determine the actual amounts of  $Mg^{++}$  normally associated with the bacterial cells. The results of such a study are shown in Table 5. In these studies, where a determination of  $Mg^{++}$  was being made, it was necessary to use some other salt solution for washing the cells in the preparative procedure. As shown in Table 5, the type of washing solution, including redistilled water alone, did not make any significant difference in the amount of  $Mg^{++}$  found associated with the cells.

It was found that a marked difference exists in the  $Mg^{++}$  content of the two micro-organisms grown in low-salt glycerol medium. S. marinorubra was generally found to contain higher quantities of  $Mg^{++}$  than S. marcescens. Younger cells of either organism contained considerably more of this cation than stationary cells, regardless of growth media. Although the  $Mg^{++}$  content of S. marinorubra did not differ appreciably when grown in 3%  $NaCl$ -glycerol medium or a low-salt glycerol medium, the  $Mg^{++}$  content of S. marcescens showed a dramatic increase when grown in 3%  $NaCl$ -glycerol medium. These results are suggestive

Table 5. Effect of Various Washing Solutions on Intracellular Magnesium Content of S. marcescens and S. marinorubra\*

Organism and Growth Phase	Medium	2X	2X	2X	2X	2X	1X
		Tris 0.05M pH 6.8	H <sub>2</sub> O	NaCl 0.85%	Na-PO <sub>4</sub> 0.05M	Na <sub>2</sub> SO <sub>4</sub> 0.1M	MgCl <sub>2</sub> 0.1M 2X NaCl 0.85%
<u>S. marcescens</u> 1	a	--	0.096	0.093	--	--	--
<u>S. marinorubra</u> 1	a	--	0.16	0.165	--	--	--
<u>S. marcescens</u> s	a	0.016	0.02	0.01	--	--	0.016
<u>S. marinorubra</u> s	a	0.10	0.116	0.09	--	--	0.098
<u>S. marcescens</u> 1	b	--	0.145	0.155	--	--	--
<u>S. marinorubra</u> 1	b	0.181	0.151	0.204	0.171	0.164	--
<u>S. marcescens</u> s	b	0.059	0.040	0.085	--	--	0.088
<u>S. marinorubra</u> s	b	0.06	0.062	0.069	--	--	0.104

\*Results are expressed in terms of milligrams Mg<sup>++</sup> per 100 mg cells (dry weight).

- a. regular glycerol media.
- b. 3% NaCl-glycerol media.

Sodium phosphate wash was at pH 6.8.

of incorporation of increased numbers of acidic groups in the cell envelope of S. marcescens when grown in the presence of 3% NaCl, and in this regard, the salt-grown cells may resemble the strict halophilic bacteria. (4) It is not known whether this is a result of selective or adaptive processes.

#### 3.4.4 Effect of pH of Washing Solution on Cation Content

The effect of pH of the washing solution on retention of intracellular  $K^+$  and  $Mg^{++}$  is shown in Table 6. For this study, logarithmic phase cells of glycerol-grown S. marcescens were washed twice in 0.85% NaCl in which the pH had been adjusted to the indicated values by the addition of HCl. It may be seen that as the pH was lowered to approximately 3, the cells lost essentially all their intracellular  $K^+$ . This loss was more than likely associated with the weakening of the chelate bonds between cell envelope and the  $Mg^{++}$  by protonation of some of the acidic groups normally involved in chelation and the resultant loss of membrane integrity caused by such displacement of  $Mg^{++}$ . At pH 2, the  $Mg^{++}$  was effectively displaced by  $H^+$ , and all the  $Mg^{++}$  was also lost from the cells. At these low pH values, the cells became extremely fragile and difficult to handle.

Table 6. Effect of pH on Retention of Intracellular  $K^+$  and  $Mg^{++}$  by Glycerol-Grown S. marcescens

<u>pH of Wash</u> 0.85% NaCl	<u>K<sup>+</sup></u> Mg/100 Mg Cells	<u>Mg<sup>++</sup></u> Mg/100 Mg Cells
6.0	0.841	0.078
5.0	0.875	0.084
4.0	0.866	0.100
3.0	0.210	0.082
2.0	0.016	0.023

### 3.5 LIGHT SCATTERING STUDIES

The use of light scattering to measure transport of materials across microbial membranes is based upon the immediate increase in the amount of light scattered by suspensions of certain (particularly gram negative) bacteria when these are subjected to sudden increases in external osmotic pressure by the addition of osmotically active substances to the suspending medium<sup>(1-3)</sup>. The magnitude of the initial plasmolytic response and resultant change in light scattered depends upon a number of factors, including: concentration of osmotically active material, species of bacteria, phase of growth, prior treatment of cells, and pH. Figure 4 demonstrates the effect of titrating a typical cell suspension with increasing concentrations of a salt such as NaCl which is not transported or slowly transported by the cells. That concentration of external salt required to elicit plasmolytic response is a fair index of the intracellular salt content of the cells, since plasmolysis will not occur except where the concentration of external salt becomes hypertonic to the interior of the cells.

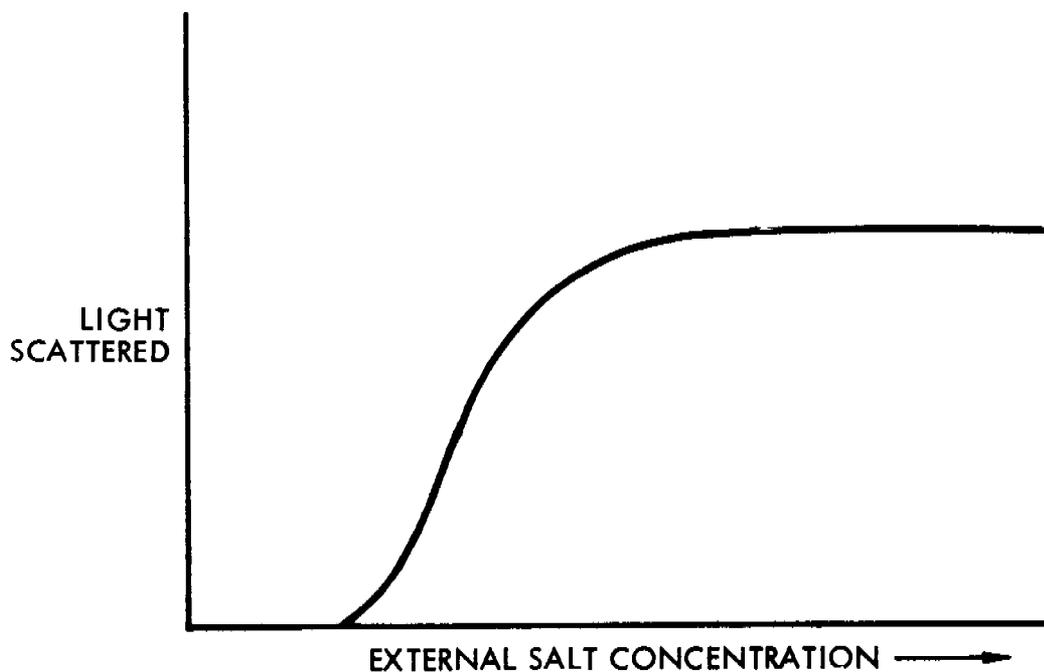


Figure 4. Light Scattering Response to Increasing Salt Concentrations

It is generally agreed that the light scattering changes result from plasmolysis, i. e., the loss of intracellular water due to the immediate osmotic effect (see Figure 5). The increase in scattered light is due to compression and concentration of the cytoplasm with associated changes in refractive index and possibly to changes in the actual size and shape of the cells. If, subsequent to the initial increase in scattered light, the cells accumulate some osmotically active solute from the external medium, the resulting osmotic effects will cause the cells to resume their original shape, size and density (deplasmolysis), and as a result of deplasmolysis, there will be a decrease in the amount of light scattered by the cells. Thus, light scattering can be used to rapidly and sensitively measure the transport of substances across the cell membranes of bacteria. This technique has the advantage of providing immediate qualitative information concerning the transport of materials across the microbial membranes, and although absolute amounts of transport are not given, the rates at which deplasmolysis occurs undoubtedly indicates the rate of transport.

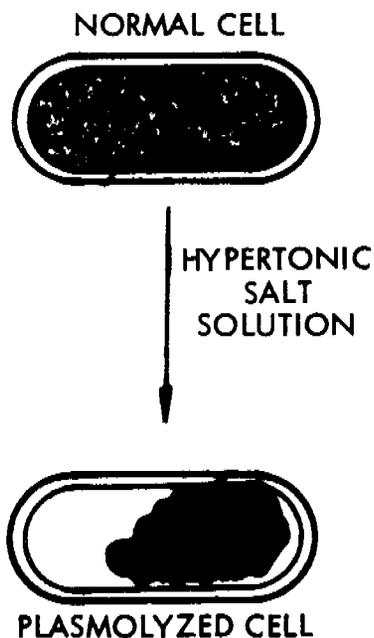


Figure 5. Plasmolysis of Bacterial Cells

Initial studies in this laboratory were directed toward the characterization of transport of the chloride salts of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Li}^+$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$  and  $\text{Sr}^{++}$  in the two test micro-organisms. It was hoped that by using a common anion the influence of the cations themselves could more readily be determined. Conditions chosen for preparation of cells and subsequent resuspension were arbitrary, and were not necessarily those optimal for deplasmolysis with any of the salts employed. This set of conditions did become, however, a standard against which later modifications of technique were measured.

It was found that the plasmolytic response was greatest in early logarithmic phase cells of S. marcescens grown in glycerol, washed twice in redistilled water and resuspended in 0.05M tris-HCl at pH 6.8. External salt concentrations of 0.08M were chosen for routine light scattering studies with S. marcescens, prepared as just described, since this concentration elicited good plasmolysis and yet allowed immediate detection of transport of salts.

Cells of S. marcescens generally were found to be fairly permeable to monovalent cations, but virtually impermeable to divalent cations. Deplasmolysis rates were greatest with KCl, and much slower with NaCl. A comparison of deplasmolysis of glycerol-grown S. marcescens and S. marinorubra is shown in Figure 6. The amount of deplasmolysis with buffered NaCl, which became more pronounced as the cells were allowed to stand at room temperature, or as the pH was raised, was probably largely due to the transport of tris, as explained in a later section. Deplasmolysis of S. marinorubra with KCl was normally very slow, but the addition of glucose resulted in rapid and complete deplasmolysis.

### 3.5.1 Effect of pH on Rates of Deplasmolysis

The effect of pH on the rate of deplasmolysis (with KCl) of S. marcescens in buffered and unbuffered suspensions was studied to find the optimal conditions for  $\text{K}^+$  transport. The results of this study are depicted in Figure 7. The data is a composite of rates obtained by water-washed cells suspended in 0.85% NaCl or in 0.05M tris-HCl in which the pH was adjusted to the desired value by the addition of HCl. These solutions were chosen on the basis of their known effect on intracellular  $\text{K}^+$  concentrations and light scattering responses, and to avoid the addition of

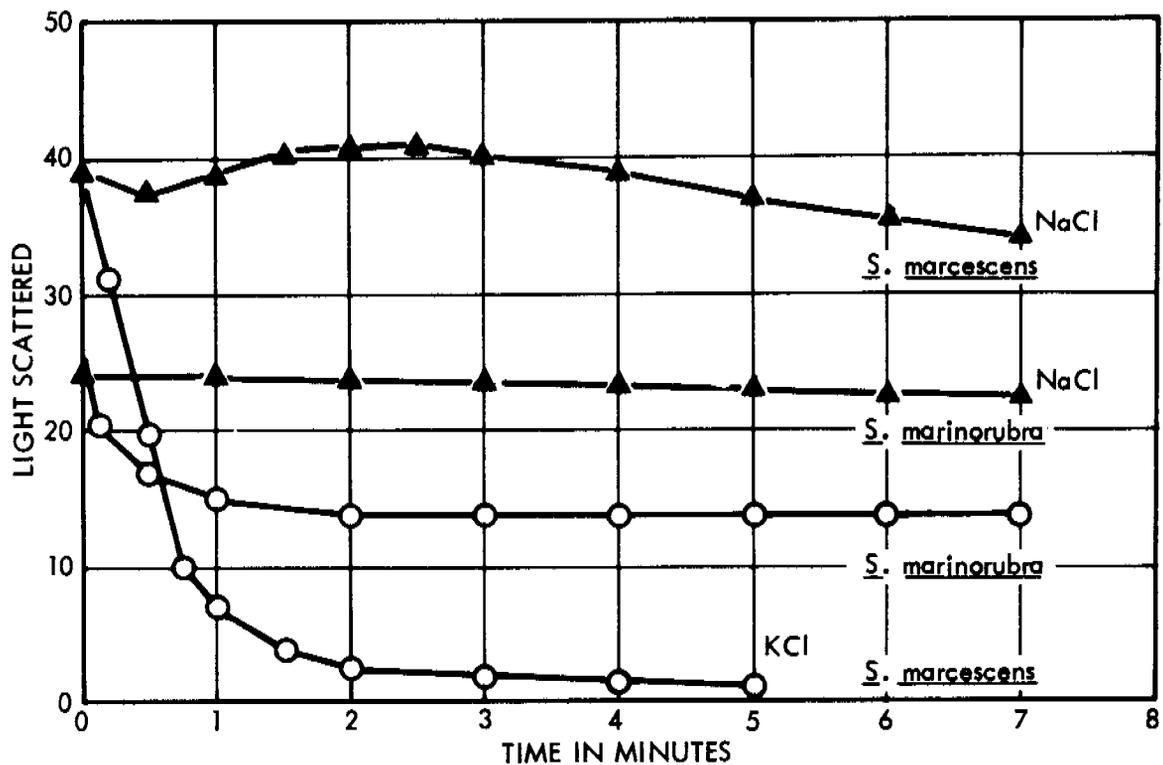


Figure 6. Light Scattering Changes Produced by 0.08M Salt Solutions with Serratia marcescens and Serratia marinorubra

unknown factors to the cell suspensions. Cells washed and resuspended in 0.01M  $MgCl_2$  gave lower rates (approximately one-half those shown on the ordinate in Figure 7), but the same general curve was observed. There is a fairly sharp pH optimum between 5.8 and 6.1, with a marked decrease in rate of deplasmolysis as the pH is varied in either direction. At pH 6.8 it can be seen that the curve is quite steep, and a small difference in pH can result in a drastic difference in rate of deplasmolysis. This undoubtedly accounts for the individual differences of separate experimental runs, or differences obtained between freshly prepared suspensions and those allowed to stand at room temperature for a period of several hours. Furthermore, at pH 6.8, in studying the effect of inhibitors on transport of  $K^+$ , it becomes imperative to closely monitor the effect of the addition of inhibitor and solvent on the pH of the cell suspension. This will be discussed further in a later section.

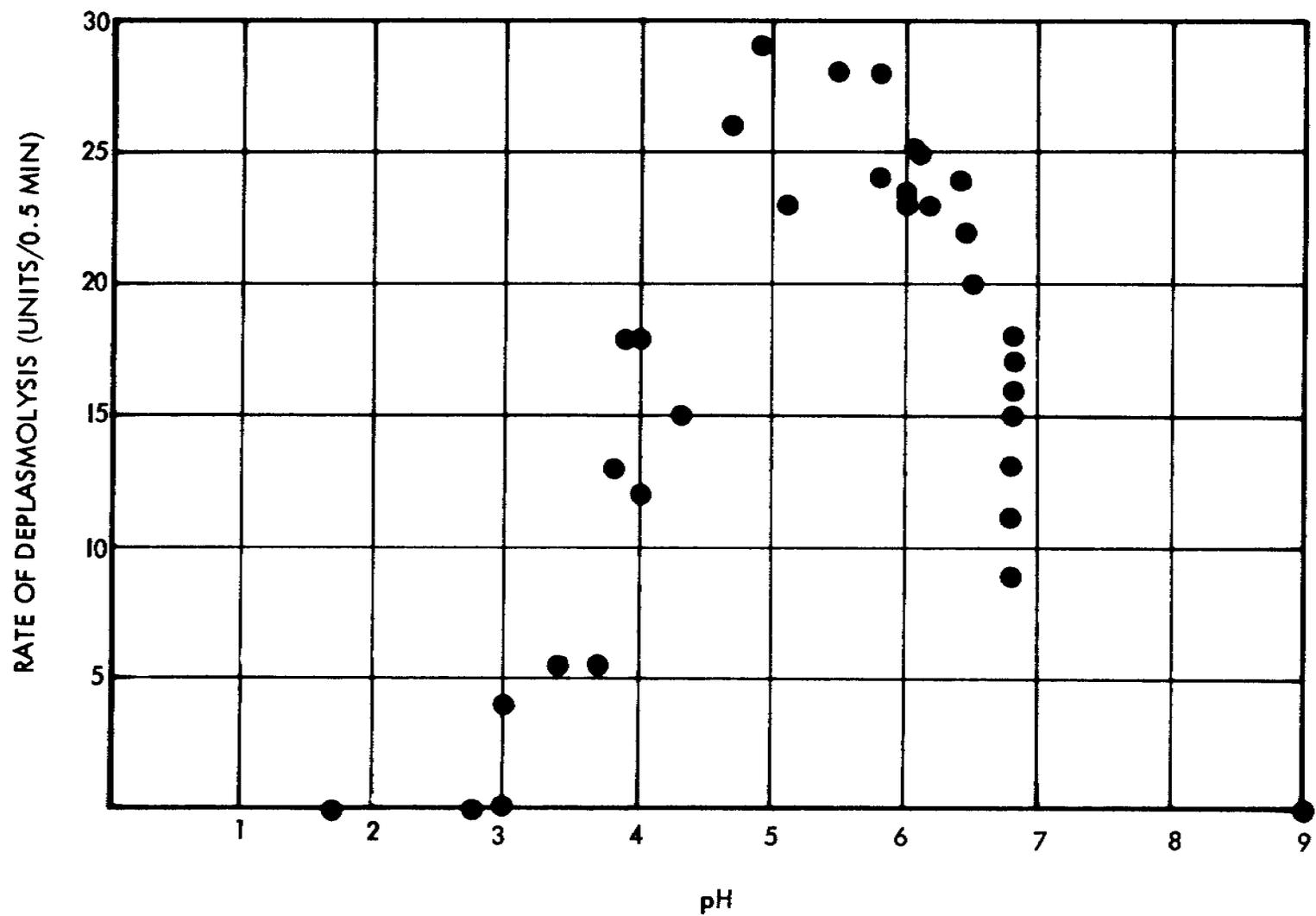


Figure 7. Effect of pH on Rate of  $K^+$  Transport

The results shown in Figure 7 with S. marcescens confirm those reported for E. coli by Schultz, et al.<sup>(15)</sup> that the transport of  $K^+$  is inhibited at low pH. Schultz, et al. also showed by radioisotope studies as well as pH determinations that extracellular  $K^+$  is exchanged at least in part for intracellular (metabolically produced)  $H^+$  in E. coli. The possibility of such an exchange phenomenon occurring in S. marcescens prompted a study which measured changes in pH during a time when  $K^+$  was known to be taken up. Approximately 30 mg of water-washed S. marcescens resuspended in 0.85% NaCl or in redistilled water (OD = 5.0) were used for the study to avoid the use of buffers. No significant change in the pH of such resting cell suspensions was caused by the addition of KCl, or by the uptake of  $K^+$  by the cells. Judging by the amount of cells present and the amount of  $K^+$  known to be taken up by such cells, if  $H^+$  was exchanged in a 1:1 ratio for  $K^+$ , the pH of the suspension should have dropped over 3 pH units. The evidence, therefore, suggests that  $H^+$  is not exchanged for  $K^+$  in S. marcescens under these conditions. The  $K^+$  is probably taken up through an active transport process, accompanied by  $Cl^-$  or some other anion, with no exchange for intracellular cations necessitated by such a process. Chloride ion is believed to be passively transported in bacteria to maintain electroneutrality.<sup>(14)</sup>

### 3.5.2 Light Scattering Studies with Salt-Grown Cells

Light scattering studies using young cells of either S. marcescens or S. marinorubra grown in 3% NaCl-glycerol media required that salt solutions of approximately isotonic strength as compared with the cells' interior be used instead of distilled water for washing and preparation. Washing such cells in distilled water causes considerable lysis due to osmotic shock as discussed in an earlier section, and thus renders the cells useless for light scattering studies. It was found that when the cells were washed with 0.1M  $MgCl_2$  or 0.05M tris, and resuspended in tris at pH 6.8, excellent plasmolytic response and typical deplasmolysis were obtained (see Figure 8). Salt-grown cells washed and resuspended in 0.1M  $MgCl_2$  were found to require higher external concentrations of KCl or NaCl to elicit maximum plasmolytic response than similar cells treated with tris. This was in agreement with atomic absorption data which showed greater retention of intracellular  $K^+$  when cells were washed with

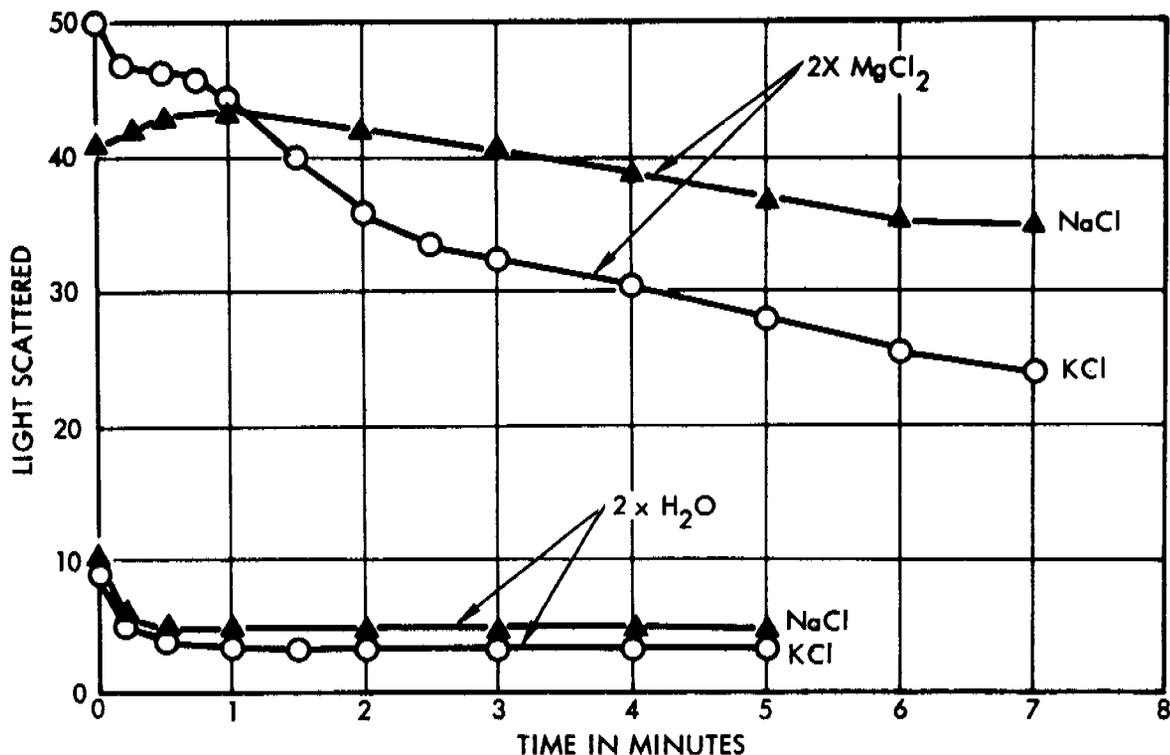


Figure 8. Effect of Washing Solution on Plasmolysis of 3% NaCl - Grown Serratia marnorubra

MgCl<sub>2</sub> rather than tris. Higher intracellular content of salts would require higher external salt concentrations to create the hypertonic conditions prerequisite for plasmolysis.

Studies with salt-grown S. marcescens show results similar to those just described for S. marnorubra, with the exception that deplasmolysis of MgCl<sub>2</sub>-washed cells occurred much more rapidly. This may be attributed to the higher glycogen reserves which are known to occur in S. marcescens as discussed earlier.

### 3.5.3 Light Scattering Studies with Stationary Cells

Washing with water has been shown to drastically alter the cells' content of salts, either in stationary phase glycerol-grown S. marcescens or in salt-grown cells of either organism. Moreover, stationary cells of S. marcescens contain considerably less Mg<sup>++</sup> than younger cells, and would not be expected to survive osmotic shock as well as the younger cells. It was no surprise, then, when water-washed stationary cells of S. marcescens grown in glycerol did not give normal plasmolysis and deplasmolysis. It was found, however, that in such stationary cells, the

addition of KCl to cell suspensions resulted in only a small plasmolysis peak, but the amount of light scattered soon fell considerably below the base line. A consideration of the amount of intracellular salts found by the atomic absorption technique and the concentration of extracellular tris buffer under these conditions indicated that such cells should indeed be plasmolyzed when they were resuspended in the tris buffer. Tris buffer itself can exert the osmotic pressure required to plasmolyze if present in hypertonic concentrations. By adjusting the sensitivity of the light scattering photometer so as to raise the original base line to a position normal for plasmolyzed cells, it was found that the addition of KCl resulted in fairly typical deplasmolysis curves. The earlier results showing a decrease of light scattered to levels below the base line were in fact indications of deplasmolysis from a condition of essentially complete plasmolysis. With proper instrumental sensitivity and through knowledge of the approximate original internal concentrations of salts, typical deplasmolysis curves and good light scattering data can be obtained with stationary cells of either organism. These light scattering results were confirmed by atomic absorption data which showed an increase in the  $K^+$  content of stationary cells suspended for a period in 0.08M KCl in tris buffer.

#### 3.5.4 Kinetic Studies: The Effect of Varying $K^+$ Concentration on the Initial Rate of Deplasmolysis

With the supporting evidence provided by atomic absorption data indicating that the deplasmolysis of S. marcescens and S. marinorubra in the presence of KCl is due to the uptake of  $K^+$ , it became possible to apply the technique of light scattering to a study of the kinetics of  $K^+$  transport by suspensions of resting cells. The effect of varying  $K^+$  concentration on the initial rate of deplasmolysis of glycerol-grown S. marcescens is shown in Figure 9. These results were obtained using cells washed twice in 0.1M  $MgCl_2$  and resuspended in 0.05M sodium bicarbonate buffer at pH 5.5. This buffer was selected because of its effective buffering capacity at the pH found to be optimal for  $K^+$  transport in S. marcescens. The  $Na^+$  present in the buffer does not interfere with the transport of  $K^+$  at pH 5.5. Prior to the addition of KCl to the cell suspension each 3 ml aliquot was plasmolyzed by the addition of 30  $\mu$ liters

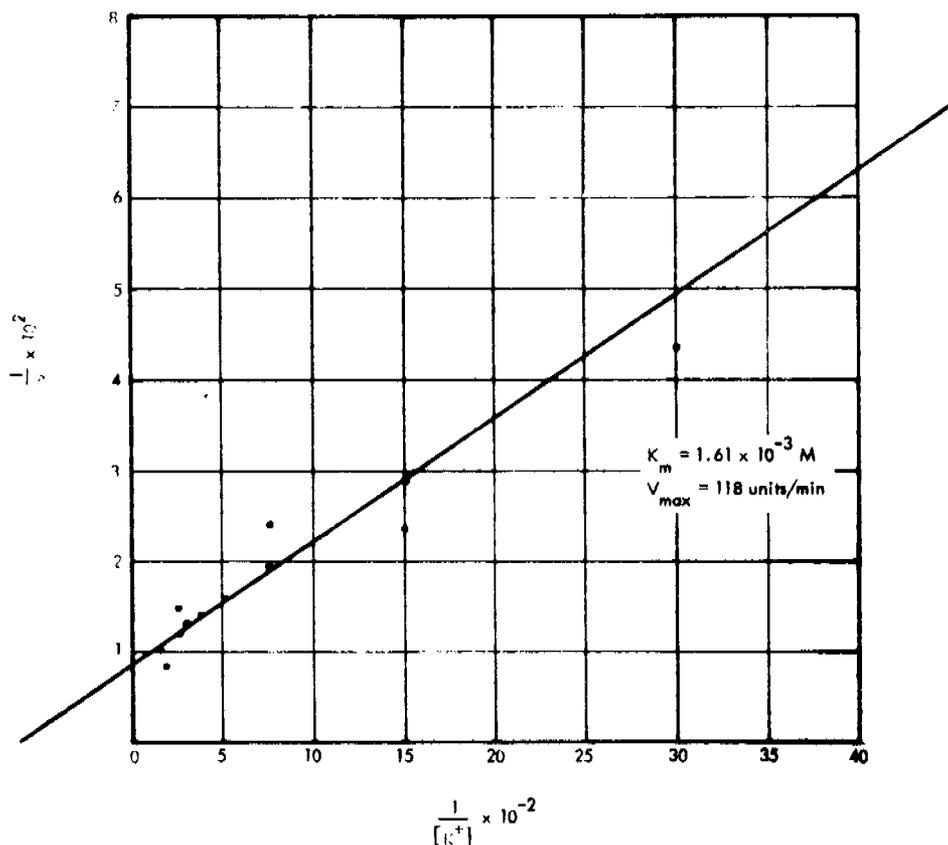


Figure 9. Effects of Varying  $K^+$  Concentration on Rate of Deplasmolysis of S. marcescens

of 4M  $MgCl_2$  ( $MgCl_2$  is not taken up by the cells and therefore deplasmolysis does not occur.) and allowed to give a stable base line. The addition of KCl resulted in deplasmolysis from a constant condition of nearly complete plasmolysis. The initial rates of deplasmolysis were found to obey typical Michaelis-Menten kinetics, as determined by the straight line graph produced by the Lineweaver-Burk plot ( $1/v$  versus  $1/S$ ). The velocity was expressed in terms of arbitrary units decrease in light scattering per unit time. From the figure it may be seen that half maximal velocity ( $K_m$ ) was obtained with  $1.6 \times 10^{-3}M$  KCl. These results were found to be reproducible when working with freshly prepared cells suspended in the bicarbonate buffer. However, bicarbonate buffer is not stable for long periods of time at this pH; thus, as the pH of the cell suspension slowly increased with time, the rates of deplasmolysis became erratic. Studies repeated in sodium phosphate buffer at pH 5.5 gave essentially the same  $K_m$  value, without the pH and stability problems associated with the bicarbonate buffer.

Future applications of this type of study will include comparative studies with S. marinorubra as well as using the technique to determine whether some of the inhibitory compounds used during the course of this study act as competitive or non-competitive inhibitors of the enzymes involved in the transport of  $K^+$ , and thus help to define the site of action of these inhibitors. By such techniques, some evidence of the chemical nature of the enzymes involved in transport may be obtained. A summary of the effect of these inhibitors on  $K^+$  transport and respiration is presented in a later section.

### 3.6 CORRELATION OF LIGHT SCATTERING AND ATOMIC ABSORPTION STUDIES

The correlation of results from light scattering and atomic absorption studies which provides the basis for the kinetic studies as described above is shown in Tables 7 and 8. It was found that when water-washed cells of glycerol-grown S. marcescens or S. marinorubra were resuspended in 0.08M KCl in 0.05M tris-HCl at pH 6.8, the internal concentration of  $K^+$  increased. The transport of  $K^+$  takes place with or without favorable concentration gradients, as shown by  $K^+$  accumulation in cases of low  $K^+$  concentrations (see Table 7).

Table 7. Uptake of  $K^+$  by Water-Washed Cells of S. marcescens

	<u>Treatment</u>	<u>Na<sup>+</sup> Mg/100 Mg Cells</u>	<u>K<sup>+</sup> Mg/100 Mg Cells</u>
I.	Tris	0.004	0.475
	Tris + 0.08M KCl	0.004	1.05
	Tris + 0.16M KCl	0.005	1.05
	Tris + 0.24M KCl	0.006	1.05
	Tris + 0.32M KCl	0.007	1.05
	Tris + 0.40M KCl	0.005	1.08
	None	0.010	0.54
II.	Tris	0.009	0.758
	Tris + 0.02M KCl	0.008	1.29
	Tris + 0.04M KCl	0.007	1.36
	Tris + 0.06M KCl	0.006	1.36
	Tris + 0.004M KCl	0.008	0.985

Table 8. Uptake of  $K^+$  by Water-Washed Cells of S. marinorubra

<u>Treatment</u>	<u>Na<sup>+</sup></u>	<u>K<sup>+</sup></u>
Tris	0.003	0.325
Tris + 2.5 mM glucose	0.012	0.300
Tris + 0.08M KCl	0.013	0.780
Tris + 0.08M KCl + 2.5mM glucose	0.007	0.902
Tris + 0.16M KCl	0.004	0.747
Tris + 0.16M KCl + 2.5mM glucose	0.002	0.975
Tris + 0.24M KCl	0.003	0.796
Tris + 0.24M KCl + 2.5mM glucose	0.003	0.926

NOTE: Glycerol-grown cells were harvested in the logarithmic phase of growth (OD = 0.7), aliquots were washed 2 times in water, suspended for ten minutes as indicated under Treatment, and subsequently recollected and washed two times in 0.1M  $MgCl_2$ . Finally, the cells were suspended in 9 ml tris + 1 ml  $HNO_3$  and held in a boiling water bath for 7 minutes. Following centrifugation, the supernatant was analyzed by atomic absorption spectroscopy. Tris was 0.05M at pH 6.8.

In the presence of NaCl, however, there is only a slight increase in the  $Na^+$  content of the cells (Tables 9 and 10). It is doubtful, then that the small increases found in atomic absorption studies can account for the rates and amounts of deplasmolysis seen with NaCl in buffered suspensions of S. marcescens, particularly at elevated pH.

The decrease in light scattering observed subsequent to the addition of NaCl, therefore, must be the result of transport of other substances in the suspension which might be accumulated. A study of the effect of increasing pH on deplasmolysis with buffered NaCl showed that as the pH was raised, the rate of deplasmolysis was markedly increased until the deplasmolysis curves resembled those obtained with KCl at lower pH values. Yet, atomic absorption data failed to reveal the uptake of significant amounts of  $Na^+$  (see Table 9). The only other substance in the suspension which might be accumulated is the tris buffer. When concentrated tris buffer alone was added to the suspension of cells in 0.05M tris

Table 9. Exclusion of Na<sup>+</sup> by Water-Washed Cells of S. marcescens

<u>Treatment</u>	<u>Na<sup>+</sup></u> <u>Mg/100 Mg Cells</u>	<u>X K<sup>+</sup></u> <u>Mg/100 Mg Cells</u>
<u>I</u>		
None	0.004	0.395
Tris	0.012	0.441
Tris + 0.08M NaCl	0.009	0.422
Tris + 0.16M NaCl	0.014	0.405
Tris + 0.24M NaCl	0.025	0.375
<u>II</u>		
Tris (pH 6.8)	0.007	0.490
Tris (pH 6.8) + 0.08M NaCl	0.027	0.456
Tris (pH 7.5) + 0.08M NaCl	0.051	0.447
Tris (pH 8.0) + 0.08M NaCl	0.041	0.396
Tris (pH 8.5) + 0.08M NaCl	0.067	0.404
Tris + 1.65 x 10 <sup>-2</sup> M NaCN (pH 11) + 0.08M NaCl	0.066	0.405

Table 10. Exclusion of Na<sup>+</sup> by Water-Washed Cells of S. marinorubra

<u>Treatment</u>	<u>Na<sup>+</sup></u>	<u>K<sup>+</sup></u>
Tris	0.010	0.217
Tris + 2.5 mM glucose	0.005	0.200
Tris + 0.08M NaCl	0.059	0.261
Tris + 2.5 mM glucose + 0.08M NaCl	0.040	0.243
Tris + 0.16M NaCl	0.097	0.191
Tris + 2.5 mM glucose + 0.16M NaCl	0.081	0.270
Tris + 0.24M NaCl	0.097	0.191
Tris + 2.5 mM glucose + 0.24M NaCl	0.121	0.270

Note: Unless otherwise noted, tris was the usual concentration (0.05M) at pH 6.8. Conditions were as described under Tables 7 and 8.

(final tris concentration became approximately 0.13M), deplasmolysis curves essentially identical to those obtained with added NaCl were observed. As the pH of the suspending and added buffer was raised, the rates of deplasmolysis became greater, typical of earlier results with NaCl in the presence of tris. The increase in rate of deplasmolysis with increasing pH is suggestive of transport of the uncharged, non-protonated, form of tris. Substitution of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) for tris did not alter the situation since this compound was also found to effect deplasmolysis of water-washed S. marcescens. The maximum rate of  $K^+$  transport occurs at pH 5.5 where the uncharged moiety of tris ( $pK = 8.1$ ) cannot interfere. Plasmolysis of S. marcescens or S. marinorubra with divalent cations was not followed by deplasmolysis<sup>(6)</sup>, despite the presence of tris, which in the presence of monovalent  $Na^+$  at least, aided deplasmolysis. The role of divalent cations in maintaining the integrity of the cell membrane and perhaps regulating the spacing between subunits of the membrane has been well established<sup>(4)</sup>, and in the presence of excess divalent cation, the available sites for transport of the uncharged tris molecule may be blocked.

### 3.7 EFFECT OF METABOLIC INHIBITORS ON $K^+$ TRANSPORT.

In an effort to elucidate the major enzymatic pathways and energy sources that contribute to ion transport, a study was made on the effects of various metabolic inhibitors on deplasmolysis and on the accumulation of  $K^+$  as determined by atomic absorption. The results of these studies are presented in Tables 11 and 12. The stimulation of deplasmolysis with NaCl, in the presence of NaCN (reported earlier) is attributed to pH effects and not to the specific presence of  $CN^-$ . If the NaCN is first adjusted to pH 6.8, the stimulatory effect is not evident. With or without adjusting the pH, atomic absorption data fails to show any significant accumulation of  $Na^+$  in the presence or absence of  $CN^-$ . As stated before, transport of tris is probably responsible for much of the observed deplasmolysis. Antimycin A, oligomycin and sodium amytal were found to have no effect on respiration or  $K^+$  transport, and are probably not allowed entry into the bacterial cells under the conditions employed. The use of higher concentrations of  $HgCl_2$  (Table 13) resulted in drastic losses of intracellular  $K^+$ , and probably causes disruption of the cell envelope as

Table 11. Effect of Inhibitors on Ion Transport and Respiration in *Serratia marcescens*\*

Inhibitor	K <sup>+</sup> Transport	Respiration
metachloro-CCP (in acetonitrile)	-	+
Sodium bisulfite 3.3 x 10 <sup>-3</sup> M	-	-
Sodium cyanide 10 <sup>-2</sup> M	-	-
Dinitrophenol 5 x 10 <sup>-4</sup> M	-	0
Dinitrophenol 1 x 10 <sup>-4</sup> M	- (slight)	0
Sodium fluoride 2 x 10 <sup>-2</sup> M	0	0
Sodium Iodoacetate 10 <sup>-2</sup> M	-	-
Sodium Arsenate 10 <sup>-3</sup> to 10 <sup>-2</sup> M	-	-
Sodium Amytal 3 x 10 <sup>-4</sup> M	0	0
Sodium Azide 1.67 x 10 <sup>-2</sup> M	-	-
Mercuric chloride	-	-

\* + stimulation

- inhibition

0 no apparent effect

Table 12. Atomic Absorption Data on Inhibition of  $K^+$  Transport

INHIBITOR	% INHIBITION			
	<u>S. marcescens</u>		<u>S. marinorubra</u>	
	Water	MgCl <sub>2</sub>	Water	MgCl <sub>2</sub>
45 $\mu$ M metachloro-CCP	100	47	64	71
$10^{-2}$ M Na <sub>3</sub> AsO <sub>4</sub>	100	97	30	35
$1.67 \times 10^{-2}$ M NaN <sub>3</sub>	67	43	13	6
$1.67 \times 10^{-2}$ M DNP	100	100	76	75
$1.67 \times 10^{-2}$ M NaF	0	--	0	--
$1.67 \times 10^{-2}$ M Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub>	30	--	--	--
$1 \times 10^{-6}$ M HgCl <sub>2</sub>	19	21	6	2
$5 \times 10^{-6}$ M HgCl <sub>2</sub>	16	--	40	44
$2 \times 10^{-4}$ M HgCl <sub>2</sub>	--	--	--	85
$1.5 \times 10^{-3}$ M HgCl <sub>2</sub>	Loss of all $K^+$			
$10^{-2}$ M NaCN	100			

Note: Glycerol-grown cells were harvested in the logarithmic phase of growth (OD = 0.8) and washed 2X in either water or 0.1M MgCl<sub>2</sub> as indicated. The pellet was resuspended for 10 minutes in 0.05M tris at pH 6.8 containing 0.08M KCl. In the case of S. marinorubra, 2.5 mM glucose was also added. This was followed by two washes in 0.1M MgCl<sub>2</sub> and subsequent analysis for intracellular cations. Inhibition was calculated on the basis of comparison with controls receiving no inhibitor. Inhibitors were all at pH 6.8 so as to eliminate pH effect. There was a 5 minute preincubation of cell suspension with inhibitor prior to addition of KCl.

Table 13. Effect of HgCl<sub>2</sub> on Cation Content of S. marcescens \*

Treatment	Na <sup>+</sup> mg/ 100 mg cells	K <sup>+</sup> mg/ 100 mg cells
Tris	.069	.772
Tris + 0.08M KCl	.069	1.081
Tris + 1.5 mM HgCl <sub>2</sub> + 0.08M KCl	.094	.051
Tris + 0.5 mM HgCl <sub>2</sub> + 0.08 M KCl	.099	.056
Tris + 0.1 mM HgCl <sub>2</sub> + 0.08M KCl	.089	.103
Tris + 0.05 mM HgCl <sub>2</sub> + 0.08M KCl	.088	.274
Tris + 5 μM HgCl <sub>2</sub> + 0.08M KCl	.070	1.040
Tris + 0.5 μM HgCl <sub>2</sub> + 0.08M KCl	.071	1.097

\* Conditions were as described under Table 7.

discussed by Rothstein.<sup>(12)</sup> Lower concentrations, approaching 10<sup>-6</sup> to 10<sup>-7</sup> M still inhibit the accumulation of K<sup>+</sup> as shown by both light scattering and atomic absorption results.

K<sup>+</sup> transport is adversely affected by practically every type of inhibitor employed, including compounds known to block glycolysis as well as respiration. A notable exception to this is NaF, shown to be a powerful inhibitor of glycolysis and respiration in many other systems. In the present study, concentrations of this compound up to 2 x 10<sup>-2</sup> M with or without preincubation did not cause significant inhibition of K<sup>+</sup> uptake or respiration.

In a study such as this, it must be considered that any given inhibitor may actually be acting in a number of sites, at one or a number of specific enzymatic steps in glycolysis or respiration, or perhaps directly with the cell envelope and/or the carrier system involved in the transport of cations. As mentioned earlier, future kinetic studies may help define the site of action of some of these compounds.

#### 4. CONCLUSIONS

1. Biological membranes can be chemically and physically treated to alter their ion transport properties.
2. For greatest retention of intracellular materials, maximum respiratory rates, transport rates, etc. for the preparation of bacterial cells for study, the use of  $MgCl_2$  or probably salts of divalent metals in general at a concentration approximately isotonic to the interior of the cell appear to be best.
3. The retention of intracellular salts is dependent, in part, upon the pH of the washing solution. Essentially all the intracellular  $K^+$  is lost to the washing solution at approximately pH 3.0.  $Mg^{++}$  associated with the cells is lost to the washing medium at approximately pH 2.0. The loss of intracellular  $K^+$  and the extreme fragility of the cells at low pH are probably related to partial or complete displacement of the  $Mg^{++}$  and other polyvalent cations bound to the cell envelope and responsible for its integrity.
4. The intracellular content of bacterial cells can be greatly affected by the nature of the medium in which they are grown and also by the procedures used to prepare them for study, particularly the type of suspending medium employed. The following relationships were observed:
  - a) S. marcescens is generally more capable of retaining its intracellular  $K^+$  during water washing than similarly grown and treated S. marinorubra.
  - b) S. marinorubra contains considerably more intracellular  $Mg^{++}$  than S. marcescens. Growth of the latter organism in the presence of 3% NaCl causes an increase in its intracellular  $Mg^{++}$  concentration.
  - c) Stationary cells of either organism contain less  $Mg^{++}$  and late stationary phase cells contain less  $K^+$  than logarithmic phase cells.

5. Growing or resting cells of both micro-organisms transport  $K^+$  against a concentration gradient and exclude  $Na^+$ . When grown in a medium containing added  $NaCl$ ,  $Na_2SO_4$ , or  $KCl$ , the intracellular  $K^+$  content increases markedly.
6. The transport of  $K^+$  occurs most readily at a pH of 5.8 to 6.1 and under specified conditions in freshly prepared cells, appears to obey Michaelis-Menten kinetics. The  $K_m$  for  $K^+$  is of the order of  $10^{-3}M$ .
7.  $K^+$  transport is inhibited by a variety of glycolytic and respiratory inhibitors as well as by anaerobiosis. It would thus appear that the process of  $K^+$  accumulation depends upon some aspect of electron transport through the respiratory chain. The possibility of concomitant reaction between inhibitory compounds and the cell envelope and/or enzymes directly involved in transport cannot be ruled out. The only inhibitor not affecting transport was  $NaF$ .
8.  $Na^+$  is not normally transported to any appreciable extent, and deplasmolysis in the presence of tris-buffered  $NaCl$ , especially at elevated pH, is probably due to transport of tris.
9. Preliminary studies fail to reveal exchanges of external  $K^+$  for intracellular  $Na^+$  or  $H^+$ .
10. S. marcescens contains considerably more glycogen than S. marinorubra and this is probably responsible for greater rates of  $K^+$  transport and respiration in the former micro-organism. Addition of an exogenous energy source such as glucose to suspensions of S. marinorubra enhances the rates of respiration and  $K^+$ , but not  $Na^+$ , accumulation.
11. The ability of cells to plasmolyze and deplasmolyze, and the magnitude of plasmolytic response, depends upon a number of factors:
  - a) Nature of washing medium and preparation procedure.
  - b) Intracellular ionic strength—after preparation procedure, and relation of intracellular to extracellular ionic strength.
  - c) Ability of the cells to retain intracellular contents in the presence of the suspending medium.

## 5. RECOMMENDATIONS

1. Continue studies to learn how to more effectively control ion transport properties and increase rates of  $K^+$  transport. Evaluate the effects of ionic strength on ability of cells to transport selected cations.
2. Use of kinetic studies to explore possible competitive and non-competitive inhibition of  $K^+$  transport by other cations and specific metabolic inhibitors.
3. Extension of these kinetic studies to include S. marinorubra grown in the presence and absence of NaCl.
4. Extensive light scattering studies with stationary cells to observe what effects the higher content of glycogen may have on rates of ion transport.
5. Use of radioisotopes of  $K^+$ ,  $H^+$ , and  $Na^+$  to detect possible exchange reactions.
6. Control the pH of organisms grown in presence of NaCl to better define the effect of final pH of the culture on cation transport.
7. Analyze products of glycerol metabolism in the presence and absence of NaCl. This will allow some idea of the pathways involved in the metabolism of glycerol and possible alterations in these pathways caused by high ionic strength.
8. Look for effects of increased ionic strength on specific enzymes involved in those pathways where NaCl or high intracellular  $K^+$  concentration may exert some controlling effect.
9. Study the effect of varying the anion of the suspending or plasmolyzing medium and perform quantitative analyses for such common anions as  $Cl^-$ ,  $PO_4^{-3}$ ,  $SO_4^{-2}$ , etc.

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