In this study the relationship between salinity and temperature changes on microbial growth, enzyme induction and substrate uptake were investigated. The obligately psychrophilic bacterium, *V. marinus* MP-1 was grown for 48 hours at 15°C in a glucose-ammonium medium (GAM) containing 0.4 M NaCl. Log-phase cells were harvested and shifted to a glutamate medium (GM) at either 4, 15, 20 or 25°C and containing 0.26, 0.4, 0.6 or 0.8 M NaCl. It was found that at any one temperature investigated (with the exception of 25°C), an increase in the amount of NaCl in GM from 0.26 to 0.4 M resulted in an increase in shifted cell growth. A further increase in NaCl to 0.6 or 0.8 M reduced the net cellular growth. Of the four salinities tested, 0.4 M NaCl was determined as being optimal for growth. Growth
temperature profile studies in a polythermostat revealed an optimal growth temperature of 12-13 C and a maximum of 20-22 C in GAM (0.4 M NaCl), GM (0.4 M NaCl), and marine broth (MB).

Cells shifted to GM at 4 C showed only low levels of induction of glutamic dehydrogenase (GDH) after ten hours in any NaCl concentration. Increasing the shift temperature to 15 C rapidly increased the induction rate of GDH with a maximum at 0.4 M NaCl (56-fold increase in ten hours). A temperature increase to 20 C in the shift medium produced lower rates of induction than at 15 but a maximum in 0.4 M NaCl was maintained (35-fold increase in ten hours). Cells shifted to GM at 25 C initially began to produce GDH but ceased to do so within 2-4 hours since all cells died within two hours after shifting.

Uptake of $^{14}$C-glutamate by shifted cells, at the various temperatures and salinities tested, revealed a maximal rate of uptake and net incorporation of label into cells at 0.26 M NaCl at 15 C. Further increases in salinity (0.4, 0.6 and 0.8 M) or alterations in temperature (4, 20, 25 C) reduced the uptake rate and net incorporation of the isotope. One exception, however, occurred at 25 C where the maximal rate of uptake and net incorporation occurred in 0.6 M.

Measurement of uptake and incorporation of $^{14}$C-proline into protein by shifted cells at various salinities and temperatures revealed essentially the same pattern as in the $^{14}$C-glutamate studies, except that maxima for both determinations occurred at 20 C.
Radiorespirometry revealed that increasing salt concentrations from 0.26 M to 0.8 M reduces cell respiration at 15 C but does not prevent it. It also showed that 2% of the $^{14}$C-glutamate available to the cells was taken up after one hour incubation in GM (0.4 M NaCl) at 15 C. Of the total amount available, only 0.2% (12% of that taken up) was respired as $^{14}$CO$_2$.

These data are interpreted to mean that the induction of GDH is directly related to the growth rate of the organism in GM. The growth rate at any one temperature, however, is salinity dependent. Sub-optimal NaCl concentrations may well be affecting some integral part of the cells structural integrity necessary for GDH synthesis, since a NaCl concentration of 0.26 M allowed a maximal rate of entry and accumulation of $^{14}$C-glutamate into cells at 15 C, as well as a maximal rate of protein synthesis and respiration, but produced sub-optimal growth rates. It would appear that growth rate limitation by lowered NaCl concentrations is not a direct consequence of any of these processes. In addition, since 0.4 M NaCl allowed maximal GDH induction and produced a maximal growth rate, it seems likely that this concentration of NaCl enhances GDH induction, thereby producing a maximal rate of growth. NaCl concentrations greater than 0.4 M generally seem to retard GDH induction by inhibiting substrate uptake, protein synthesis, and respiration, thus inhibiting growth.

Metabolic inhibitor studies revealed that energy generation is
necessary for $^{14}$C-glutamate uptake at 15 C in GM (0.4 M NaCl).

The chloride ion (Cl$^-$) seems to be required for the uptake of $^{14}$C-glutamate at 15 C in GM (0.4 M NaCl).

by

Thomas Edwin Staley

A THESIS submitted to Oregon State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

June 1971
APPROVED:

Redacted for Privacy

Professor of Microbiology and Oceanography
in charge of major

Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented  August 31, 1970

Typed by Donna L. Olson for  Thomas Edwin Staley
ACKNOWLEDGEMENTS

The authors wishes to express his sincere appreciation and gratitude to:

Professor Richard Y. Morita for his patience and confidence in my work.

The Public Health Service for financial support under Trainee-ship 5-T01-GM00704.

My Marine Microbiology cohorts (Bob, Frank, George, Jan, Janet, Joe, Kala, Larry, Mary Faith, Pat, Paul, and Yusan--in order of decreasing solipsistic grandeur) for an extraordinary array of physical and metaphysical experiences.

My Doctoral Committee--Professor P. R. Elliker, Professor R. Y. Morita, Professor M. E. Morgan, Associate Professor J. E. McCauley and Associate Professor J. L. Fryer.

My parents, whose unfailing support and personal sacrifices throughout the years has enabled me to confidently and continually pursue my educational and professional goals.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>Organism and Maintenance Conditions</td>
<td>13</td>
</tr>
<tr>
<td>Growth and Preparation of Cells</td>
<td>13</td>
</tr>
<tr>
<td>Medium Shifting Procedure for Glutamic Dehydrogenase (GDH) Induction</td>
<td>15</td>
</tr>
<tr>
<td>Methods of Enzyme Production and Assay</td>
<td>16</td>
</tr>
<tr>
<td>Thermostability of GDH</td>
<td>19</td>
</tr>
<tr>
<td>Protein Determination</td>
<td>20</td>
</tr>
<tr>
<td>Standardization of Cell Suspensions</td>
<td>20</td>
</tr>
<tr>
<td>Optimum Growth Temperature</td>
<td>20</td>
</tr>
<tr>
<td>Uptake of $^{14}$C-Glutamate by Whole Cells</td>
<td>21</td>
</tr>
<tr>
<td>Incorporation of $^{14}$C-Glutamate into Protein by Whole Cells</td>
<td>22</td>
</tr>
<tr>
<td>Uptake and Incorporation into Protein of $^{14}$C-Proline by Whole Cells</td>
<td>22</td>
</tr>
<tr>
<td>Salt Specificity for $^{14}$C-Glutamate Uptake</td>
<td>23</td>
</tr>
<tr>
<td>Respiration Studies</td>
<td>24</td>
</tr>
<tr>
<td>Uptake Inhibition Studies</td>
<td>25</td>
</tr>
<tr>
<td>Phase Microscopy</td>
<td>25</td>
</tr>
<tr>
<td>Dry Weight Determinations on Shifted Cells</td>
<td>26</td>
</tr>
<tr>
<td>Reagents and Biochemicals</td>
<td>26</td>
</tr>
<tr>
<td>RESULTS</td>
<td>27</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>85</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>99</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>102</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Growth of <em>V. marinus</em> MP-1 upon shifting cells from GAM to GM at 15 C containing various molarities of NaCl.</td>
</tr>
<tr>
<td>2.</td>
<td>Growth of <em>V. marinus</em> MP-1 upon shifting cells from GAM to GM at 20 C containing various molarities of NaCl.</td>
</tr>
<tr>
<td>3.</td>
<td>Growth of <em>V. marinus</em> MP-1 upon shifting cells from GAM to GM at 25 C containing various molarities of NaCl.</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of increased concentrations of NaCl in the assay buffer on the GDH activity of a CFE (7.0 mg protein/ml) prepared with wash buffer.</td>
</tr>
<tr>
<td>5.</td>
<td>Efficacy of using various suspending solutions in the preparation of cell-free extracts for GDH activity.</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of salinity and temperature changes on the specific activity (U/mg protein) of GDH from <em>V. marinus</em> MP-1 ten hours after shifting cells from GAM to GM.</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of salinity and temperature changes on the uptake of $^{14}$C-glutamate by <em>V. marinus</em> MP-1.</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of NaCl on the evolution of $^{14}$CO$_2$ by <em>V. marinus</em> MP-1 in GM (3.0 mM glutamate) at 15 C containing 0.2 µC $^{14}$C-glutamate/ml cell suspension.</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of various metabolic inhibitors on the uptake of $^{14}$C-glutamate (0.2 µC/ml cell suspension) by cells of <em>V. marinus</em> MP-1 in GM (3.0 mM glutamate at 15 C.</td>
</tr>
<tr>
<td>10.</td>
<td>Comparison of natural and experimental parameters under which <em>V. marinus</em> MP-1 was exposed.</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Flow diagram for monitoring the induction of GDH in <em>V. marinus</em> MP-1 on shifting cells from GAM to GM containing various molarities of NaCl.</td>
<td>17</td>
</tr>
<tr>
<td>2.</td>
<td>Growth of <em>V. marinus</em> MP-1 at 15 C in GAM and upon shifting to GM containing various concentrations of NaCl.</td>
<td>28</td>
</tr>
<tr>
<td>3.</td>
<td>Change in mass (dry weight) of <em>V. marinus</em> MP-1 cells cultured in GAM for 48 hours at 15 C and shifted to GM at 15 C containing various concentrations of NaCl.</td>
<td>29</td>
</tr>
<tr>
<td>4.</td>
<td>Growth of <em>V. marinus</em> MP-1 cultured in GAM for 48 hours at 15 C and shifted to GM at 20 C containing various concentrations of NaCl.</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>Change in mass (dry weight) of <em>V. marinus</em> MP-1 cells cultured in GAM for 48 hours at 15 C and shifted to GM at 20 C containing various concentrations of NaCl.</td>
<td>34</td>
</tr>
<tr>
<td>6.</td>
<td>Growth of <em>V. marinus</em> MP-1 cultured in GAM for 48 hours at 15 C and shifted to GM at 25 C containing various concentrations of NaCl.</td>
<td>37</td>
</tr>
<tr>
<td>7.</td>
<td>Change in mass (dry weight) of <em>V. marinus</em> MP-1 cells cultured in GAM for 48 hours at 15 C and shifted to GM at 25 C containing various concentrations of NaCl.</td>
<td>38</td>
</tr>
<tr>
<td>8.</td>
<td>Growth of <em>V. marinus</em> MP-1 in MB after 24 (O—O) and 48 hours (Δ—Δ).</td>
<td>41</td>
</tr>
<tr>
<td>9.</td>
<td>Growth of <em>V. marinus</em> MP-1 in GM containing 0.4 M NaCl after 24 (O—O) and 48 hours (Δ—Δ).</td>
<td>42</td>
</tr>
<tr>
<td>10.</td>
<td>Growth of <em>V. marinus</em> MP-1 in GAM after 56 (O—O) and 76 hours (Δ—Δ).</td>
<td>43</td>
</tr>
</tbody>
</table>
11. Effect of pH on the percent maximal activity of GDH using a potassium phosphate-0.05 M buffer in the assay procedure (the amount of activity obtained at pH 9.5 was taken as 100%).

12. Effect of pH on the percent maximal activity of GDH using a Tris-HCl buffer, 0.05 M (○-○) and 0.5 M (△-△) in the assay procedure (the amount of activity obtained at pH 8.5 and 9.5 in 0.05 M and 0.5 M buffer, respectively, was taken as 100%).

13. Effect of pH on the percent maximal activity of GDH using a Tris-SO\(_4\) buffer, 0.05 M (○-○) and 0.5 M (△-△) in the assay procedure (the amount of activity obtained at pH 8.5 and 9.5 in 0.05 M and 0.5 M buffer, respectively, was taken as 100%).

14. Effect of diluting CFE in wash buffer on its GDH activity in 43.5 μ mol Tris-HCl (pH 9.0), 0.75 μM NAD\(^{+}\) and 200 μM glutamate per ml at 15 C.

15. Effect of heating a CFE (5.8 mg protein/ml) prepared with wash buffer on GDH activity (○-○-0 C, △-△-6.5 C, □-□-15 C, ○-○-19 C, ▽-▽-25 C). The amount of activity obtained immediately upon preparation of the CFE was taken as 100%.

16. Thermostability of GDH at 2 C in cell-free extracts prepared in wash buffer (○-○) containing 0.4 M NaCl (8.8 mg protein/ml) and in potassium phosphate (△-△), 0.05 M, pH 7.6 (10.0 mg protein/ml). The amount of activity obtained immediately upon preparation of the CFE was taken as 100%.

17. Induction of GDH in \textit{V. marinus} MP-1 on shifting cells from GAM to GM at 4 C containing various molarities of NaCl.

18. Induction of GDH in \textit{V. marinus} MP-1 on shifting cells from GAM to GM at 15 C containing various molarities of NaCl.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>19.</td>
<td>Induction of GDH in <em>V. marinus</em> MP-1 on shifting cells from GAM to GM at 20°C containing various molarities of NaCl.</td>
</tr>
<tr>
<td>20.</td>
<td>Induction of GDH in <em>V. marinus</em> MP-1 on shifting cells from GAM to GM at 25°C containing various molarities of NaCl.</td>
</tr>
<tr>
<td>21.</td>
<td>Relationship between OD and the number of viable cells per ml of wash buffer.</td>
</tr>
<tr>
<td>22.</td>
<td>Time course of $^{14}$C-glutamate (0.2 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 4°C at various NaCl concentrations.</td>
</tr>
<tr>
<td>23.</td>
<td>Time course of $^{14}$C-glutamate (0.2 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.</td>
</tr>
<tr>
<td>24.</td>
<td>Time course of $^{14}$C-glutamate (0.2 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 20°C at various NaCl concentrations.</td>
</tr>
<tr>
<td>25.</td>
<td>Time course of $^{14}$C-glutamate (0.2 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 25°C at various NaCl concentrations.</td>
</tr>
<tr>
<td>26.</td>
<td>Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 4°C at various NaCl concentrations.</td>
</tr>
<tr>
<td>27.</td>
<td>Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.</td>
</tr>
<tr>
<td>28.</td>
<td>Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) uptake by <em>V. marinus</em> MP-1 cells in GM (3.0 mM glutamate) at 20°C at various NaCl concentrations.</td>
</tr>
</tbody>
</table>
29. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) uptake by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 25°C at various NaCl concentrations.

30. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) incorporation into cellular protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 4°C at various NaCl concentrations.

31. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) incorporation into cellular protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.

32. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension incorporation into cellular protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 20°C at various NaCl concentrations.

33. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) incorporation into cellular protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 25°C at various NaCl concentrations.

34. Incorporation of $^{14}$C-glutamate (0.2 µC/ml cell suspension) into protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.

35. Uptake of $^{14}$C-glutamate (0.2 µC/ml cell suspension) at 15°C containing 0.4 M of various salts (Ac = acetate).

36. Effect of replacing 0.4 M NaCl with the same molarity KCl, NaAc, or KAc in GM (3.0 mM glutamate) at 15°C on the OD of *V. marinus* MP-1 cells.

37. Effect of various metabolic inhibitors on the uptake of $^{14}$C-glutamate (0.2 µC/ml cell suspension) in GM (0.4 M NaCl) containing 3.0 mM glutamate at 15°C by *V. marinus* MP-1 cells.
38. Effect of various metabolic inhibitors on the uptake of 14C-glutamate (0.2 µC/ml cell suspension) in GM (0.4 M NaCl) containing 3.0 mM glutamate at 15°C by V. marinus MP-1 cells.

39. Diagramatic representation of possible mode of GDH induction inhibition in GM (0.26 M NaCl) at 15°C containing 3.0 mM glutamate after one hour incubation.
EFFECTS OF SALINITY AND TEMPERATURE CHANGES ON THE INDUCTION OF GLUTAMIC DEHYDROGENASE IN THE MARINE PSYCHROPHILIC BACTERIUM, \textit{Vibrio marinus} MP-1

INTRODUCTION

Enzymatic induction in bacteria has long been the subject of much intensive investigation. Data, to date have indicated that the normal response of bacteria to a shifting physical or chemical environment is rapid and predictable. Most of these studies have, however, been done with mesophilic bacteria whereby cells were exposed to various changes in their organic milieu and the resulting metabolic rearrangements monitored as precisely as possible.

It is the purpose of this study, then, to determine the effect of changing salinities and temperatures on the growth, enzymatic induction, and substrate uptake by the obligately psychrophilic, marine bacterium, \textit{Vibrio marinus} MP-1.

Information on these processes is important from several aspects. The significance of marine bacteria in the metabolism of the sea (93) has often been questioned due to their apparent low numbers in the water column and minimal metabolic rates under the \textit{in situ} conditions of high salinity and low temperature, usually combined with high hydrostatic pressure. There are, however, reports indicating that the heterotrophic bacterial population in the water column may
range from 0 to $10^5$ organisms/liter (64). The uppermost sediments of estuaries and certain sea bottoms have particularly high populations of heterotrophic bacteria. Oppenheimer (64) reports $5 \times 10^3$ to $1.5 \times 10^8$ cells/g of wet sediment from a Texas estuary. Morita and ZoBell (56) found populations of viable aerobic bacteria ranging from $10^4$-$10^5$ cells/g of wet red clay sediment at a depth of 4484 m in the mid-Pacific. These reports, and numerous others, definitely indicate that the original assumption regarding the paucity of bacteria in the marine environment, both estuarine and deep-sea, is no longer valid.

The metabolic capability of marine microorganisms has also been demonstrated to be considerable, even at very low temperatures. Morita and Albright (53) have shown that V. *marinus* MP-1 produced culture densities of $9 \times 10^9$ cells/ml in 24 hours at 3 C. Sinclair and Stokes (77) reported culture densities of $1.8 \times 10^9$ cells/ml in 170 hours with *Pseudomonas fluorescens* at 10 C. Numerous other reports attest to the relatively high metabolic capabilities of bacteria, particularly psychrophiles, at low temperatures.

With the knowledge that marine bacteria can and do contribute to the overall nutritional cycling of the sea, it is important to determine the extent to which this occurs. The rate of metabolic activity of a bacterium, and therefore the extent to which it participates in nutrient cycling, is a function of the chemical and physical parameters existing at the time when nutrients present themselves. Consider, for
example, the broad range of salinities and temperatures that microorganisms in tidal mud flats must successfully endure diurnally. It is also interesting to speculate on the physiological reaction of marine bacteria subjected to an influx of nutritionally rich and relatively warm fresh-water of terrestrial origin, or conversely, on the effect of increased salinity and colder temperatures of oceanic waters on fresh-water organisms (such as the important pollution organisms) swept out in rivers into the sea. Other areas of the sea where microorganisms might be exposed to wide variations in salinity and temperature would be in regions of upwelling or sinking or at cold and warm current convergences.

All of these examples point out the fact that marine microorganisms are constantly vulnerable to salinity, temperature, and pressure changes which may drastically affect the functioning of the cells. A knowledge of the results of these changes on the metabolic potentiality of marine microorganisms under near-environmental conditions is of paramount importance if we are to understand and more wisely participate in the dynamic economy of the oceans.
REVIEW OF LITERATURE

The existence of indigenous marine bacteria in oceanic waters, both inshore and open, and marine sediments has been firmly established (34, 55, 63, 64, 65, 94). Taxonomic analysis of some marine benthic isolates from the Oregon-Washington continental shelf and slope revealed that *Pseudomonas* (71%) was the predominant genus followed by *Achromobacter* (9%), *gram-variable rods* (6%), *Aeromonas* (5%), *Bacillus* (3%) and miscellaneous (5.5%) (89). Quigley and Colwell (70) reported that 95% of the isolates from deep-sea sediments of the South Pacific to be pseudomonads and the remainder aeromonads when subjected to computer taxonomic analysis. *Vibrio marinus* MP-1 was isolated from the water column at a depth of 1200 m off the Oregon Coast (55).

Although it is generally accepted that marine bacteria are stenohaline and stenothermic, Wiebe and Liston (90) have presented data indicating that this is not the case with some marine sediment isolates. Fifty percent of the strains grew at 35 C of which 44% of these would grow only in sea water medium at this temperature. Only 14% of their isolates required sea water for growth on initial isolation (a criterium originally proposed by ZoBell and Upham to define a marine bacterium (95)). Also, only 1.5% of their isolates were considered obligate psychrophiles (i.e. no growth above 28 C). They
summarized their findings as follows, "In general, it appears as though marine bacteria are well adapted to the marine environment, but are physiologically versatile, i.e. capable of growth under conditions at temperature and salinity not commonly found in the sea" (90).

The organism used in this study, *Vibrio marinus* MP-1, was reported to grow from -1 (the lowest tested) to 20 C with an optimum at 15-16 C (an obligate psychrophile) and to lose viability by heating at 28.8 C for 6.25 hours (55). It, like the majority of marine bacteria, is heterotrophic.

The effect of salt (NaCl) on microbial growth has received considerable attention (14, 15, 19, 20, 30). Larsen (38) has proposed several hypotheses pertaining to growth inhibition by salt: (a) deprivation of needed water by the organism, (b) direct action on structure or function of the cell surface, (c) osmotic damage (water diffusing out), (d) Na⁺ interference directly with metabolism, therefore, no osmotic effect. Pratt and Austin (68) found that the growth rate of selected marine bacteria, and not the total crop, was regulated by the concentration of NaCl. They found that Mg⁺⁺, K⁺, sucrose and lactose exerted a sparing effect on the Na⁺ concentration but could not replace the Na⁺ requirement. Matches and Liston (45) reported that a salt concentration in growth media of 3% or greater prevented growth of some *Salmonella* species at 8 C but at 37 C, 7-8% was required (i.e., increased temperatures diminish the inhibitory effect of
NaCl). Johnson and Johnson (30) have shown that the limiting range of NaCl concentrations permitting growth of a halophilic green alga Dunaliella salina could be altered depending on the concentration in the original growth medium. Cells previously grown in media containing 7.5% NaCl grew in 1-22% NaCl media, while those previously grown in 22% were able to grow in 2-28% NaCl. Cells grown in sub-optimal salt concentrations had distinct lag phases, smaller total cell yields and somewhat decreased maximal growth rates. Ritchie (72) reports that the mold Eurotium halophilicum is able to grow at 30 C in media containing 20% NaCl, but not at the same temperature if the NaCl concentration is lowered to 10%. Stanley and Morita (79) found that they could increase the maximal growth temperature of Vibrio marinus MP-1 from 10.5 to 20 C by increasing the salt concentration in a defined medium from 0.2 to 0.4 M. A further increase in salt to 0.6 M reduced the maximal growth temperature to 19 C. Flannery and Durio (15) were able to show that increased concentrations of NaCl (1.5, 2.0, and 3.0 M) relieved the depressant effect of high cysteine substrate on oxidation at 0.5 and 1.0 M NaCl, suggesting a higher rate of pyruvate utilization with increased concentrations of NaCl.

MacLeod and his collaborators have done considerable work on the Na\(^+\) requirement of a marine pseudomonad (40, 41, 42, 84). In summary, they have found that Na\(^+\) does not stimulate the activity of enzymes from the pseudomonad, but it does help in maintaining the
stability of enzymes. Also, Na⁺ was found to be required for the uptake of the non-metabolizable substrate, α-amino-isobutyric acid, as well as for maintaining the integrity of the cytoplasmic membrane and mucoproteptide layer in the cell wall. Other investigators (35, 61, 78) using halophilic organisms think that Na⁺ is required for the maintenance of cell membrane integrity by binding to neighboring negatively charged groups on the membrane, thus decreasing the mutual electrostatic repulsion. Stevenson (80) has reported that NaCl is required for the functioning of a preformed glutamate transport system in Halobacterium salinarum, presumably due to its necessity for energy production (DNP severely reduced the uptake). Similarly, Drapeau and MacLeod (11) found Na⁺ to be required for the functioning rather than the formation of a transport system in the marine pseudomonad, since chloramphenicol did not prevent uptake of substrate by cells grown in the absence of that substrate. Frank and Hopkins (16) found that Na⁺ stimulated the uptake of glutamate by Escherichia coli B, while Kahana and Avi-Dor (31) observed no stimulation of glutamate oxidation by sodium in broken cell preparations of the same organism, whereas the cation did stimulate glutamate oxidation by intact cells.

Organisms have recently been classified according to their growth-temperature characteristics. Thermophiles are those organisms capable of growing best between 40 and 100 C, while mesophiles are able to do so from 20-40 C. Psychrophiles are able to grow best from
Although the effect of temperature on the growth of microorganisms has been the subject of numerous reviews (5, 9, 12, 13, 28), relatively little data has been reported on psychrophiles. Numerous proposals have been presented to explain the phenomenon of psychrophily on a biochemical basis. There are many reports implicating abnormally thermolabile enzymes as the cause of psychrophily (69, 81, 82). Mathemeier (46) has reported that certain enzymes of the tricarboxylic acid cycle and glycolytic pathway from the obligate psychrophile, *V. marinus* MP-1 to be conspicuously thermal labile (succinic dehydrogenase was 50% inactivated at 16 °C for one hour). Similarly, Langridge and Morita (36) have shown malic dehydrogenase from *V. marinus* MP-1 to be stable to heating between 0 and 15 °C intracellularly, but to be considerably labile in cell-free extracts even at 0 °C. Farrell and Rose (13) have proposed that psychrophily may be due to the synthesis or accumulation of metabolic poisons at superoptimal temperatures. Nashif and Nelson (58), however, gave evidence indicating that psychrophiles were unable to function at elevated temperatures due to damage or destruction of metabolic control, since their pseudomonad synthesized lipase at 15 °C or less but not at 30 °C even though the optimal temperature for activity was 40 °C. Upadhay and Stokes (86) have demonstrated that mesophilic and
facultative psychrophilic *E. coli* strains were unable to synthesize formic hydrogenlyase above 20 C, even though the enzyme from the psychrophilic strain was optimally active at 30 C and inactive at 45 C. Other investigators attribute psychrophily to the loss or change of permeability of the cells at restrictive growth temperatures (12, 74). It should be apparent that no one explanation is sufficient, as of yet, to explain the psychrophilic nature of microorganisms.

Bacteria have been shown to possess at least two basic mechanisms whereby the synthesis of a particular enzyme can be controlled to meet the cells' requirements. Constitutive control is a type of regulation whereby, ideally, the enzyme comprises a constant fraction of the cellular protein, irrespective of the growth conditions (18). Another type of enzymatic regulation is inducible control. With this mode of control, concentrations of some enzymes in the cells (expressed as the amount of enzyme per mg cell of protein or specific activity) can vary strikingly, by factors of hundreds or thousands, depending on the nutritional conditions (66).

The model system upon which most of the current induction theory is based comes from data on β-galactosidase induction in *E. coli*. Horiuchi *et al.* (27) have reported on the effect of temperature on the rate of β-galactosidase synthesis in a temperature sensitive (ts) mutant of *E. coli*. They inferred that the repressor substance was thermolabile in the ts mutant and was, therefore, unable to stop the
synthesis in the enzyme at elevated temperatures. Marr et al. (44) postulated that the effect of temperature is on the concentration of repressor for β-galactosidase in E. coli and not on the equilibrium between repressor and its site of action. Ng et al. (59) have postulated that the synthesis of tryptophanase at 35°C but not at 20°C was due to the over production of repressors at the low temperature and not an interference with the induction process, per se. Gray and Clark (22) have reported the repression of glutamic dehydrogenase in cells grown in the combined presence of threonine and lysine but not with glutamate or amino acids of the glutamate family. Bannerjee and Liston (2) have presented evidence indicating that the temperature at which cells are grown determines their subsequent inducibility. Cells of their psychrophilic pseudomonad grown at 22°C could be induced for creatinase while those grown at 8°C could not, even though 8°C grown cells could produce the enzyme adaptively. Weimer (91) found that the amount of gelatin hydrolyzed by V. marinus MP-41 increased with increasing temperatures from 5 to 24°C. She interpreted this as induction of gelatinase, since only short incubation periods were used preventing cell proliferation. Rhodes and Payne (71) have found that the induction of resting cells of Pseudomonas natriegens (a marine bacterium) to the oxidation of lactose and mannitol was dependent on the presence of Na⁺ with marine levels of Mg⁺ and K⁺ in the suspending medium. McElroy (49) has
reported that the concentration of NaCl in the growth medium determines the rate of synthesis of luciferase. At 1% NaCl, very little enzyme is found by Achromobacter fischeri, but when cells are shifted to 3% NaCl medium, a rapid synthesis of luciferase, and thus light intensity occurs. These are the only references encountered that implicate a Na$^+$ requirement in the formation of inducible enzymes.

Due to this paucity of information of the role of Na$^+$ in the formation of induced enzyme systems, this investigation was undertaken in order to determine the relationship between salinity and temperature changes on growth, enzyme induction, and amino acid uptake of V. marinus MP-1.

An induction scheme involving glutamic dehydrogenase (GDH) was selected because of the enzyme's strategic position in the metabolic pathway (oxidatively deaminating glutamic acid to $\alpha$-ketoglutaric acid, a key intermediate in the TCA cycle) and because glutamic acid is frequently found in marine waters. Siegal and Degens (76) reported from 1.6 to 2.7 $\mu$g/l of dissolved glutamic acid in Buzzards Bay sea water, however, most of it was in the combined, rather than the free form. Park (67) has reported a concentration of $>$1 mg/m$^3$ of glutamic acid in deep-sea water. Johannes and Webb (29) reported that live zooplankton incubated at 7-25.6 C released 2.4-30.5 mg $\alpha$-amino nitrogen/gm dry weight/day.

GDH has been reported in a wide variety of animals (8, 17, 25,
and microorganisms (3, 10, 22, 39, 65, 75). The majority of glutamic dehydrogenases require NAD$^+$ as a cofactor and are relatively heat stable. Although there are few reports on the effect of NaCl on the functioning of this enzyme, data on other enzymes may well applicable to the GDH from the marine psychrophile currently under investigation.

The effect of NaCl on halophile enzymes has received considerable attention (4, 14). Nachum and Bartholomew (57) have shown that the temperature for maximal activity of an exocellular amylase from a Halobacterium sp. increases with increasing NaCl concentrations (5, 15 and 25% NaCl, maximal activity occurred at 25, 40 and 55°C, respectively). Holmes and Halvorson (26) found that malic dehydrogenase (MDH) extracted in 4 M NaCl from the extreme halophile, Halobacterium salinarium was inactivated when the salt was removed but could be reactivated by 2.5 M NaCl or 4 M KCl or sodium formate. Salts both activated and stabilized the enzyme. Lanyi and Stevenson (37) also reported a stimulation of enzyme activity by 0.5 to 1.5 M NaCl using catalase from the extreme halophile, Halobacterium cutirubrum. One study on GDH from the extreme halophile, Halobacterium halobium showed it to require 1 to 2 M NaCl for stability over 24 hours and 0.6 M NaCl for good activity (1). Miller (50) reported that glucose-6-phosphate dehydrogenase from V. marinus MP-1 was protected from thermal inactivation at 44°C by NaCl.
MATERIALS AND METHODS

Organism and Maintenance Conditions

The obligately psychrophilic bacterium, *Vibrio marinus* MP-1 (ATCC 15381) used in these experiments was isolated from the North Pacific Ocean by Morita and Haight (55) and described by Colwell and Morita (7). Stock cultures were grown on Marine Agar 2216E (Difco) slants for three days at 15°C and then stored at 2°C. In order to maintain purity of the stock cultures, the organism was streaked monthly on Marine Agar 2216E and typical colonies removed to slants.

Growth and Preparation of Cells

Starter cultures of *V. marinus* MP-1 were grown at 15°C for 24 hours in test tubes containing 10 ml of marine broth (MB). This medium contained 5.0 g Bacto-peptone, 1 g yeast extract, 0.1 g FePO$_4$ and 26.3 g Rila salts per liter. The medium was boiled, cooled to room temperature and filtered through Whatman #1 filter paper to remove undissolved FePO$_4$ and a fluffy, white precipitate. The pH of MB after autoclaving was 7.2.

Cells used in the shift experiments were grown at 15°C in 2.8 liter Fernbach flasks with 500 ml of a defined glucose-ammonium medium (GAM) containing per liter, 2.2 g K$_2$HPO$_4$ (13 mM), 0.25 g
KH$_2$PO$_4$ (1.8 mM), 0.12 g MgSO$_4$·7H$_2$O (0.52 mM), 23.4 g NaCl (400 mM) and 2.5 g NH$_4$Cl (4.7 mM). This basal salts medium was then autoclaved and temperature equilibrated to 15°C. Ten ml of a 25% (w/v) filter-sterilized glucose solution (final concentration in GAM was 14 mM) and 0.5 ml of a filter-sterilized vitamin solution (400 mg nicotinamide, 100 mg thiamin·HCl, 100 mg pyridoxime·HCl, 100 mg calcium-D-pantothenate, 25 mg riboflavin and 20 mg biotin per 100 ml) were added to the above medium. One-half ml of starter culture was then added to the medium as the inoculum. The final pH of GAM was 7.4.

The cultures were incubated for 48 hours at 15°C in an incubator shaker (Model R26, New Brunswick Scientific Co.) and shaken at 70 strokes/minute with an amplitude of 3.0 cm to ensure adequate aeration. Cells from each 500 ml culture were then harvested in a refrigerated centrifuge (Sorvall RC2-B) at 10,400 x g at 2°C and the supernatant decanted. The sedimented cells were resuspended with 40 ml of cold (iced) wash buffer [2.2 g K$_2$HPO$_4$ (13 mM), 0.25 g KH$_2$PO$_4$ (1.8 mM), 0.12 g MgSO$_4$·7H$_2$O (0.52 mM), 23.4 g NaCl (400 mM) per liter] and washed by repeated centrifugation at 7710 x g for five minutes at 2°C. The wash procedure was then repeated once again.
Medium Shifting Procedure for Glutamic Dehydrogenase (GDH) Induction

Four liters of a 48 hour GAM grown culture (500 ml per flask) were prepared as above and the cells suspended to a total volume of 40 ml with cold wash buffer. The shift medium, glutamate medium (GM), was composed of the same basal salts medium as GAM, except that monosodium glutamate (final concentration in GM, 30 mM) replaced the glucose and NH$_4$Cl as the sole carbon and nitrogen sources, respectively. To eight Fernbach flasks, each containing 400 ml GM equilibrated to 4, 15, 20 or 25 C, was added 4 ml of this washed cell suspension (the Fernbach flasks contained GM-0.26, 0.4, 0.6 or 0.8 M NaCl in duplicate). Upon shifting the GAM cells to GM, 100 ml portions (200 ml total in each salinity) were removed after 2, 4, 7 and 10 hours. The samples were immediately centrifuged at 2 C, washed twice as before, and suspended to a final volume of 5 ml with cold wash buffer.

The remaining 8 ml of washed cells not added to the shift medium was centrifuged and the sedimented cells suspended to a volume of 5 ml. This was taken as the uninduced sample (i.e. no exposure to glutamate).

The 5 ml samples prepared above were held on ice and treated immediately with an ultrasonic system (Bronwill Biosonic III) equipped with a 3/4 titanium probe tip for 15 seconds at 80% maximum power.
The resulting material was centrifuged at 30,900 x g for 30 minutes at 2 C and the cell-free extracts (CFE) removed for assay.

The induction flow diagram is shown in Figure 1.

Methods of Enzyme Production and Assay

Glutamic dehydrogenase (GDH) was assayed by a modification of the Strecker procedure (83). This enzyme catalyzes the following reversible reaction,

\[
\text{L-glutamate} + \beta\text{-NAD}^+ \rightleftharpoons \alpha\text{-ketoglutarate} + \text{NH}_3 + \beta\text{-NADH}_2
\]

The reduction of nicotinamide adenine dinucleotide (NAD\(^+\)) was measured by following the increase in OD at 340 m\(\mu\) on a spectrophotometer (Gilford Model 2000) using a 1 cm light path, full scale absorbance of 0.1, ratio at 1.0, speed at 1:1 (#2) and equipped with a water-jacketed chamber. All enzyme assays were run at 15 C.

Each assay cuvette contained 2.6 ml of Tris-HCl buffer (0.05 M, pH 9.0 using 1.0 M HCl), 0.1 ml of NAD\(^+\) (0.02 M) and 0.2 ml of monopotassium glutamate (1.0 M) or 0.2 ml of water in the blank. The final concentrations per ml of assay solution were as follows: Tris-HCl (43.5 \(\mu\)M), NAD\(^+\) (0.75 \(\mu\)M), glutamate (200 \(\mu\)M). One-tenth of a ml of freshly prepared CFE was added to each cuvette equilibrated at 15 C, mixed by inversion and replaced into the spectrophotometer. The rate of NAD\(^+\) reduction was determined by measuring the slope of the linear portion of the reaction curve. The
Growth in GAM for 48 hours
15 C 0.4 M NaCl

Wash

Resuspend

GM 0.26 M NaCl
GM 0.4 M NaCl
GM 0.6 M NaCl
GM 0.8 M NaCl

Sample 2, 4, 7, 10
Hours After Shift

Wash, Sonicate (CFE)

GDH Assay

Figure 1. Flow diagram for monitoring the induction of GDH in _V. marinus_ MP-1 in shifting cells from GAM to GM containing various molarities of NaCl.
units of enzyme per 0.1 ml of CFE were calculated from the formula,
\[ U \text{ (units/0.1 ml)} = \Delta OD_{340nm} \text{ per minute} \times 10^3. \]
Each enzyme was assayed in duplicate.

**GDH assay studies in buffers containing various salt concentrations** were undertaken. Solutions were prepared using the composition of the wash buffer except that various amounts of NaCl were used (0.0, 0.26, 0.4, 0.6, 0.8 M). In place of the Tris-HCl, 0.05 M (pH 9.0) buffer usually used in the assay, 2.6 ml of each of these buffers were substituted and the assay run at 15°C as previously described.

Cell-free extracts were prepared by suspending cells in various solutions. The cells were grown in GAM, 15°C, for 48 hours, shifted to GM (0.4 M NaCl) for 10 hours as before and suspended to a volume of 5 ml with the following solutions: (1) wash buffer, (2) Tris-HCl, 0.05 and 0.5 M (pH 9.0), (3) Tris-HCl, 0.05 and 0.5 M (pH 9.0) with 10 mM β-mercaptoethylamine, (4) Tris-HCl, 0.05 and 0.5 M (pH 9.0) with 10 mM β-mercaptoethylamine and 10 mM EDTA, (5) potassium phosphate, 0.05 M (pH 7.6). The suspensions were then subjected to ultrasonic treatment, centrifuged, and assayed in the usual manner.

The cell-free extracts prepared above were held at 2°C and assayed periodically up to 76 hours.

Cell-free extracts were prepared by various methods. Cells grown in GAM, 15°C, for 48 hours and shifted to GM (0.4 M NaCl) for
10 hours were prepared as before. Cell-free extracts were prepared by suspending the cells to a total volume of 5 ml with (1) wash buffer followed by the usual ultrasonic treatment, (2) 0.5 ml wash buffer plus 4.5 ml Triton X-100 lysing solution (180 mg Triton X-100, 360 µM Tris-$\text{SO}_4$, 0.8 µM ethylenediaminetetraacetic acid (EDTA), pH 8.6), (3) wash buffer followed by heating at 30°C for one hour, (4) cold distilled water. Also, an equal amount of cells was pelleted and five drops of toluene mixed with the cell paste. The paste was then brought up to 5 ml with wash buffer. All of the above preparations were then centrifuged and the supernatants assayed as before.

The specific activity of the cell-free extracts was determined by dividing the number of units of enzyme per ml of CFE by the mg of protein per ml of extract.

As an enzyme control, beef liver L-glutamic dehydrogenase, Type I in ammonium sulfate (Sigma) stored at 2°C was diluted 1/200 with cold 0.05 M potassium phosphate buffer, pH 7.6 and assayed as above at 15°C.

Thermostability of GDH

The susceptibility of GDH to thermal denaturation was evaluated by heating 3 ml of CFE (in duplicate) containing 5.8 mg protein per ml in the polythermostat and assaying after various periods of exposure to a given temperature. As a control, some of the CFE was kept
on ice and then assayed.

**Protein Determination**

The protein content of cell-free extracts was determined by the Lowry (40) procedure using bovine serum albumin (BSA) as the standard.

**Standardization of Cell Suspensions**

In order to ensure the use of cell suspensions containing the same number of viable cells, a concentrated cell suspension of washed 48 hour GAM cells was diluted with wash buffer at 15°C and the optical density recorded on a colorimeter (Bausch & Lomb Spectronic 20) at 450 μm. Concomitantly, the same suspension was diluted in sterile wash buffer at 15°C, plated on Marine Agar 2216E plates at the same temperature, incubated at 15°C for four days and the colonies counted.

**Optimum Growth Temperature**

Duplicate tubes (18 x 150 mm) containing 10 ml of GAM, GM, or MB were equilibrated in a polythermostat (55) at temperatures ranging between -1.0 and 28°C. To each tube was added 0.05 ml of a 24 hour culture grown at 15°C in MB. The change in optical density (OD) was recorded on a colorimeter at 525 μm after 1-3 days of incubation.
using an uninoculated tube as a blank.

**Uptake of $^{14}$C-Glutamate by Whole Cells**

Five hundred ml of a 48 hour GAM culture were harvested as above and diluted with cold wash buffer until a 1/50 dilution in wash buffer gave an optical density reading of 0.44-0.46 at 450 mp on a colorimeter using 1.27 cm diameter glass tubes and water as a blank. One ml of the adjusted concentrated cell preparation thus obtained was added to 99 ml of uptake media (giving a concentration of $5 \times 10^7$ cells/ml) equilibrated at 4, 15, 20 or 25 C and containing the same ingredients as the GM previously described except only 1/10 the usual amount of cold monosodium glutamate (3 mM) was used. Once the cells were evenly suspended in these solutions, 10 ml were removed to temperature equilibrated 125 ml Erylenmeyer flasks in the New Brunswick Incubator Shaker. Each flask, containing 0.2 ml of UL-$^{14}$C-L-glutamic acid solution (New England Nuclear Corporation) at a concentration of 10 $\mu$C/ml (specific activity, 197 mc/mM), was quickly shaken to thoroughly mix the isotope and 0.5 ml samples taken every 15 minutes for one hour. The 0.5 ml samples were pipetted into 5 ml of cold wash buffer in a 15 ml Millipore scintered glass filter apparatus (Millipore Corporation) and suctioned through a 0.45$\mu$m pore size cellulose-acetate membrane filter (Millipore) to collect the labeled cells. The membrane filters was washed once with 5 ml of
cold wash buffer, removed to scintillation vials and dried under heat lamps a minimum of two hours. Ten ml of scintillation fluid [5.0 g 2, 5-diphenyloxazole (PPO), 0.3 g dimethyl-1, 4-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP) per liter of toluene, w/v] were added to each vial after which they were assayed on a scintillation computer (Nuclear-Chicago Mark I Liquid Scintillation Computer, Model 6868) for ten minutes and converted to disintegrations per minute.

Incorporation of $^{14}$C-Glutamate into Protein by Whole Cells

The procedure for determining the effect of NaCl on the incorporation of $^{14}$C-glutamate into cellular protein was essentially the same as that used for $^{14}$C-proline incorporation studies described below. The uptake suspension contained 0.2 µC of $^{14}$C-glutamate per ml of GM (0.26, 0.4, 0.6, or 0.8 M NaCl) at 15°C.

Uptake and Incorporation of $^{14}$C-Proline by Whole Cells

The procedure used was essentially that used for the $^{14}$C-glutamate uptake studies except that each flask contained 0.1 ml of a 5 µC/ml UL-$^{14}$C-proline (New England Nuclear Corporation) solution (specific activity not determined) plus 10 ml of cell suspensions giving a final concentration of isotope of 0.05 µC/ml uptake suspension.

One-half ml samples were removed every 15 minutes and
processed as in the $^{14}$C-glutamate uptake experiments. To assess the amount of incorporation of $^{14}$C-proline into cellular protein, the procedure described by Kennell (33) was employed with some modification. One ml of cell suspension was removed from the flasks every 15 minutes and mixed with 1 ml of 20% (w/v) trichloroacetic acid (TCA) on ice. One ml of cold TCA (5%) was added to each tube and held on ice for at least 30 minutes. The tubes were covered with a marble and placed in a heated water-bath at 80° C for 30 minutes. The solutions were cooled to room temperature and filtered through microfiber glass disc prefilters, type AP20 (Millipore Corporation) soaked in 10% TCA, to remove the hot TCA insoluble material. The tubes were rinsed with 8 ml of cold 10% TCA and also poured through the prefilters which were then rinsed with 4 ml cold TCA and then 10 ml of cold ethanol (70% v/v). The prefilters were dried under heat lamps for at least two hours and counted as previously described.

Salt Specificity for $^{14}$C-Glutamate Uptake

The specificity of NaCl for the uptake of $^{14}$C-glutamate of 15 C by shifted cells was assessed by replacing the 0.4 M NaCl in the GM shift medium with 0.4 M of KCl, NaAc or KAc (pH 7.3, 7.4, 7.5, respectively). The osmotic pressure due to the salts alone was calculated in each case to be 22.2 atm ($\pi = g/MRT$, where $\pi$ is the osmotic pressures in atm, $V$ the volume in liters, $g$ the weight of the
solute in grams, \( M \) the molecular weight, \( R \) the gas constant, 
\((0.082 \text{ liter-atm per mole per degree})\) and \( T \) the absolute temperature. The ionic strength \( (\mu) \) of these solutions (again due only to the \( 0.4 \text{ M salt used} \)) was calculated to be \( 0.4 \) \((\mu = \frac{1}{2} \sum mZ^2 \), where \( \mu \) is the ionic strength, \( m \) the molarity, \( Z \) the charge of the ion).

It should be noted that the cold glutamate was added as the monopotassium salt \( (3.0 \mu\text{M}) \) and not as the monosodium salt as in all other uptake experiments.

The uptake suspension contained \( 0.2 \mu\text{C/ml of } ^{14}\text{C-glutamate.} \)

**Respiration Studies**

The ability of whole cells, grown in GAM for 48 hours and shifted to the GM uptake solution \( (3.0 \text{ mM glutamate}) \) containing various molarities of \( \text{NaCl} \), to respire \( ^{14}\text{CO}_2 \) at \( 15 \text{ C} \) was determined by using the radiorespirometric method of Wang \( (87) \). Ten ml uptake suspensions were prepared as usual, placed in \( 125 \text{ ml respiration flasks} \) and equilibrated with shaking at \( 15 \text{ C} \). Air was pumped to the surface of the uptake suspension at the rate of approximately \( 40 \text{ cc/minute}. \) The experiment was initiated by adding \( 0.2 \text{ ml of a } 10 \mu\text{C/ml } ^{14}\text{C-glutamate solution with a } 1.0 \text{ ml syringe directly into the uptake suspension. The effluent gases containing the } ^{14}\text{CO}_2 \) were passed through sintered glass filters to effect a sparging action in \( 10 \text{ ml of ethanol-ethanolamine trapping solution (25:1 v/v)}. \) The radioactive \( \text{CO}_2 \) was
collected in this manner for one hour at which time 1 ml of the trapping solution was removed and added to 10 ml of scintillation fluid and counted.

**Uptake Inhibition Studies**

Cells grown for 48 hours in GAM at 15 °C were shifted to GM (0.4 m NaCl) (3.0 mM cold glutamate) containing various metabolic inhibitors as well as 0.2 µC of $^{14}$C-glutamate/ml uptake suspension at 15 °C. Uptake flasks contained either NaN$_3$ ($10^{-3}$ M), chloramphenicol (CAP, 10 µg/ml uptake suspension), 2,4-dinitrophenol (DNP, $10^{-3}$ M), KCN ($10^{-3}$ M), K$_4$As$_2$O$_5$ ($6.2 \times 10^{-2}$ M) or no inhibitor at all. The NaN$_3$, DNP, KCN and K$_4$As$_2$O$_5$ were weighed and added to the mix solution. Chloramphenicol was added to the mix solutions as a 1% solution (w/v) in 95% ethanol.

One-half ml samples were taken every 15 minutes, filtered and treated as before.

**Phase Microscopy**

Cells were observed by use of a Leitz Ortholux microscope equipped with a Heine phase contrast condenser #74, P v Fl 70/1.15 n objective and green light filter.
Dry Weight Determinations on Shifted Cells

Dry weight determinations on shifted cells were performed by removing 2-90 ml portions from each flask, washing once with cold wash buffer (total volume 40 ml), centrifuging at 7710 x g for five minutes and decanting away the wash supernatant. The pellet surface was then rinsed with distilled water and resuspended with distilled water to a volume of 1.0 ml. One-half ml duplicate fractions were put in preweighed aluminum tares, heated at 300 C for six hours, until a constant weight was obtained, dessicated and weighed.

Reagents and Biochemicals

All chemicals used in this study were reagent grade. NAD\(^+\), NADP\(^+\), beef liver L-glutamic dehydrogenase, and BSA were obtained from the Sigma Chemical Company.
RESULTS

The growth curve of *V. marinus* MP 1 at 15 C in GAM as measured by an increase in OD is shown in Figure 2. The generation time was calculated to be approximately four hours. After the culture reached an OD of 0.4-0.5 in 48 hours it contained approximately $8 \times 10^7$ cells/ml. The change in OD upon shifting cells described above to a new medium, GM, at 15 C containing various amounts of NaCl with glutamate as the sole C and N source is also plotted in Figure 2. It can be seen that the amount of NaCl in the shift medium is not only affecting the initial OD (increased NaCl increased the absorbance of the cells giving higher OD readings) of the suspension, but also the subsequent growth rate of the organism (Table 1). No growth was detected in ten hours after shifting to GM containing 0.26 and 0.8 M NaCl. Growth was detected only within six hours after shifting to GM (0.6 M NaCl), while within four hours there was a marked OD increase in GM (0.4 M NaCl) with a generation time of eight hours. It should be mentioned that the decrease in OD occurring when cells are shifted from GAM (0.4 M NaCl) to GM (0.4 M NaCl) was due to cell loss during the wash procedure. Figure 3 shows the increase in mass (dry weight) per 100 ml of cells growing in GM at 15 C after medium shifting. Almost no change in the dry weight was detected in GM (0.26 M NaCl) and GM (0.8 M NaCl) while the mass of the cells in GM (0.4 M
Figure 2. Growth of *V. marinus* MP-1 at 15 C in GAM and upon shifting to GM containing various concentrations of NaCl.
Figure 3. Change in mass (dry weight) of *V. marinus* MP-1 cells cultured in GAM for 48 hours at 15°C and shifted to GM at 15°C containing various concentrations of NaCl.
Table 1. Growth of *V. marinus* MP-1 upon shifting cells from GAM to GM at 15°C containing various molarities of NaCl.

<table>
<thead>
<tr>
<th>NaCl concentration in GM</th>
<th>Initial OD increase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GT (hr)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>OD Inc. (10 hr)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Cell mass Inc. (10hr)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Viability Inc. (10hr)&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Morphology (10hr)&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.26 M</td>
<td>none</td>
<td>*</td>
<td>none</td>
<td>1.2 X</td>
<td>none</td>
<td>rods</td>
</tr>
<tr>
<td>0.4 M</td>
<td>4</td>
<td>8</td>
<td>1.7 X</td>
<td>2.6 X</td>
<td>3.0 X</td>
<td></td>
</tr>
<tr>
<td>0.6 M</td>
<td>6</td>
<td>10</td>
<td>1.3 X</td>
<td>1.5 X</td>
<td>1.3 X</td>
<td>ovals</td>
</tr>
<tr>
<td>0.8 M</td>
<td>none</td>
<td>*</td>
<td>0.8 X</td>
<td>none</td>
<td>0.5 X</td>
<td>spheres</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hours after shifting as measured by ΔOD at 450 μμ.

<sup>b</sup> Generation time by OD at 450 μμ.

<sup>c</sup> OD increase ten hours after shift (1.7 X 1.7-fold increase and 0.8 X 80% of original OD remaining).

<sup>d</sup> Cell mass (dry weight) increase ten hours after shift.

<sup>e</sup> Viability increase ten hours after shift (0.5 X 50% original number of viable cells remaining).

<sup>f</sup> Morphology ten hours after shift.

<sup>*</sup>No detectable growth after ten hour observation.
NaCl) and GM (0.6 M NaCl) increased 2.6 and 1.5-fold, respectively.

Plate counts of cells shifted to 15°C medium revealed essentially no increase in viable cells in GM (0.26 M NaCl), a 3- and 1.3-fold increase in GM (0.4 M NaCl) and GM (0.6 M NaCl), respectively, and a 50% reduction in GM (0.8 M NaCl) within ten hours after shifting.

Phase microscopy of cells shifted to GM at 15°C revealed that at 0.6 and 0.8 M NaCl, the cells tended to become ovals during the shift period and became entirely spherical in ten hours in GM (0.8 M NaCl). Cells resuspended in GM (0.26 M NaCl) and GM (0.4 M NaCl) showed essentially no morphological change within ten hours after shifting.

The results of media shifting at 15°C on growth of cells are summarized in Table 1.

Washed cells grown in 15°C in GAM as described above and shifted to GM at 4°C containing various amounts of NaCl gave a negligible change in OD even ten hours after shifting. Only in GM (0.4 M NaCl) did a detectable change in OD (0.26 to 0.29 ten hours after shifting) occur. Dry weight determinations confirmed the fact that only cells suspended in GM (0.4 M NaCl) were capable of measurable growth, increasing their cell mass from 2.8 to 4.5 mg/100 ml culture in ten hours.

The morphology of 4°C shifted cells remained essentially constant (short, slightly curved rods and occasional spiraled forms) at all four salinities tested until after seven hours, at which time the cells
began to form into ovals and spheres in GM (0.8 M NaCl).

Plate counts made on 4 C shifted cells showed a constant number of viable cells per ml in all four salinities over the entire ten hour shift period.

Cells grown in GAM for 48 hours at 15 C and suspended in GM containing various concentrations of NaCl were tested at 20 C. Figure 4 shows the change in OD obtained on shifting cells to GM at 20 C. The results obtained were nearly identical to those at 15 C. No growth was obtained in GM (0.26 M NaCl) or GM (0.8 M NaCl). The usual drop in OD occurred in GM (0.8 M NaCl) and stabilized after 5-6 hours incubation. Also, slight growth was detected in GM (0.6 M NaCl), while in GM (0.4 M NaCl), the cells gave detectable growth within four hours after shifting with a generation time of approximately 12 hours. Dry weight determinations (Figure 5) indicated essentially the same trend as the OD measurements. No significant cell mass increase occurred in GM (0.26 M NaCl) and GM (0.8 M NaCl), while a slight increase (2.9 to 4.3 mg/100 ml of culture medium) was detected in GM (0.6 M NaCl). The dry weight of cells suspended in GM (0.4 M NaCl) increased from 2.7 to 6.6 mg/100 ml culture medium within ten hours after shifting.

Phase microscopy of cells shifted to GM at 20 C revealed that there was essentially no observable change in cellular morphology in GM (0.26 M NaCl) and GM (0.4 M NaCl) even ten hours after shifting.
Figure 4. Growth of _V. marinus_ MP-1 cultured in GAM for 48 hours at 15 C and shifted to GM at 20 C containing various concentrations of NaCl.
Figure 5. Change in mass (dry weight) of *V. marinus* MP-1 cells cultured in GAM for 48 hours at 15°C and shifted to GM at 20°C containing various concentrations of NaCl.
However, after 7-10 hours shifting, cells suspended in GM (0.6 and 0.8 M NaCl) showed a tendency to become oval.

Viability determinations on cells shifted into GM at 20°C showed a 60% loss within four hours after shifting to GM (0.26 M NaCl), while in GM (0.4 M NaCl) a 30% loss was obtained within the same time period. These two suspensions retained their 40 and 70% viabilities, respectively, throughout the remainder of the shift period. Cells suspended in GM (0.6 M NaCl) and GM (0.8 M NaCl) showed a 75 and 85% loss in viability, respectively, within ten hours after shifting.

The results of medium shifting at 20°C on cell growth are summarized in Table 2.

Cells grown in GAM for 48 hours at 15°C and shifted to GM containing 0.26, 0.4, 0.6 or 0.8 M NaCl at 25°C were examined. Figure 6 shows the change in OD that occurred. There was a slight reduction (0.47 to 0.44 and 0.54 to 0.50) in OD in GM (0.26 M NaCl) and GM (0.4 M NaCl), respectively, within the ten hour shift period. Cells suspended in GM (0.6 M NaCl) and GM (0.8 M NaCl) showed a more drastic decrease in OD in the same time from 0.60 to 0.48 and 0.65 to 0.45, respectively.

Results of dry weight determinations (Figure 7), gave similar results to those of the OD measurements. Only slight losses (approximately 2 mg) occurred in ten hours in GM (0.26, 0.4 and 0.6 M NaCl)
Table 2. Growth of V. marinus MP-1 upon shifting cells from GAM to GM at 20 C containing various molarities of NaCl.\textsuperscript{a}

<table>
<thead>
<tr>
<th>NaCl concentration in GM</th>
<th>Initial OD increase</th>
<th>GT (OD)</th>
<th>OD Inc. (10hr)</th>
<th>Cell mass Inc. (10hr)</th>
<th>Viability Inc. (10hr)</th>
<th>Morphology (10hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.26 M</td>
<td>none</td>
<td>*</td>
<td>none</td>
<td>1.3 X</td>
<td>0.4 X</td>
<td>rods</td>
</tr>
<tr>
<td>0.4 M</td>
<td>4</td>
<td>12</td>
<td>1.4</td>
<td>2.4 X</td>
<td>0.7 X</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.6 M</td>
<td>7</td>
<td>13</td>
<td>1.2</td>
<td>1.5 X</td>
<td>0.3 X</td>
<td>ovals</td>
</tr>
<tr>
<td>0.8 M</td>
<td>none</td>
<td>*</td>
<td>0.9</td>
<td>0.9 X</td>
<td>0.1 X</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Abbreviations same as in Table 1.

\*No detectable growth after ten hour observation.
Figure 6. Growth of V. marinus MP-1 cultured in GAM for 48 hours at 15 C and shifted to GM at 25 C containing various concentrations of NaCl.
Figure 7. Change in mass (dry weight) of V. marinus MP-1 cells cultured in GAM for 48 hours at 15°C and shifted to GM at 25°C containing various concentrations of NaCl.
while cells suspended in GM (0.8 M NaCl) lost nearly twice that amount of material (3.5 mg per 100 ml culture). It seems rather apparent that leakage of cellular materials has occurred at 25 C, being most marked in 0.8 M NaCl.

Viability studies indicated that in all four salinities at 25 C, death had occurred to all cells within two hours after shifting.

Again, phase microscopy of shifted cells revealed that the cells began to form ovals and became small spheres around seven hours after shifting in all four salinities tested.

Table 3 summarizes the data on the growth of _V. marinus_ MP-1 upon shifting GAM cells grown at 15 C to GM at 25 C containing 0.26, 0.4, 0.6 or 0.8 M NaCl.

The maximal and optimal temperatures for growth in GAM, GM and MB are given by Figures 8-10. In all three media tested, the optimum temperature for growth lay between 12-13 C while the maximal temperature allowing detectable growth by OD measurements was approximately 20 C. Poor growth was obtained in both defined media (GAM and GM) while growth was considerably better in the undefined medium, MB.

Several buffer systems at various pH values were initially tried to ascertain which one would give the maximal GDH activity. Figure 11 shows the results obtained by using a potassium phosphate buffer, 0.05 M at various pH values. The optimal pH lay between 8.5 and
Table 3. Growth of *V. marinus* MP 1 upon shifting cells from GAM to GM at 25 C containing various molarities of NaCl.\(^a\)

<table>
<thead>
<tr>
<th>NaCl concentration in GM</th>
<th>Initial OD increase</th>
<th>GT (OD)</th>
<th>OD Inc. (10 hr)</th>
<th>Cell mass Inc. (10hr)</th>
<th>Viability Inc. (10hr)</th>
<th>Morphology (10hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.26 M</td>
<td>none</td>
<td>*</td>
<td>none</td>
<td>0.8 X</td>
<td>0.0 X</td>
<td>spheres</td>
</tr>
<tr>
<td>0.4 M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.9 X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.8 M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.9 X</td>
<td>0.8 X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>0.8 M</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.7 X</td>
<td>0.6 X</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

\(^a\)Abbreviations same as in Table 1.

\(*\)No detectable growth after ten hour observation.
Figure 8. Growth of V. marinus MP-1 in MB after 24 (○-○) and 48 hours (Δ-Δ).
Figure 9. Growth of *V. marinus* MP-1 in GM containing 0.4 M NaCl after 24 (■—■) and 48 hours (△—△).
Figure 10. Growth of *V. marinus* MP-1 in GAM after 56 (○—○) and 76 hours (△—△).
Figure 11. Effect of pH on the percent maximal activity of GDH using a potassium phosphate-0.05 M buffer in the assay procedure (the amount of activity obtained at pH 9.5 was taken as 100%).
10.0 in this buffer. Figure 12 shows the values obtained using 0.05 and 0.5 M Tris-HCl buffer at various pH values. The optimal pH in this buffer was 8.5 for 0.05 M and 9.5 for 0.5 M Tris-HCl. Figure 13 shows the maximal activity occurring at pH 9.0 in 0.05 and 0.5 M Tris-Cl4. All five buffers at pH 9.0 were then tested and it was found that Tris-HCl, 0.05 M and 0.5 M, pH 9.0 allowed the maximal GDH to be expressed. Tris-HCl buffer, 0.05 M, pH 9.0 was chosen for all subsequent GDH assays.

By using various concentrations of glutamate and NAD+ along with the Tris-HCl, 0.05 M, pH 9.0 buffer, it was possible to determine a range of enzyme activities over which there would be no inhibition due to substrate or cofactor limitation. When using 43.5 mM Tris-HCl (pH 9.0), 0.75 mM NAD+ and 200 mM glutamate per ml of reaction mixture at 15 °C, a linear response was obtained between units/0.1 ml CFE and dilution, over a range from 10-90 U/0.1 ml (Figure 14). Hereafter, the GDH in cell-free extracts will be referred to as GDH.

A CFE giving an average of 68 U/0.1 ml with NAD+ as the cofactor, failed to show any reduction of an equimolar amount of NADP+. NAD+ was therefore found specific for the GDH under investigation.

Figure 15 shows the effect of heating a CFE (5.8 mg protein/ml) on its GDH activity. It is evident that the enzyme is quite stable at 0 °C for ten hours and then gradually loses its activity with time. Increased
Figure 12. Effect of pH on the percent maximal activity of GDH using a Tris-HCl buffer, 0.05 M (O—O) and 0.5 M (Δ—Δ) in the assay procedure (the amount of activity obtained at pH 8.5 and 9.5 in 0.05 M and 0.5 M buffer, respectively, was taken as 100%).
Figure 13. Effect of pH on the percent maximal activity of GDH using a Tris-\(\text{SO}_4\) buffer, 0.05 M (○○) and 0.5 M (△△) in the assay procedure (the amount of activity obtained at pH 8.5 and 9.5 in 0.05 M and 0.5 M buffer, respectively, was taken as 100%).
Figure 14. Effect of diluting CFE in wash buffer on its GDH activity in 43.5 μM Tris-HCl (pH 9.0), 0.75 μmolar NAD$^+$ and 200 μM glutamate per ml at 15 °C.
Figure 15. Effect of heating a CFE (5.8 mg protein/ml) prepared with wash buffer on GDH activity (C—○—0 C, △—△ - 6.5 C, □—□—15 C, ○—○—19 C, ▼—▼—-25 C). The amount of activity obtained immediately upon preparation of the CFE was taken as 100%.
temperatures result in a more rapid decrease in activity (at 19 and 25 C, nearly 50% loss in activity occurred within seven hours). The CFE used in the above experiment was prepared in the usual manner using the wash buffer (containing 0.4 M NaCl) as the suspending solution for the cells prior to ultrasonic treatment. Freezing of the above extract at -20 C overnight and subsequent thawing resulted in complete loss of GDH activity.

Figure 16 shows the effect of holding the extracts prepared above at 2 C on the GDH activity. Eighty-two percent of the GDH activity remained after 24 hours at 2 C in the wash buffer solution, while 46% remained in the same period in the phosphate buffer. Both preparations continued to lose activity after 24 hours at 2 C.

Due to the stabilizing effect of NaCl in the wash buffer on the GDH activity, it was used as the suspending solution in all subsequent CFE preparations.

The substitution of wash buffers containing various concentrations of NaCl for the usual Tris-HCl buffer, 0.05 M (pH 9.0), used in the GDH assay system resulted in complete loss of activity as indicated by Table 4. Wash buffer containing no NaCl did yield an activity of 20 U/0.1 ml CFE while the usual Tris-HCl buffer gave an activity of 40 U/0.1 ml CFE. The CFE contained 7.0 mg protein/ml.

Table 5 shows that only the wash buffer and potassium phosphate 0.05 M (pH 7.6) are effective suspending solutions for preparing active
Figure 16. Thermostability of GDH at 2 C in cell-free extracts prepared in wash buffer (O-O) containing 0.4 M NaCl (8.8 mg protein/ml) and potassium phosphate, (Δ-Δ), 0.05 M, pH 7.6 (10.0 mg protein/ml). The amount of activity obtained immediately upon preparation of the CFE was taken as 100%.
Table 4. Effect of increased concentrations of NaCl in the assay buffer on the GDH activity of a CFE (7.0 mg protein/ml) prepared with wash buffer.

<table>
<thead>
<tr>
<th>Assay buffer</th>
<th>U/0.1 ml CFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash buffer - 0.0 M NaCl</td>
<td>20</td>
</tr>
<tr>
<td>Wash buffer - 0.26 M NaCl</td>
<td>0</td>
</tr>
<tr>
<td>Wash buffer - 0.40 M NaCl</td>
<td>0</td>
</tr>
<tr>
<td>Wash buffer - 0.60 M NaCl</td>
<td>0</td>
</tr>
<tr>
<td>Wash buffer - 0.80 M NaCl</td>
<td>0</td>
</tr>
<tr>
<td>Tris-HCl - 0.05 M pH 5.0a</td>
<td>40</td>
</tr>
</tbody>
</table>

*a This buffer was the one used throughout the study in the routine GDH assay procedure.

Table 5. Efficacy of using various suspending solutions in the preparation of cell-free extracts for GDH activity.

<table>
<thead>
<tr>
<th>Suspending solution</th>
<th>U/0.1 ml CFE</th>
<th>mg protein/ml CFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash buffer - 0.4 M NaCl</td>
<td>36</td>
<td>8.8</td>
</tr>
<tr>
<td>Tris-HCl, 0.05 M (pH 9.0)</td>
<td>0</td>
<td>9.3</td>
</tr>
<tr>
<td>Tris-HCl, 0.5 M (pH 9.0)</td>
<td>0</td>
<td>12.0</td>
</tr>
<tr>
<td>Tris-HCl, 0.05 M (pH 9.0) + 10 mM β-mercaptoethylamine</td>
<td>0</td>
<td>9.0</td>
</tr>
<tr>
<td>Tris-HCl, 0.5 M (pH 9.0) + 10 mM β-mercaptoethylamine</td>
<td>0</td>
<td>11.5</td>
</tr>
<tr>
<td>Tris-HCl, 0.05 M (pH 9.0) + 10 mM β-mercaptoethylamine + 10 mM EDTA</td>
<td>0</td>
<td>10.0</td>
</tr>
<tr>
<td>Tris-HCl, 0.5 M (pH 9.0) + 10 mM β-mercaptoethylamine + 10 mM EDTA</td>
<td>0</td>
<td>11.0</td>
</tr>
<tr>
<td>Potassium phosphate, 0.05 M (pH 7.6)</td>
<td>59</td>
<td>9.0</td>
</tr>
</tbody>
</table>
cell-free extracts by ultrasonic treatment. The use of Tris-HCl (0.05 and 0.5 M, pH 9.0), with or without β-mercaptoethylamine (10 mM), and with or without ethylenediaminetetraacetic acid (EDTA, 10 mM) completely destroyed the GDH activity.

It should be mentioned that the cells immediately lysed upon suspension in all of the Tris-HCl solutions before ultrasonic treatment. Only cells suspended in wash buffer, followed by ultrasonic treatment, produced an active CFE. Lysing the cells by Triton X-100, heating for one hour at 30 C in wash buffer, toluene extraction, or cold distilled water did not yield active preparations. Ultrasonic treatment in wash buffer was therefore chosen as the method for preparing all subsequent cell-free extracts.

It should be mentioned that the ultrasonic treatment of 5 ml cell suspensions for 15 sec at 80% maximum released 90-95% of the cellular protein (up to 9-10 mg/ml) as determined by subsequent identical treatment of cellular debris.

Figure 17 demonstrates the induction (increase in specific activity) of GDH prepared from 48 hour GAM cells grown at 15 C after shifting to GM at 4 C containing various quantities of NaCl. A 21- and 24-fold increase in GDH was evident in 0.6 and 0.8 M NaCl media, respectively, within the ten hour induction period. A 4- and 16-fold increase in GDH was detected in GM (0.26 and 0.4 M NaCl), respectively, within the same time period.
Figure 17. Induction of GDH in *V. marinus* MP-1 on shifting cells from GAM to GM at 4 C containing various molarities of NaCl.
As indicated in Figure 18, the rate of GDH induction in cells grown for 48 hours in GAM at 15 C and shifted to GM at 15 C at various salinities is apparently greatest in 0.4 M NaCl. Within ten hours after shifting, a 56-fold increase in the concentration of GDH occurred in 0.4 M NaCl, while in 0.26, 0.6 and 0.8 M NaCl, an increase of 21-, 44- and 9-fold was detected, respectively.

Figure 19 shows the effect of various amounts of NaCl in GM at 20 C upon the induction of GDH in shifted 48 hour GAM (15 C) grown cells. Final specific activities (ten hours after shifting) were 6, 35, 29 and 8 U/mg protein in GM (0.26, 0.4, 0.6 and 0.8 M NaCl), respectively.

Cells grown in GAM for 48 hours at 15 C and shifted to GM at 25 C containing various amounts of NaCl showed an initial rapid induction (Figure 20) of GDH (within two hours after shifting) followed by an immediate decline (four hours). Little or no GDH was detected in cells held at 25 C longer than four hours at any salt concentration used.

The results of salinity and temperature changes on the specific activity of GDH ten hours after shifting are summarized in Table 6.

The relationship between cell density (OD) and the number of viable cells in suspension is plotted in Figure 21. Cells used in the uptake studies were routinely adjusted, so that a 1/50 dilution in wash buffer of the washed, concentrated cell suspension gave an OD of 0.45
Figure 18. Induction of GDH in *V. marinus* MP-1 on shifting cells from GAM to GM at 15 C containing various molarities of NaCl.
Figure 19. Induction of GDH in *V. marinus* MP-1 on shifting cells from GAM to GM at 20°C containing various molarities of NaCl.
Figure 20. Induction of GDH in *V. marinus* MP-1 in shifting cells from GAM to GM at 25°C containing various molarities of NaCl.
Figure 21. Relationship between OD and the number of viable cells per ml of wash buffer.
corresponding to approximately $5 \times 10^7$ cells/ml (40 µg dry weight/ml).

Table 6. Effect of salinity and temperature changes on the specific activity (U/mg protein) of GDH from V. marinus MP-1 ten hours after shifting cells from GAM to GM.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Molarity of NaCl in GM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
</tr>
</tbody>
</table>

The concentration of glutamate in GM was 30 mM. When this concentration of substrate was used in the flasks along with $^{14}$C-glutamate at a final concentration of 0.2 µC/ml of uptake suspension, virtually no uptake of the isotope could be detected. Therefore, the concentration of glutamate was lowered to one-tenth the original concentration to give 3.0 mM glutamate/ml uptake suspension. Lowering the glutamate concentration from 30 to 3.0 mM resulted in a measurable rate of $^{14}$C-glutamate uptake.

Figure 22 shows the time course of $^{14}$C-glutamate uptake by 48 hour GAM cells grown at 15 °C and shifted to GM (3.0 mM glutamate) at 4 °C containing various concentrations of NaCl. It is evident that as the concentration of NaCl is increased in the GM uptake medium, a decrease in the rate of uptake and the ultimate intracellular concentration of $^{14}$C-glutamate occurs. The uptake of $^{14}$C-glutamate by 48
Figure 22. Time course of $^{14}$C-glutamate (0.2 $\mu$C/ml cell suspension) uptake by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 4°C at various NaCl concentrations.
hour GAM cells grown at 15 C and shifted to GM (3.0 mM cold glutamate) at 15 C containing NaCl in various concentrations is depicted in Figure 23. Increased amounts of NaCl in the uptake suspension caused a progressive decrease in the rate of uptake as well as the net amount of isotope taken up. The results of shifting 48 hour GAM cells grown at 15 C to GM (3.0 mM cold glutamate) at 20 C containing various amounts of NaCl are plotted in Figure 24. Again, as the amount of NaCl is increased in the GM uptake medium, the rate of uptake and the total amount of isotope taken up are reduced, except with 0.26 and 0.4 M NaCl which gave nearly identical results at this temperature.

The time course of $^{14}$C-glutamate uptake by 48 hour GAM cells grown at 15 C and shifted to GM (3.0 mM glutamate) at 25 C containing various concentrations of NaCl is represented by Figure 25. As with all the previous uptake experiments, an increase of NaCl in the GM uptake solution resulted in a decreased rate of uptake and net intracellular concentrations of the isotope. These data did, however, differ in one important aspect. The uptake rate at 0.26 M NaCl was considerably below that of all the other salinities. This was not observed at any other shift temperature.

The results of the $^{14}$C-glutamate experiments with GAM cells shifted to GM containing different concentrations of NaCl at various temperatures are summarized in Table 7. It can be seen that the
Figure 23. Time course of $^{14}$C-glutamate (0.2 µC/ml cell suspension) uptake by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.
Figure 24. Time course of $^{14}$C-glutamate (0.2 μC/ml cell suspension) uptake by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 20°C at various NaCl concentrations.
Figure 25. Time course of $^{14}$C-glutamate (0.2 µC/ml cell suspension) uptake by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 25°C at various NaCl concentrations.
Table 7. Effect of salinity and temperature changes on the uptake of $^{14}$C-glutamate by *V. marinus* MP-1.

<table>
<thead>
<tr>
<th>Temperature ($^\circ$C)</th>
<th>Molarity of NaCl in GM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>Rate ($x10^3$)</td>
<td>Net uptake b</td>
</tr>
<tr>
<td>Rate ($x10^3$)</td>
<td>Net uptake</td>
</tr>
<tr>
<td>Rate ($x10^3$)</td>
<td>Net uptake</td>
</tr>
<tr>
<td>Rate ($x10^3$)</td>
<td>Net uptake</td>
</tr>
<tr>
<td>4</td>
<td>2.0</td>
</tr>
<tr>
<td>15</td>
<td>8.8</td>
</tr>
<tr>
<td>20</td>
<td>3.1</td>
</tr>
<tr>
<td>25</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
</tr>
</tbody>
</table>

*a* Initiate rate of uptake of $^{14}$C-glutamate in disintegrations per minute. (It is assumed that no growth took place during the one hour sample period with the liberation of $^{14}$CO$_2$.

*b* Net uptake refers to the total amount of $^{14}$C-glutamate taken up by 0.5 ml of the cell suspension after 60 minutes incubation expressed in disintegrations per minute.

*c* Percent of highest net isotope uptake at one particular salinity.
most rapid rate of uptake occurred at 15 C in 0.26 M NaCl. Any further increase in salinity reduced the rate of uptake except at 25 C where the maximal rate occurred in GM (0.6 M NaCl). Also, decreasing or increasing the temperature from 15 C resulted in a lowered rate of uptake in all salinities tested.

As indicated by Figure 26, the uptake rate and total amount of $^{14}$C-proline incorporated into cells grown at 15 C in GAM and shifted to GM at 4 C was affected by the concentration of NaCl in the shift medium. As the molarity of NaCl increased, the uptake rate decreased as did the net $^{14}$C-proline taken up. Cells in GM (0.26 M NaCl) took up nearly four times (about 27% of the total amount of isotope available) the amount of $^{14}$C-proline taken up by cells suspended in GM (0.8 M NaCl) at which salinity only very slight uptake occurred. Cells in GM (0.4 M NaCl) and GM (0.6 M NaCl) took up nearly equivalent amounts of the isotope [about 75% of that in GM (0.26 M NaCl)].

The results obtained for the uptake of $^{14}$C-proline at 15 C (Figure 27) are much like those for 4 C. The only noticeable difference is that at a high NaCl concentration (0.8 M), the rate and net amount of isotope uptake has increased.

Shifting the temperature to 20 C markedly increased the rate and total uptake of $^{14}$C-proline [35% more than at 15 C in GM (0.26 M NaCl)] by cells. Again, as in the two previous experiments, an
Figure 26. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) uptake by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 4°C at various NaCl concentrations.
Figure 27. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) uptake by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.
increase in NaCl concentration in GM resulted in decreased rates of uptake and total isotope saturation at the end of the 60 minute test period. These results are plotted in Figure 28.

When the temperature of the shift medium is further raised to 25 C, the rate of uptake and net uptake by cells were reduced from that obtained at 20 C (Figure 29). As with previous experiments the rate of uptake and total accumulated $^{14}$C-proline was decreased at 25 C as the salinity was increased with one notable exception. With cells shifted into GM (0.26 M NaCl), practically no uptake occurred. In all other experiments at 4, 15 and 20 C, this salinity gave the maximal rate of uptake and net accumulation.

The results for $^{14}$C-proline incorporation into cellular protein by whole cells shifted to GM containing various NaCl concentrations at different temperatures are essentially the same as those for $^{14}$C-proline uptake (Figures 30-33). In general, as the salinity was increased in the GM shift medium, the rate of and total incorporation of $^{14}$C-proline into protein decreased. However, as with the uptake experiments, cells placed in GM (0.26 M NaCl) at 25 C showed a much lower rate of and net incorporation into protein than in 0.4 M NaCl, which was the maximum at 20 C.

Also, an increase in temperature at any one salinity generally increased the rate of and net incorporation of $^{14}$C-proline into protein with a maximum at 20 C. Raising the temperature to 25 C
Figure 28. Time course of $^{14}$C-proline (0.05 μC/ml cell suspension) uptake by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 20°C at various NaCl concentrations.
Figure 29. Time course of $^{14}$C-proline (0.05 $\mu$C/ml cell suspension) uptake by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 25 C at various NaCl concentrations.
Figure 30. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) incorporation into cellular protein by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 4°C at various NaCl concentrations.
Figure 31. Time course of $^{14}$C-proline (0.05 µC/ml cell suspension) incorporation into cellular protein by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.
Figure 32. Time course of $^{14}$C-proline (0.05 μCi/ml cell suspension) incorporation into cellular protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 20°C at various NaCl concentrations.
Figure 33. Time course of $^{14}$C-proline (0.05 $\mu$C/ml cell suspension) incorporation into cellular protein by *V. marinus* MP-1 cells in GM (3.0 mM glutamate) at 25°C at various NaCl concentrations.
reduced these two measurements.

At least at 4 and 20 C, the ratio of $^{14}$C-proline incorporated into cellular protein to that taken up was from 50-84%. Similar comparisons at 15 and 25 C could not be made since uptake and incorporation experiments were done separately.

When cells are shifted to GM at 15 C containing various molarities of NaCl, the incorporation of $^{14}$C-glutamate into protein is affected by the salinity of the suspending medium. Figure 34 shows that the rate of incorporation and total incorporation of label was maximal at 0.4 M NaCl. Cells suspended in 0.6 M NaCl incorporated $^{14}$C-glutamate at nearly the same rate as in 0.4 M NaCl but only to 83% of the total amount incorporated at that salinity. Cells in 0.26 M NaCl showed a slightly reduced rate from that of 0.6 M NaCl as well as the total incorporation (71% of net $^{14}$C-glutamate incorporated at 0.4 M NaCl). Further increase in NaCl in GM to 0.8 M greatly decreased the rate and net $^{14}$C-glutamate incorporated (35% of the net $^{14}$C-glutamate incorporated at 0.4 M NaCl) into protein.

As the data indicates in Table 8, shifted cells are respiring at 15 C. As the NaCl concentration increases in the GM shift solution, the rate of respiration progressively decreases, until at 0.8 M NaCl, only 62% of the amount of $^{14}$CO$_2$ evolved at 0.26 M was given off.

Disregarding the amount of $^{14}$CO$_2$ dissolved in the medium and of that remaining in the atmosphere immediately above the cell
Figure 34. Incorporation of $^{14}$C-glutamate (0.2 µC/ml cell suspension) into protein by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 15°C at various NaCl concentrations.
suspension, approximately 0.2% of the total radioactivity in the reaction vessel was evolved as $^{14}$CO$_2$ at 15°C in 0.4 M NaCl within one hour. Under the same conditions 1.8% of the total radioactivity available was incorporated into the cells in the same time period.

Cells suspended in GM containing 0.4 M of either NaCl, KCl, NaAc or KAc showed different rates of uptake of $^{14}$C-glutamate (Figure 35). Media containing NaCl gave the most rapid rate of uptake with KCl being nearly as effective. NaAc and KAc, however, did not allow any uptake of the label. Since NaAc could not substitute for NaCl in the uptake experiment, it would appear as though the chloride ion is specifically required for uptake. It should be remembered that all uptake solutions contained K$^+$. 

Table 8. Effect of NaCl on the evolution of $^{14}$CO$_2$ by V. marinus MP-1 in GM (3.0 mM glutamate) at 15°C containing 0.2 μC $^{14}$C-glutamate/ml cell suspension.

<table>
<thead>
<tr>
<th>Molarity of NaCl in GM</th>
<th>DPM$^a$</th>
<th>Percent maximal label respired</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.26</td>
<td>913</td>
<td>100</td>
</tr>
<tr>
<td>0.40</td>
<td>882</td>
<td>97</td>
</tr>
<tr>
<td>0.60</td>
<td>812</td>
<td>89</td>
</tr>
<tr>
<td>0.80</td>
<td>570</td>
<td>62</td>
</tr>
</tbody>
</table>

$^a$These values represent the dpm collected in 1 ml of ethanol-ethanolamine after incubating the cells for 60 minutes.

OD measurements revealed no change within 60 minutes in NaCl.
Figure 35. Uptake of $^{14}$C-glutamate (0.2 µC/ml cell suspension) by V. marinus MP-1 cells in GM (3.0 mM glutamate) at 15 C containing 0.4 M of various salts (Ac = acetate).
and KCl, but a slight reduction of about 0.1 OD unit in NaAc and KAc in the same time period (Figure 36).

Plating studies demonstrated that viability was maintained in all suspending media except in that containing 0.4 M KAc. All of the cells died within 30 minutes after shifting in 0.4 M KAc.

The inclusion of various metabolic inhibitors into the uptake solution of GM (0.4 M NaCl) at 15 C resulted in altered uptake data from that obtained without the use of inhibitors as a control. Figures 37 and 38 show that KCN caused a 50% reduction of $^{14}$C-glutamate taken up in 60 minutes while DNP and KAr nearly completely inhibited uptake. No inhibiting effects were obtained with CAP and only a slight reduction with NaN$_3$.

The summary of the results of the inhibitor studies are given in Table 9.

<table>
<thead>
<tr>
<th>Inhibitor$^a$</th>
<th>Net uptake$^b$</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3500$^c$</td>
<td>0</td>
</tr>
<tr>
<td>CAP</td>
<td>3400</td>
<td>3</td>
</tr>
<tr>
<td>NaN$_3$</td>
<td>2700</td>
<td>22</td>
</tr>
<tr>
<td>KCN</td>
<td>1600</td>
<td>54</td>
</tr>
<tr>
<td>KAr</td>
<td>998</td>
<td>72</td>
</tr>
<tr>
<td>DNP</td>
<td>713</td>
<td>80</td>
</tr>
</tbody>
</table>

$^a$Inhibitor concentration was $10^{-3}$M for all except CAP (10 µg/ml uptake suspension)

$^b$Net uptake is defined as the total amount of label taken up in 60 min.

$^c$Average of two experiments with no inhibitor.
Figure 36. Effect of replacing 0.4 M NaCl with the same molarity KCl, NaAc, or KAc in GM (3.0 mM glutamate) at 15 C on the OD of V. marinus MP-1 cells.
Figure 37. Effect of various metabolic inhibitors on the uptake of $^{14}$C-glutamate (0.2 µC/ml cell suspension) in GM (0.4 M NaCl) containing 3.0 mM glutamate at 15 C by V. marinus MP-1 cells.
Figure 38. Effect of various metabolic inhibitors on the uptake of $^{14}$C-glutamate (0.2 µC/ml cell suspension) in GM (0.4 M NaCl) containing 3.0 mM glutamate at 15 C by V. marinus MP-1 cells.
DISCUSSION

Physical and chemical parameters resembling those possibly encountered in oceanic waters, especially in nearshore environments, were simulated in the growth conditions used in these experiments (Table 10). The effect of changing two important parameters, salinity and temperature, on the growth, enzyme induction, and substrate uptake of an obligately psychrophilic, marine bacterium, *V. marinus* MP-1, was investigated. All the cells used in this investigation were grown for 48 hours at 15 C in GAM, subsequently shifted to new medium, GM, at various temperatures and salinities, and the above events monitored.

It should be mentioned that a linear rate of GDH induction was not obtained at any salinity or temperature tested. The rates of synthesis seemed to be second-order, i.e. following a geometric progression, thus indicating an ever increasing rate of enzyme synthesis. This observation was probably due to the fact that the cells were constantly growing and adapting themselves to preferentially synthesized GDH. Nevertheless, the cells were shown to be inducible for GDH. The relative rates of induction (U/hour) are taken to mean the net increase in enzyme units/mg protein/10 hours, regardless of the initial rate of synthesis.

It was observed that the salinity of the suspending medium at
Table 10. Comparison of natural and experimental parameters under which *V. marinus* MP-1 was exposed.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Salinity $^a$</th>
<th>Temperature $^\circ$C</th>
<th>Hydrostatic pressure (atm)</th>
<th>Osmotic pressure (atm)</th>
<th>Ionic strength</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea water</td>
<td>35.5</td>
<td>3.2</td>
<td>120</td>
<td>25.5</td>
<td>0.5</td>
<td>8.1</td>
</tr>
<tr>
<td>GAM</td>
<td>29.9</td>
<td>15</td>
<td>1</td>
<td>23.1</td>
<td>0.4</td>
<td>7.4</td>
</tr>
<tr>
<td>GM-0.26</td>
<td>21.8</td>
<td>4, 15, 20, 25</td>
<td>1</td>
<td>16.5</td>
<td>0.3</td>
<td>7.4</td>
</tr>
<tr>
<td>GM-0.4</td>
<td>29.9</td>
<td>&quot;</td>
<td>1</td>
<td>26.2</td>
<td>0.4</td>
<td>7.4</td>
</tr>
<tr>
<td>GM-0.6</td>
<td>36.6</td>
<td>&quot;</td>
<td>1</td>
<td>29.4</td>
<td>0.6</td>
<td>7.4</td>
</tr>
<tr>
<td>GM-0.8</td>
<td>55.3</td>
<td>&quot;</td>
<td>1</td>
<td>41.8</td>
<td>0.8</td>
<td>7.4</td>
</tr>
</tbody>
</table>

$^a$Does not include organic compounds.
15 C definitely affected the induction of GDH. This interference with GDH induction seems to be attributable to events happening under two sets of circumstances depending on whether the NaCl concentration in the shift medium is sub- or supra-optimal.

In the former case, i.e. when the cells were exposed to NaCl concentrations below the optimal for GDH induction (0.26 M NaCl), the cells seemed to undergo some alteration in their structural integrity, either cellular or macromolecular, which was vital for induction. This conclusion was drawn from the fact that the substrate uptake rate, as well as the rate of protein synthesis, was maximal at that salinity. Therefore, the organisms were quite capable of getting the substrate into the cells as well as synthesizing GDH, yet induction occurred at a very slow rate. Also, the cells seemed to be quite able to supply any energy needed for GDH induction, since respiration was shown to occur maximally at this salinity (12% of the amount of $^{14}$C-glutamate taken up by the cells, which itself amounted to 2.0% of the total radioactivity available, was respired as $^{14}$CO$_2$ after one hour at 15 C in 0.26 M NaCl). However, some interesting explanations can be postulated from the data. Figure 39 indicates several possible levels at which sub-optimal salt (NaCl) concentrations might reduce the induction rate of GDH. Penetration (uptake) or energy (respiration) level inhibition would not seem tenable for the reasons previously discussed. Also, a transcriptional, translational, or folding, level
Figure 39. Diagramatic representation of possible mode of GDH induction inhibition in GM (0.26 M NaCl) at 15°C containing 3.0 mM glutamate after one hour incubation. 1. penetration (uptake), 2. energy (respiration), 3. metabolic conversion, 4. repressor (inducer), 5. replication, 6. transcription, 7. translation, 8. folding.

Percent of total available.
inhibition would likewise seem unlikely, since protein synthesis was shown to occur at a high rate and because active GDH was synthesized, but at a low rate (Figure 18).

The three steps at which sub-optimal salt might be acting could be at the metabolic conversion, repressor (inducer), or replication, level. The first of these, the metabolic conversion level, seems tenable, if, in fact, the actual small molecular weight molecule doing the inducing is derived from glutamate by one or more chemical reactions. If these reactions were stopped, an uninterrupted repression of GDH would continue. Another, and more appealing explanation would involve a direct repression level inhibition. This would be possible in at least three ways, (a) the rate of repressor synthesis might be increased (repressor may or may not be a protein), (b) the affinity of the repressor for the operator gene might be increased, or (c) the interconversion of inactive and active repressor by the inducer might be altered (24). Also, sub-optimal salt may be preventing the replication of DNA.

Although no data were presented on the penetration of Na\(^+\) and/or Cl\(^-\) into the cells, reports of other investigators using a marine pseudomonad indicate that the intra-/extracellular Na\(^+\) concentration ratio is unity (84). If this is indeed the case with _V. marinus_ MP-1, then cells grown in GAM at 0.4 M NaCl should leak Na\(^+\) and accumulate water (swell) when placed into GM (0.26 M NaCl). Swelling was
not observed by phase microscopy but was detected by OD measurements. It seems doubtful that Na\(^+\) is immediately penetrating cells shifted to a different concentration of NaCl. The OD of a cell suspension decreased immediately on shifting cells to solutions of lower salinities, indicating an osmotic effect. This effect is only possible where the solute molecule is unable to penetrate a semi-permeable membrane. In this case the higher Na\(^+\) concentration inside the cell is apparently not allowed to equilibrate with that outside, resulting in an uptake of water causing swelling (OD would decrease).

In the foregoing discussion on the effects of sub-optimal NaCl concentrations on induction, it was assumed that the major pathway for glutamate catabolism was by oxidative deamination, mediated by GDH, to \(\alpha\)-ketoglutarate. This compound would then be further oxidized through the TCA cycle. This assumption was based on the fact that the growth data at 15 C generated a series of curves similar to those for GDH induction (compare Figures 2 and 18), i.e. the growth of the cells was directly proportional to the amount of GDH synthesized. There have, however, been other pathways of glutamate catabolism demonstrated in microorganisms. Evidence recently obtained in this laboratory seems to indicate that a change in the salinity of the suspending medium alters the percent participation of different \(^{14}\)C-glucose catabolic pathways in \textit{V. marinus} MP-1.

The explanation for GDH induction inhibition at 15 C by
supra-optimal NaCl concentrations is considerably clearer. At 0.6 and 0.8 M NaCl the induction of GDH (and therefore, presumably, growth) seemed to be directly related to the rate of substrate uptake. As the concentration of NaCl increased from 0.4 to 0.6 or 0.8 M, the uptake systems were progressively impaired (Table 7). As a consequence, the respiration rate was decreased (Table 8), as was the rate of protein synthesis (Figure 31). Exactly what biochemical processes were effected is difficult to say. However, a physical rearrangement of cell structure was implicated since it was observed that cells suspended in salt concentrations greater than those in which they were grown, characteristically increased in their absorbancy. Such an increase in OD of cell suspensions might well be due to an increase in cell density (compression of the total volume of the cell) or merely shrinking of the cytoplasmic membrane, but not the entire cell. This difference was not resolved by phase microscopy. If the latter situation actually occurred, such an osmotic effect would be indirect evidence that Na$^+$ is not immediately, and freely penetrating the cells. Matula and MacLeod (47) have recently presented evidence that the OD increases produced in suspensions of a marine pseudomonad by adding NaCl was due to an interaction of salts with components of the cell envelope, causing a contraction of the envelopes and shrinkage of the cells. They felt that the OD increase was not due to an osmotic effect, since this organism presents no osmotic barrier to NaCl (84).
A similar conclusion was drawn from experiments with a terrestrial pseudomonad (48).

In general, the same effects as those described for cells shifted to GM at 15 C occurred with cells shifted to GM at 4 C. At 4 C the induction rate was considerably reduced, although detectable, over that at 15 C, particularly at 0.4 and 0.6 M NaCl. This reduction in induction rate at 4 C appears to be merely the result of lowered chemical reaction rates. This was further evidenced by reduced rates of $^{14}$C-glutamate uptake and growth, even under the optimal salinity conditions, 0.4 M NaCl, compared to those at 15 C. It should be noted that as with the cells shifted to GM at 15 C the maximal apparent uptake rate of $^{14}$C-glutamate and $^{14}$C-proline occurred at the lowest salinity. It might also be of interest to note that uptake and incorporation curves for $^{14}$C-proline at 4 C are very similar to those obtained at 15 C.

Due to the relative slow rates of GDH induction and growth at 4 C, and a corresponding reduction in the resolution of relatively small changes in specific activity of the extracts (Figure 17), very little can be said concerning the effect of NaCl on induction at this temperature.

At 20 C the effect of the NaCl concentration in GM on GDH induction and growth was essentially the same as that obtained at 15 C, except that somewhat lower rates of induction and growth were
obtained. This result was not particularly surprising in view of the fact that 20-22°C was found to be maximal for growth (Figure 9) in GM (0.4 M NaCl). A maximum growth temperature of 20°C was previously reported for the same organism by Morita and Haight (55).

As in the 15°C experiments, the apparent uptake rate of C-glutamate was maximal at 0.26 M NaCl, as was the apparent rate of $^{14}$C-proline uptake and incorporation into protein. It seems that the induction rate decrease at 20°C over that at 15°C was due to a thermal alteration of substrate transport. An increase in salt from 0.4 M to 0.6 or 0.8 M at 20°C does protect the transport system against this thermal alteration, but apparently decreases the porosity of the membrane to solute passage as occurred at 4 and 15°C.

One interesting point that should be mentioned was the fact that the optimal temperature for $^{14}$C-proline uptake and incorporation into protein was 20°C at all four salinities tested. This is in contrast to the uptake of $^{14}$C-glutamate where the optimal temperature was 15°C at all salinities. Cooper (6) previously reported the same finding with V. marinus MP-1 when she noted that protein synthesis at 20°C was equal to or greater than that at 15°C. Likewise, Morita and Albright (53) found a stimulation in protein synthesis over that at 15°C when cells of V. marinus MP-1 were subjected to a temperature of 21°C. Based on these different optima for uptake of two different solutes, it seems as though this organism has at least two thermally
Tai and Jackson (85) have recently shown, however, that the solute-transporting and energy-yielding systems of an obligate psychrophile, Micrococcus cryophilus were unrelated to the maximal growth temperature. Interpretation of the effect of NaCl on induction at 20 C is further complicated by the fact that cells lost their viability at different rates depending on the salinity (Table 2), but seemed to continue to increase in mass over the ten hour sample period.

It seems doubtful that GDH would be synthesized at a normal rate at 20 C and inactivated by thermal denaturation intracellularly, even at the lower salinities, since only a 50% reduction in activity was found in a CFE heated for nine hours at 19 C (Figure 15). One would expect a higher thermal stability with enzymes heated within the cells than in cell-free extracts (36).

At 25 C there does not appear to be any significant effect of salinity on induction. There is an initial induction at all salinities but all the levels drop to background activities within four hours after shifting. Although the cells are apparently able to take up the substrate, synthesize protein, and respire at this temperature, irreversible thermal damage occurred to the cells within two hours after shifting cells to 25 C. Salt concentrations from 0.26 to 0.8 M did not protect the cells from thermal death at 25 C. The higher salt concentration, 0.8 M NaCl, seemed to cause increased leakage at 25 C as
indicated by the dry weight determinations. Haight and Morita (23) have previously demonstrated thermal induced leakage by heating _V. marinus_ MP-1 at 22.3°C for 95 minutes.

The data on the uptake and incorporation of $^{14}$C-glutamate and $^{14}$C-proline must be interpreted with caution. The true uptake rate should represent the total amount of substrate passing through the cell membrane per unit time. Uptake studies, characteristically, do not take into consideration that amount of label respired as $^{14}$CO$_2$ during the experiment. It has recently been shown in our laboratory that as much as 70-80% of the total amount of $^{14}$C-glutamate taken up by _V. marinus_ MP-1 at 0.6 M NaCl between 5-20°C is respired as $^{14}$CO$_2$. The apparent uptake rate (that amount of label accumulated in whole cells per unit time), was negligible. This was not found to be the case with GAM grown cells shifted to GM (0.4 M NaCl) at 15°C, the optimum for growth and induction. Under these conditions, only 12% of the $^{14}$C-glutamate entering the cells was respired as $^{14}$CO$_2$. Therefore, the apparent uptake rate of $^{14}$C-glutamate, at least at 15°C, very nearly approximates the true uptake rate. It would seem reasonable to assume that the same situation exists at 4°C. At 20 and 25°C, however, the effect of respiration may significantly affect the true uptake rate. This seems particularly applicable to the data in Figures 25, 29, and 33 for $^{14}$C-glutamate uptake, $^{14}$C-proline uptake and $^{14}$C-proline incorporation into protein, respectively, in 0.26 M
NaCl at 25 C. The drastically reduced rates of apparent uptake and incorporation at this salinity and temperature might very well be a reflection of an increase in respiration under these conditions, thus possibly producing a constantly low, intracellular label pool. These assumptions could easily be tested by running respiration studies concomitantly with uptake studies at 20 and 25 C. Robinson and Morita (73) have demonstrated that exposure of _V. marinus_ MP-1 cells to temperatures above 20 C brought about a decrease in the rate of respiration. This would seem to indicate that the reduced rate of apparent uptake observed at 20 and 25 C is probably close to the real uptake rate.

The three main difficulties encountered in interpreting kinetic data on induction are (a) substrate penetration variability, (b) the use of whole cells respiration techniques in assessing enzyme concentrations, and (c) the use of non-gratuitous inducers which allow not only the induced synthesis of a particular enzyme, but also growth (65). In these studies the first two difficulties were eliminated by following substrate penetration with $^{14}$C-glutamate under induction conditions and assaying the enzyme by using cell-free extracts. The difficulties encountered in the use of a non-gratuitous inducer, as glutamic acid, were partially overcome by monitoring growth during the induction period by OD, mass and viability measurements. However, much more significant and precise kinetic data may well be obtained in
future experiments with the use of a non-gratuitous inducer.

Unlike many previous reports concerning the activation of enzymes by near environmental concentrations of NaCl from marine (37, 46, 51), halophilic (37, 57) and non-halophilic (60) bacteria, no such activation was encountered with GDH from V. marinus MP-1. Enzymatically active cell-free extracts tested in 0.22, 0.35, 0.46 and 0.6 M NaCl wash buffer, which were close to the salinities under which shifted cells were subjected, were completely inhibited. In addition, cell-free extracts prepared in the complete absence of Na⁺ (phosphate buffer, Figure 16) were considerably active in the Tris-HCl assay buffer. The only Na⁺ available to the enzyme might have been carried over from the GAM growth medium. This amount might be enough to function catalytically, however.

As noted by other workers (46, 51), NaCl seems to have a stabilizing effect on enzyme activity. This was also observed with the GDH investigated here (Figure 16).

The data obtained on the salt specificity for $^{14}$C-glutamate uptake at 15°C are rather interesting. Since all the solutions contained salts in a concentration of 0.4 M, the resulting osmotic pressures were equivalent. However, the apparent rate of uptake was drastically different. NaCl gave the most rapid rate of uptake with KCl the next best. Substitution for NaAc for NaCl completely inhibited $^{14}$C-glutamate uptake. This result was interpreted to mean that the
Cl\(^{-}\) ion is essential for \(^{14}\)C-glutamate uptake. This is in contrast to numerous reports indicating a Na\(^{+}\) ion specificity for uptake (11, 16, 42, 43, 80). Again, the uptake rates have been referred to as the "apparent uptake rates" because respiration studies were not done under the same conditions. It seems doubtful, however, that a change in the respiration rate of cells in NaAc over that in NaCl would be sufficient to account for the large difference in the apparent uptake rates observed.

The metabolic inhibitor studies indicated that the uptake of \(^{14}\)C-glutamate was by an active transport process and not simple diffusion. Inhibitors of respiration, NaN\(_{3}\), KCN, KAr and DNP (88) showed various degrees of uptake inhibition with DNP being the most effective. Again, respirations studies will have to be performed under the same conditions to confirm these results, but it is doubtful that respiration could account for the large difference between the apparent uptake rates in the presence and absence of DNP.

Chloramphenicol (CAP) was only slightly inhibitory to the apparent uptake process, presumably indicating that protein synthesis is not required for uptake. However, we do not have conclusive evidence that the CAP has actually entered the cells.
SUMMARY

Experimental procedures were set up whereby the effects of temperature and salinity changes on growth, enzyme induction, and substrate uptake of an obligately psychrophilic bacterium, _V. marinus_ MP-1, could be assessed.

Observations were made on the effect of changing the salinity and temperature of a glutamate shift medium (GM) on the growth, enzymatic induction, and substrate uptake of cells grown in glucose-ammonium medium (GAM) at 15°C. Cells used in these experiments were grown in GAM for 48 hours at 15°C and shifted to GM containing 0.26, 0.4, 0.6, or 0.8 M NaCl at 4, 15, 20 or 25°C.

It was found that at all temperatures tested (except 25°C) cellular growth was maximal in GM (0.4 M NaCl). In GM (0.8 M) NaCl growth was completely inhibited.

Cellular growth was maximal at 15°C in all salinities tested. At 25°C, the cells actually decreased in mass presumably due to leakage of cellular constituents. Optimal growth temperature experiments performed at 2 to 3°C increments demonstrated that this organism had a temperature optimum between 12-13°C in both the defined (GAM and GM) and complex media (MB). In all the media the maximal growth temperature was 20-22°C.

The pH for optimal GDH activity was 9.0 at 15°C. GDH was
also found to require NAD$^+$ specifically as a cofactor. The enzyme retained 100% of its original activity for ten hours at 0 C, but lost all activity upon freezing (5.8 mg protein/ml). NaCl was slightly effective in stabilizing the enzymatic activity. Ultrasonic treatment in wash buffer was the most effective manner of preparing cell-free extracts with GDH activity.

The induction of GDH at 4 C yielded a 14 to 24-fold increase in specific activity ten hours after shifting. Induction at 15 C gave a 56-, 44-, 21- and 9-fold increase in specific activity in GM (0.4, 0.6, 0.26 and 0.8 M NaCl), respectively. The rate of induction at 15 C paralleled the growth of shifted cells at 15 C. Similar results were obtained for induction at 20 C, except that lower levels of GDH were found ten hours after shifting (35-, 29-, 6-, and 8-fold in 0.4, 0.6, 0.26 and 0.8 M NaCl, respectively). Again, these results indicate that the rate of induction is directly related to the growth rate. Only a small initial increase in specific activity (3-12 U/mg protein) occurred by shifting the cells at 25 C, followed by even lower levels thereafter. Hence, the induction process appears to be operative at 25 C but cannot be enhanced due to cell death at this temperature.

The uptake rate and net incorporation of $^{14}$C-glutamate by shifted whole cells is dependent on the salinity of the uptake solution. At 4, 15, and 20 C the maximal rate of isotope uptake and net incorporation into whole cells occurred at the lowest salinities (0.26 M
NaCl) and decreased progressively as the NaCl concentration increased. However, at 25 C, shifted cells showed a maximal uptake rate and net incorporation at 0.4 and 0.6 M and not a 0.26 M NaCl. The optimal temperature for uptake of $^{14}$C-glutamate at all salinities was 15 C. The chloride ion (0.4 M) was found to be necessary for the uptake of $^{14}$C-glutamate at 15 C. The uptake and incorporation of $^{14}$C-proline into protein by whole cells was similarly shown to be dependent on the salinity of the uptake solution. Again, as the salt concentration increased, the rate of uptake and net incorporation into protein of label decreased at all temperatures except at 25 C. At this temperature, as with $^{14}$C-glutamate, the maximal rate of uptake and incorporation into protein occurred at 0.4 M NaCl and not 0.26 M. The optimal temperature for uptake and incorporation of $^{14}$C-proline at any one salinity was found to be 20 C.

Respiration studies showed that increasing salt concentrations from 0.26 M NaCl decreased the rate of evolution of $^{14}$CO$_2$ at 15 C.

Metabolite inhibition studies revealed that $^{14}$C-glutamate uptake at 15 C was 80% inhibited by DPN, 72% by KAr, 54% by KCN, 22% by NaN$_3$ and only 3% by CAP.

These studies seem to indicate that the environmental conditions of high salinity (0.6 M NaCl) and low temperature (4 C) are severely restricting the potential metabolic activity of _V. marinus_ MP-1 in the open ocean.


15. Flannery, W. L. and S. N. Durio. Effect of sodium chloride on cysteine utilization by Vibrio costicolus. (Abstract) PI02, p. 120. 1963.


