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Title: GLUCOSE UPTAKE AND CATABOLISM IN THE MARINE PSYCHROPHILIC BACTERIUM, VIBRIO MARINUS

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The effects of various physical parameters on the binding, uptake, and catabolism of glucose in the marine psychrophilic bacterium, Vibrio marinus, were studied. It was shown that shortly after the cell was exposed to labeled glucose, the radioactive label became rapidly associated with the cell fraction. It was also shown that much of this activity was readily removed when the cells were exposed to an acidic environment. Since this release was not associated with leakage and the release was practically instantaneous, it appeared that this loss was related to the removal of loosely bound glucose. In order to reduce the chance of error in measuring substrate uptake, this loosely bound glucose was routinely removed by acidification prior to cell activity assay.

The requirements for specific ions in the uptake of glucose were studied. It was found that Na^{+}, Li^{+}, and Mg^{++} would permit

uptake but that K^+, Rb^+, and NH_4^+ would not. Of the various different salts of Na^+ studied, all permitted glucose uptake. This was the same pattern that was seen when the effects of specific ions on cell growth were studied.

The salinity limits of growth were defined, and their relationship to patterns of glucose uptake and respiration was observed. It was shown that there were no significant pattern shifts associated with the maximum growth salinity but that there were significant changes associated with the region of minimum growth salinity. When the effects of altering salinity on the uptake and respiration of specifically labeled glucose were studied, several patterns of potential significance were observed. The total uptake of glucose, as measured by the first, second, third and sixth carbon activities, showed that all portions of the molecule were used for energy and cell material at 0.15 M NaCl but at higher salinities, part of the molecule was released by the cells in some form other than CO_2.

Three basic patterns associated with the first and sixth carbons were observed as the salinity was increased from 0.0 M to 1.0 M NaCl. In the 0.0 M to 0.15 M range, the amount of CO_2 released from both carbons increased at approximately the same rate. In the 0.15 M to 0.30 M range, there was a decrease in the CO_2 associated with both of these carbons and a concomitant increase in the amount of cell activity associated with both of these carbons. As the salinity

was increased above this point, the CO_2 associated with the sixth carbon remained relatively constant while that associated with the first carbon increased markedly. This shift in the C_6/C_1 ratio was shown to be relatively unaffected by changes in temperature over a wide range.

Glucose Uptake and Catabolism in the Marine Psychrophilic Bacterium, Vibrio marinus

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GLUCOSE UPTAKE AND CATABOLISM IN THE MARINE PSYCHROPHILIC BACTERIUM, *VIBRIO MARINUS*

INTRODUCTION

As more is known about the marine ecosystem, it has become increasingly apparent that bacteria are potentially important in the mineralization process, in the concentration of various organic and inorganic molecules, in the control of Eh and in many other processes. In order to understand these and other bacterial functions, a great deal must be learned about the factors affecting the uptake, utilization and disposition of available nutrients. This type of knowledge could be extremely important in the interpretation of reactions of marine organisms to unusual environmental conditions or to anticipate the reaction of the marine biosphere to proposed large scale alterations of the marine environment. To obtain this type of information, two basic approaches are currently available; by directly observing substrate uptake, *in situ*, by all organisms present, or by selecting representative organisms and observing their nutrient requirements in pure culture. The latter approach has been used in this series of experiments.

While studying the effects of salinity on the obligate marine psychrophile *Vibrio marinus*, Stanley and Morita (1968) showed that salinity affected the maximum growth temperature. It was also

reported that certain cations permitted growth whereas others did not. These and other observations suggested that both the type and amount of salt available to this organism might have an effect on substrate uptake and catabolism. If such cause-effect relationships were established, this could have important implications concerning this organism's ability to function under diverse ionic conditions.

Requirements for specific ions have been demonstrated in other marine organisms (e.g., the work of MacLeod and his associates and Payne and his associates). In most of these studies, done on species of the genus Pseudomonas, the general effects of specific ions on substrate uptake and metabolism have been studied. There are however, many aspects of salinity and metabolism in marine bacteria which are yet to be explored. For example, it is quite possible that different salinities might have an effect on the type of substrate used, the speed of utilization, the type of catabolic pathways involved, the amount of substrate used for energy, cell material, or metabolic by-product production. It is the purpose of this study to determine the specific effects of the ionic environment on some of these parameters in V. marinus.

Apparently little is known about the mode of glucose-cell surface attachment and what effects the environment might have on this interaction. As a result, the role of surface bound glucose in substrate uptake studies is generally ignored. If "uptake" is defined

as the total substrate that has entered the cell, i. e. passed through the cell envelope, then that substrate still clinging to the outside of the cell must be accounted for. If changes in uptake relative to another parameter are to be measured then, at the very least, loosely bound substrate must be maintained at a constant level. Otherwise any observed change might be due to changes in surface binding rather than to uptake. It is also the purpose of this paper to explore some of the parameters affecting surface binding and to discover a technique that would reduce errors generated by surface binding.

REVIEW OF LITERATURE

Effects of Salt on Growth

The ionic environment of the bacterial cell has been shown to be of great importance to the cell's function, especially in marine bacteria. While trying to detect qualities which are unique to marine bacteria, several investigators have looked at the specific ionic requirements of these bacteria as compared to closely related terrestrial forms. Much of the early work in this area was done by MacLeod and his associates. In 1954 MacLeod, Onofrey and Norris studied the specific organic and inorganic requirements of 33 marine bacteria representing at least three genera. They found that all organisms studied required K^{+}, and Mg^{++} and that most required Na^{+} and Ca^{++} for growth. In a subsequent report, MacLeod and Onofrey (1957) looked at the ionic requirements for six of the previously studied strains. Of the anions studied, all strains required $PO_4^{\equiv}$ and $SO_4^{=}$. More recently, Hidaka (1964, 1965) and Hidaka and Saki (1968) reported that the majority of the marine strains studied had salinity optima in the 5-7% range whereas most terrestrial forms had optima near 0.5%. Hidaka (1964) classified bacteria found in the marine environment as terrestrial, marine, or halophilic according to their salinity requirements. Of the 275 strains tested, 32% were marine, 50% terrestrial, and 18% halophilic. In

a more recent study, Simidu and Hasuo (1968) looked at the effects of altering the salinity of the isolation medium on the number of viable bacteria recovered from fresh marine fish. They showed a maximum number of colonies at 2.5% NaCl.

According to Drapeau and MacLeod (1965), the primary roles of ions in the growth medium of marine bacteria are: (1) the maintenance of intracellular solute concentration, (2) the requirement for cellular integrity (lysis prevention) and (3) the requirement for nutrition and metabolism.

Intracellular Ionic Concentrations

Both Na^+ and K^+ have been shown to be important for growth in marine bacteria. The specific roles of these and other ions have been investigated. MacLeod and Onofrey (1957) showed that the concentration of sodium was the same in the cells of the marine bacterium they were studying as that in the medium. They discovered, however, that the cells apparently accumulated potassium ions. In their study of the internal concentrations of sodium and potassium in a non-halophilic bacterium, Christian and Waltho (1961) reported that there was an accumulation of potassium and that generally the sodium concentration was less within the cells than in the growth medium.

Takacs, Matula, and MacLeod (1963) looked at the internal Na^+ and K^+ concentrations of a marine pseudomonad under various

conditions. They found that at the concentrations tested, the K^+ within the cells was double that found in the suspending medium. This was found to be affected somewhat by the Na^+ concentration. Using atomic absorption spectroscopy, light scattering and chemical analytical techniques, Gale, Dittman and Goldner (1970) looked at the effects of NaCl on the concentration of Mg^{++} and K^+ in two Serratia species. The accumulation of K^+ within the cell was energy dependent but was not directly linked with Na^+ exchange except in damaged cells. This was different from the situation observed in Escherichia coli where the accumulation of K^+ depended on the rapid exchange of H^+ for K^+ and a slower process involving the exchange of Na^+ for K^+ (Epstein and Schultz, 1966).

In an extreme example of K^+ accumulation by cells, Gochnauer and Kushner (1971) reported that the internal K^+ concentration in Halobacterium halobium was 31 - 38% of the cell's dry weight. The fact that these cells can maintain this concentration in a K^+ free medium for extended periods of time indicate that the ions were bound to some degree to the cell's protein. In marine yeasts, Norkrans (1968) showed that the internal K^+ concentration was the highest in the most halotolerant yeasts and that the K^+ concentration decreased with increasing Na^+ concentration.

Ionic Requirements for Cell Integrity

The importance of the ionic environment to the marine bacterium becomes quite apparent when it is suspended in distilled water. Lysis normally takes place very rapidly. MacLeod and Matula (1961) reported that Mg^{++} and Ca^{++}, at the levels normally found in sea water, eliminated the Na^{+} requirement for lysis prevention in a marine bacterium. These authors reported that the lower limit of salt requirement was species specific (1962). Of the monovalent cations studied, Na^{+} and Li^{+} were shown to be the most effective in preventing lysis. In all but one organism, Mg^{++} and other divalent cations prevented lysis at 0.05 M or less. The similarities in the patterns seen in all organisms tested suggested that the same type of mechanism might be acting in all cases. They also pointed out that as the lysis point was approached, no morphological changes were seen. This is in contrast to the results of Abram and Gibbons (1961). While studying lysis in halophilic strains, they observed a shift from rod to spherical forms as the salinity was decreased to the point of lysis. Korngold and Kushner (1968) looked at the effects of changing the ionic environment on a psychrophilic bacterium. In this organism, NaCl alone would not prevent lysis. Magnesium was shown to be required as well. They also showed that Mg^{++}, Na^{+}, and Li^{+} could help prevent lysis but that K^{+} could not. They

suggested that the divalent cation component of the cell wall might be related to the requirement for divalent cations in lysis prevention.

The electron microscopy work of Buckmire and MacLeod (1965) showed that there might be a direct salt-membrane interaction involved in the lytic phenomenon. At 0.5 M NaCl, a normal two unit membrane was seen in the cell envelope preparation. At 0.01 M NaCl however, the two membranes become separated and hexosamine containing material from the mucopeptide layer was released. This was observed whether or not the preparation was heated. The authors, therefore, concluded that this was a direct salt effect rather than the result of lysozyme activity. They also observed that as the salt concentration was reduced, the pH of the medium increased. These observations led them to speculate that there was a salt dependent mucopeptide layer which required the shielding of negative charges by cations in order to preserve hydrogen and hydrophobic bonding between units. They suggested that at low salinities, there are not enough cations present to shield these negative charges. The weakened cross linkages coupled with intracellular osmotic pressure was enough to cause cell lysis.

In a recent study on cell leakage in a marine Pseudomonas species type IV, DeVoe et al. (1970) reported that 260 nm absorbing material was released unless NaCl, $MgSO_4$ and KCl were present

in combination.

The work of Buckmire and MacLeod (1970) with a marine pseudomonad suggested that the effects of salinity on lysis is different from the effects of sucrose on the same system and that, to a degree, the extent to which a solute can penetrate the cell affects its ability to prevent lysis.

Change in OD and Plasmolysis

In many cases, the OD of a cell suspension will increase rapidly as the solute concentration increases. This increase is often followed by a slow decrease in OD (Henneman and Umbreit, 1964 and Matula and MacLeod, 1969). The decrease (but not the initial increase) could be stopped by omitting K^+ from the suspending medium or by using various metabolic inhibitors such as DNP, NaN_3, PCMB and $HgCl_2$. Since, in all cases studied, the NaCl concentration within the cell was essentially the same as that found outside the cell, they concluded that the OD changes were not due to osmotic effects. The OD of isolated cell envelopes increased with increasing NaCl concentration without the addition of $MgSO_4$. The authors speculated that the increase in OD was caused by an interaction between the salt and the cell envelope causing a contraction of the cell envelope. It was also noted that the concentration of NaCl that caused maximum shrinkage was the optimum concentration for macromolecular leakage

prevention and for optimum growth. Similar observations have been made by Matula and MacLeod in another organism (1969) and by Haight and Morita (unpublished data) in Vibrio marinus.

The importance of K^+ in "second phase" OD changes was also noted by Matula et al. (1970). In this study glutamate (a metabolizable substrate for the organism) enhanced the uptake of K^+ and also affected the speed and extent of this change.

Thompson, Costerton, and MacLeod (1970) looked at the effects of K^+ on deplasmolysis. The results of their study led them to conclude that under the conditions studied, K^+ loss or gain affected conditions of plasmolysis and deplasmolysis respectively. The effects of NaCl on OD was attributed to its ability to control porosity of the cytoplasmic membrane.

Cation Involvement in Transport

There are two possible effects of salinity on the transport of materials into the cell. It is possible that the membrane is altered structurally so as to effect porosity and substrate attachment. It is also possible that one of the enzyme systems associated with transport is affected by salt; either as a direct salt-enzyme interaction, or indirectly through enzyme induction. Because of the complexity of the problem, there is some difficulty in separating out these alternatives. MacLeod et al. (1958) looked at the effect of various media

on the oxidation of substrates. They showed that both Na^+ and K^+ were required for the oxidation of the substrate studied. It was concluded that Na^+ formed complexes with the substrate before it could cross the cytoplasmic membrane.

While studying Pseudomonas natriegens, Payne (1960) showed that Na^+ was required for permease activity associated with glucuronate uptake and that K^+ was required for "oxidative mechanisms." The concentration of K^+ required for growth was shown to be only 0.01 that of Na^+. He also showed that Li^+, Cs^+, and Rb^+ could not replace Na^+ and K^+ as growth requirements. Subsequent work by Rhodes and Payne (1962) has shown that Mg^{++} concentration may affect the integrity of the cell to the point that at very low concentrations some, but not all, substrates may be transported into the cell.

While studying the uptake of glutamate by a Halobacterium sp., Stevenson (1966) showed that NaCl was required for its active uptake. When either the cation or anion was replaced, very little uptake was seen. In 1969 Wong, Thompson, and MacLeod looked at the effects of varying NaCl concentration on the uptake and release of the non-metabolizable substrate α aminoisobutyric acid (AIB). They found that the uptake of AIB specifically required Na^+ but that the ion requirement for the retention of AIB could be partially fulfilled by other ions (also shown by Drapeau, Matula and MacLeod, 1966). When cells containing AIB were exposed to the inhibitors KCN, or

2, 4 dinitrophenol, AIB was lost in the presence of NaCl but not LiCl. From their data, they concluded that Na^+ functioned in two ways, by facilitating the uptake and by allowing the release of AIB. Using a similar system, Costerton et al. (1967) studied the retention of AIB in protoplasts. The concentration of NaCl required for optimum uptake equalled that for maximum retention. They hypothesized that NaCl acts to maintain the structural integrity of the cytoplasmic membrane.

The requirement for Na^+ and K^+ for the uptake of two substrates and their nonmetabolizable antalogs have also been studied (Drapeau, Matula and MacLeod, 1966) using D-galactose with its antilog fucose and alanine with its antilog AIB. They found that the optimum concentration of salt required for the uptake of these two groups was different. These data plus competitive inhibition studies indicate that two separate systems are involved in the transport of these two groups. The fact that the effects of Na^+ on uptake are principally located in the cytoplasmic membrane rather than the cell wall was illustrated in the work of DeVoe et al. (1970). They used cells that had the outermost layers of the cell envelope removed and still retained their natural shape (mureinoplasts).

Frank and Hopkins (1969) observed that Escherichia coli grown on glutamate was greatly affected by Na^+ concentration. The amount of growth was significantly increased in the presence of Na^+. By

using antalogs of glutamate and noncompetitive inhibitors, he was able to show that NaCl enhanced transport rather than respiration. Stevenson (1966) found that sodium was also required for the uptake of glutamate by Halobacterium salinarium.

Using the spheroplasts of a marine bacterium, Rhodes and Payne (1967) showed that both NaCl and K^+ had to be present along with a metabolizable substrate for metabolic swelling to occur. Studies with inhibitors showed that swelling also required energy and ATPase.

Effects of Salt on Enzyme Activity

In most of the above mentioned studies, the effects of salinity on the cell have been shown to be primarily on cell structure. There have been many other studies in which salt-enzyme interactions have been shown. MacLeod et al. (1958) noted that K^+ was required for the activation of certain TCA enzymes isolated from a marine pseudomonad. (Much of the literature in the area has recently been reviewed by Suelter (1970).) In a more recent set of papers (O'Brien and Stern, 1969a and b), the requirement for Na^+ in the utilization of citrate was studied using Aerobacter aerogenes. In this case, only Na^+ could satisfy the requirement when citrate was the sole carbon-energy source under anaerobic conditions. Enzyme analysis showed that oxalacetate decarboxylase required sodium for activation (this

enzyme is essential for citrate utilization under anaerobic conditions). In another study by Pratt and Happold (1960), NaCl was required for indole production in both cell extracts and whole cells of a marine bacterium. Apparently one of the enzymes involved in the conversion of tryptophane to indole required NaCl for activation.

While studying the ionic requirements of the marine pseudomonad B 16, Tomlinson and MacLeod (1957) determined that there were at least three types of specific functions related to Na^+ and K^+ in the oxidation of exogenous substrates. One requiring Na^+, one requiring Na^+ or K^+ and one requiring only K^+. It was subsequently shown (Payne, 1960) that K^+ was required for oxidation but not the uptake of the substrate tested.

Cation activation of membrane bound ATPase has been studied in several bacteria. Hafkenscheid and Bonting (1969) found a Mg^{++} requiring ATPase in E. coli that was activated by K^+ or Na^+. They also showed that when the cell preparation was treated by sonication, there was a loss of monovalent cation activation. In contrast to these findings, Drapeau and MacLeod (1963) showed that Mg^{++} or Ca^{++} could be used to activate ATPase in two marine bacteria and that neither Na^+ nor K^+ affected activity. The same type of enzyme activation was found by Hayashi et al. (1970) in a halophilic Vibrio. In this case, it was a membrane bound 5' nucleotidase that was involved. This pattern was again observed by Thompson, Green and

Happold (1969) in a membrane bound nucleotidase in a marine bacterium.

There have also been cases reported in which either monovalent or divalent cations can activate enzymes interchangeably. Such was the case with catalase activity from a halophilic organism (Laugi and Stevenson, 1969). There are also cases where there is a nonspecific salt requirement for enzyme activation; for example, the activation of isocitrate dehydrogenase found in a marine bacterium (Macleod, Hori and Fox, 1960). The fact that there are many enzymes that are activated by monovalent cations (often Na^+ or K^+) has led to much speculation as to the specific interactions involved.

Effects of Salinity on Enzyme Induction

Another possible effect of salt is on the induction and synthesis of protein. Payne (1960) notes that the rate of glucuronate oxidative enzyme induction was affected by the concentration of NaCl. Maximum rates of induction and oxidation were achieved only when K^+ was also present. While studying the uptake of labeled mannitol into a marine bacterium, Payne (1967) concluded that Na^+ was required for both the synthesis and function of the "entry mechanism" for this substrate. This work has since been expanded (Webb and Payne, 1971). The authors concluded that the synthesis of a mannitol binding protein was almost nonexistent in cells which were not exposed to Na^+. They also

noted gross effects of Na^+ and K^+ on the synthesis of protein, RNA and DNA. While studying the growth of a marine psychrophile, Staley (1971) showed that salinity in the form of NaCl, affected the fate of glutamate uptake in Vibrio marinus. He concluded that glutamate dehydrogenase induction was affected.

Major Pathways of Glucose Catabolism

Much of the current knowledge in the field of carbohydrate metabolism in microorganisms has been reviewed in a recent article by Anderson and Wood (1969).

Most studies on microorganisms show initial glucose catabolism taking place via one of three major pathways: the Embden-Meyerhof (EMP), the Entner-Doudoroff (ED), and the hexose monophosphate (HMP) pathways. By far the majority of the reports show EMP and HMP participation. In a group of Bacillus species, the range for the EMP pathway was 75-94%, and 6-25% participation for the HMP pathway (Bulla, 1970). Wang et al. (1958) reported ranges of 65-97% and 3-35% respectively in a group of nine different bacterial species.

Wang et al. (1958) pointed out that there were no known cases where the EMP and ED pathways were operating at the same time. This still apparently holds true if one considers only glucose which is catabolized for its energy. There have been several cases where the enzymes for both the EMP and ED pathways have been shown to be

present in the same organism (Katznelson and Zagallo, 1957 and Matin and Rittenberg, 1970). Matin and Rittenberg (1970) went one step further to show that the main pathway for energy production was the ED pathway. They also showed that the EMP and HMP pathways were also functioning but were used exclusively for the synthesis of biosynthetic intermediates.

Altered Glucose Pathways Related to Shifts in the Environment

There have been relatively few studies made on the effects of environmental changes on the relative participation of various catabolic pathways. Palumbo (1967) and Palumbo and Witter (1969) showed that the level of substrate used can alter the relative participation of the ED and HMP pathways in Pseudomonas fluorescenes. Wang and Krackov (1962) reported that Bacillus subtilus grown in glucose-salts medium, catabolized glucose via the ED and HMP pathways (65% and 35% respectively). When a nondefined medium was used, this figure was changed to 80% and 20% respectively. The altered glucose catabolism due to altered nutritional components in the growth medium has also been reported (Matin and Rittenberg, 1970) in Thiobacillus intermedius. The mechanism for control in this case was thought to be on the level of enzyme synthesis. In another study involving similar effects in Streptococcus faecalis,

the controlling mechanism involved the FDP inactivation of 6 phosphogluconate dehydrogenase (Brown and Wittenberg, 1971).

The alteration of the relative participation of glucose pathways by other environmental factors has been reported in very few cases. Porath and Mayber (1964) reported that salinity affected the relative release of CO_2 from specifically labeled glucose in pea root tips. They reported that the C_6/C_1 ratio decreased with increasing salinity. Subsequent work on the same system showed that this shift was related to a shift in the HMP pathway (Porath and Mayber, 1969).

Methods of Pathway Determination

There are currently available several methods for the determination of the relative participation of known metabolic pathways in bacteria. Of these, two seem to be the most effective; enzyme analysis and the monitoring of substrate utilization. Of the two, only the second can give direct information about the workings of the living cell (Keele, Hamilton and Elkan, 1969 and Matin and Rittenberg, 1970).

Monitoring of cell growth, respiration, and end product production using unlabeled substrates and intermediates coupled with inhibitor studies, has been the traditional method of approach. One of the inherent problems with this approach is that the rates of uptake and respiration are almost impossible to separate. By employing the

method developed by Wang et al. (1956 and 1958) using specifically labeled substrates and radiorespirometry, some information can be obtained about relative pathway participation and rate of glucose catabolism in bacteria.

To determine the relative participation of major glucose catabolic pathways using Wang's equations, six basic assumptions have to be made (Wang and Krackov, 1961). The equations for pathway determination were modified by Katz and Wood (1963) to circumvent some of the assumptions they found to be questionable. Under certain conditions, namely when there is a large amount of glucose being catabolized via the HMP pathway, the equations of Wang should theoretically give low values. They reasoned that there is some recycling associated with this pathway resulting in a randomization of the second and third carbons into the first three carbons of glucose 6 phosphate. They also pointed out that "with glucose ^{14}C, it [recycling] causes the ^{14}C concentration of the glucose 6 C to be less than that of the inflowing labeled glucose." By modifying these equations to account for this phenomenon and by looking at levels of respiration before the substrate is significantly reduced, they hoped to circumvent some of these possible errors. They pointed out that the change in substrate concentration during the course of the experiment might in itself cause a shift in pathways.

This method was checked using another method involving C2

glucose randomization and glycogen analysis (Chefurka, Horie and Robinson, 1970) and was found to be consistent when compared to either the Katz and Wood or to the Wang model. For most practical work, the simple equations of Wang and Krackov (1962) are still used (Höfer, 1967).

This technique has been applied to a large variety of organisms with a large diversity of relative pathway participation. For example, it has been used to study glucose metabolism in insects (Chefurka, Horie and Robinson, 1970); yeasts (Höfer, 1967); pea root tips (Porath and Mayber, 1964); and a large number of bacterial species (Wang and Krackov, 1962; Wang et al., 1958; Palumbo, 1969; Bulla et al., 1970; Keele, Hamilton and Elkan, 1968; and Brown and Whittenberg, 1971).

METHODS

Organism and Cultural Conditions

Vibrio marinus MP-1 ATCC 15381 (Morita and Haight, 1964, and Colwell and Morita, 1964) which was used in these experiments is an obligate marine psychrophilic bacterium. Stock cultures were kept in 5 ml portions of ASW medium at 5 C. The ASW medium was prepared by adding Bacto-peptone, 10.0 g; yeast extract, 3.0 g; Rila Marine Mix (Rila Products), 18 g; and NaCl, 17.0 g; to one liter distilled H_2O. The medium was adjusted to pH 7.3 with 1.0 N NaOH and sterilized by autoclaving for 15 minutes. The stock cultures were initially incubated at 15 C for 24 hours before transferring to 5 C. The purity of the stock culture was checked periodically by assaying growth at room temperature. Culture purity was also checked by reisolating the strain on ASW streak plates.

Unless indicated to the contrary, all cells used in these experiments were grown on SOS medium. SOS medium contained the following: $(NH_4)_2SO_4$, 2.0 g; K_2HPO_4, 4.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.123 g; NaCl, 23.36 g; trace element solution, 1.0 ml and 1.0 liter distilled H_2O. The above solution was adjusted to pH 7.3 with NaOH and then sterilized in the autoclave for 15 minutes. Just before inoculating the culture with a 0.1% inoculum from the

stock culture, 100 ml glucose solution and 1.0 ml vitamin solution was added aseptically. All components were cooled to 5 C at the time of inoculation. The trace element solution contained $FeCl_2$, 10.0 mg; $MnSO_4 \cdot 7H_2O$, 70 mg; and 100 ml distilled H_2O. The vitamin solution, which was filter sterilized before use, was made up of nicotinamide, 400 mg; thiamine, 100 mg; pyridoxal HCl, 100 mg; Ca-D-pantothenate, 100 mg; riboflavin, 25 mg; biotin, 1 mg; and 1.0 liter distilled H_2O. The glucose solution (autoclaved separately for 15 minutes) was prepared by adding glucose (2.5 g) to 100 ml distilled H_2O.

Except in cases indicated, all cells were grown at 15 C for 48 hours on a reciprocal shaker (stationary phase). In all cases, 250 ml of medium was used in a Ferbach flask.

Harvesting and Washing Cells

In all cases the cells were harvested by centrifugation at 16, 300 x g for 10 minutes at 2 C in a RC2B Sorvall refrigerated centrifuge. The supernatant was decanted and the remaining pellet was suspended in salt buffer. This procedure was followed twice and then the cells were suspended in the buffer to be tested. The salt buffer used in these experiments was SOS medium without glucose or vitamin solution. All reagents were kept at 0 C. The cell suspensions had an OD of 0.6 at 600 nm using 0.5 M NaCl salt buffer as a reference on a Bausch and Lomb Spectronic 20 colorimeter.

Radiorespirometry Techniques

The techniques and equipment used were basically those first described by Wang et al. (1958) as the "microscale" technique. Four and one half ml of cell suspension was added to a 50 ml single side arm incubation flask. One half ml of the substrate to be tested was placed in the side arm. The side arm was then sealed with a serum bottle rubber cap. The flask was then attached to the CO_2 collection assembly which was then mounted on the refrigerated Warburg apparatus (Gibson Medical Electronics). All CO_2 collecting assemblies were then connected to the air manifold which was, in turn, connected to a source of filtered air. The temperature of the water bath was 15 C unless otherwise stated. The air flow rate was 50-60 ml per minute as measured by a flowmeter (Kontes Glass Co.).

The CO_2 absorbing material used was a 2:1 mixture of 100% ethanol:monoethanolamine (Baker Chemical Co.). In experiments where the collection time was one hour or less, 10 ml of this mixture was used but for longer collection periods, 20 ml was used. The system was equilibrated for 15 minutes before the substrate was added to the cell suspension from the side arm. The time at which the substrate was added was considered to be time 0 in all experiments. The rate of air flow was checked every 15 minutes and after

each CO_2 sample was taken. A continuous air flow was maintained by the use of a three way valve and two CO_2 traps as described by Wang et al. (1958).

At the end of the incubation period, the cell suspension was acidified to pH 2.0 with 0.11 ml of 2 N HCl. A one ml portion was then removed to be used in the cell radioactivity determination. The rest was centrifuged 36,400 x g at 2 C for 20 minutes. The resulting supernatant was analyzed further.

The cell suspension was diluted ten times with cold salt buffer (pH 2.0). One ml portions of the diluted suspension were filtered quickly through a Millipore filter apparatus fitted with a membrane filter (Millipore Corp. 0.045 μ). A minus 25 psi vacuum was used to facilitate filtration and a 10 ml wash of pH 2.0 cold salt buffer was used to remove supernatant activity from the filter. The filters were then dried under heat lamps for at least 30 minutes before placing them into scintillation vials for counting.

Radioactivity Determinations

In preparing samples for both CO_2 and cell activity determinations, 10 ml of scintillation fluor made up of 2, 5-diphenyloxazole (PPO), 5.0 g; dimethyl-1, 4-bis-2-(5-phenyloxazolyl)benzene (POPOP), 0.3 g; and one liter toluene was added to scintillation vials. The dried filters were placed directly into the fluor for

counting cell activity. CO_2 activity was measured by adding 1.0 ml ethanol-monoethanolamine-$^{14}CO_2$ directly into the fluor. A different fluor made up of scintillation grade naphalene, 100 g; PPO, 7.0 g; POPOP, 0.3 g; and one liter dioxane was used in supernatant and substrate activity determinations (all aqueous samples). Ten ml fluor and 0.1 ml aqueous sample were used in these determinations. All scintillation fluor reagents except the solvents, were purchased from the Packard Instrument Co.

All samples were assayed on a Nuclear Chicago Mark I liquid scintillation computer. The counts were made for 10 minutes giving total counts in the range of 10^3 to 10^4. The average background counts were 17 cpm ± 2. The instrument was calibrated to give the maximum counts on the main channel. The determination of the counting efficiencies required to convert CPM to DPM was routinely made by the channels-ratio method as described by Bush (1963). A chemical quench curve was established using the standard toluene and chloroform technique described by Wang and Willis (1965). This quench curve was checked periodically by spiking samples with standard ^{14}C toluene (New England Nuclear Corp.). Good agreement was found between the two methods. Separate quench curves were established for toluene and dioxane fluors.

The percent of activity recovered for any given sample was determined by dividing the total DPM for the fraction being tested

by the activity of the substrate used at the beginning of the experiment.

Cell Growth and Viability Determinations

The determination of the effects of salt concentration on the growth of MP-1 at 15 C was made by inoculating the organisms into SOS of varying NaCl concentrations. One hundred ml portions of SOS medium in 250 ml Erylenmeyer flasks were inoculated with 0.1 ml of the stock culture. All flasks were incubated at 15 C for 63 hours on a reciprocating shaker. Total growth at the end of the incubation period was determined by measuring the optical density (OD) at 600 nm on a Spectronic 20 colorimeter (Bausch and Lomb).

The effects of incubating cells at different salinities on cell viability under conditions encountered during the uptake and respiration studies, was determined by the following technique. Cell suspensions were treated in the identical manner as that normally used during respiration experiments except no labeled substrate was added. Cell viability was determined by spreading 0.1 ml portions onto cold ASW plates. Two dilutions of each sample were made using cold 30% salt buffer. The plates were incubated at 15 C for 24 hours before they were counted. The results were reported in terms of the percent reduction in the original population at any given salinity over the duration of the experiment.

Uptake and Release of Activity from Cell Fractions

The effects of varying pH on cell radioactivity was determined by subjecting identical cell fractions retained on membrane filters to 10 ml portions of cold 0.45 M salt buffer at varying pH's. The cell preparation and radioactivity determinations were made using previously described techniques.

The release of activity from acidified cells was determined by monitoring the cell activity after the cell suspension was acidified. The cell suspension was prepared as described before. UL ^{14}C glucose (0.01 μc) was added to 10 ml of cell suspension and the resulting mixture was incubated for 6 minutes at 0 C. The suspension was split into two equal parts. One aliquot was not acidified and acted as the control. The other aliquot was acidified with 0.11 ml 2 N HCl to reduce the pH to 2.0. Immediately after acidification, the first sample was taken (time 0). The acidified samples were washed with 10 ml salt buffer at pH 2.0 while the control was washed with 10 ml salt buffer at pH 7.3.

The uptake of UL ^{14}C glucose into the cells with time was measured by periodically checking cell activity after substrate addition. One half ml of UL ^{14}C glucose with an activity of 1.0 μc/ml was added to 20 ml of cell suspension with an OD of 0.1 at 600 nm. Duplicate samples of 1.0 ml each were taken at each time. Each

sample was washed with 10 ml acidified salt buffer and assayed as previously described.

The effects of various cations and anions on glucose ^{14}C uptake were studied in stationary phase cells grown in ASW broth. In the cation experiments, only chloride salts at 0.4 M were used in an ammonium phosphate buffer system. The buffer was prepared by adding 0.01 M solutions of $(NH_4)_2 HPO_4$ and $(NH_4)H_2PO_4$ in a 6:25 ratio. The final pH of all buffers was 7.3. In the anion experiments, only sodium salts were used. In these experiments, a sodium phosphate buffer was used that was prepared in the same manner as the ammonium phosphate buffer. In all cases, the cell suspension was incubated at 0 C for 20 minutes before the cells were filtered and washed with cold acidified salt buffer.

Effects of Salinity on Leakage of 260 and 280 nm Absorbing Materials

Stationary phase cells were used. The washed cell suspension was divided into 3 ml fractions which were centrifuged to remove the cells. The cells were resuspended into 3 ml of salt buffer of varying salinity. The suspensions were incubated with shaking for 30 minutes at 15 C. The suspensions were then centrifuged to remove the cells and the supernatants were assayed for 260 nm and 280 nm absorbancy on a Beckman DB spectrophotometer. Two separate determinations were made using the same original cell suspension.

OD Changes Associated with Lowered pH

Cells were harvested and suspended as previously described. The cell suspension was divided into three aliquots; one in which the cells were lysed by sonication, a second which was acidified to pH 2.0 and incubated for 10 minutes at 15 C and a third which was not acidified. All fractions were centrifuged at 36,400 x g for 10 minutes. The resulting supernatants were analyzed for absorbancy at 260 nm and 280 nm on a Gilford Model 2000 spectrophotometer. The absorbancy of the first fraction was used as the maximum absorbancy possible. The last fraction was used as the minimum. The percent of change with acidification was calculated by dividing the absorbancy difference between the acidified and the nonacidified sample by the maximum absorbancy of the sample treated by sonication.

Salinity Effects on the Radioactivity Associated with Cells

Experiments in which the effects of salinity, either in the form of NaCl or $MgCl_2$, on the radioactivity associated with cells were carried out in the same manner. The cells were washed and suspended in the salt buffer to be tested. UL ^{14}C glucose was added to these suspensions and the cells were then incubated at 0 C for 20 minutes. The cell activity was assayed as previously described.

The effects of salinity on the cell activity were also studied with the use of specifically labeled glucose. In these experiments, the total cell activity was determined only once at the end of a respiration experiment. All of the labeled glucose used in these experiments (C 1, 2, 3, 3-4, and 6) was obtained from the New England Nuclear Corp. In preparing these labels for use, the ethanol was evaporated off and a volume of glass distilled water was added to make up stock solutions with activities of 1.0 μc/ml (specific activities in the range 5 to 14 mc/mM). These stock solutions were kept frozen and were used to make the working solution by diluting them with distilled water as required. The method of assay was the same as that previously described.

Respiration Studies

In the studies using four labels (illustrated in Figure 10), the duration of CO_2 collection was three hours. In these studies, the cell activity and the activity remaining in the supernatant were also determined at the end of the sampling period. The cells that were used were in late log phase. In the experiments using just the first and sixth carbons, two hour samples were taken and stationary phase cells were used.

In the respiration rate studies, stationary phase cells were used and one hour samples were taken. In some cases, such as in the

temperature and respiration studies, the amount of the activity recovered is expressed in terms of the total activity recovered during the "relative time unit" (RTU) as defined by Wang et al. (1956). This is the point at which there should be no more glucose available to the cell in its original form.

The effects of increasing osmotic pressure with glycerol on the pattern of CO_2 released from the first and sixth carbons was studied by adding glycerol (0.55 M) to the 0.15 M NaCl buffer.

Assay of Enzyme Activity in Cell-free Extracts

The cells used in this experiment came from a 500 ml stationary phase culture grown in ASW broth at 15 C. The cells were harvested and washed as previously described and suspended in a 0.1 M Tris (hydroxymethyl)aminomethane buffer at pH 8.0. The cells were lysed by treatment for 15 seconds in an ice water bath with a Bronwill Biosonic III at maximum power. The cell fragments were removed by centrifugation at 68,100 x g for 15 minutes at 2 C. The resulting supernatant was assayed within three hours for enzymatic activity. The activity of the fructosediphosphate aldolase (4.1.2.13) was measured using the standard aldolase assay described by Christian (1955). The other two enzymes studied were assayed using slight modifications of this technique. All reactions were monitored at 340 nm with a Gilford model 2000 spectrophotometer. All principal

reagents used in these experiments were obtained from the Sigma Chemical Co.

The measurement of glyceraldehyde phosphate dehydrogenase (1.2.1.12) activity was made using the aldolase procedure except no enzyme was added to the reaction mixture. The hexosephosphate isomerase (5.3.1.9) activity was assayed by using the aldolase technique except dihydroxyacetone phosphate (prepared as suggested by the Sigma Chemical Co. from the dimethylketal Di-monocyclohexylamine monohydrate salt) was used instead of fructose-1,6-diphosphate. The specific activities were determined by dividing the change in OD per minute by the mg protein per sample. The protein determination was made using the standard Biuret method.

Acid and Neutral Volatile Products

Four and one half ml of a heavy cell suspension of stationary cells were added to one half ml of 0.1 μc/ml UL ^{14}C glucose. The cell suspension was incubated as previously described under "respiration studies." At the end of the incubation period, the mixture was centrifuged for 10 minutes at 16,300 x g. The resulting supernatant was decanted and kept for further analysis. The pellet was resuspended in 5 ml SOS and acidified to pH 2.0. The cells were then removed by centrifugation and the resulting supernatant was removed from the pellet. Both supernatants were distilled until one half of the

original volume was removed. The distillants were retained for radioactivity determinations. The residues were acidified to pH 1.0 with concentrated sulfuric acid and then steam distilled.

RESULTS

The salinity limits of growth for Vibrio marinus, MP-1 had to be established for SOS medium. The growth of MP-1 at 15 C in this medium at various salinities is given in Figure 1. The lower limit for growth as measured by OD at 600 nm was 0.275 M NaCl, the upper limit was 0.70 M NaCl, and the optimum was near 0.50 M NaCl.

Once the salinity range of growth was established, the effects of salinity on cell leakage were studied. The effects of salinity on the release of 260 nm and 280 nm absorbing material from washed cells is shown in Figure 2. In the 0.0 M to 0.1 M NaCl range, the optical densities were relatively constant and are thought to represent the maximum loss of cell integrity. As the salinity was increased from 0.1 M to about 0.3 M, the cellular integrity increased markedly as evidenced by OD at 260 and 280 nm. As the salinity was increased above 0.3 M NaCl, the amount of material released was relatively constant and close to what would be considered background for the system.

Figure 3 shows the effects of varying buffer salinity on the viability of suspended cells subjected to four hours incubation in the Warburg apparatus at 15 C. The results are expressed in terms of percent of viable cells remaining after four hours incubation relative

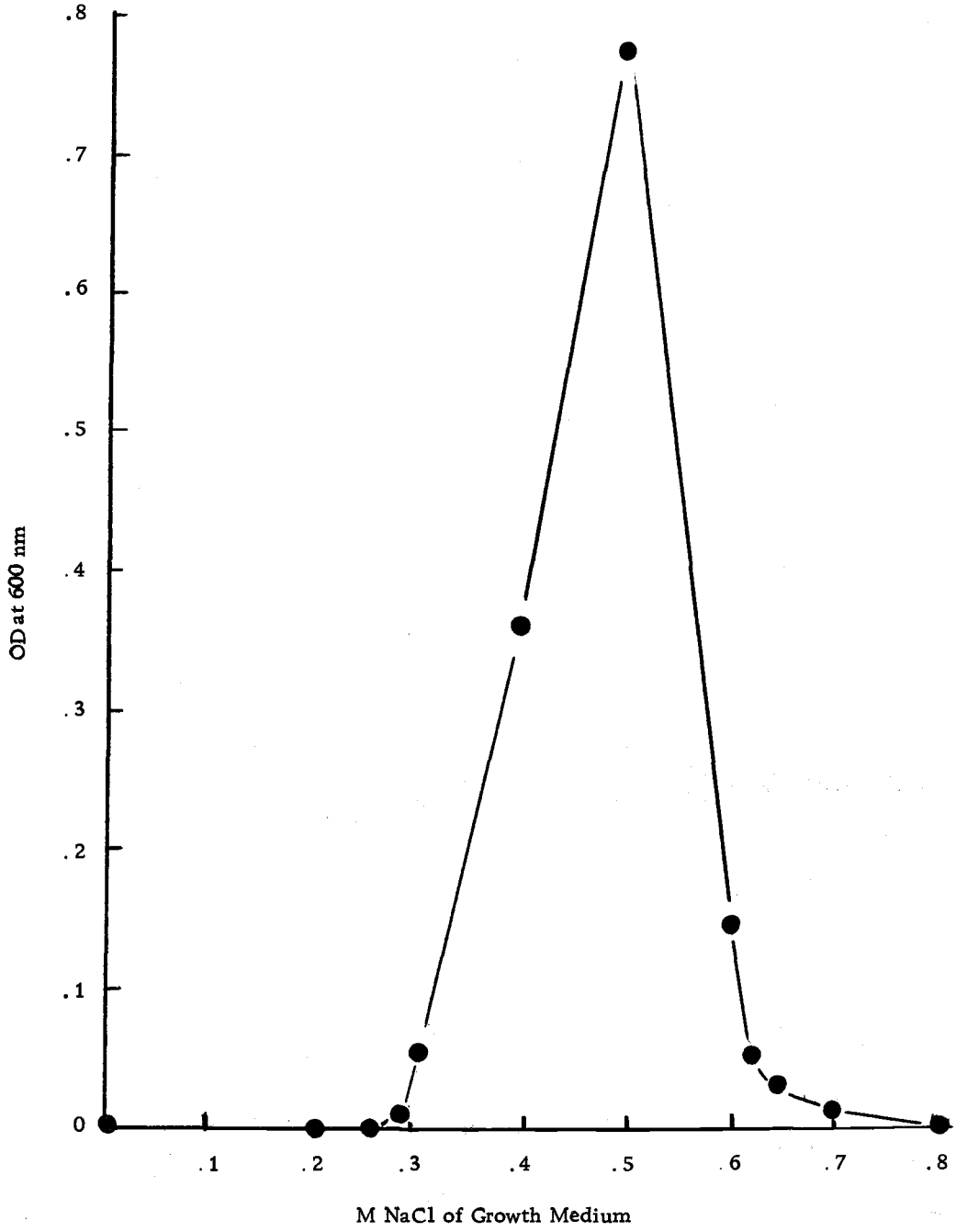


Figure 1. The effects of various salinities on the growth of V. marinus MP-1. Total growth was measured by observing the OD at 600 nm. The cells were incubated at 15 C for 63 hours in SOS medium.

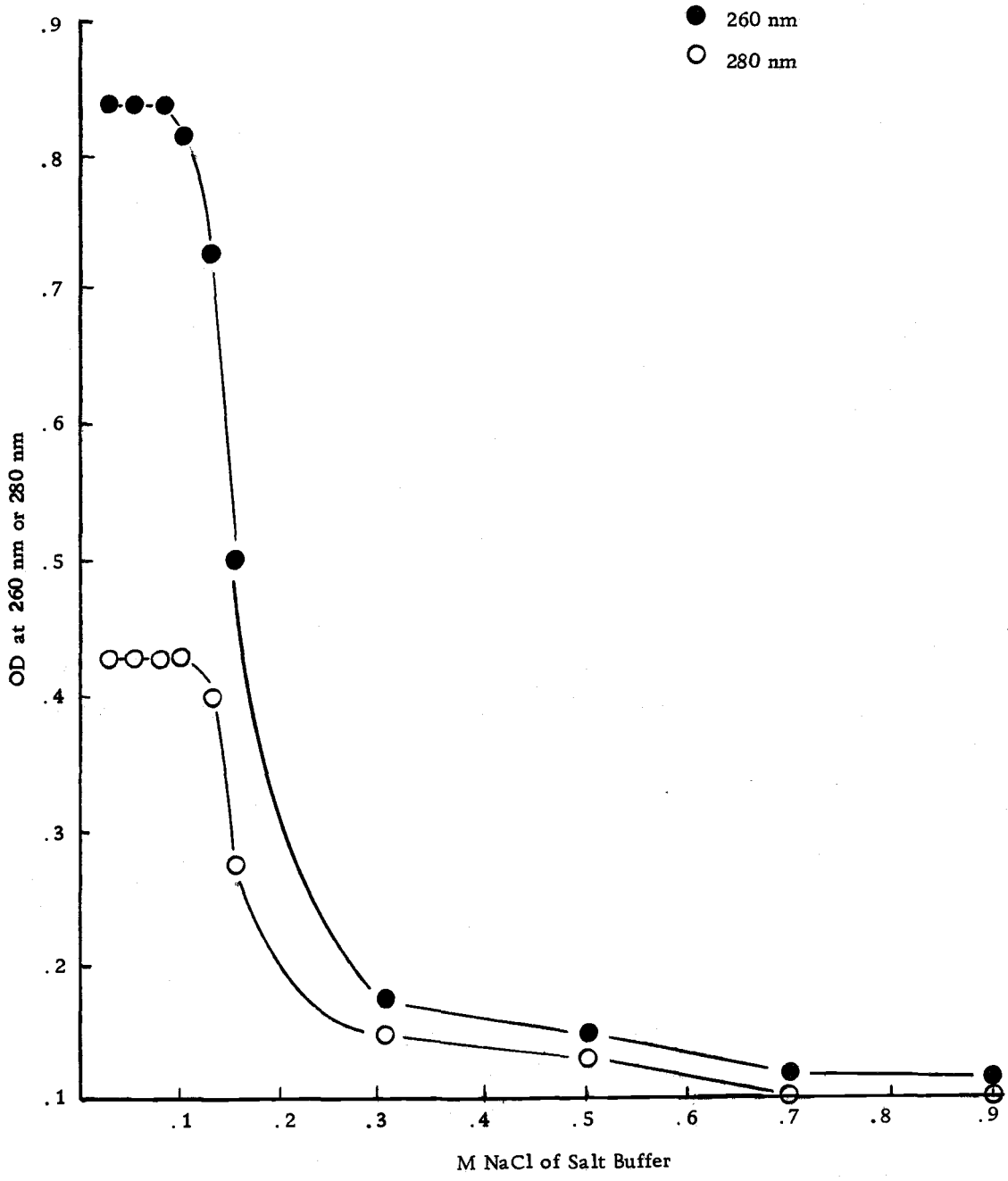


Figure 2. Release of 260 nm and 280 nm absorbing material from V. marinus MP-1 cells suspended in buffers (pH 7.3) containing various concentrations of NaCl. The cells were incubated at 15 C for 30 minutes with shaking.

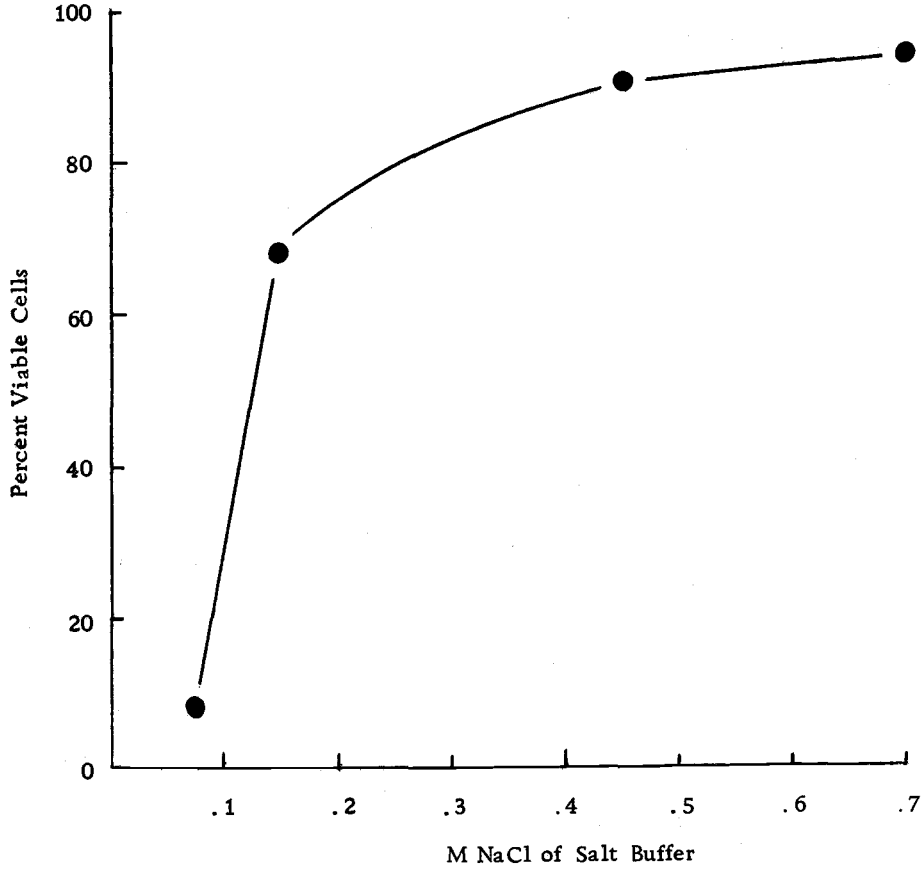


Figure 3. Viability of V. marinus MP-1 suspended in buffers (pH 7.3) containing various concentrations of NaCl. The cell suspension was incubated for 4 hours at 15 C in the buffer to be tested. Cell viability was determined by counting colonies on ASW agar plates prepared by the spread plate technique. The plates were incubated for 24 hours at 15 C before counting.

to the viable cells present at the same salinity at time zero. At 0.075 M NaCl, the lowest salinity tested, there was a marked reduction in the percent of viable cells present after the incubation period. It is quite possible that, at this low salinity, the reduction in viability might greatly alter the amount of glucose taken up and respired. After 0.15 M NaCl, there was a gradual increase in the percent of survivors until, at 0.70 M NaCl, there was no measurable loss.

Both the salinity and the pH of the salt buffer used to wash labeled cells altered the amount of activity associated with the cells. The effect of salinity on cell activity was shown in cells that had been labeled with UL ^{14}C glucose for 20 minutes at 0 C. These cells were washed with cold salt buffer containing 0.70, 0.15, or 0.00 M NaCl. The percent of activity recovered in the cell fraction was 31.8, 19.7, and 18.3% respectively.

Figure 4 shows the effects of altering the pH of the salt buffer wash on the amount of activity associated with the cells. As the pH was increased from 1.0 to 2.0, there was a gradual increase in activity. This rate of increase was greatly accelerated as the pH was increased to 3.0. There was a slight leveling off as the pH was increased from 3.0 to 5.0 with an increase in slope again in the region of 5.0 to 6.0. As the pH was increased above 6.0, the rate of increase became less. Using phase microscopy, the morphology of the cells suspended in the buffer at pH 2.0 were compared with

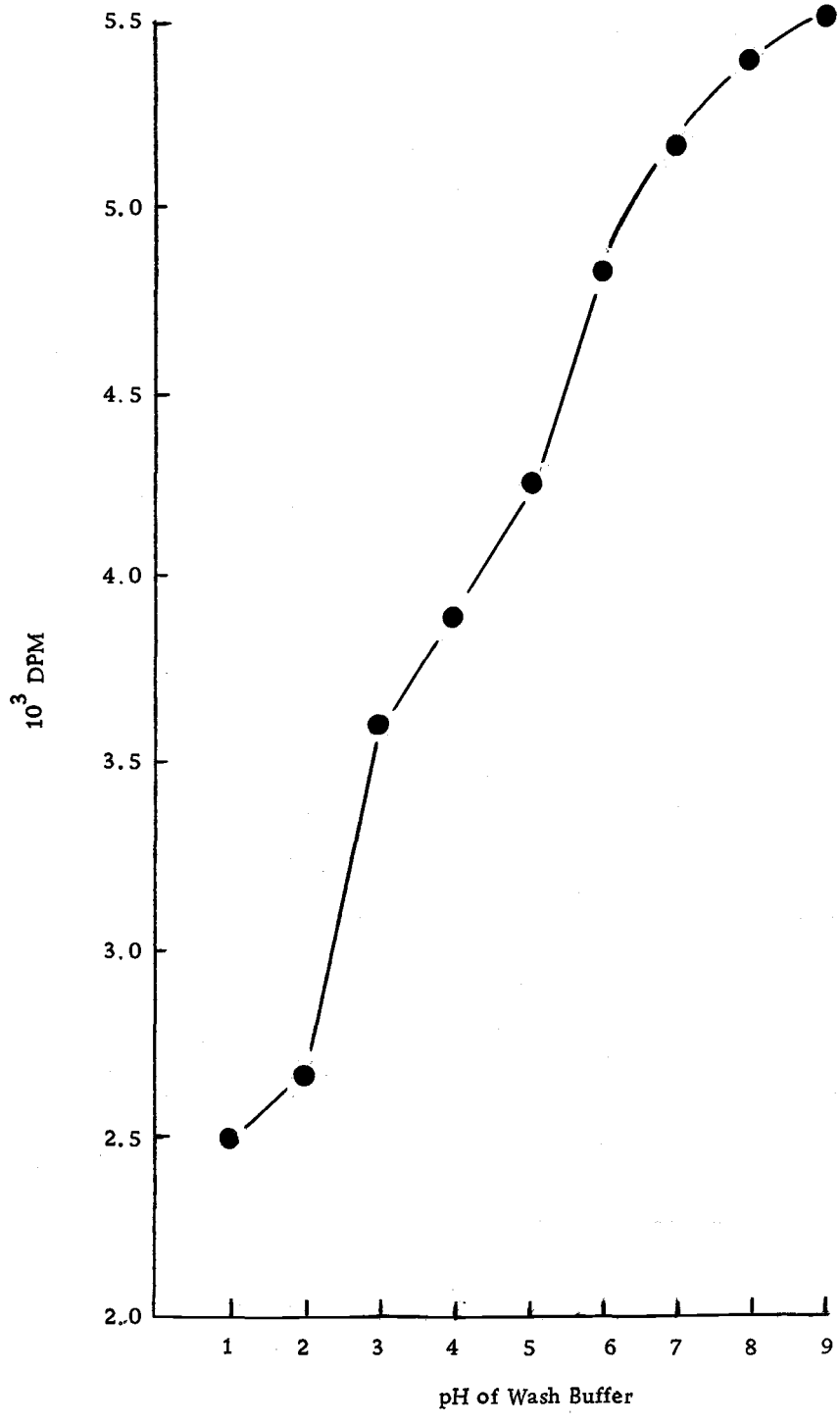


Figure 4. Effects of altering wash buffer (pH 7.3) on the radioactivity associated with the cells. The cells were exposed to UL ^{14}C glucose for 3 minutes at 0 C and then filtered and washed with 10 ml of 0.45 M salt buffer at various pH's.

those at pH 7.3; no noticeable changes were seen. The effects of decreasing pH on cell integrity were further checked by looking at the effects of reduced pH on turbidity and the appearance of 260 nm and 280 nm absorbing material. After being acidified for 10 minutes, the optical density (OD) of the cell suspension at 600 nm was 0.25 as opposed to 0.23 for the nonacidified cells. The OD of the acidified cell suspension supernatant fluid was also measured at 280 nm. The OD at this wavelength was 0.29 as opposed to 0.21 for nonacidified cells. This represented a 0.5% raise in terms of the total 280 nm absorbing material present relative to a sample containing lysed cells. Using the same samples, the increase in 260 nm absorbing material in the acidified sample was 4.3%.

The reliability of the assay methods used was measured by comparing the total activity added to the cell suspension at the beginning of the experiment in the form of utilizable substrate with the total percent recovered in the three main fractions after the incubation period (CO_2 collected, supernatant and cells). In order for these to consistently total 100%, the methods must be relatively accurate. The resulting totals over a range of glucose concentrations were consistent and within the experimental error normally tolerated in this type of experiment. An exception to this consistency was found in the percent activity recovered in the cells at 9.0×10^{-7} M glucose. This figure can be safely disregarded because the actual counts were

too close to the background figure to be significant. Because of the techniques used, the actual counts in the cell samples were ten times lower than that found in the other two samples.

Table 1. Effects of labeled glucose concentration on the percent of activity recovered in $^{14}CO_2$, cell, and supernatant fractions.

Glucose Concentration	$^{14}CO_2$	Cells	Supernatant	Total
4.45×10^{-4} M	7.6	57.5	29.3	94.4
4.45×10^{-5} M	7.4	63.2	23.8	94.4
9.00×10^{-6} M	7.0	67.2	24.4	98.6
9.00×10^{-7} M	7.2	135.0	28.2	166.3

The cells were incubated for a total of 7 hours. $^{14}CO_2$ samples were taken hourly. Supernatant and cell activity were assayed at the termination of the experiment. The cells were incubated with shaking at 15 C in the radiorespirometer.

Figure 5 shows the time required for the cell to lose radioactivity after it has been acidified to pH 2.0. The time between the addition of the acid and the point shown as time 0 on the graph was about 15 seconds. The initial loss of activity may have been even more rapid but, using this technique, 15 seconds was the minimum dead time possible. This dramatic initial drop was followed by a gradual decrease in activity after two minutes incubation.

The uptake of UL ^{14}C glucose at 0 C using 0.45 M NaCl salt buffer is shown in Figure 6. Under these conditions, the maximum

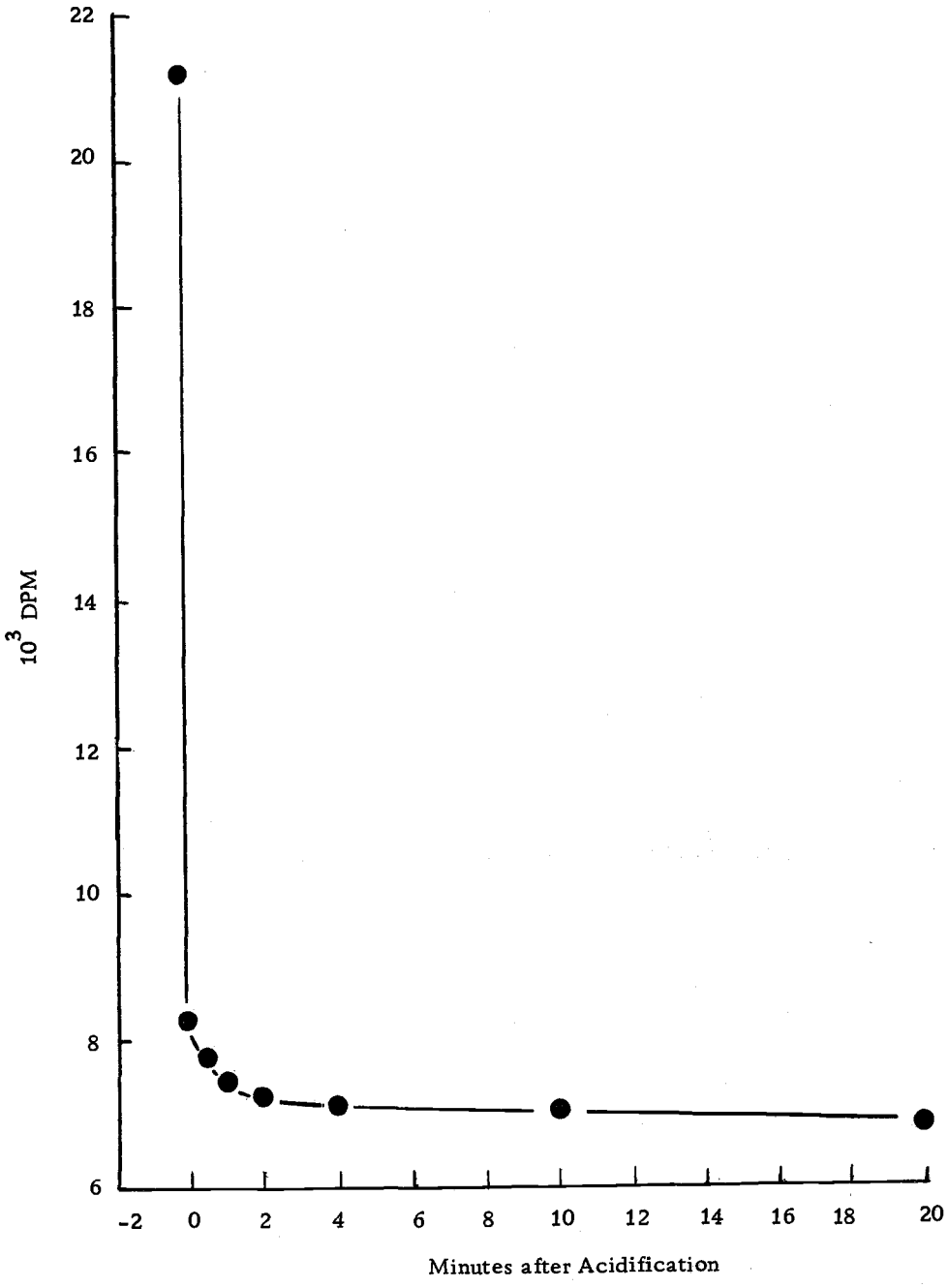


Figure 5. Loss of radioactivity with time from cells after acidification. The cells were incubated with UL ^{14}C glucose for 3 minutes at 0 C. The level of cell activity was assayed just prior to and at intervals after the cell suspension was acidified to pH 2.0.

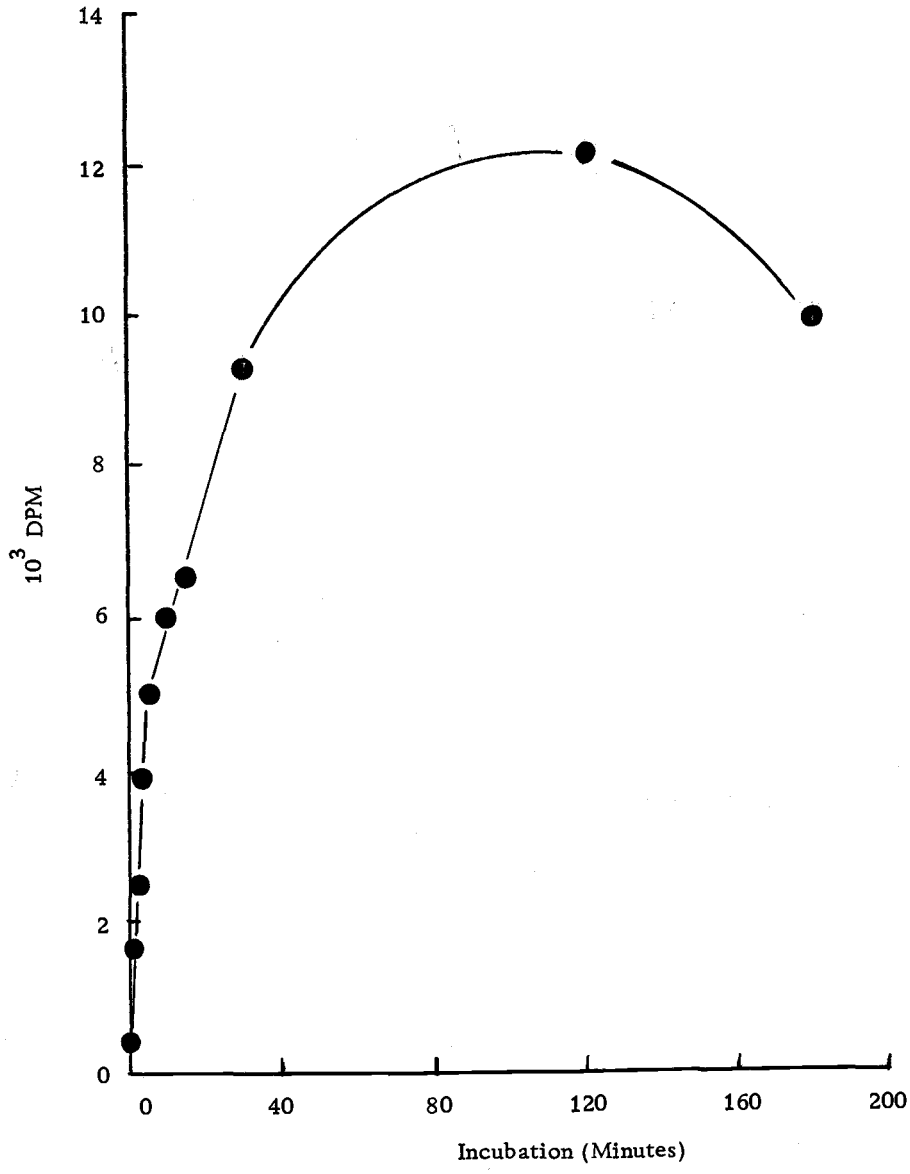


Figure 6. Uptake of UL ^{14}C glucose into cells incubated at 0 C. A 0.45 M NaCl salt buffer at pH 7.3 was used for the cell suspension. The cells were washed with a buffer at pH 2.0 before assaying for cell activity.

activity was seen in the cells after they had been incubated for 120 minutes. The initial uptake of glucose into the cells was rapid with one third of the total activity obtained after 1.5 minutes incubation and one half of the maximum activity obtained after 11 minutes incubation.

The effects of various cations and anions on the uptake of UL ^{14}C glucose are given in Figure 7. All of the sodium salts tested allowed about the same amount of uptake with the exception of iodide. The level of activity achieved with iodide, even though reduced, was high enough to be interpreted as active uptake. Of the cations tested (all chloride salts), sodium, and lithium allowed the greatest uptake. $MgCl_2$ showed the ability to allow active uptake but at a reduced level (about one half that for Na^+ and Li^+). The activity associated with K^+, Rb^+, and NH_4^+ was considered to be at the baseline for this system i.e., no active uptake present.

The effects of salinity in the form of NaCl on the uptake of UL ^{14}C glucose in ASW grown cells is shown in Figure 8. There was very little uptake shown in the 0.10 M to 0.15 M region but there was a drastic increase in cell activity as the salinity was increased from 0.15 M to 0.30 M. This was followed by a region with a gradual decrease in activity up to 0.9 M. As can be seen in Figure 1, the region of the most radical change was also the region of minimum growth salinity. This same type of pattern, only shifted slightly to

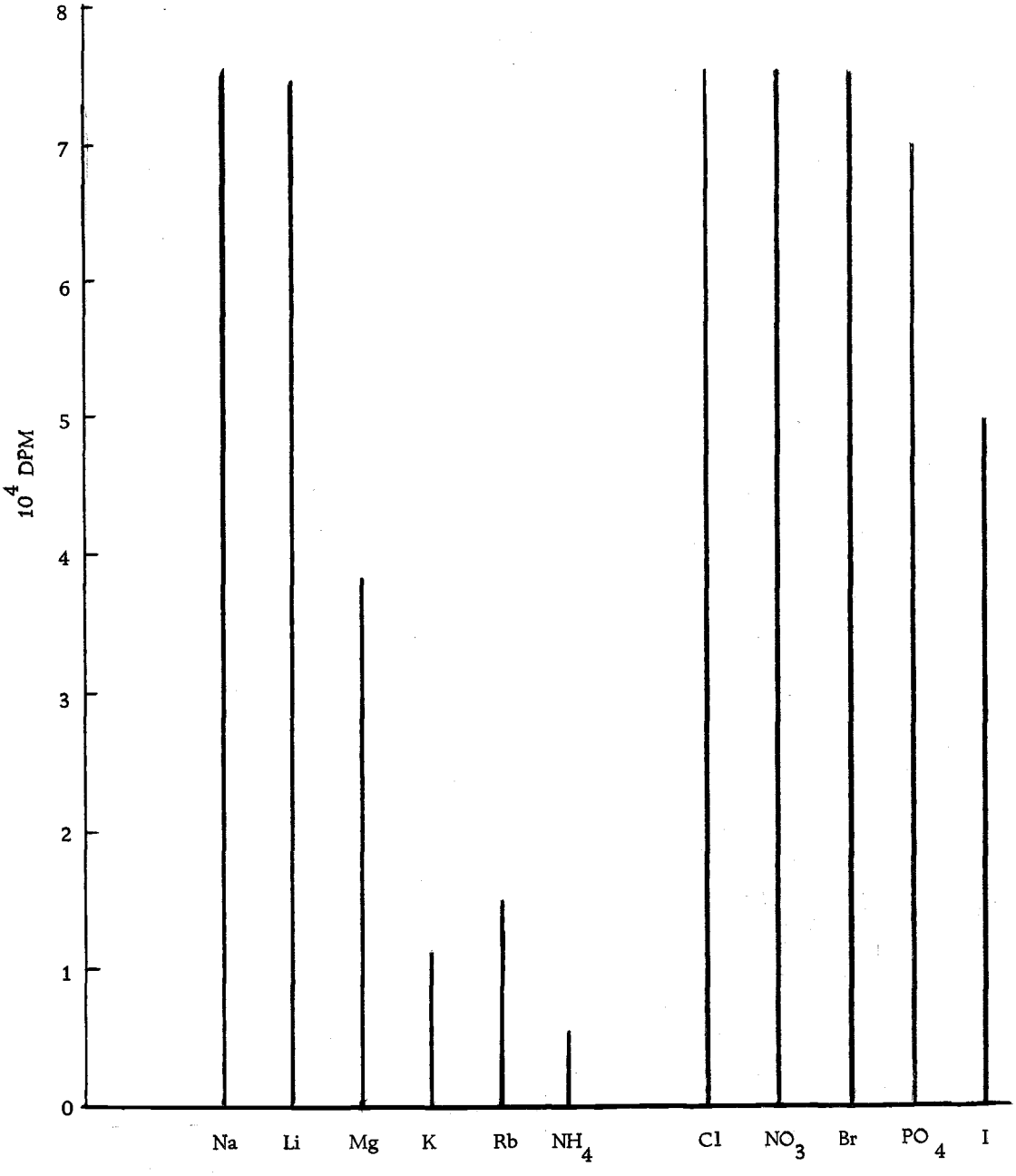


Figure 7. The effects of various cations and anions on UL ^{14}C glucose uptake by MP-1. Buffers containing various salts of sodium in 0.01 M sodium phosphate or chlorine salts in 0.01 M ammonium phosphate were used. The cell suspension was incubated for 20 minutes at 0 C. Both buffers had a pH of 7.3 and all samples were washed with acidified phosphate buffer (pH 2.0).

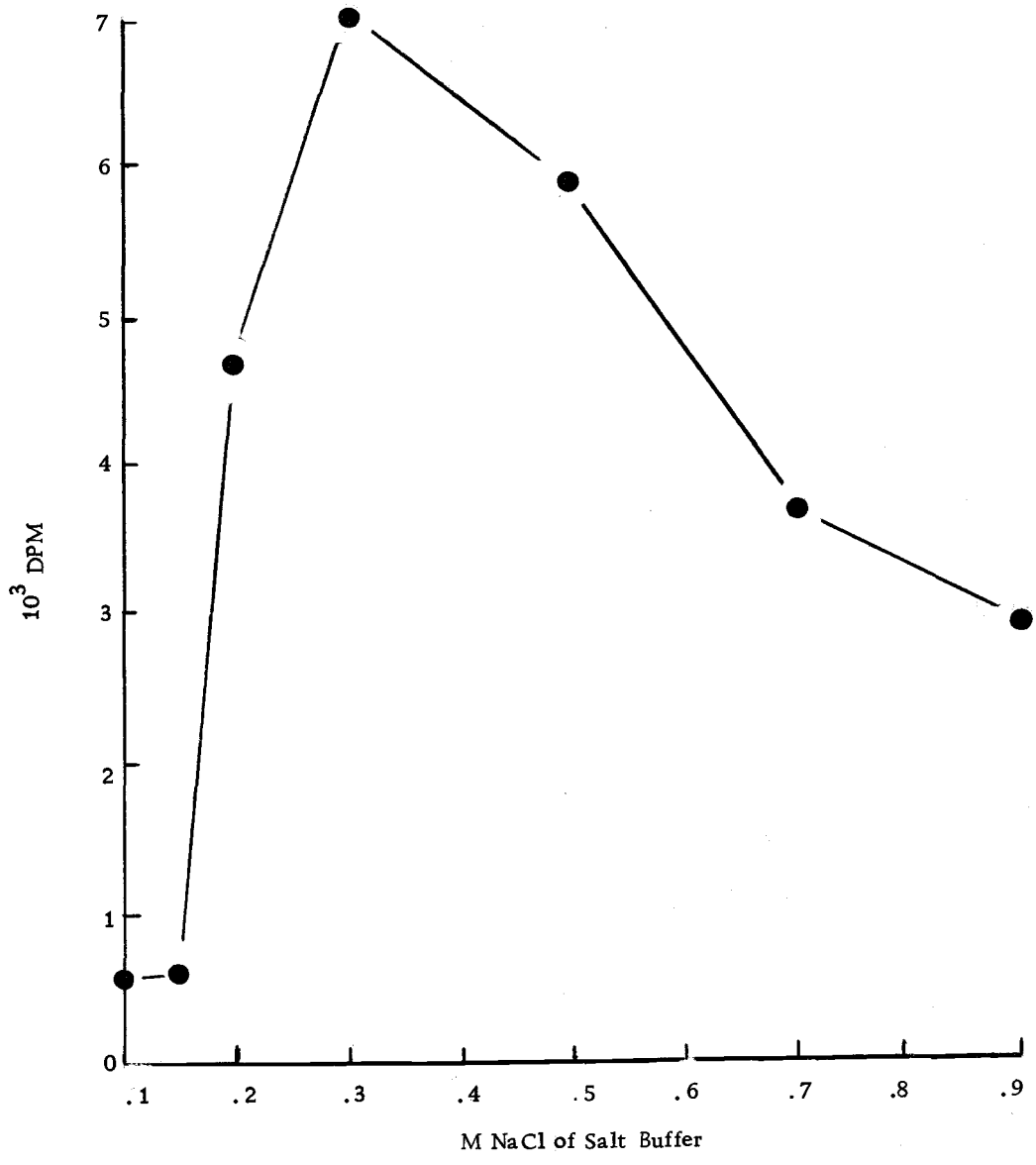


Figure 8. The effects of various NaCl concentrations on the uptake of UL ^{14}C glucose into the cells. ASW grown, stationary phase cells were suspended in 0.45 M salt buffer at pH 7.3 and incubated at 0 C for 20 minutes.

the left, was seen when the increase in salinity was generated by increased concentrations of $MgCl_2$ (Figure 9).

A series of studies was made in which the effects of salinity on the uptake and respiration patterns of specifically labeled glucose carbons were studied. The patterns generated by cell activity at various salinities are given in Figure 10. In all specifically labeled glucose carbons studied, there was a rapid increase in cell activity as the salinity was increased from 0.15 M to 0.30 M and a gradual decrease as the salinity was increased above that point. The cell activity associated with the first, second, and sixth carbons of glucose was very close to being the same for all salinities tested. In the case of the third carbon, however, the activity was significantly lower at all salinities except 0.9 M NaCl. In this same set of experiments, the amount of activity given off as CO_2 was also measured. Figure 11 shows the percent of activity recovered as CO_2 for each of the four labels tested over a range of salinities. Here, as was seen in the cell activities, there was a rapid change in the pattern in the 0.15 M to 0.30 M region. There was also a gradual change as the salinity was increased above that point. The level of activity from the first, second, and sixth carbons was quite similar; however, that of the third carbon was significantly higher. Another pattern was evident in log phase cells which was even more pronounced in stationary phase cells (Figure 11). This

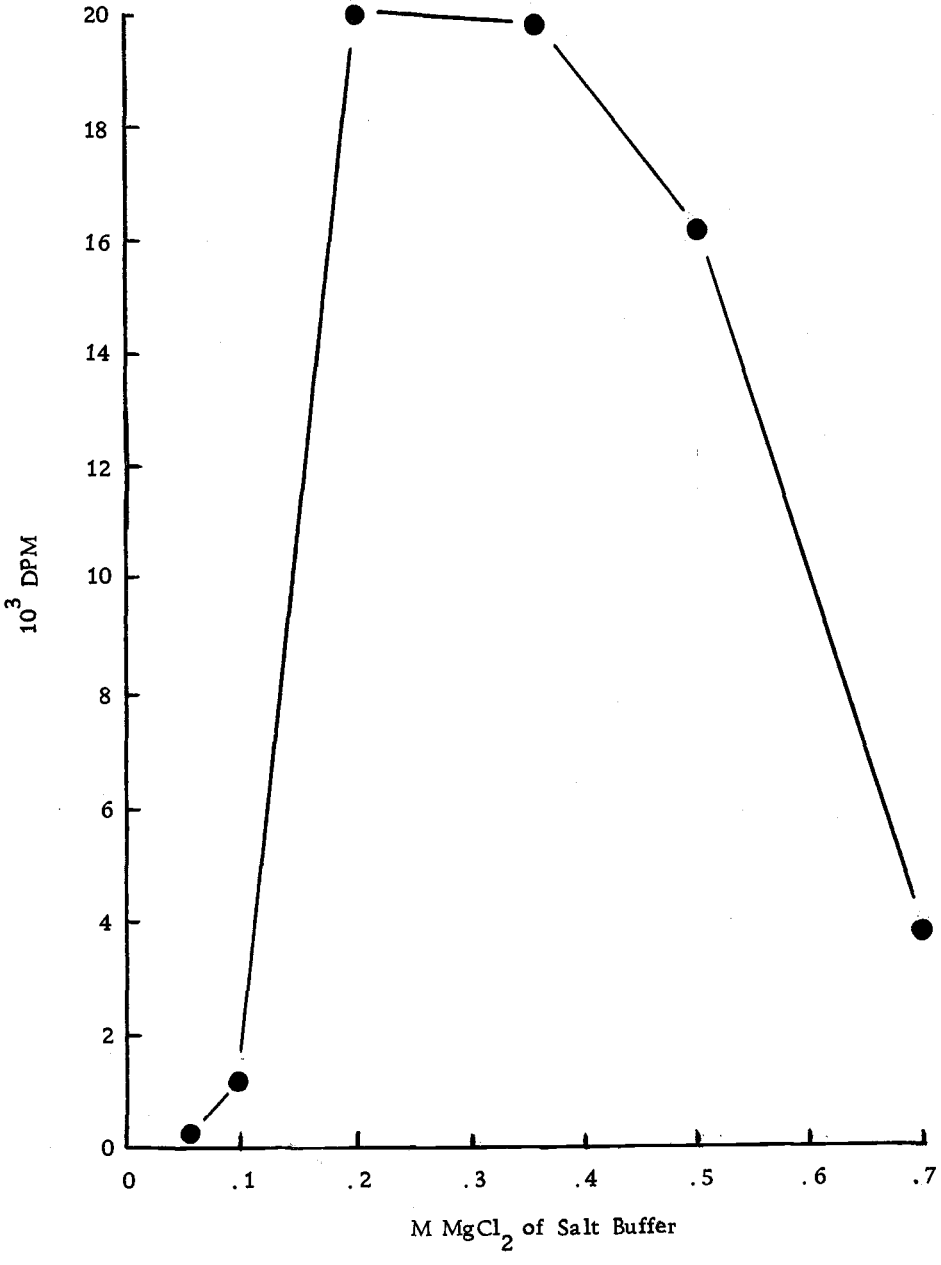


Figure 9. The effects of various $MgCl_2$ concentrations on the uptake of UL ^{14}C glucose into the cells. Cells suspended in 0.45 M salt buffer at pH 7.3 were incubated for 20 minutes at 0 C.

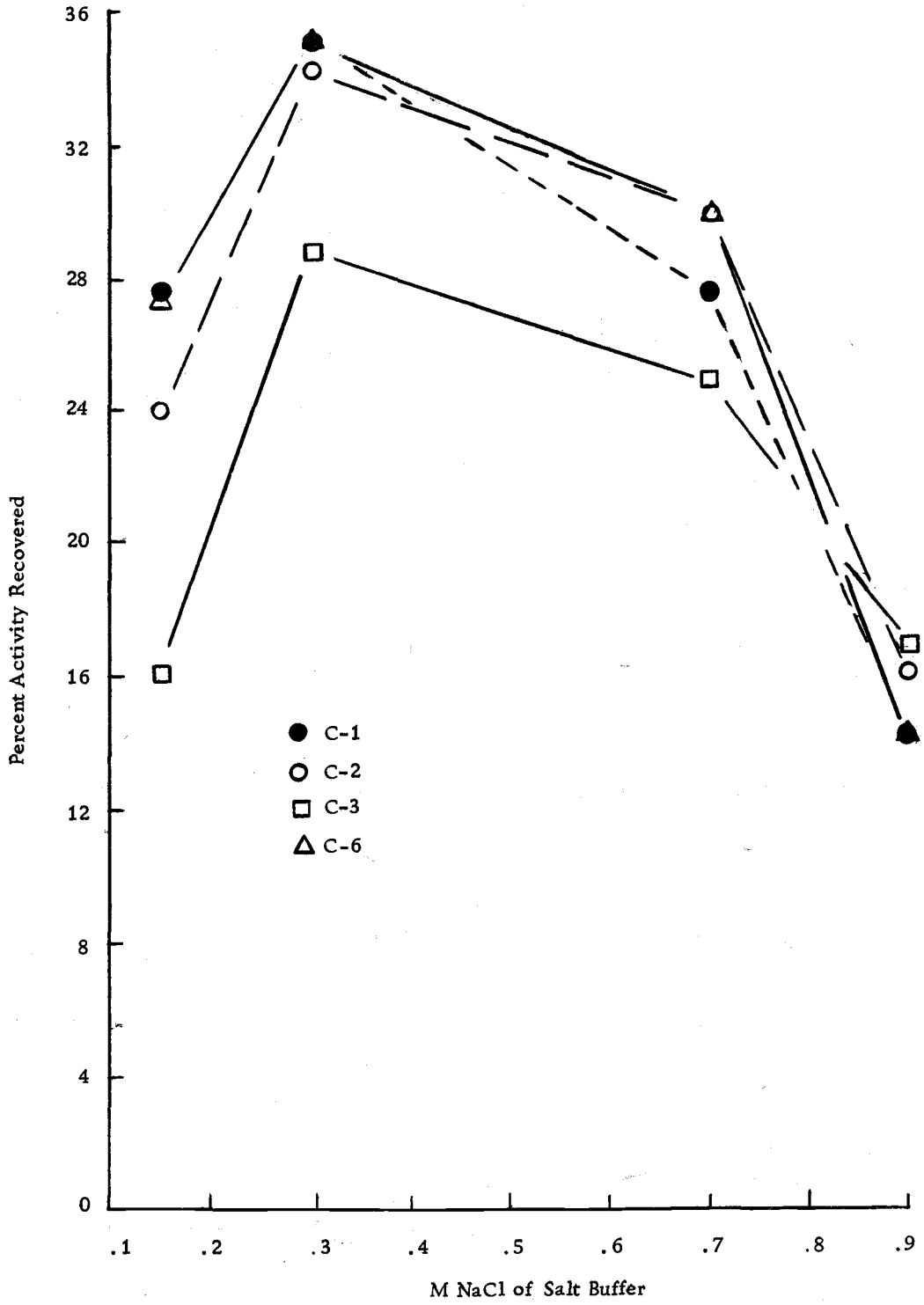


Figure 10. The effects of salinity on the cell activity associated with specifically labeled glucose. Cell suspensions were incubated for 3 hours at 15 C with shaking in the radiorespirometer. Log phase cells were used. The cells were suspended in SOS medium with no unlabeled glucose.

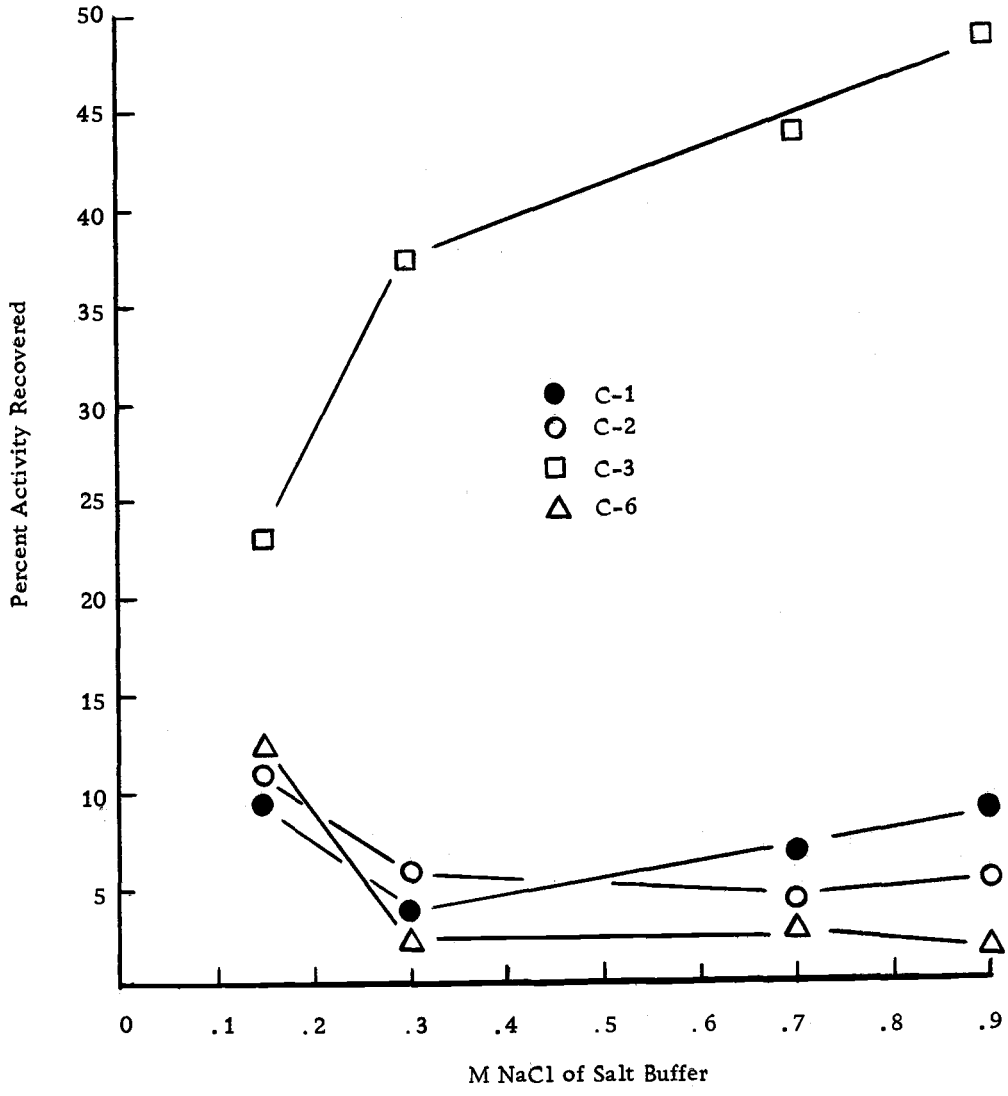


Figure 11. The effects of salinity on the $^{14}CO_2$ released from specifically labeled glucose carbons. The same cells and conditions were used as in the cell activity experiments described in Figure 10.

pattern involved a decrease in the ratio of CO_2 recovered from the sixth carbon relative to that recovered from the first as the salinity was increased from 0.3 M to 0.9 M NaCl. When this phenomenon was investigated in greater detail (Figure 12), the ratio of C_6/C_1 was roughly unity from 0.05 M to about 0.25 M NaCl. When the salinity was increased above that point, the C_6/C_1 ratio decreased until it was 0.2 at 1.0 M. This decrease was relatively constant both throughout and beyond the salinity range for growth. With the higher resolution and the wider range of this study, it became apparent that two other patterns existed. There was a rapid increase in the CO_2 activity seen as the salinity was increased from 0.05 M to 0.15 M NaCl. This seems to parallel the pattern of cell viability shown under the same conditions as given in Figure 3. There was also a pronounced decrease in the release of radioactive CO_2 as the salinity was increased from 0.15 M to 0.3 M NaCl.

Cell free extracts of MP-1 were analyzed for three enzymes of the EMP pathway, fructosediphosphate aldolase, hexosephosphate isomerase, and glyceraldehydephosphate dehydrogenase. All enzymes showed significant specific activities of 2.3, 0.65, and 0.96 respectively.

The release of CO_2 with time from the first and sixth carbons is given in Figures 13 and 14. In this case, only four representative salinities were studied. There was an apparent shift in the relative

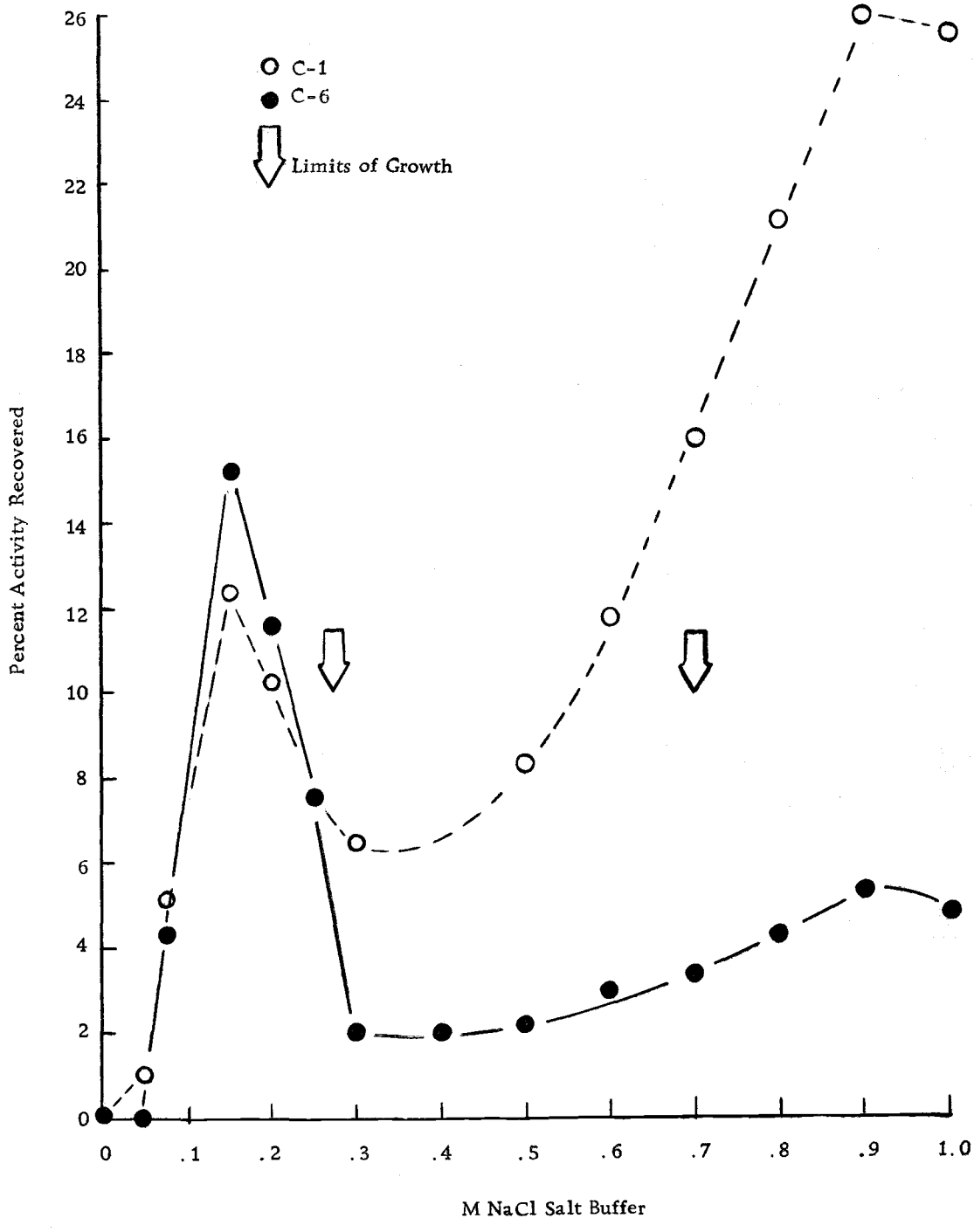


Figure 12. The effects of salinity on the $^{14}CO_2$ released from the first and sixth carbons of glucose. Stationary phase cells were used. The cells were incubated for 2 hours in the radiorespirometer at 15 C.

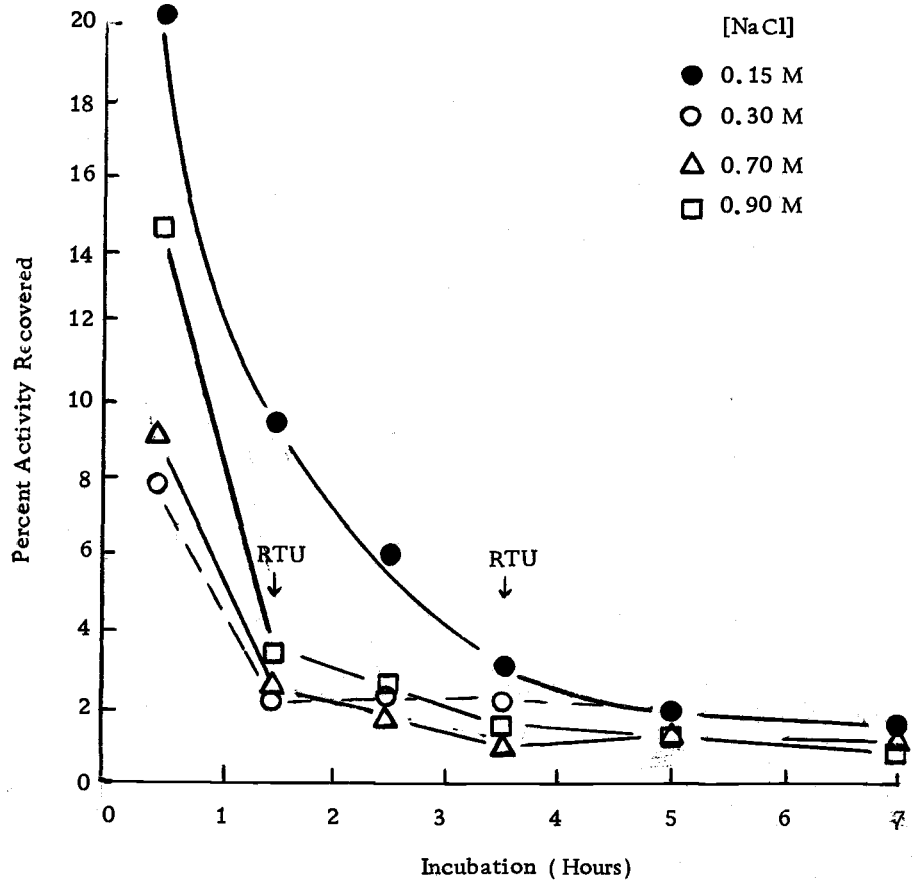


Figure 13. $^{14}CO_2$ evolution with time from glucose labeled on the first carbon at four salinities. Stationary phase cells were used. The cells were incubated for a total of 8 hours with samples being taken periodically. The results are given as the percent recovered for the time span observed; represented as a percentage for the average of the sampling time. The relative time unit (RTU) is as defined by Wang et al. (1958).

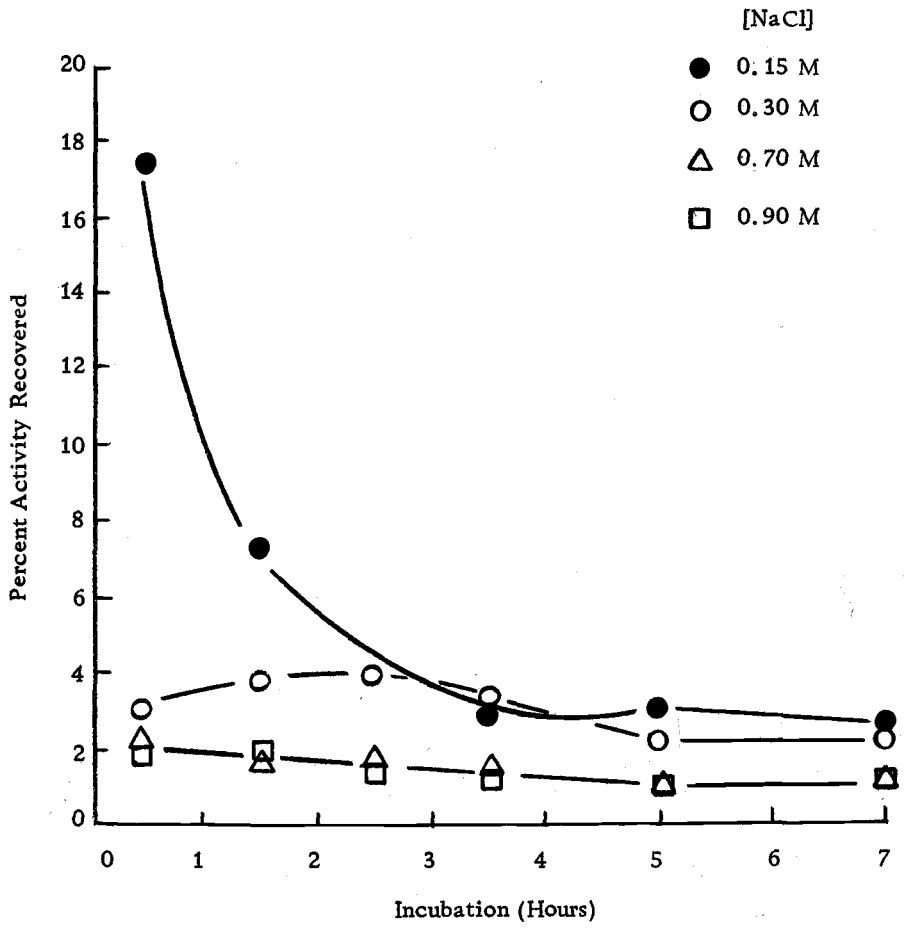


Figure 14. $^{14}CO_2$ evolution with time from ^{14}C glucose labeled on the sixth carbon. The conditions were the same as those described in Figure 13.

time unit (RTU) as defined by Wang et al. (1958) when the salinity was decreased below 0.30 M NaCl (evident only in C-1). This same shift was seen when the salinity increase was caused by the addition of LiCl instead of NaCl (Figure 15). According to Wang's theory, this should reflect a reduced uptake and/or respiration rate at the lower salinities. It should also be noted that, for the sixth carbon there was no large initial release of CO_2 when the salinities were 0.3 M NaCl or above. This was not the case with the first carbon.

The effects of using other salts to cause the shift in the C_6/C_1 ratio were also studied. Figure 15 shows the effects of changing salinities using LiCl. The resulting patterns closely resemble those for NaCl as shown in Figures 13 and 14. The same type of pattern was observed when NaBr or $NaNO_3$ was used in the same way.

The total amount of glucose taken up by the cells at various salinities using specific labels was studied. Patterns such as those shown in Figure 9 were observed. The total uptake in this case was the sum of the activity collected at CO_2 and the activity associated with the cells. At 0.15 M NaCl, the amount of activity taken up by the cells in the form of all carbons tested was about equal. This was also the case for all carbons except C-3 at the other salinities tested. There was, however, a much larger apparent uptake of the third carbon at the salinities above 0.15 M NaCl. Most of the observed difference was generated at salinities above 0.3 M. The apparent

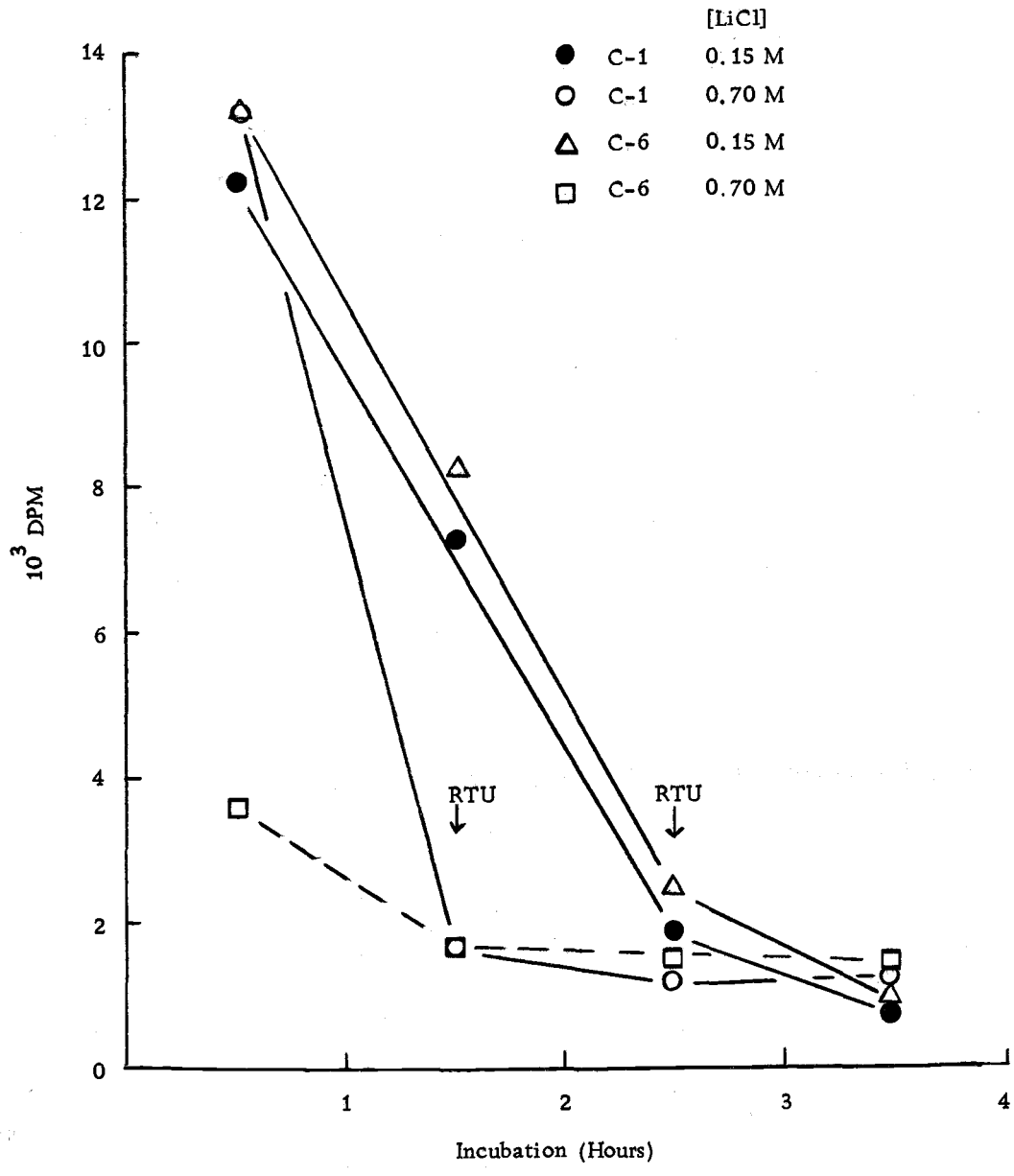


Figure 15. $^{14}CO_2$ evolution with time from ^{14}C glucose labeled on the first and sixth positions at two salinities using LiCl instead of NaCl.

uptake of the other carbons decreased as the salinity increased. The resulting differential is expressed in Figure 16 as the difference between the uptake of the third and the average uptake of the other carbons tested.

When supernatant fluid of cells grown on high concentrations of UL ^{14}C glucose was analyzed, no activity could be found associated with the volatile neutral and volatile acid fractions. Under the conditions of this study, 44.3% of the activity was associated with the cells, 21.8% of the activity was recovered as CO_2 and 33.9% of the activity remained in the supernatant.

The percent of activity recovered as CO_2 from the first and sixth carbons at two extreme salinities was studied at various temperatures. The temperature range studied was from 5 C (near the temperature at which the organism was originally isolated) to 25 C (5 C above its maximum growth temperature). As can be seen in Table 2, the ratio of the CO_2 released from C_6/C_1 was constant over the entire temperature range. With the exception of the study made at 25 C and at 0.15 M NaCl, all of the total recoveries were relatively stable.

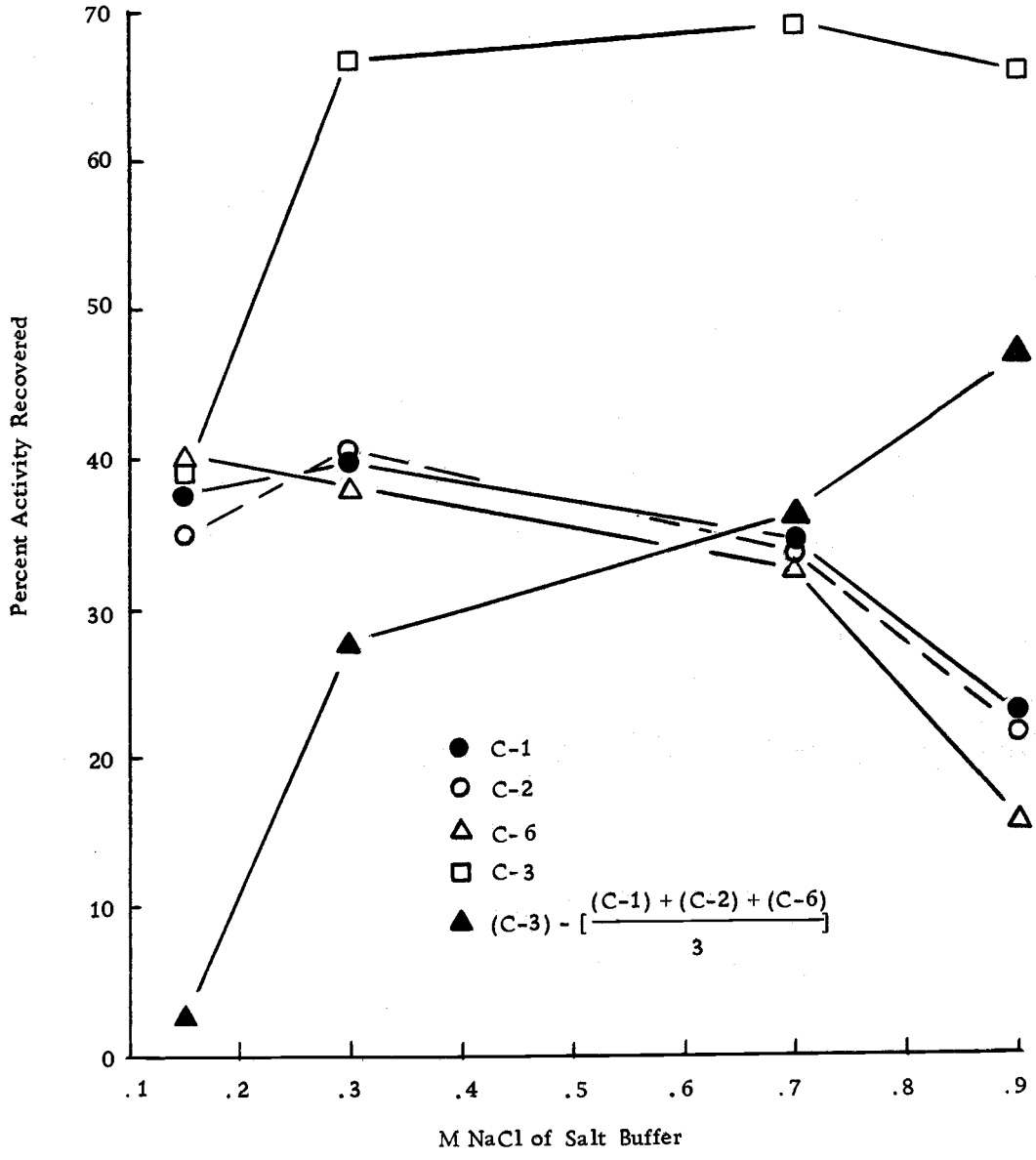


Figure 16. The effects of salinity on total substrate uptake using specifically labeled glucose. The sum of activities recovered as $^{14}CO_2$ and cell activity as given in Figures 10 and 11.

Table 2. Effects of temperature on the shift in C_6/C_1 ratios.

Temperature of Incubation	NaCl Concentration	Percent Activity Recovered		Ratio C_6/C_1
		Carbon 6	Carbon 1	
5 C	0.15 M	16.1	17.0	0.94
15 C	0.15 M	10.8	13.8	0.95
20 C	0.15 M	17.3	18.4	0.94
25 C	0.15 M	7.3	7.1	1.03
5 C	0.70 M	6.0	17.6	0.56
15 C	0.70 M	4.1	14.1	0.34
20 C	0.70 M	6.7	11.8	0.56
25 C	0.70 M	9.3	13.4	0.69

Cells were suspended in either 0.15 M or 0.70 M SOS medium. ^{14}C glucose was labeled on either the first or sixth carbon. The $^{14}CO_2$ was collected for 4 hours but that determined as the percent recovered was the sum collected over the relative time unit (RTU) as defined by Wang et al. (1958).

DISCUSSION

While studying the effects of salinity on the growth of four marine bacteria, Stanley and Morita (1968) observed that the salinity of the growth medium can affect the maximum growth temperature in these organisms. When incubated in a nondefined medium, MP-1 had a minimum growth salinity of approximately 0.2 M NaCl and a maximum growth salinity of 0.7 M. When the same organism was grown in a glucose salts medium (SOS), the maximum growth salinity was the same but there was an increase in the minimum salinity to 0.275 M (Figure 1). The apparent shift in the minimum salinity for growth under more demanding growth conditions may reflect a requirement for salt in one of the metabolic pathways. Under less demanding substrate conditions, as one finds in the nondefined medium, certain critical intermediates may be supplied which otherwise would be synthesized from glucose. Under these conditions, if there was a salt requiring enzyme involved in its synthesis, the organism could require a higher salinity.

With the exception of the salinity-growth study, all the studies were done on washed cells suspended in a salt buffer for varying lengths of time. It was important to determine the effects of salinity on the viability of the cells suspended in this buffer. A study of salinity effects on leakage (Figure 2) showed that a great deal of

260 nm and 280 nm absorbing material was lost in the 0.1 M to 0.3 M range with the greatest loss in the 0.1 M to 0.2 M range. It is reasonable to assume that under these conditions at least part of this leakage reflected cell lysis. As can be seen in Figure 3, there was a significant reduction in the number of viable cells, after incubation for four hours, as the salinity was reduced from 0.7 M NaCl. The reduction in apparent viability was particularly significant below 0.15 M. Since most of the studies were made at salinities of 0.15 M and above, only this region is of concern. The 30% drop in apparent viability at 0.15 M NaCl would theoretically be a problem if the system was saturated i.e., the substrate concentration would be high enough that the number of cells present would dictate the rate of uptake and respiration. Preliminary studies showed that at the substrate concentrations used, even a ten fold dilution did not appreciably affect the rate of uptake. Likewise, a study of the effects of varying substrate concentration showed that the system was not saturated (Table 1).

In most radioactive tracer experiments, it is very important to be able to account for all of the activity at the end of the experiment. In some of the pilot studies, a great deal of variability was found in the total activity recovered. Detailed studies of the three main fractions most often assayed (CO_2, supernatant and cells) showed that the variability was associated with the cell fraction. Since standard methods for the determination of radioactivity in

cells was followed, there was some reason for concern. The effects of varying either the pH or the salinity of the buffer used to wash the cells were studied. Both of these variables affected the apparent activity associated with the cells. Since salinity was one of the variables to be studied, this posed an important problem. It has been demonstrated in this laboratory that MP-1 cells previously exposed to radioactive uricil, rapidly lost much of their activity when washed with an acid solution (F. J. Hanus, personal communication). It was also observed that after the initial loss in activity, there was no further significant loss even after an extended period of time. It was also known that uptake studies done using a technique described by Harrison, Wright and Morita (1971) gave reproducible results over a wide range of conditions. In this technique, the cell suspension was acidified prior to assaying.

Keeping these facts in mind, it seemed quite possible that much of the labeled substrate might be loosely bound to the cell and that this may be removed, to varying degrees depending on the physical conditions, during the washing process. In order to stabilize the system, it seemed desirable to strip the cells of the loosely bound substrate before assaying cell activity.

It can be shown that the amount of activity associated with the cell is directly related to the pH of the wash buffer (Figure 4). It is not known what significance, if any, the slightly different slopes

have in the graph. One thing seems fairly certain, at pH 2.0 the amount of activity being lost is near a leveling-off point. For this reason, a buffer of this pH was tested. When this acid wash was used in conjunction with the other assay techniques, good reproducibility and total accountability was achieved over a wide range of substrate concentrations (Table 1).

It might be argued that what is seen here is not the removal of substrate from the outside of the cell but a leakage of materials from within. This possibility has been ruled out. There was no significant release of 280 nm absorbing material and very little release of 260 nm material when the cells were acidified. There was also very little change in cell suspension turbidity and no morphological changes noted in acidified cells. If the 50% reduction in radioactivity observed at pH 2.0 was due only to leakage, it seems reasonable to assume that at least some change would have occurred in one of these parameters. Also if this release was due to leakage, it would seem reasonable that the loss of activity would take place over an extended period of time. The rate at which activity was lost from the cells after acidification was studied. The results showed that the majority of the activity was lost in the first 15 seconds (Figure 5). There was little loss after that period. One would predict from this that as the cells are incubated, less and less of the activity associated with the cells, in this easily removable form, would be present. This is

what has been observed.

It is also reasonable to assume that attachment does not require living or even whole cells. When the debris from cells treated by sonication was exposed to labeled glucose, there was attachment of glucose which was removable by acid treatment. It then follows that the presence of cell debris as well as whole but inactive cells would have the capacity to bind substrate; removing it from the suspending medium. This would affect the apparent total percent of activity taken up and respired by the population.

Very little is known of the type of bond involved in the attachment of glucose to the cell. This attachment was completely inhibited by a very low concentration of formaldehyde in MP-1. Once the glucose had become attached, however, the formaldehyde did not affect the level of cell activity.

The fact that both pH and salinity can affect glucose binding may give some clue as to the type of interaction involved. This phenomenon might be explained in terms of polyelectrolyte theory (Kotin, 1963). According to this theory, the surface of the cell would act as a polyanion. The negative charges would be subject to shielding by the cations of a salt. Thus the salinity could affect the degree of charge shielding and in turn, the degree of substrate binding. This theory might also explain why the attached substrate is so rapidly lost when the cells are acidified (Figure 5). Such a

mechanism was used by Buckmire and MacLeod (1965) to explain membrane integrity changes and resulting lysis at low salinity. Regardless of the mechanism involved, the rate of binding and uptake is relatively rapid (Figure 6).

While studying the effects of salinity on growth, Stanley and Morita (1968) reported that certain cations would allow growth in MP-1 and that others would not. This might be due primarily to a cation requirement for the uptake of nutrients. The effect of varying the cation and anion content of the suspending medium on the uptake of UL ^{14}C glucose was studied (Figure 7). Of the anions studied, only one, iodide, showed a reduced ability to facilitate glucose uptake. Of the cations tested, only Na^+, Li^+, and Mg^{++} allowed the active uptake of glucose. Mg^{++} was apparently not as effective as Na^+ and Li^+ in promoting the uptake of glucose. Even though the molarity of Mg^{++} used in this experiment was one half that of the other cations studied, this should not have significantly affected the results (Figure 9). The levels of activity seen for K^+, Rb^+, and NH_4^+ are considered to be background for the system. This is the same pattern that Stanley and Morita (1968) demonstrated in their growth experiments. It may be more than a coincidence that this same type of pattern has been observed in several monovalent cation-activated enzyme systems (Evans and Sorger, 1966). They observed that in most systems studied, K^+, Rb^+, and NH_4^+ are activators and Na^+ and Li^+ are not.

Some enzymes, notably those isolated from holophilic organisms, are activated by Na^+ and Li^+ but not by K^+, Rb^+, nor NH_4^+.

The requirement for a specific cation for the uptake of substrate has also been observed by others. MacLeod et al. (1958) showed that Na^+ was required for substrate transport by a marine bacterium. Wong, Thompson and MacLeod (1969) showed that Na^+ may be required for both uptake and retention of substrate. Specific cation requirements for the uptake of substrate and related phenomena have also been reported by others (Stevenson, 1966; Frank, and Hopkins, 1969; DeVoe et al., 1970; Drapeau, Matula and MacLeod, 1966; and Payne, 1960).

As has been shown in Figure 1, salinity is a very important factor in the growth of this organism. It is possible that some salinity effects may be related to substrate uptake and shifts in the major pathways of nutrient catabolism. Glucose was chosen as the substrate to be tested for two reasons. First, glucose can be used as a sole carbon-energy source by this organism. Secondly, well established methods using specifically labeled glucose, are available for the determination of the types and relative participation of main glucose catabolic pathways (Wang et al., 1958).

One of the first parameters to be analyzed was the effect of salinity on the activity associated with cells i. e., how much activity was retained by the cells after a set incubation time. Figure 8 shows

the resulting pattern. If one superimposes this pattern over the salinity versus growth curve (Figure 1), one is struck by several obvious differences. The latter curve is relatively symmetrical about an optimum and the former is not. In the region of minimum growth salinity, there is a rapid change in cell activity with increasing salinity, yet at the maximum growth salinity, there is no rapid change. It would appear from these data, that as far as the uptake and retention of glucose is concerned, the region of minimum growth salinity is more critical than the maximum.

In order to determine whether or not this pattern was specific for the sodium ion, a salinity profile was done using $MgCl_2$. As can be seen in Figure 9, the same type of pattern was generated only shifted to the left. This same type of pattern was reported by Kelly and Brock (1969) when the effects of salinity on the uptake of thiamine were studied.

To determine the major pathways of glucose catabolism and their relative importance, a series of studies was initiated in which the effects of salinity on the patterns of respiration and cell activity associated with specific carbons of glucose were studied. Figure 10 shows the patterns associated with cell activity. The same general patterns were seen that were observed when UL ^{14}C glucose was used. One feature of this study which is of particular interest is the difference between the cell activity associated with the first,

second, and sixth carbons and that found for the third. If all of these carbons were treated in the same manner by the cell, one would expect that they would all show the same pattern. The possible significance of this difference will be discussed later.

A look at the respiration patterns for each of the carbons studied show some important trends (Figure 11). Here, as in the cell activity profiles, there is a rapid change in pattern as the salinity is increased through the area of the minimum growth salinity while relatively little change is associated with the increase through the region of maximum growth salinity. Here again, there is a difference in the level and pattern between the third and the first, second, and sixth carbons. There is also another trend shown which is of potential importance. As the salinity is increased from 0.3 M to 0.9 M, there is a decrease in the ratio of CO_2 released from the sixth and the first carbons (C_6/C_1 ratio). Another factor which is of theoretical importance is the relative level of CO_2 evolved from each carbon.

In order to obtain a better picture of the first and sixth carbon respiration patterns, a high resolution study was made using stationary phase cells. Figure 12 shows the results of that study. The same patterns that have been seen before were observed in a more clearly defined form. The pattern can be broken down into at least three main phases. The first phase from 0.0 M to 0.15 M NaCl shows a rapid increase in respiration as the salinity of the suspending

buffer is increased. It should be noted that the level of CO_2 from each of these carbons is roughly equal until the minimum salinity of growth is reached. The second phase from 0.15 M to 0.3 M shows a sharp decrease in the amount of CO_2 released from both carbons. The third phase from 0.3 M to 1.0 M NaCl, shows a gradual increase in the CO_2 from the first carbon but a fairly constant release from the sixth carbon as the salinity is increased. The theoretical significance of these three phases will be discussed later.

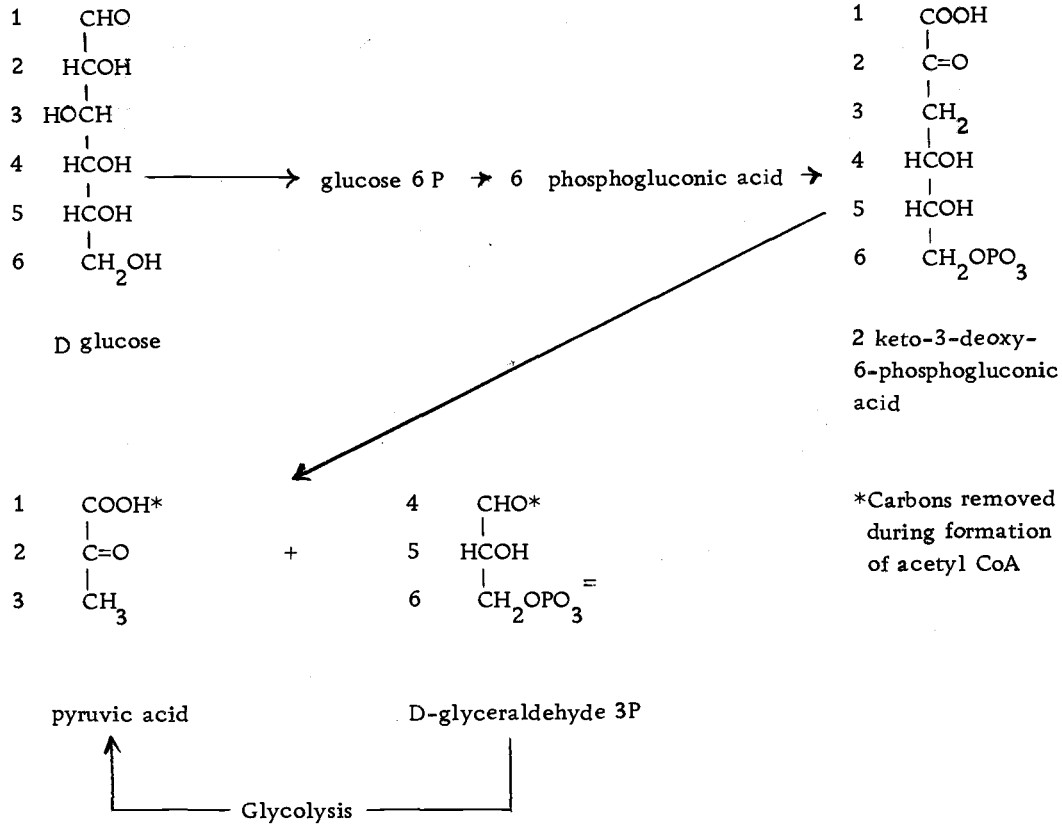
It seems possible that the length of the sampling time coupled with a shift in the RTU as defined by Wang et al. (1956) and Stern, Wang and Gilmour (1959) might have generated at least part of the pattern seen below 0.3 M NaCl. In order to check out this possibility, the rate of CO_2 evolution from both of these carbons was studied at four different representative salinities (Figures 13 and 14). As one can see in Figure 13, there was a shift in the RTU at the lowest salinity tested. This shift is even more dramatically shown in another study in which LiCl was used (Figure 15). Even though it is felt that this shift is significant, it is not sufficient to cause the pattern seen in Figures 11 and 12. As a matter of fact, this shift would, if anything, tend to enhance the trends. It is also important to note that the shift in the RTU affected both carbons equally; therefore, it would not affect the ratio of C_6 to C_1 in the form of $^{14}CO_2$.

According to Wang et al. (1956) and others (Katz and Wood,

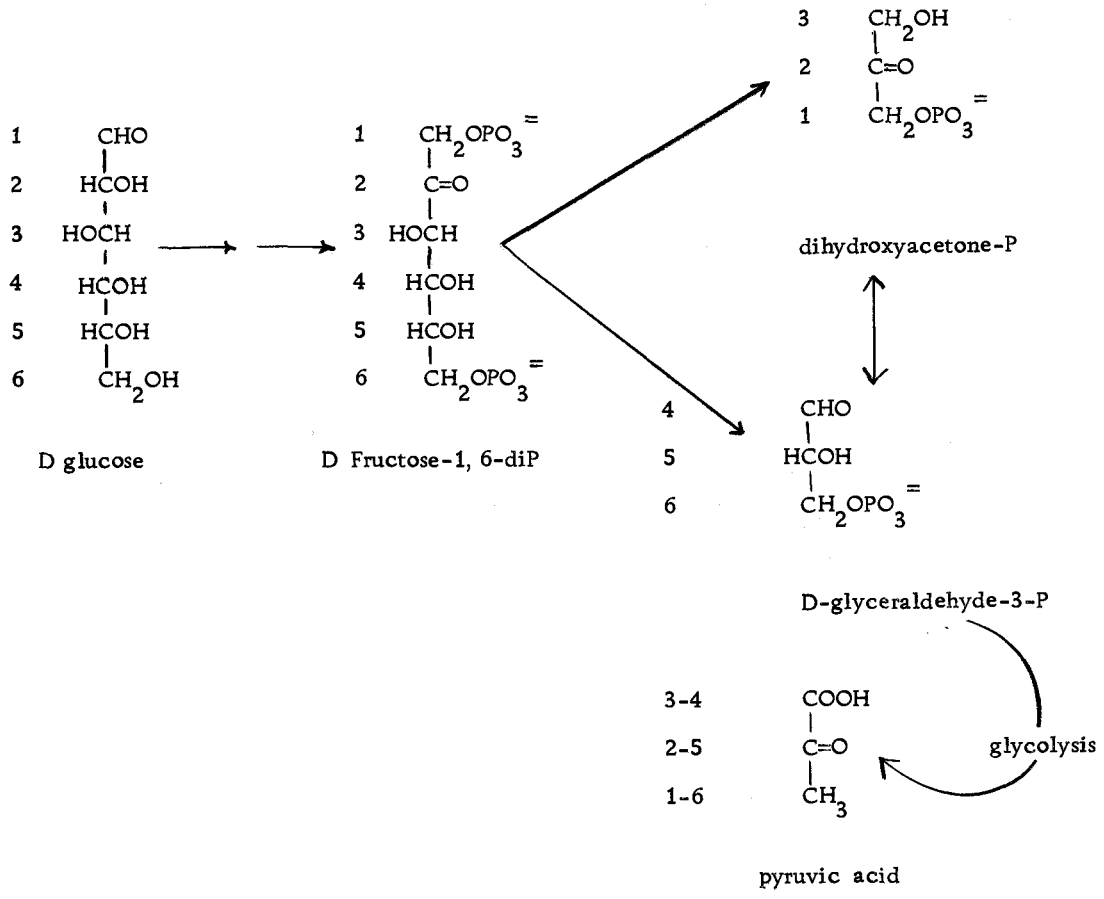
1963), one should be able to obtain information about the relative participation of various glucose catabolic pathways using the types of data given in this study. This of course is subject to the assumptions outlined by Wang et al. (1956) and Wang and Krackov (1962). In order to interpret these data, one must determine whether the main pathway of glucose catabolism is the Entner-Doudoroff (ED) or the Embden-Meyerhoff-Parnas (EMP) pathway. In the vast majority of the cases studied, these pathways have been shown to be mutually exclusive. Recently discovered exceptions to this rule have been reported by two groups (Matin and Rittenberg, 1970 and Keele, Hamilton and Elkan, 1968). Matin and Rittenberg (1970) showed the presence of key enzymes from both pathways in Thiobacillus intermedius. They concluded that the ED pathway was used primarily for energy production and the EMP and HMP pathways were used for the production of biosynthetic intermediates. Keele, Hamilton and Elkan (1968) came to the same conclusion while studying these pathways in Rhizobium japonicum. There are no reports to the author's knowledge where the ED pathway has been shown to be used for biosynthetic products and the EMP pathway used for energy production. As will be seen in the following argument, this would be the only other reasonable alternative to the more common EMP-HMP involvement.

The key to the identification of the pathway present lies in the

analysis of the relative release of CO_2 from glucose labeled in the first, third, fourth and sixth carbons. If one assumes the presence of an active TCA cycle and the sole utilization of the ED pathway, the CO_2 released from carbon 1 = 4 > 2 = 5 > 3 = 6 (Wang et al., 1956 and Spangler, 1966). This of course also assumes that some intermediates are utilized in biosynthesis, otherwise the CO_2 released from all carbons would be equal. The logic of this becomes evident when the fate of each carbon is traced through the pathway as shown below.



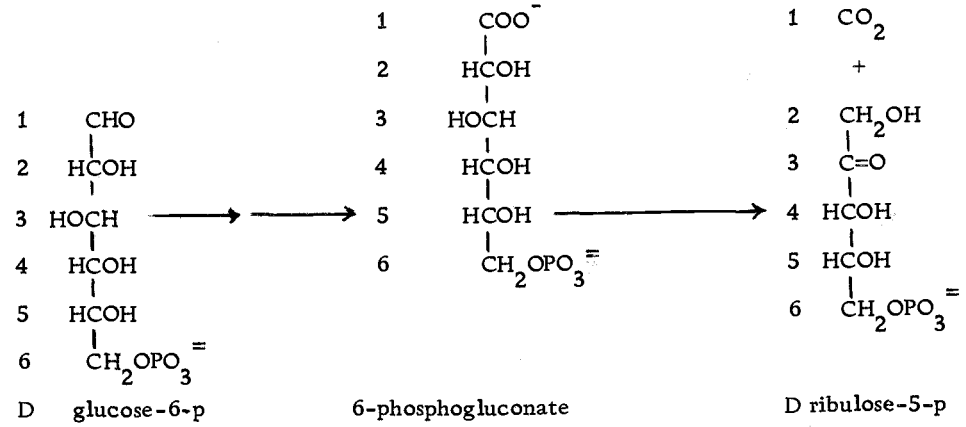
If on the other hand the EMP pathway was the only one being utilized, the pattern of the CO_2 released from each of the carbons should be 3 = 4 > 2 = 5 > 1 = 6 (Spangler, 1966). Here again, if the specific carbons are followed through the pathway, the reasoning behind this assumption becomes apparent.



In this organism, the CO_2 released from the third and fourth carbons was very close to being equal. The release from the third carbon was much greater than that given off by the first and sixth

carbons (Figure 11). These data suggest that MP-1 was using the EMP pathway as the main route of glucose catabolism. This conclusion is supported by the fact that the following three enzymes showed relatively high specific activities in crude cell-free extracts: fructose-diphosphate aldolase, hexosphosphate isomerase, and glyceraldehyde-phosphate dehydrogenase. All three of these enzymes are present in the EMP pathway.

If the EMP pathway were the only one operating for glucose catabolism, one would expect the CO_2 from the first and the sixth carbons to be equal. As can be seen in Figure 12, the amount of CO_2 released from the first carbon was greater than that from the sixth at salinities above 0.275 M. According to Wang et al., 1956; Höfer, 1967; and Bulla et al, 1970, this difference reflects the amount of glucose being catabolized via the hexose monophosphate pathway (HMP). If one follows the fate of the first carbon through this pathway, it becomes apparent that this carbon is removed in the process.



According to several observers (Wang and Krackov, 1962 and Bulla et al., 1970) the CO_2 from carbon 1 > 2 > 6 if both the EMP and the HMP pathways are operating. As can be seen in Figure 11, this is the relative sequence seen at the higher salinities in MP-1.

Katz and Wood (1962) and others (Chefuvka, Horie and Robinson, 1970) have pointed out that the estimation of the HMP pathway in absolute terms was not accurate using Wang's method when relatively large quantities of glucose were catabolized via that route. They suggested that there was a built-in error which would tend to underestimate HMP participation. They reasoned that every time a glucose molecule completed the cycle, the sixth carbon would remain in the fructose 6 phosphate pool while the original first carbon was removed. Thus, using respiration data from labeled glucose alone, one would not be able to distinguish between this molecule and the label on glucose following the glycolytic scheme. Since, at the optimum salinity, the percent of the glucose catabolized through this pathway was only about 12% of that going through the EMP pathway, this dilution factor was not considered important. When comparing relative results as we are here, this should not generate a variable error.

When evaluating a changing C_6/C_1 ratio, investigators have usually resorted to one of three possible explanations (Carlier and Van Assche, 1968 and Landau, Bartsch and Williams, 1966): (1) that there is a randomization of the label between the first and sixth

carbons (2) that there is a change in the participation of a third pathway such as the glucuronic acid pathway, which would alter the amount of CO_2 released from the sixth carbon (3) that there is a change in the participation of the HMP pathway. If the decreased C_6/C_1 ratio with increasing salinity was an artifact of the method due to a decreased randomization of the first and sixth carbons, one would expect the type of pattern shown in Figure 17A. If, on the other hand, it was due to a decreased participation of a pathway such as the glucuronic acid pathway, one would expect the type of pattern seen in Figure 17B. If the decrease in this region was due to the increased participation of the HMP pathway, one would expect the pattern shown in Figure 17C. When one compares these patterns with that found in Figure 12, it appears that the last alternative best fits the data. This certainly does not prove that a salinity mediated HMP pathway shift has taken place, but from the data given, it is the most plausible explanation at present. If one assumes that the differential between the first and second carbons reflects the relative participation of the HMP pathway, the fact that this difference disappears at the minimum salinity for growth may be significant. It is generally recognized that the HMP pathway is an important source of metabolic intermediates and NADPH (Sanwell, 1970). This then leads to the possibility that this organism's ability to grow at low salinities may be limited by the activity of the HMP pathway. There have been several reports

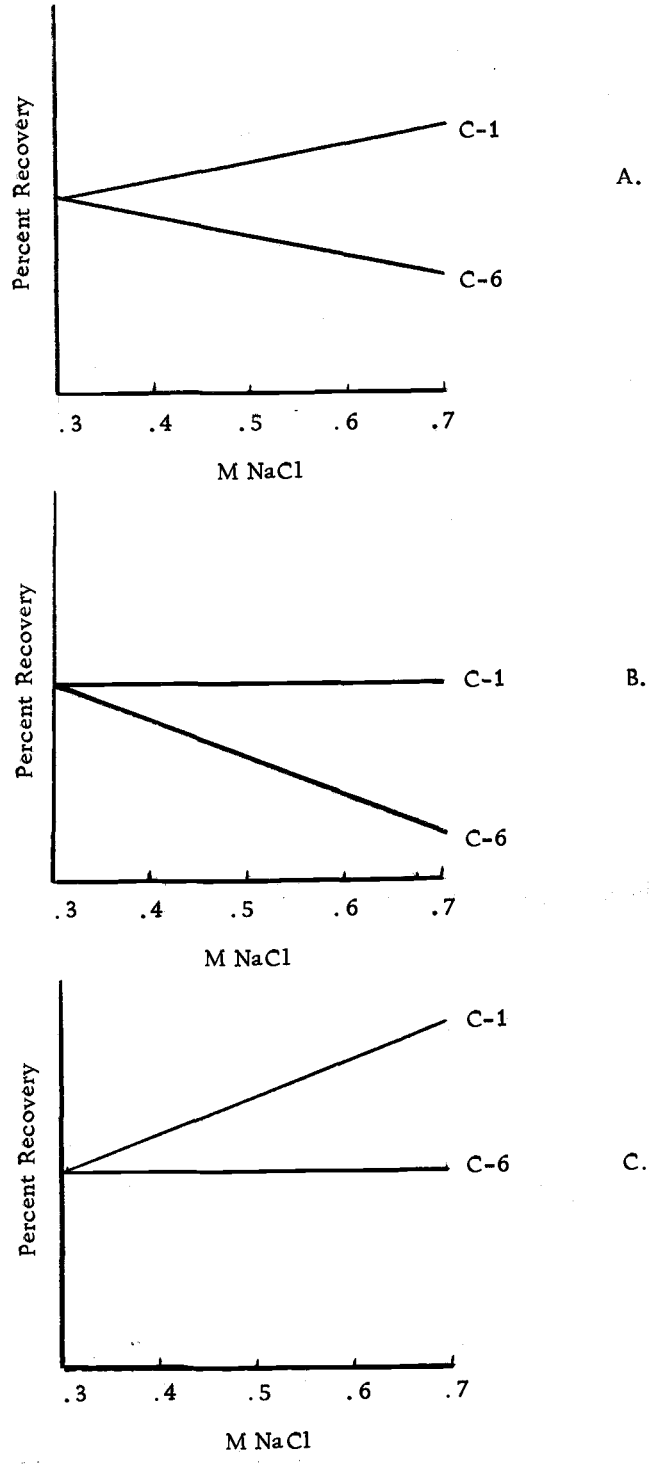


Figure 17. Theoretical curves of CO_2 evolution from the first and sixth carbons of glucose at various salinities.

of metabolic pathway shifts associated with shifts in salinity (O'Brien and Stern, 1969 a and b; Rhodes and Payne, 1962; Watson, 1970; Webb and Payne, 1971). There has been one report of a salinity induced shift in a glucose catabolic pathway (Porath and Mayber, 1964). This study which was done on pea root cells, showed a decrease in the C_6/C_1 ratio with increasing salinity. Subsequent work has confirmed that this shift was due to a salinity induced shift in the HMP pathway (Porath and Mayber, 1970).

This may explain the pattern seen in the third phase of the graph (Figure 12), but how about the first two? It would appear from the viability data (Table 1), that the rapid increase in the CO_2 evolved from both carbons in the 0.05 M to 0.15 M range reflects an increase in the cell's ability to take up and respire glucose as viability increases.

In the second phase, from 0.15 M to 0.3 M, there was also an increase in viability but a decrease in the CO_2 evolved from the first, second and sixth carbons. The CO_2 from these carbons decreased in a region where the viability, CO_2 from carbon three, and the cell activity associated with all carbons were increasing with increasing salinity. If one assumes that all of the glucose being metabolized is being routed through the EMP-TCA scheme, this pattern can best be explained by an increase in substrate uptake and increased EMP participation accompanied by one of two phenomena.

One possibility is that, with increasing salinity, there is an increasing number of C1-2 and C5-6 units being drained off into biosynthetic pathways instead of being utilized as energy. Another possibility is that there is an increasing number of C1-2 and C5-6 units being removed from the cell as metabolic by-products. An answer to this problem may lie in data given in Figure 16. Here the total uptake of glucose into the cell is represented by the sum of the total CO_2 evolved and cell activity assayed at the end of the studies described previously (Figures 10 and 11). Assuming that labeling a specific carbon does not affect the way the molecule is handled by the cell and that there are no labeled compounds being excreted by the cell, these figures should be identical for all carbons, as they are at 0.15 M NaCl. As the salinity is increased above 0.15 M, there is a difference generated between the activity associated with the third carbon and the activity associated with the other carbons tested. The most likely explanation for this difference is that after the third and presumably fourth carbons are decarboxylated, the remaining two carbon fragments (C1-2 and C5-6) are subject to biosynthetic pathways which eventually lead to their removal from the cell. It appears from these data that, in this region, salinity plays an important role in the activation of some biosynthetic pathway involved with this removal.

It was originally thought that this two carbon unit may be one of

the common two carbon metabolic by-products such as ethanol or acetate. Distillations of the supernatant were made in order to assay for both neutral and acid volatiles; none were found.

All of the salinity shift studies up to this point had been done with NaCl. Since many other studies with marine organisms have shown specific ionic requirements for various functions (Drapeau, Matula and MacLeod, 1966; Payne, 1960; MacLeod et al., 1958; Webb and Payne, 1971; Rhodes and Payne, 1962; DeVoe et al., 1970; Wong et al., 1969), the specificity for Na^+ or Cl^- on the C_6/C_1 ratio shift was tested. The effect of replacing NaCl with LiCl was studied. The resulting patterns (Figure 15) were almost identical to that found for NaCl. In order to test the anion requirement, the C_6/C_1 ratio shift was studied using NaBr and $NaNO_3$. Here again, the same type of pattern was observed. It would therefore appear that this shift was not dependent on either of these ions.

It also seemed possible that this shift may be in response to some osmotic effect. When glycerol was used to increase the osmotic pressure, no shift was observed.

There have been many studies made in the past in which the effects of both salinity and temperature on a given parameter have been investigated in marine organisms. In many cases, both salinity and temperature affect the same parameter in some way; often with one offsetting or enhancing the effect of the other (Stanley and Morita,

1968). With this thought in mind, it was felt that temperature might also effect the C_6/C_1 ratio shift caused by increasing salinities. The effect of varying temperature on the C_6/C_1 ratio at two extreme salinities was studied. The lowest temperature studied was 5 C, the temperature at which this organism was first isolated (Morita and Haight, 1964). The highest temperature studied was 5 C above its maximum growth temperature. As can be seen in Table 2, the C_6/C_1 ratio did not vary significantly over this wide temperature range. While studying the effects of temperature on major glucose catabolic pathways, Palumbo (1967) was not able to detect any pathway shifts in another gram negative organism.

The actual percent of activity recovered under each condition studied remained surprisingly constant with one exception. When cells were suspended in 0.15 M NaCl and subjected to an incubation temperature of 25 C, there was a noticeable decrease in the CO_2 evolved from both carbons while maintaining the same ratio. Cells incubated at the same temperature but suspended in a buffer containing 0.7 M NaCl showed no decrease. The increased salt in this case apparently protected the cell against thermal damage to some extent. This is really not surprising if one looks again at the data of Stanley and Morita (1968) or at the work of Iandolo and Ordal (1966).

SUMMARY

Vibrio marinus (MP-1), a marine psychrophilic bacterium, can be grown in a glucose-salts medium. In order to gain information about how this substrate is utilized by this organism under various ionic conditions, a series of studies was initiated. In early experiments, the amount of activity recovered always totaled less than the amount of labeled glucose added at the beginning of the experiment. This discrepancy varied depending upon experimental conditions. After further work, it became apparent that the greatest error occurred in the cell fraction. It was subsequently determined that most of this variation was due to the loss of loosely bound radioactive material presumably in the form of glucose. This activity was instantaneously removed by acidification of the cells to pH 2.0. The amount of activity released from the cell was a function of either pH or salinity and was not related to cell leakage.

When the effects of various ions on the uptake of glucose were studied, it was found that Na^+, Li^+, or Mg^{++} would permit uptake but that K^+, Rb^+, or NH_4^+ would not. All anions studied permitted glucose uptake. This is the same pattern of ionic dependency reported for growth.

In general, the salinity of the suspending medium was shown to have the greatest effect on glucose uptake and respiration in the

region of minimum growth salinity (0.275 M). Studies were made with glucose labeled on carbons 1, 2, 3, 3-4, and 6. Several trends were noted. At 0.15 M NaCl, the total apparent activity taken up by the cells was the same for all carbons. This would be expected if no activity was lost from the cell except in the form of CO_2. At higher salinities, however, the activity associated with the third carbon was different from that obtained from the first, second, and sixth carbons. It would appear that this difference reflected the release of some by-product or by-products containing the first, second, fifth and sixth carbons.

More extensive experiments monitoring CO_2 released from the first and sixth carbons showed three basic patterns, as the salinity was increased from 0.0 M to 1.0 M NaCl. In the range from 0.0 M to 0.15 M NaCl, the amount of CO_2 associated with both of these carbons increase about the same amount with increasing salinity. This closely resembles the viability profile of cells suspended in media of the same salinity range. This increase probably reflects an increase in cell activity. In the second range, from 0.15 M to 0.30 M NaCl, the amount of activity associated with both of these carbons decreases and the amount of activity associated with the cells increases. This along with the patterns seen in the third carbon indicates that as the salinity of the medium increases, more of the total glucose taken up by the cell is converted into biosynthetic

products via the Krebs cycle.

As the salinity was increased above 0.3 M NaCl, another pattern was observed which was of potential importance. The CO_2 associated with the sixth carbon remained relatively constant, but that from the first carbon increased significantly with increasing salinity. This increase was interpreted as a reflection of an increased hexose monophosphate pathway participation in glucose catabolism. If the differential between the CO_2 from these two carbons represents hexose monophosphate (HMP) pathway participation, it is significant that there is no differential found in the region of minimal growth salinity. One may infer from this that the HMP pathway is shut down in this region. The different C_6/C_1 ratios produced at different salinities was also found when LiCl, NaBr, or $NaNO_3$ was used instead of NaCl. It was also shown that these ratios were not significantly altered by changes in incubation temperatures.

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