STUDIES ON THE FUNCTION OF SODIUM IN MARINE BACTERIA

by

Gabriel Rosaire Drapeau

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

Department of Agricultural Bacteriology,
Macdonald College of McGill University,
Montreal.

August 1962.

TABLE OF CONTENTS

Page

Page

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Dr. R. A. MacLeod, Department of Agricultural Bacteriology, Macdonald College, for suggesting this study, and for his continued inspiration and guidance throughout the course of this investigation and in the preparation of the manuscript.

Appreciation is also extended to Mr.T.Matula for assistance in photography, and to Mr.F.Takacs for advice on the radioactive experiment and flame photometry.

Sincere thanks are given to the National Research Council, Ottawa, for the financial assistance which made this investigation possible.

INTRODUCTION

A number of marine bacteria have been found to have a specific requirement for sodium for growth and for metabolism. The specific function of this monovalent ion for the cells, however, is still unknown.

The aim of this project is to study the function of sodium in a Gram-negative marine pseudomonad. Three different approaches were taken to investigate this problem. The first one consisted in comparing the biochemical properties of a Na^+ requiring marine bacterium and some non-Na^+ requiring mutants derived from it. Warburg respirometer and spectrophotometric experiments were used to establish similarities and differences between the parent organism and some of its non-Na^+ requiring mutants.

An attempt has also been made to transform the non-Na^+ requiring mutants to strains with a Na^+ requirement using deoxyribonucleic acid isolated from the parent Na^+ requiring strain. Such a transformation would establish more clearly the genetic relationship between the two types of organisms and would have a

bearing on the evolutionary relationship between marine and terrestrial bacteria.

A third and more direct approach to the study of the function of sodium consisted in determining if an adenosinetriphosphatase could be found in the protoplast membrane of marine bacterial cells which was specifically activated by a combination of Na^+ and K^+. The search for a sodium and potassium activated adenosinetriphosphatase was stimulated by recent findings that such an enzyme appears to be linked to the transport of sodium and potassium across the membrane of animal cells.

GENERAL REVIEW OF LITERATURE

Distribution of Bacteria in the Sea

Although marine microbiology is regarded as being a new and relatively undeveloped science, one of the first accurately described species of bacteria, Spirochaeta plicatilis, was isolated from sea water more than a hundred years ago by Ehrenberg (1838). Cohn (1865) isolated and described the marine-dwelling Beggiatoa mirabilis and Warming (1875) described Beggiatoa minima. A year later Warming (1876) described Thiospirillum violaceum and Thiospirillum rosenbergii.

The earliest studies in marine microbiology were concerned with the distribution of bacteria in the sea. Expeditions were made by Certes as early as 1884, by Fischer in 1886, and many others before the turn of the century. By 1900, it was already known that bacteria are widely distributed in the seas, whether they be in the temperate, tropical or arctic zones, and that in the water itself they follow the distribution of the plankton organisms. The highest number of microorganisms were found in coastal areas and at the convergence of ocean currents, seemingly due to the

presence of larger quantities of organic nutrients in these areas. More bacteria were found at depths of 40 to 50 meters than in surface waters. At depths exceeding 40 to 50 meters the bacterial population decreased with depth. Bottom deposits were found to contain a very high number of microorganisms irrespective of the depths the samples were taken (ZoBell 1946).

Importance of Bacteria in the Sea

Studies were not only limited to the geographical distribution of bacteria in the sea. A considerable amount of information is also available concerning the role played by marine microorganisms in the sea.

Marine bacteria catalyse the transformation of carbon, nitrogen, hydrogen, sulfur, phosphorus, and other elements thereby influencing the chemical composition and certain physico-chemical properties of sea water, bottom sediments and other marine materials. Extensive reviews on the role of bacteria in the sea have been published by ZoBell (1946) and Wood (1958).

Bacteria in the sea have often been claimed to be important geological agents. One of the first geochemical processes to be studied by micro-

biologists was calcium carbonate precipitation, which Drew (1912) attributed to the activities of denitrifying bacteria in subtropical seas. Iron and manganese concretions in the White Sea were attributed by Butkevich (1928) to bacterial activity also. It is generally agreed by geologists that petroleum has been formed in marine sediments from the transformation of the organic remains of plants and animals. Bacteria may contribute to the process by modifying sedimentary conditions (ZoBell, 1952). In highly reducing environments, the microbial modification of organic matter may result in residues relatively richer in hydrogen and poorer in oxygen, nitrogen, sulfur, and phosphorus.

The most important activity of bacteria in the sea is the decomposing of organic matter (Waksman 1934). Although large quantities of organic matter are washed into the sea from the land and much more is produced in the sea by photosynthetic plants, the organic content of sea water is rarely more than 5 mgm. per liter. Most marine bottom deposits contain less than 5 per cent organic matter. Because of the effectiveness of bacteria in decomposing organic matter, the ocean has been described as being the world's largest and most efficient septic tank.

Carbohydrates, lipides, and proteins are

rapidly attacked by marine bacteria. In addition to the carbon cycle demonstrated in the sea, other cycles such as the nitrogen cycle, sulfur cycle, and a phosphate regeneration system are also known to be present (ZoBell 1946).

Characteristics of Marine Bacteria

Although much information is available on the biochemical, chemical, and physico-chemical activities of bacteria in the sea, little is known about whether or not bacteria in the sea are significantly different from those found on land and in fresh water.

The question of the existence of specific marine bacteria was raised by Stanier (1941). He stated that, "until this ecological problem is settled one way or the other, work on marine bacteriology apart from studies on gross transformation of matter has very little point". Such criteria as temperature range and salt tolerance led investigators like Fischer (1894) to affirm the existence of specific marine bacteria. According to Stanier, "the possibility still existed that such organisms are only varieties of similar soil forms. The existence of specific marine bacteria necessarily implies that organisms carrying on a certain process in the cycle of matter in the ocean differ from

those carrying on the same process in the soil".

When Stanier made this statement in 1941, relatively few studies had been made of the physiology of marine bacteria. To the known cultural and morphological characteristics of marine bacteria, new characteristics such as susceptibility to lysis and especially sodium requirements have been added and there are now indications that there may well be definite criteria for distinguishing marine bacteria from terrigenous ones.

Cell Morphology.

About 95 per cent of the bacterial species isolated from the sea are Gram-negative rods (ZoBell and Upham, 1944). Tyler et al. (1960) made 96 isolations of cultures which were all Gram-negative rods or, in 14 instances, vibrio to spiral forms. ZoBell (1946) reported that marine bacteria are smaller than those which occur in milk, sewage, fresh water, or soil. They are actively motile non spore-formers and belong mainly to the genera Pseudomonas, Vibrio, Flavobacterium, and Photobacterium. Pleomorphism appears to be common and chromogenicity is encountered in 70 per cent of the isolates.

Physiological Characteristics

As a group, marine bacteria are more weakly saccharolytic and probably more strongly proteolytic than either soil or fresh-water bacteria. Very few marine bacteria produce acid from sucrose, maltose, xylose, glycerol, mannite or salicin, and gas producers have not been reported from the high seas (ZoBell 1946). On the other hand, ZoBell (1946) reported that three-fourths of marine bacteria hydrolyse gelatin. Similarly, Tyler *et al*. (1960) reported that 76 out of their 96 isolates hydrolysed gelatin.

Temperature Tolerance

The ability of marine bacteria to grow at near zero or sub-zero temperatures is not a unique property of marine bacteria, since microorganisms from other habitats are endowed with this ability, but it seems to be more common among marine bacteria. ZoBell and Conn (1940) comparing their results with those reported for the percentage survival of bacteria in river water revealed that, whereas the majority of marine bacteria succumb when held for 10 minutes at 30 to 40° C. the average thermal death point of fresh-water bacteria is about 10 degrees higher. Most marine bacteria grow

best at temperatures ranging from 12 to 25° C. Nearly all species of marine bacteria grow slowly at 0 to 4° C., this being the temperature range of approximately 80 per cent of the ocean by volume.

Susceptibility to Lysis

One of the most striking dissimilarities between marine bacteria and terrigenous species is the susceptibility of the former to lysis in media of low solute concentration. This phenomenon was first observed by Harvey (1915) who reported that distilled water was unsatisfactory for washing marine luminous bacteria. This lytic problem has been studied recently by Pratt and Riley (1955). Tomlinson and MacLeod (1957) observed a correlation between lysis of a marine bacterium and its capacity to oxidize L-alanine. Tyler et al. (1960) found that the majority of their 96 isolates were susceptible to loss of optical density in distilled water. Further studies on lytic susceptibility have shown that marine bacteria have a weak cell wall. By adding penicillin to growing cells, Boring and Pratt (1961) observed the formation of protoplasts which were osmotically stable in a medium containing a salt concentration optimum for growth. Similarly, MacLeod and Matula (unpublished observations) have shown that protoplasts prepared

with lysosyme had about the same osmotic fragility as the whole cells.

Sodium Requirement of Bacteria in the Sea

The use of sea-water media for the growth of marine bacteria has been recommended by Berkeley (1919), Korinek (1926), and Lipman (1926). This recommendation was made after it had been observed by different workers that many more bacteria develop on sea-water media than on fresh-water media especially when the samples of sea-water or marine mud were collected at places remote from possibilities of terrigenous contamination. There was also evidence that the requirement for sea water was unstable. After prolonged laboratory cultivation on sea-water media, ZoBell and Upham (1944) found that marine bacteria had developed the capacity to grow in fresh-water media. It appeared then that marine bacteria lose comparatively readily the one characteristic which has been used to distinguish them from land forms. On the basis of these observations ZoBell and Upham (1944) defined marine bacteria as: "Bacteria from the sea which upon initial isolation require sea water in the medium for growth". Paradoxically, however, they and other workers failed to train marine bacteria to grow in media prepared with fresh water. Korinek was the first

one to report this observation in 1927.

In 1935, Voroshilova and Dianova indicated that a number of marine bacteria failed to grow if NaCl was omitted from the medium or if NaCl was replaced by KCl. Later, MacLeod et al.(1954) noted that three organisms which required a sea-water medium for growth all required the presence of sodium. It was also found that the level of Na^+ in the medium affected both the rate and extent of growth. This requirement for Na^+ proved to be highly specific since it could not be replaced by any one of a number of related ions, or by compounds such as sucrose, added to increase the osmotic pressure of the medium. When the requirements of the organisms for K^+ were determined, it was also found that only one-hundredth as much K^+ as Na^+ was needed. Payne (1958) (1960) also demonstrated a specific requirement for Na^+ for growth and metabolism in a marine pseudomonad. Tyler et al. (1960) studied the mineral requirement of 96 marine isolates. Only four of the isolates grew to a measurable turbidity in the liquid medium to which no sodium was added but containing K^+ and Mg^{++}. It was concluded that all 96 isolates required Na^+ for good growth. Unfortunately, no attempt was made to compensate for the large change in ionic strength which occurred when Na^+ salts were omitted from the medium.

Consequently, one is faced with a paradox concerning the salt requirement of marine bacteria. On the one hand ZoBell and Upham (1944) claimed that marine bacteria were able to grow on fresh-water media after primary isolation on sea-water media, and on the other hand, a number of workers had failed to train marine bacteria to grow in fresh-water media or in media containing no added NaCl. This was further complicated by the more recent findings of Pratt and Waddell (1959). By plating heavy suspensions of marine bacteria on trypticase medium prepared without added Na^+, they obtained colonies which they assumed were mutants of marine bacteria which no longer required Na^+ for growth.

This state of confusion, however, has become clearer due to the findings of MacLeod and Onofrey in 1961. Unlike Pratt and Waddell (1959), they were unable to obtain colonies of organisms by plating on trypticase agar medium containing no added Na^+, but found that one of the organisms could be trained to grow well on this medium by serially streaking onto the surface of plates of the medium containing progressively lower concentrations of NaCl. This adapted organism, when tested on a chemically defined medium, however, could still be shown to require Na^+ for growth. A flame photometric analysis of the trypticase medium prepared without added NaCl

revealed a concentration of 0.028 M Na^+ present as contaminant. The concentration of Na^+ in this medium as a contaminant was high enough to permit growth in a chemically defined medium after long incubation. On a chemically defined medium which contained only 6.5×10^{-5}M as a contaminant, growth never occurred when the concentration of added Na^+ was less than 0.02 M (MacLeod and Onofrey, 1961). These observations provide evidence that marine bacteria are able to adapt to a slightly lower Na^+ concentration but not to growth on a sodium-free medium. The conditions under which ZoBell and Upham (1944) and others were cultivating their organisms unquestionably influence the results. They were using complex media which contained peptone and agar, two major sources of Na^+ contamination. It is likely, then, that their medium contained enough Na^+ as a contaminant to permit growth of their isolates after primary isolation on a sea-water medium.

Whether or not all marine bacteria require sodium for growth is not known. The fact that upon initial isolation sea-water media are required for a majority of the organisms studied and that a specific requirement for Na^+ has been demonstrated in some species is a good indication that marine bacteria could be distinguished from terrestrial bacteria on this basis.

This could, therefore, be the answer to Stanier's question regarding the existence of specific marine bacteria.

PART I

COMPARISON OF BIOCHEMICAL PROPERTIES OF MARINE BACTERIUM B-16 AND SOME NON-Na^+ REQUIRING MUTANTS DERIVED FROM IT

INTRODUCTION

The first objective of this thesis was to compare the biochemical properties of marine bacterium B-16 and some non-Na^+ requiring mutants derived from it since it was of interest to determine if any fundamental differences in metabolism of the two types of cells could be detected which would enable the function of Na^+ to be localized.

LITERATURE REVIEW

Function of Sodium in Marine Bacteria

The function of sodium in marine bacteria is not known. Indeed, their metabolism has been little investigated. Marine luminous bacteria have been extensively studied, but primarily for the purpose of investigating the mechanism of bioluminescence. After the MacLeod group had established a sodium requirement for growth in some marine species, they began to investigate the function of sodium in three marine organisms, two pseudomonads and a cytophaga. The first problem that had to be solved was to determine whether or not marine bacteria require Na^+ for the primary purpose of maintaining osmotic stability, since Na^+ salts constitute approximately 80 per cent of the salts in sea water. It was found, however, that sucrose had a very limited sparing action on the Na^+ requirement even though this compound was not metabolized by the cells (MacLeod and Onofrey, 1957) The ability of Li^+, K^+, Rb^+, and Cs^+ to replace or spare the requirement for Na^+ was was also determined. None of the ions permitted growth when added in place of Na^+ to the Na^+ deficient medium although Li^+, K^+ and to a lesser extent, Rb^+ had slight sparing actions for all three organisms after short

incubation periods at sub-optimum levels of Na^+. Cs^+ on the other hand, was found to be inhibitory. Payne (1958) confirmed the findings of MacLeod and Onofrey that neither sucrose, erythritol, nor equimolar concentrations of Li^+, Rb^+, and Cs^+ replaced the specific requirement for Na^+ for growth. It is thus clear that the function of Na^+ for these organisms is not primarily to balance the internal osmotic pressure of the cells.

Metabolic studies showed that Na^+ and K^+ were essential for the oxidation of substrates such as L-alanine by cell suspensions of the marine bacteria. A requirement for Na^+, K^+ and Mg^{++} to maintain the metabolic activity of the cells on storage was also demonstrated (Tomlinson and MacLeod 1957). When the quantitative requirements for Na^+ and K^+ for maximal rate of oxidation of substrates were determined, it was observed that the optimal Na^+ concentration varied with the substrate present in the Warburg vessel. For example, for the oxidation of citrate and malate more Na^+ was required than for the oxidation of acetate (MacLeod et al. 1958). Due to the findings of Jardetzky and Wertz (1956) that common metabolites form complexes with monovalent ions, it was concluded that the differences in the Na^+ requirement of the suspensions with substrate observed could be accounted for best by assuming that the di- and tri-

basic acids, but not the monobasic acids, formed complexes with Na^+ before crossing the cell membrane.

When the requirement for K^+ for the oxidation of substrates by cell suspensions was determined no variation with substrate could be detected.

Payne also found Na^+ and K^+ to be required for the oxidation of glucuronate by his marine pseudomonad. He concluded that K^+ was required for oxidation and Na^+ for the formation of an induced penetration mechanism.

The enzymes of the tricarboxylic acid cycle of these marine bacterial cells have been examined for ion requirements using cell-free extracts of the organisms. None was found to require Na^+ specifically although one required K^+ and two others required media of appropriate ionic strength for optimal activity, (MacLeod et al. 1958, MacLeod and Hori, 1960).

Non-Na^+ requiring mutants

Reference has been made previously to the fact that all attempts to train marine bacterium B-16 to grow in a chemically defined medium containing less than 0.02 M Na^+ were unsuccessful. Upon ultra-violet irradiation of Na^+ requiring cells mutants were obtained which

grew on a medium containing no added Na^+. A total of 14 mutants were isolated and these grew on a chemically defined medium which contained no added Na^+ and only 5×10^{-5} M Na^+ present as a contaminant. These organisms have therefore been referred to as non-Na^+ requiring mutants.

MATERIALS AND METHODS

Organisms

The parent organism used in this study is referred to as B-16. It has been isolated from marine clams and tentatively identified as a species of Pseudomonas.[1] It is capable of growing on either acetate, succinate, citrate, glycerol and glucose as sources of carbon and energy and NH_4^+ as the source of nitrogen. It does not form acid or gas from glucose, arabinose, maltose, sucrose, dulcitol or lactose, under anaerobic conditions. It can oxidize glucose aerobically, if adapted to it by including glucose in the medium during growth. It produces H_2S, liquifies gelatin, hydrolyses starch, does not reduce nitrate to nitrite and is indole negative. The organism is Gram-negative, rod-shaped and motile by means of a single polar flagellum. The mineral requirements for maximal growth of this organism were found to be Na^+, 0.1 to 0.3 M, K^+, 0.01 to 0.1 M. These ions could not be replaced by Li^+, Rb^+, nor Cs^+. Mg^{++} was found to be required. It was not replaceable by Ca^{++} although the latter could be shown to spare the

---

[1] The tentative identification of this organism was made by Dr.Einar Leifson, Loyola University, Chicago.

the Mg^{++} requirement. There was no specificity for halides on the growth of this organism. Only the rate of growth could be shown to be reduced by lack of added halide and then only for a brief period in the life of the culture. An absolute requirement for Fe^{++} has been demonstrated and could not be replaced by either Ni^{++} or Co^{++} (MacLeod and Onofrey 1954, 1957).

The three mutants used in this study were chosen from the 14 isolates which grew on a Na^{+} free medium upon ultraviolet irradiation of B-16. The 14 isolates had been previously compared morphologically and in biochemical tests with the original culture of organism B-16 and with B-16 adapted to grow in trypticase agar prepared without Na^{+} (MacLeod and Onofrey, 1961). It had been established in this previous investigation that the isolates were all Gram-negative pleomorphic motile rods indistinguishable morphologically from the parent culture. All the cultures behaved in the same way as B-16 except in the gelatin liquifaction and nitrate reduction tests. Unlike B-16 they could not liquify gelatin and some were capable of reducing nitrate. Another difference between the parent and the mutants lay in their capacity to lyse. Marine bacterium B-16 is known to lyse rapidly when growth has ceased or when it is suspended in distilled water (MacLeod

and Matula, 1961). The mutant cells, however, did not lyse detectably under the same conditions. It was observed in the case of the three mutants examined in detail that although they were able to grow in a Na^+ free medium they were stimulated by the addition of 0.02 M Na^+ to the medium.

The nitrate reduction test served as a basis for differentiating the three isolates examined in this thesis. Mutant A-1 could not reduce nitrate to nitrite, Mutant B-1 reduced nitrate weakly, and Mutant C-2 reduced it strongly.

Media

The organisms were carried on a trypticase agar medium containing (in grams per liter) B.B.L. trypticase, 10 gms: agar 15 gms: dissolved in a salt mixture containing: KCl, 0.73: $MgCl_2$, 2.5 gms: and $FeSO_4(NH_4)_2SO_4$, 0.025 gm. (MacLeod et al. 1960). For the growth of the parent culture, organism B-16, 12.7 gms of NaCl were also added to the medium.

Cells for metabolic studies were grown in a liquid medium containing 8 grams of nutrient broth and 5 grams of yeast extract dissolved in a liter of the same salt solution as was employed in the trypticase agar

medium. This is the same medium as was used by MacLeod et al. (1958) in the study of the oxidative ability of organism B-16 except that to prevent precipitation of free metal ions, the medium was supplemented with 0.5% K^+ citrate to provide a metal buffering action (Chabereck et al. 1955). Citrate was omitted, however, and replaced at the same level by malate or fumarate when attempts to adapt the cells to oxidize these substrates were made.

Growth Conditions

Cells were grown at 25 C in a two-liter flask containing 250 ml of the liquid medium and shaken for 24 hours on a rotary shaker running at the rate of 200 revolutions per minute with a stroke of two inches. The flasks were inoculated with 10 ml of an 18 hour old culture grown under the same conditions. In the case of the parent organism B-16, the flasks were inoculated directly from the agar-slant culture.

Recovery of the Cells

At the end of the 24 hour period (15 hours for B-16), the cells were harvested by centrifugation at 4° C. in a Lourdes refrigerated centrifuge at 12,500 x G. Twenty minutes were required to sediment the cells in

the case of B-16 and only 10 minutes in the case of the mutants. The supernatant liquid after centrifugation was removed from the cell pad and the cells were resuspended in 0.05 M $MgSO_4$. This operation was repeated twice more.

Measurement of the Cells

In order to be able to add the same amount of cells to each Warburg flask (7 mgs, dry weight) the dry weight of cells in the washed bacterial suspension was determined by turbidity using a previously calibrated curve. This calibrated curve was prepared originally in the following manner. Cells of the organism harvested from 200 ml of medium were washed and finally suspended in 100 ml of 0.05 M $MgSO_4$ (Suspension A). From this suspension 2.0 to 2.5 ml were accurately measured out and further diluted to 100 ml in 0.05 M $MgSO_4$ to make suspension B. For measurement of turbidity, a series of dilutions from zero to one-in-ten were made with Suspension B. These dilutions gave a series of turbidity readings falling within the range of the scale of the Coleman Junior nephelometer. For dry weight determination, 10 ml samples of suspension A were centrifuged at 50,000 x G in a Spinco ultracentrifuge for 20 minutes. The supernatant liquid was removed and the cell pad was dried in an oven at 60° C. for 24 to 30 hours before

being transferred to a dessicator where it was held until its weight was constant. Total nitrogen was determined on aliquots of the same suspension by the micro-Kjeldahl method. From the dry weight and nitrogen determinations and the dilutions used to obtain the turbidity readings it was possible to relate either the dry weight or the nitrogen content of the cells in suspension to the nephelometer readings and hence to construct suitable curves.

Oxidation of Various Substrates

Standard Warburg respirometer experiments were carried out using the "direct method" described by Umbreit et al. (1959). Flask contents were the same as those used by MacLeod et al. (1958) in previous studies of the oxidation of substrates by cell suspensions of organism B-16.

To determine the effect of Na^+ on the oxidation of substrates by the various cell suspensions, a pair of flasks containing the substrate under investigation was prepared. Na^+ as NaCl was added to one member of the pair at a level which had previously been established as optimum for the oxidation of the particular substrate by organism B-16 (MacLeod et al. 1958). Flasks prepared

with and without Na^+ but containing no added substrate served as endogenous controls.

Preparation of Cell-free Extracts

Cells were washed and suspended in 0.05 M $MgSO_4$ and treated in an MSE sonic oscillator. Seven minutes treatment was required for B-16 and 10 minutes for the mutants to obtain suitable cell-free extracts. The extracts prepared were centrifuged at 50,000 x G in a Spinco ultracentrifuge for 30 minutes to remove debris and any unbroken cells.

Measurement of Enzyme Activity in Cell-free extracts

Fumarase was measured spectrophotometrically in a Zeiss Ikon spectrophotometer by observing the increase in optical density at 240 mu with malate as substrate, (Racker, 1950). Each cuvette contained: TrisCl 30 umoles (pH 8.0); Tris-malate, 10 umoles; $MgCl_2$, 16.6 umoles, in a final volume of 2 ml. Malic dehydrogenase activity was determined by following the rate of reduction of TPN (triphosphopyridine nucleotide) spectrophotometrically at 340 mu (Ochoa et al. 1948). The reaction mixture contained: TrisCl, 30 umoles (pH 8.0); Tris-malate, 10 umoles; KCl, 333 umoles;

$MgCl_2$, 23.3 umoles; TPN, 1 umole; in a final volume of 2 ml. In both cases, 0.1 ml of an appropriate dilution of the cell-free extract was added to start the reaction.

Protein was estimated by the biuret method (Gornall et al. 1949) with crystalline pepsin (Worthington Biochemical Corporation) as a standard.

RESULTS

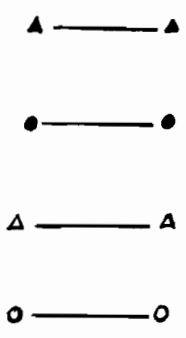
Warburg Manometric Determinations

The capacity of the three non-Na^+ requiring mutants to oxidize seven substrates known to be oxidized by resting cells of organism B-16 have been investigated. In addition, the effect of Na^+ on the oxidation has been determined. The results were compared with those obtained with the parent culture at the same time. Since the three mutants behaved identically in these studies, the results with only one of them have been recorded. In order to facilitate the comparison of the oxidative ability of B-16 and that of mutant B-1, the figures for each substrate have been arranged side by side.

Acetate is readily oxidized by both organisms (Figures 1 A and B). It can be seen that Na^+ is required for oxidation of this substrate by B-16. Mutant B-1, on the other hand, does not require Na^+. A slightly higher oxygen uptake has been demonstrated in the absence of the ion. One should note also the stimulation of endogenous respiration by Na^+ in the cells of B-16. It cannot be ascertained that a similar stimulation occurs in mutant B-1 also, since the endogenous respiration is very low in this organism.

Figure 1.

Oxidation of acetate by washed cell suspensions of marine bacterium B-16 (Fig.1A) and mutant B-1 (Fig.1B) in the presence and absence of NaCl.



▲——▲ acetate + NaCl

●——● acetate only

△——△ no acetate + NaCl

○——○ no acetate, no NaCl

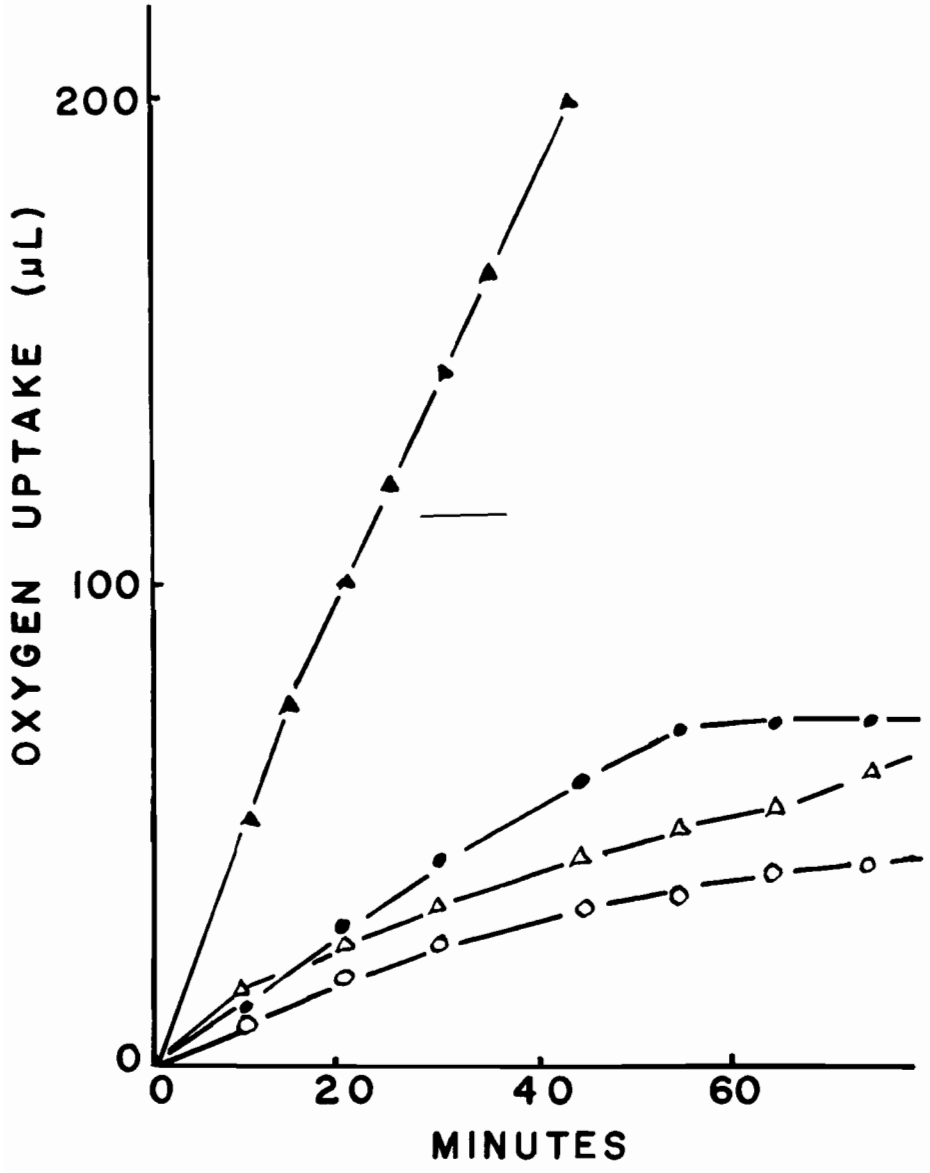


Figure 1 A

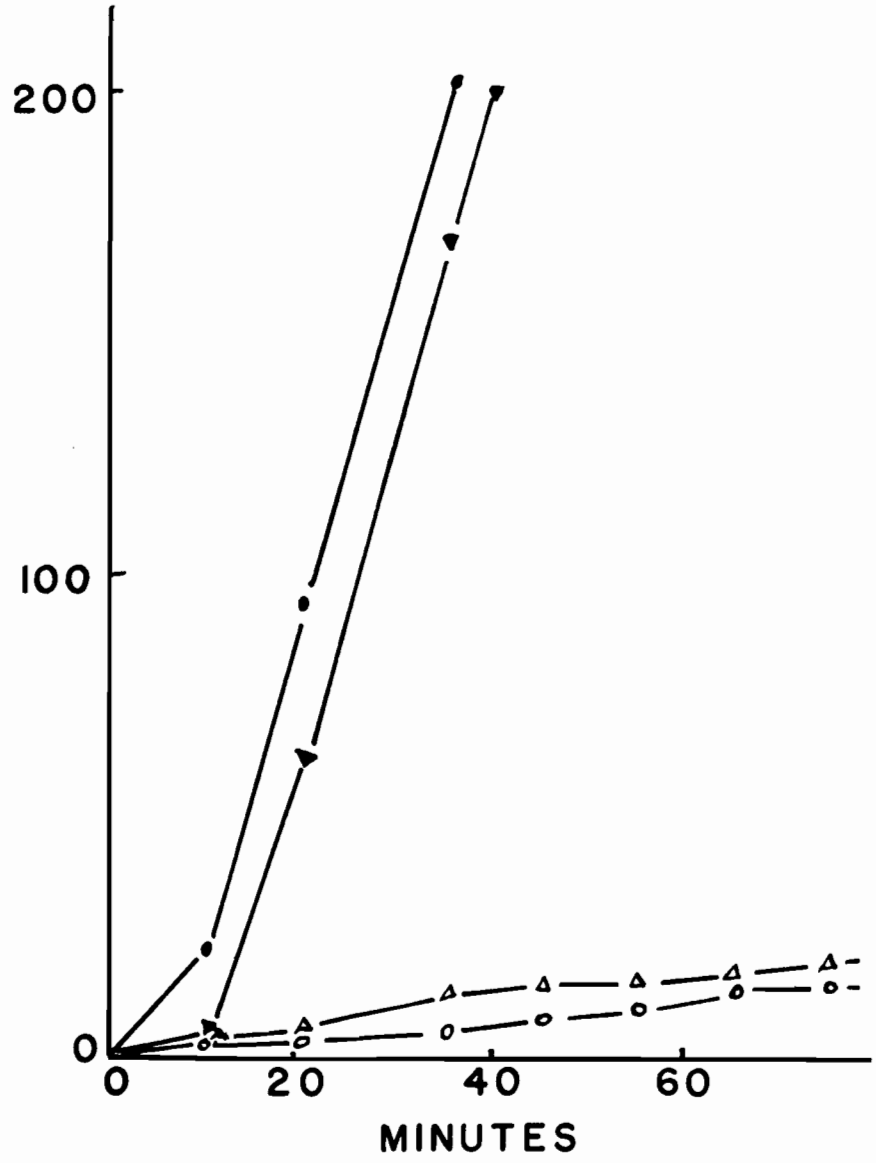


Figure 1 B

Propionate is more readily oxidized by resting cells of B-16 than by cells of mutant B-1 (Figures 2 A and B). Again, Na^+ is required for the oxidation of the of the substrate by B-16 but not by mutant B-1. In the case of the mutant the presence of Na^+ strongly inhibited propionate oxidation.

Butyrate is oxidized more rapidly by B-1 than by the parent (Figures 3 A and B). Na^+ is not required for the oxidation of this substrate by the mutant and, furthermore, shows a small inhibitory effect.

The di- and tricarboxylic acids are not as readily oxidized by cells of the mutant as the parent. This is evident in Figures 4 A and B where the capacity of B-16 and the mutant to oxidize succinate is compared. It can be seen that succinate is attacked much less rapidly by the mutant. Whereas B-16 required Na^+ for the oxidation, in the case of the mutant, Na^+ inhibited the oxidation.

To obtain cell suspensions of B-16 able to oxidize citrate and malate, cells had to be adapted first and this could be accomplished by incorporating the substrate into the growth medium (MacLeod *et al.* 1958). Cells of the mutant also did not oxidize citrate when harvested from a medium containing no added citrate,

Figure 2.

Oxidation of propionate by washed cell suspensions of marine bacterium B-16 (Fig.2A) and mutant B-1 (Fig.2B) in the presence and absence of NaCl.

▲ ——— ▲ propionate and NaCl

● ——— ● propionate only

△ ——— △ no propionate and NaCl

○ ——— ○ no propionate, no NaCl

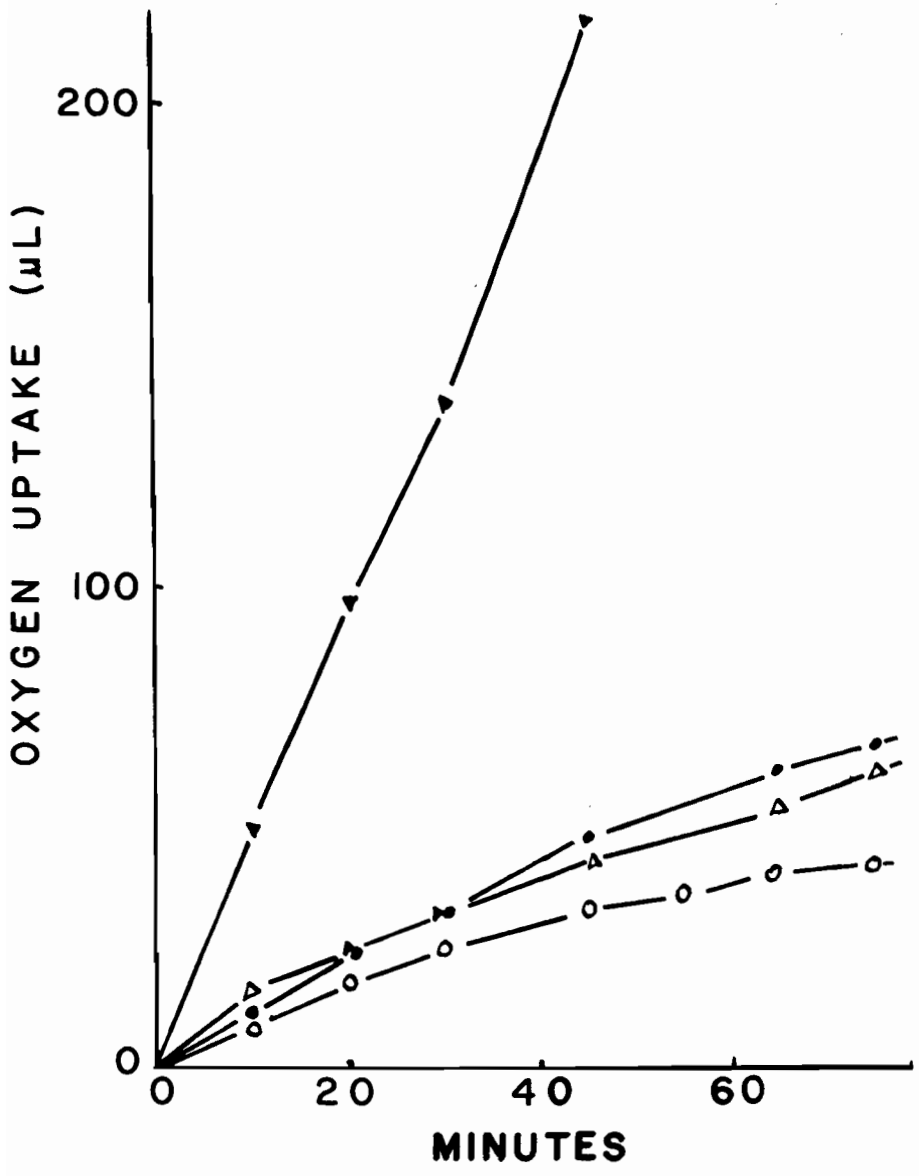


Figure 2 A

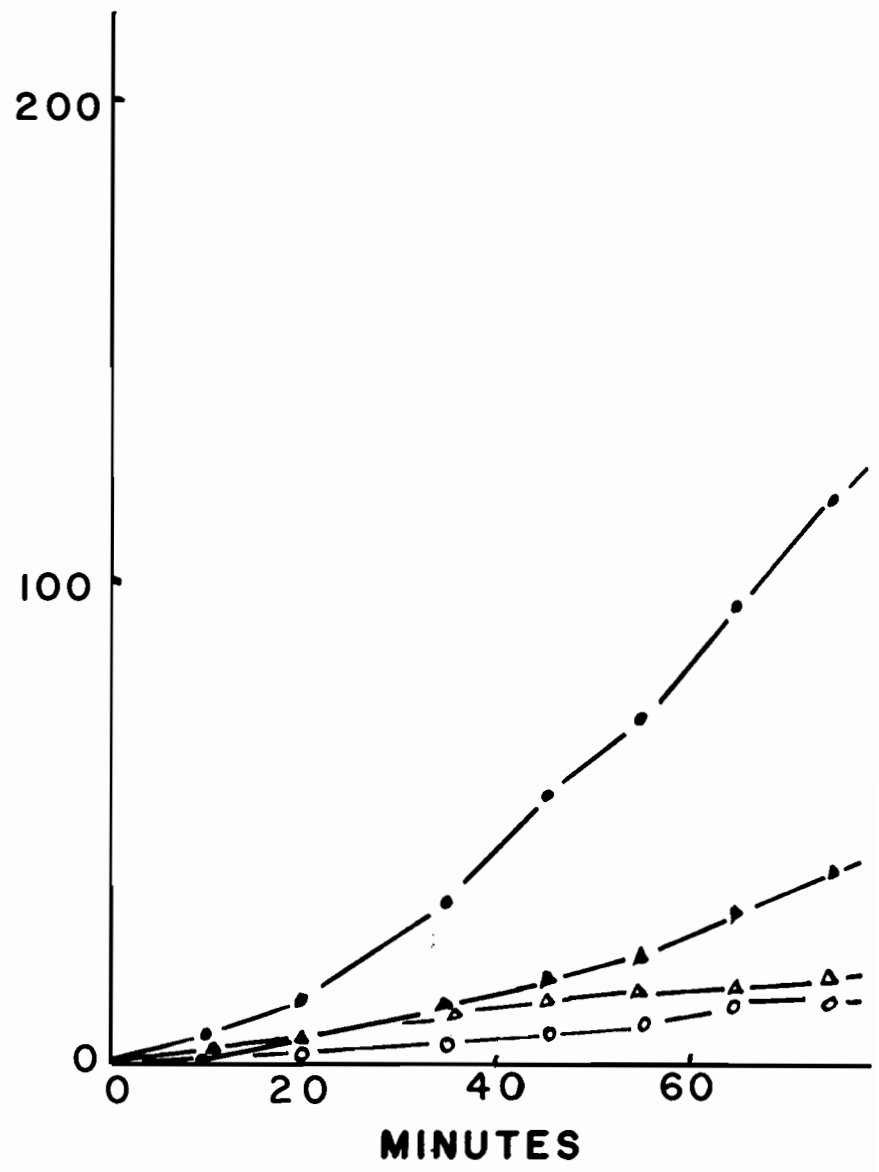


Figure 2 B

Figure 3.

Oxidation of butyrate by washed cell suspensions of marine bacterium B-16 (Fig.3A) and mutant B-1 (Fig.3B) in the presence and absence of NaCl.

▲——▲ butyrate and NaCl

●——● butyrate only

△——△ no butyrate and NaCl

○——○ no butyrate, no NaCl

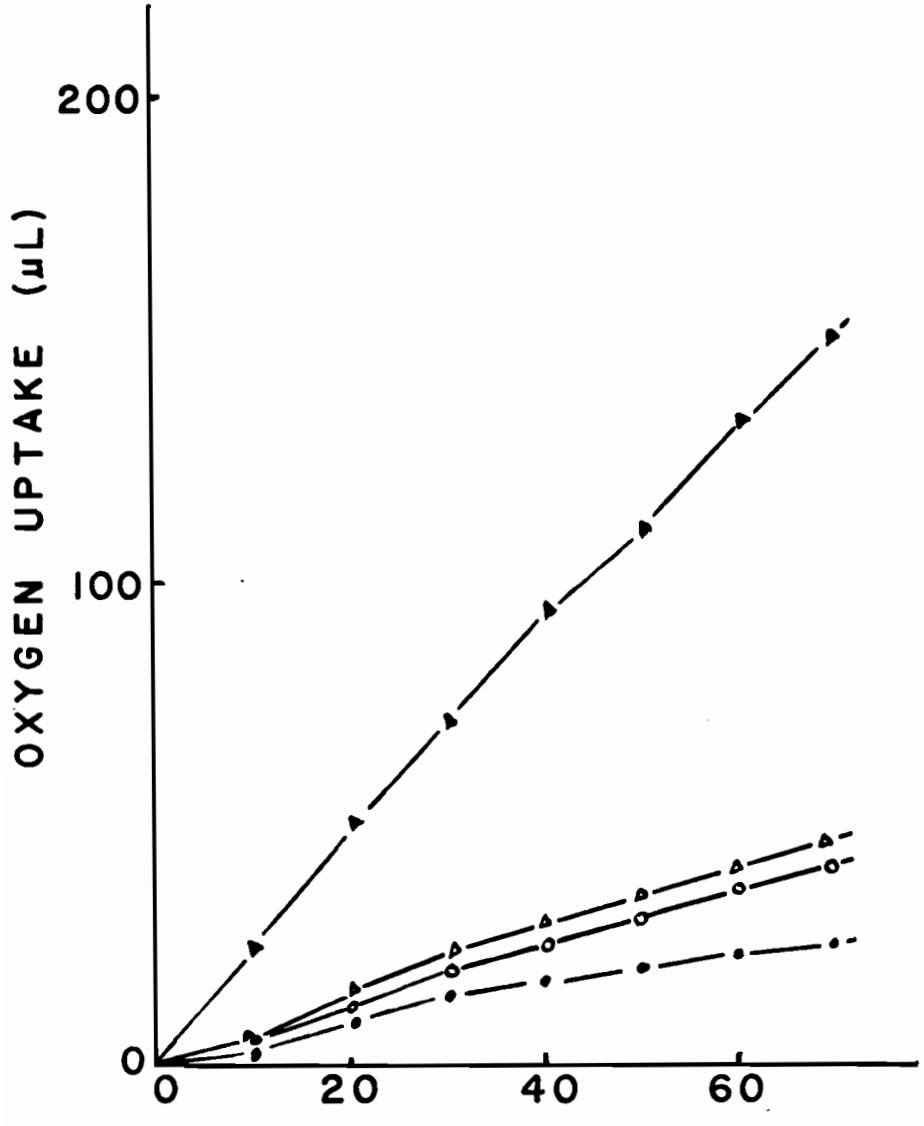


Figure 3 A

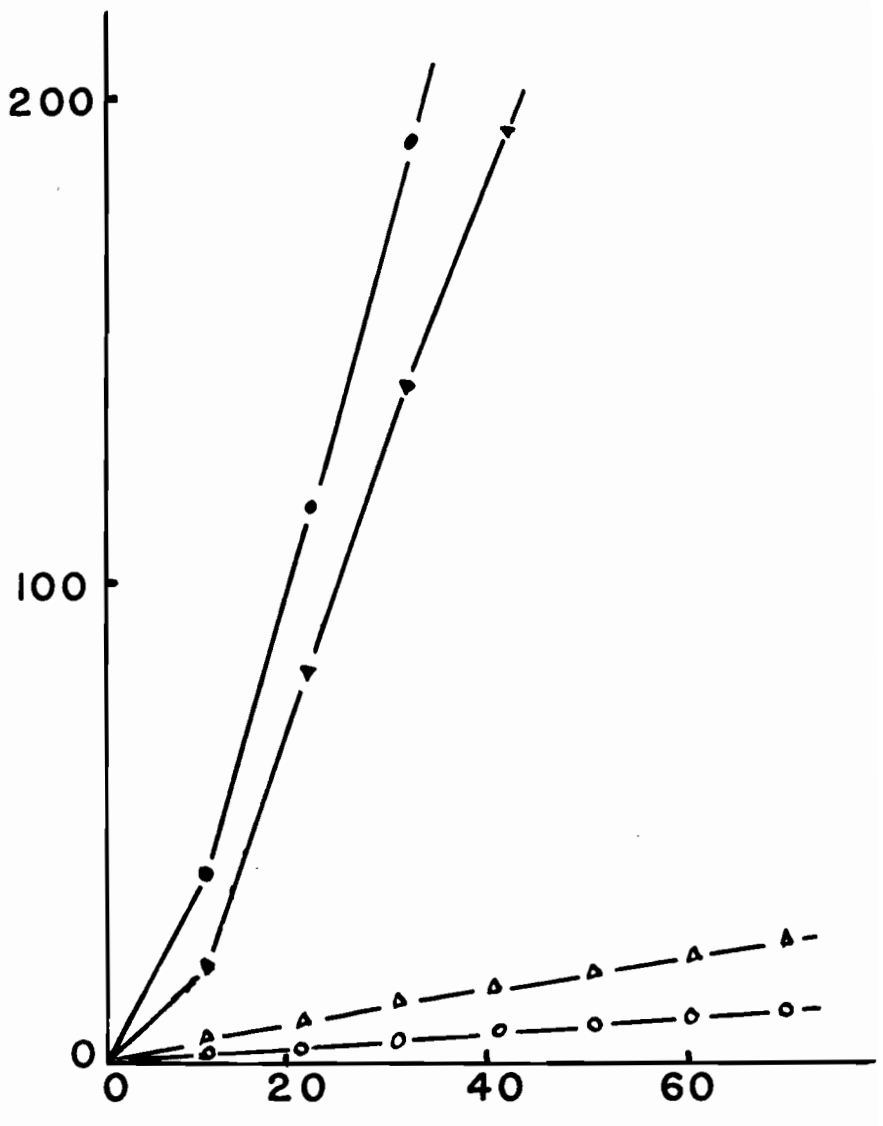


Figure 3 B

Figure 4.

Oxidation of succinate by washed cell suspensions of marine bacterium B-16 (Fig.4A) and mutant B-1 (Fig.4B) in the presence and absence of NaCl.

▲——▲ succinate and NaCl

●——● succinate only

△——△ no succinate and NaCl

○——○ no succinate, no NaCl

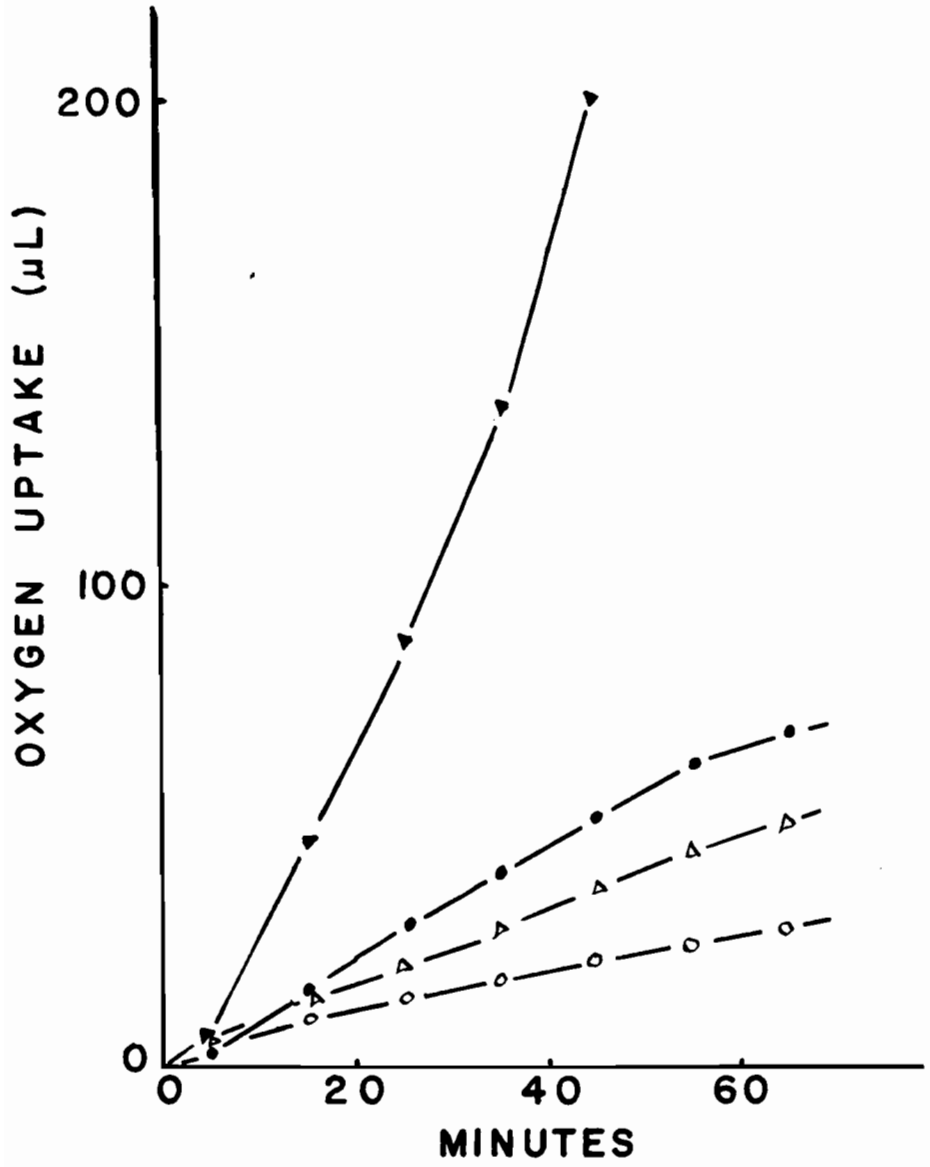


Figure 4 A

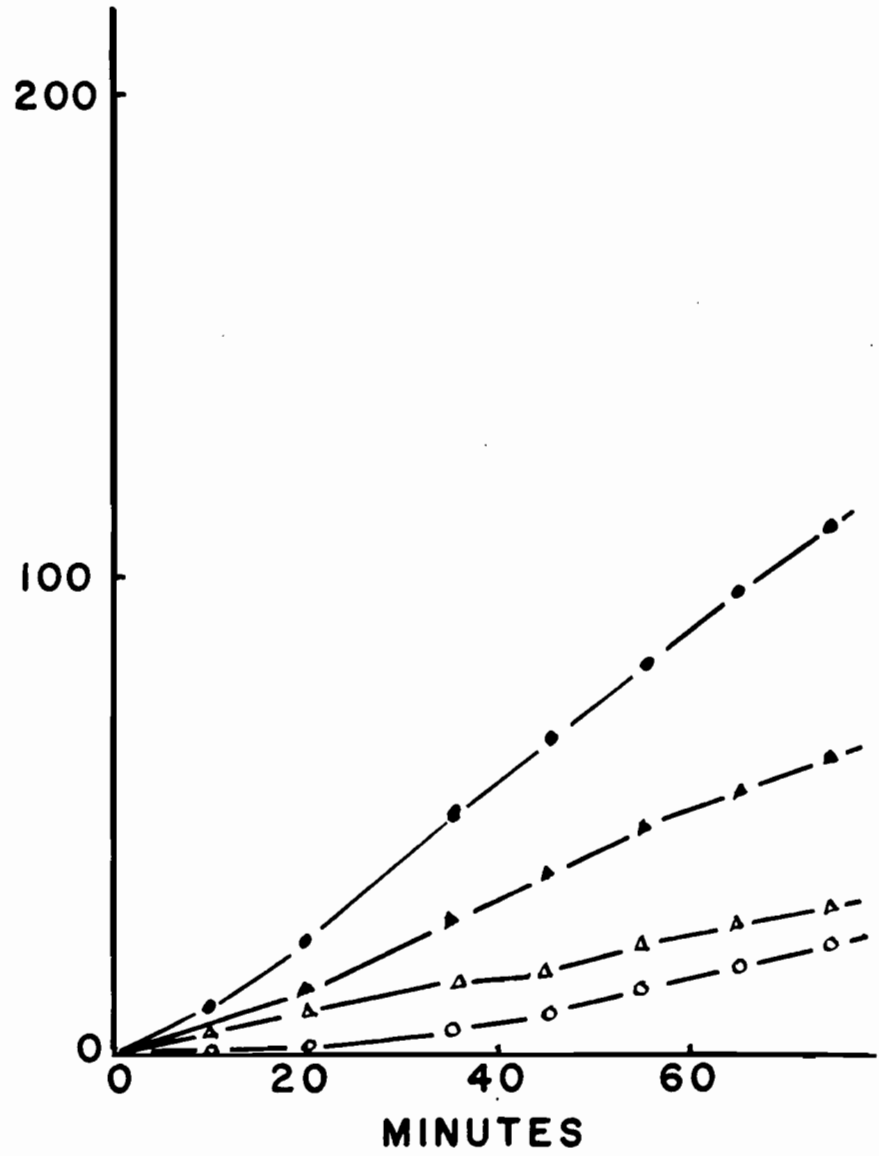


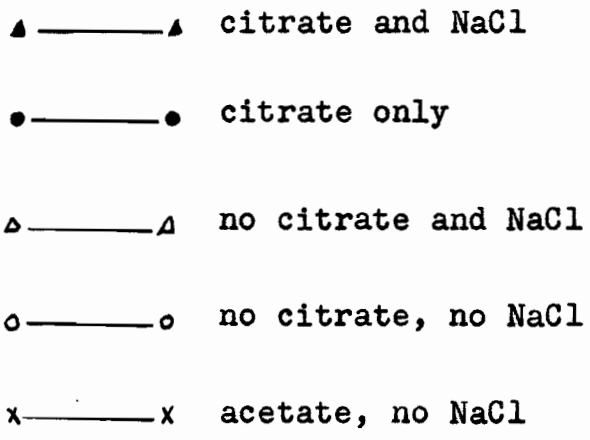
Figure 4 B

(Figure 5). That the cells were metabolically active is indicated by their ability to attack acetate. Like the parent culture, cells of the mutant grown in a medium containing citrate developed a capacity to attack citrate oxidatively (Figure 6B). The mutant attacked the substrate much less rapidly than the parent (Figure 6 A). Also, the results show that Na^+ was required for the oxidation of citrate by B-16. Na^+ inhibited the oxidation by the mutant.

The parent culture, B-16, could attack fumarate without adaptation and malate after adaptation. The mutant B-1 could not be induced to attack either of these compounds. Figure 7 A and B compares the ability of B-16 and the mutant to attack fumarate. Cells of the mutant harvested from a medium containing fumarate did not attack this compound, though as the response to propionate shows, the cells were metabolically active. B-16, on the other hand, in the presence of NaCl, attacked fumarate readily. Similarly, a preparation of mutant cells grown in the presence of malate showed little if any capacity to oxidize malate though they were able to oxidize succinate (Figure 8 B). B-16 oxidized succinate if NaCl was present (Figure 8A).

Figure 5.

Oxidation of citrate by a washed cell suspension of mutant B-1 prepared from cells grown in a medium containing no added citrate.



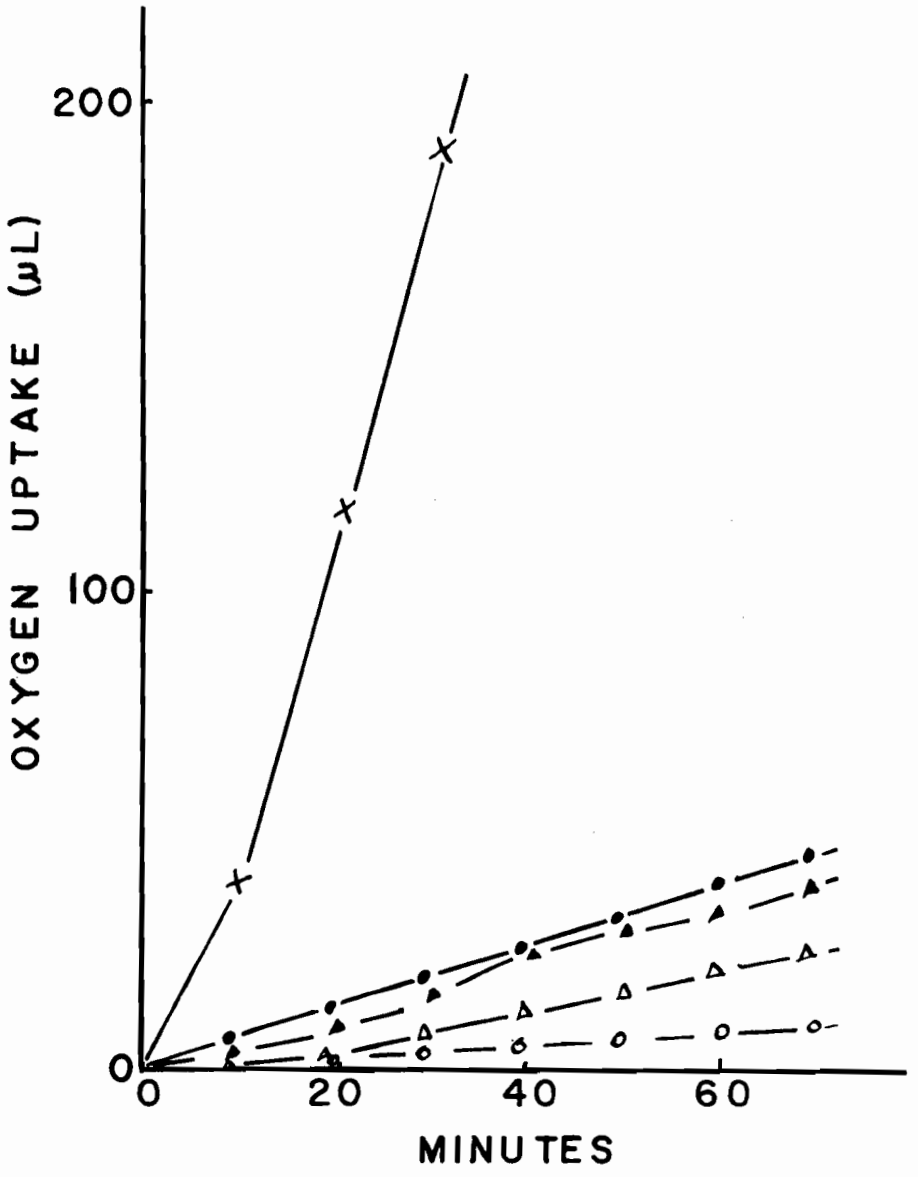


Figure 5

Figure 6.

Oxidation of citrate by a washed cell suspension of marine bacterium B-16 (Figure 6A) and mutant B-1 (Figure 6B) prepared from cells grown in a medium containing added citrate.

▲——▲ citrate and NaCl

●——● citrate only

△——△ no citrate and NaCl

○——○ no citrate, no NaCl.

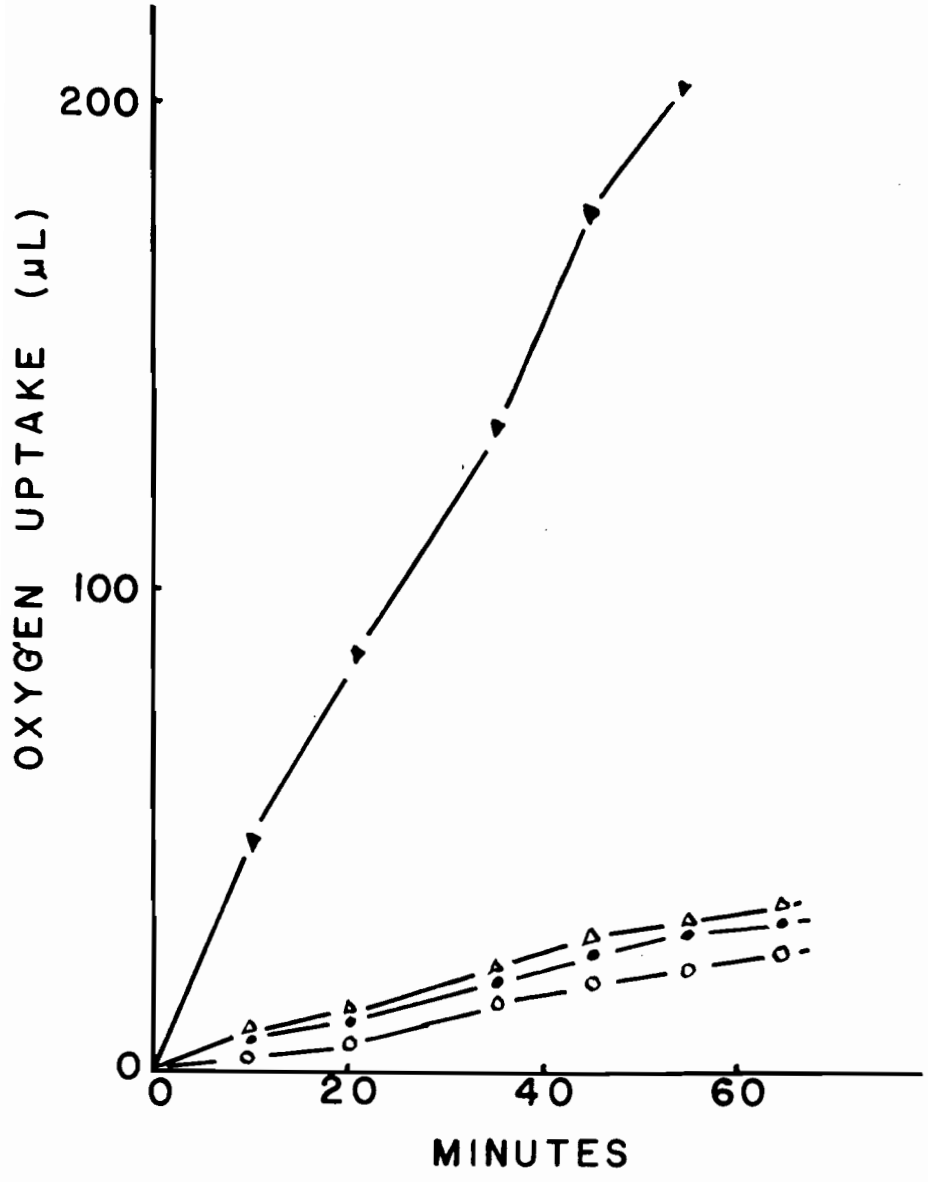


Figure 6 A

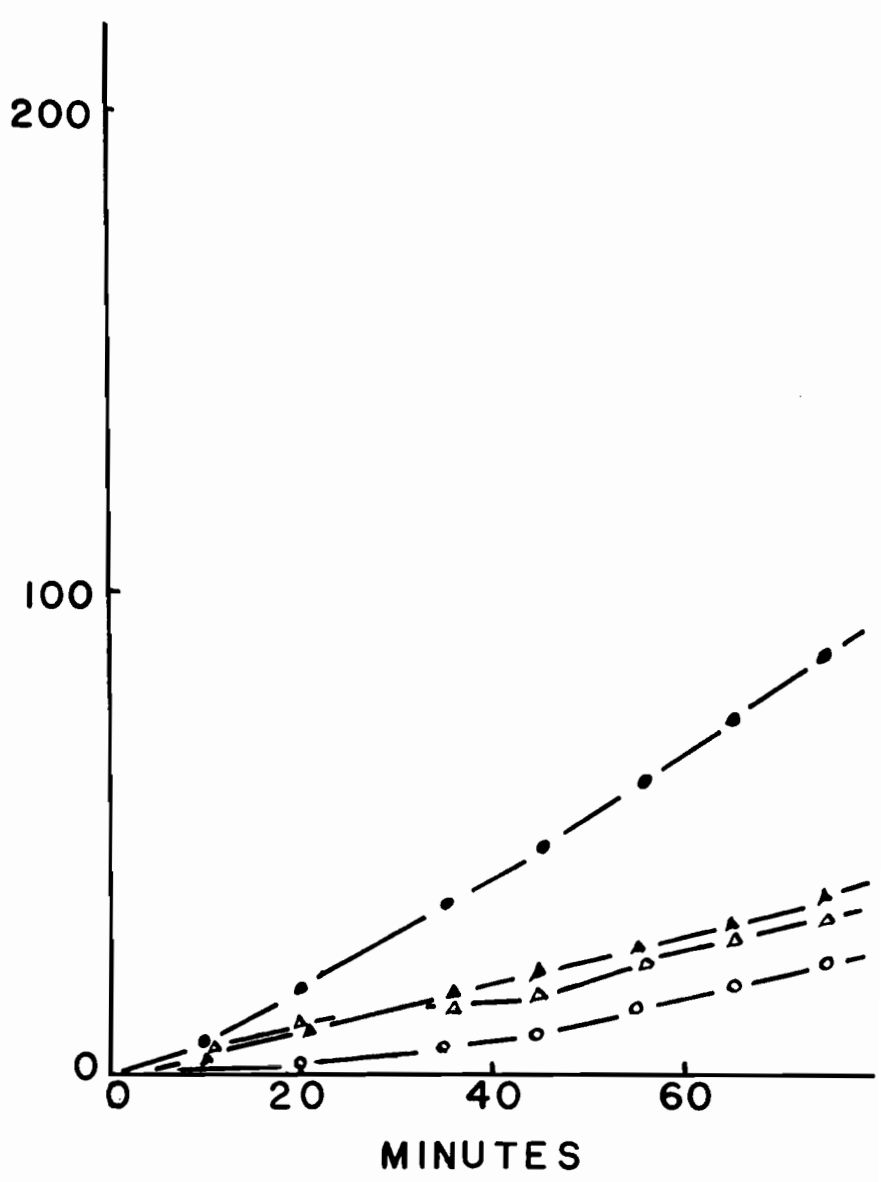


Figure 6 B

Figure 7.

Oxidation of fumarate by washed cell suspensions of marine bacterium B-16 (Fig.7A) and mutant B-1 (Fig.7B) in the presence and absence of NaCl.

▲——▲ fumarate and NaCl

●——● fumarate only

△——△ no fumarate and NaCl

○——○ no fumarate, no NaCl

×——× propionate only

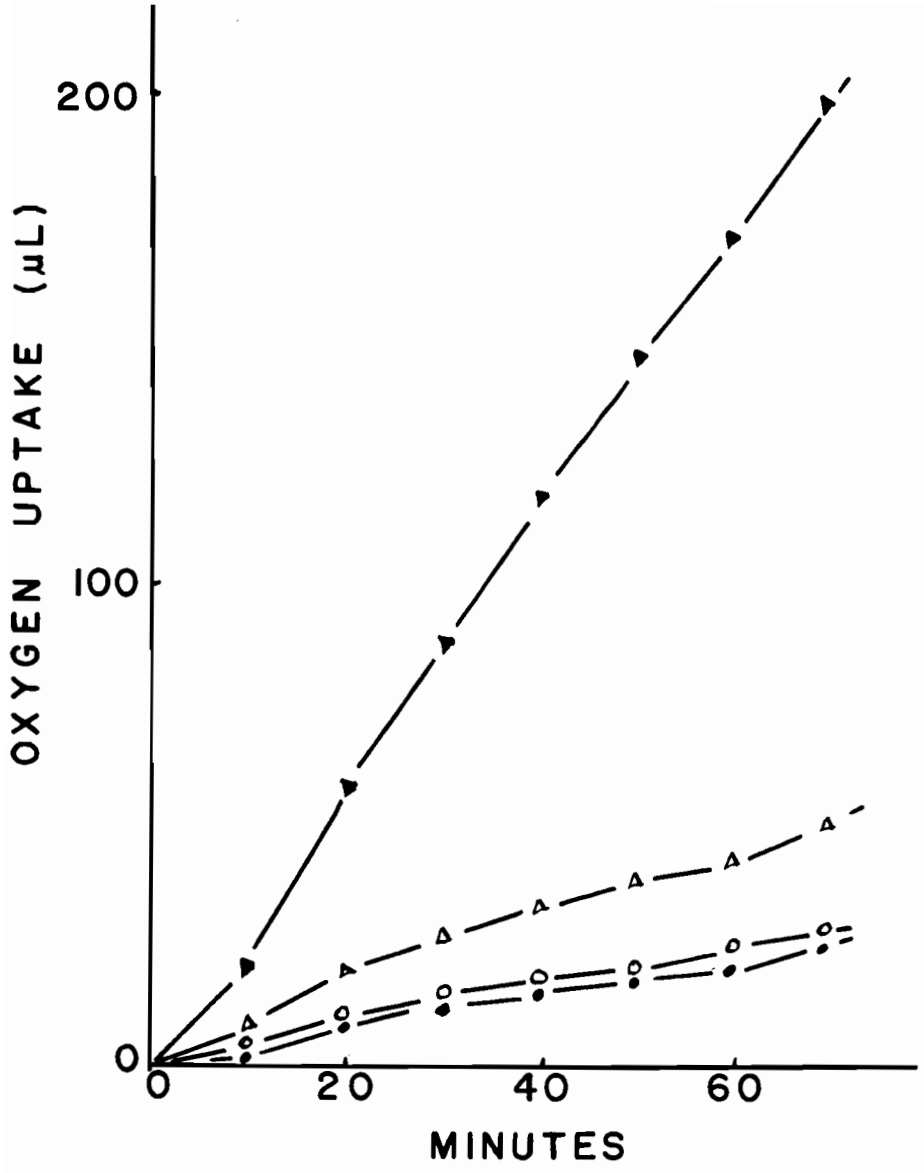


Figure 7 A

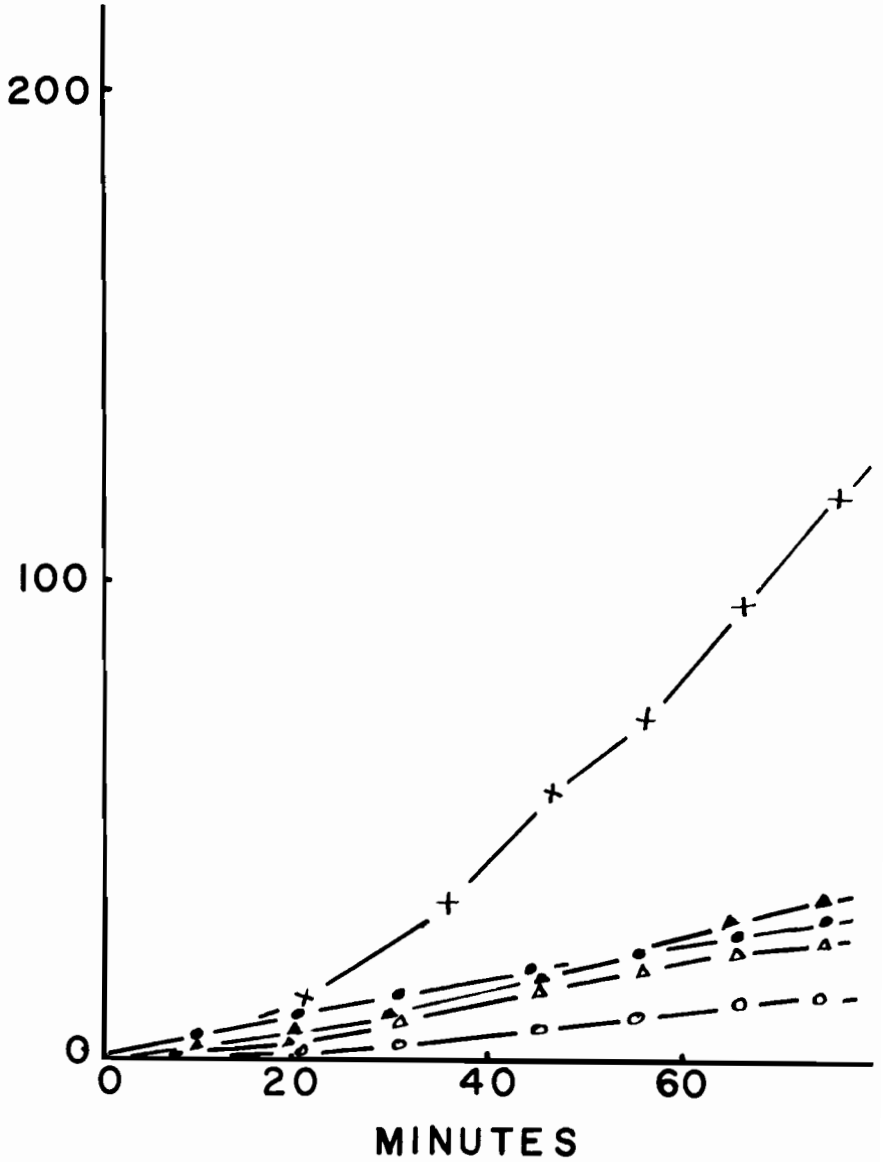


Figure 7 B

Figure 8.

Oxidation of malate by washed cell suspensions of marine bacterium B-16 (Fig.8A) and mutant B-1 (Fig.8B) in the presence and absence of NaCl.

▲——▲ malate and NaCl

●——● malate only

△——△ no malate and NaCl

○——○ no malate, no NaCl

×——× succinate only

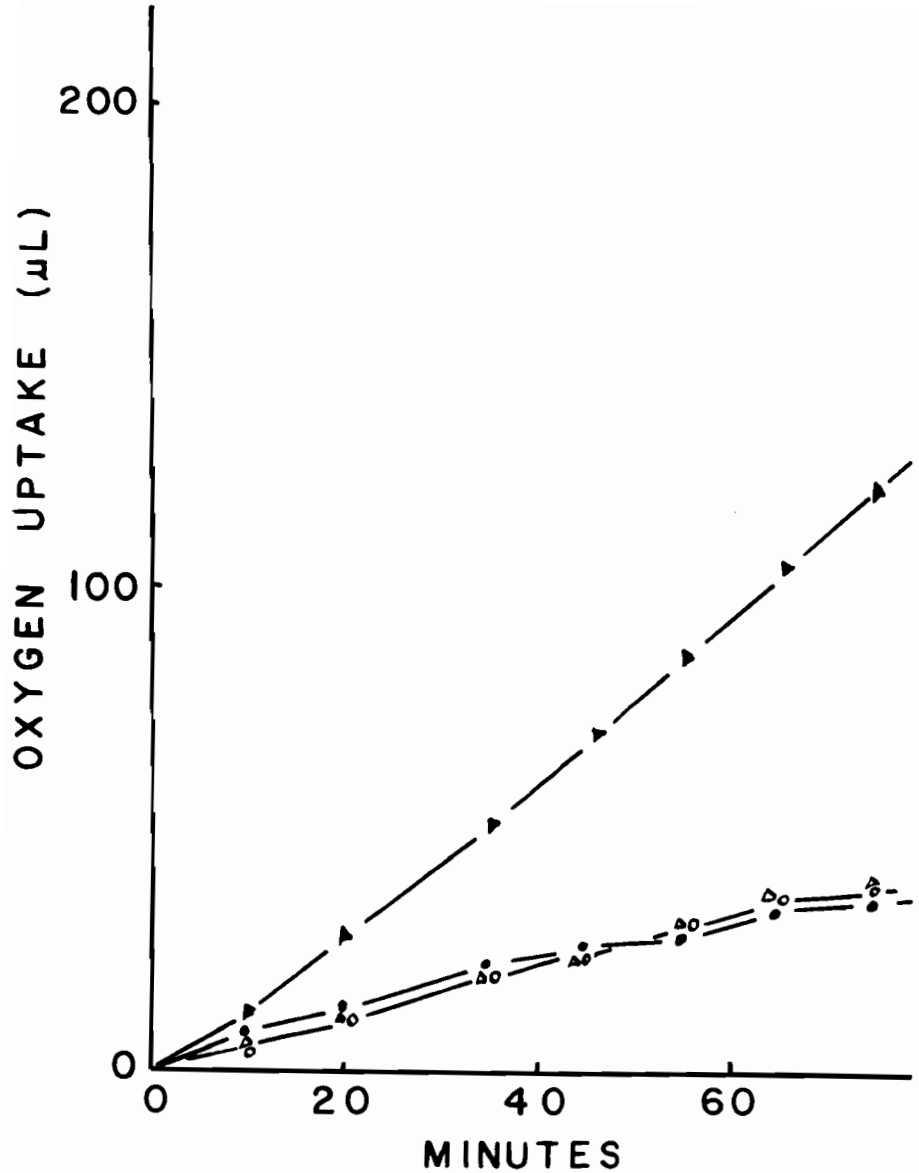


Figure 8 A

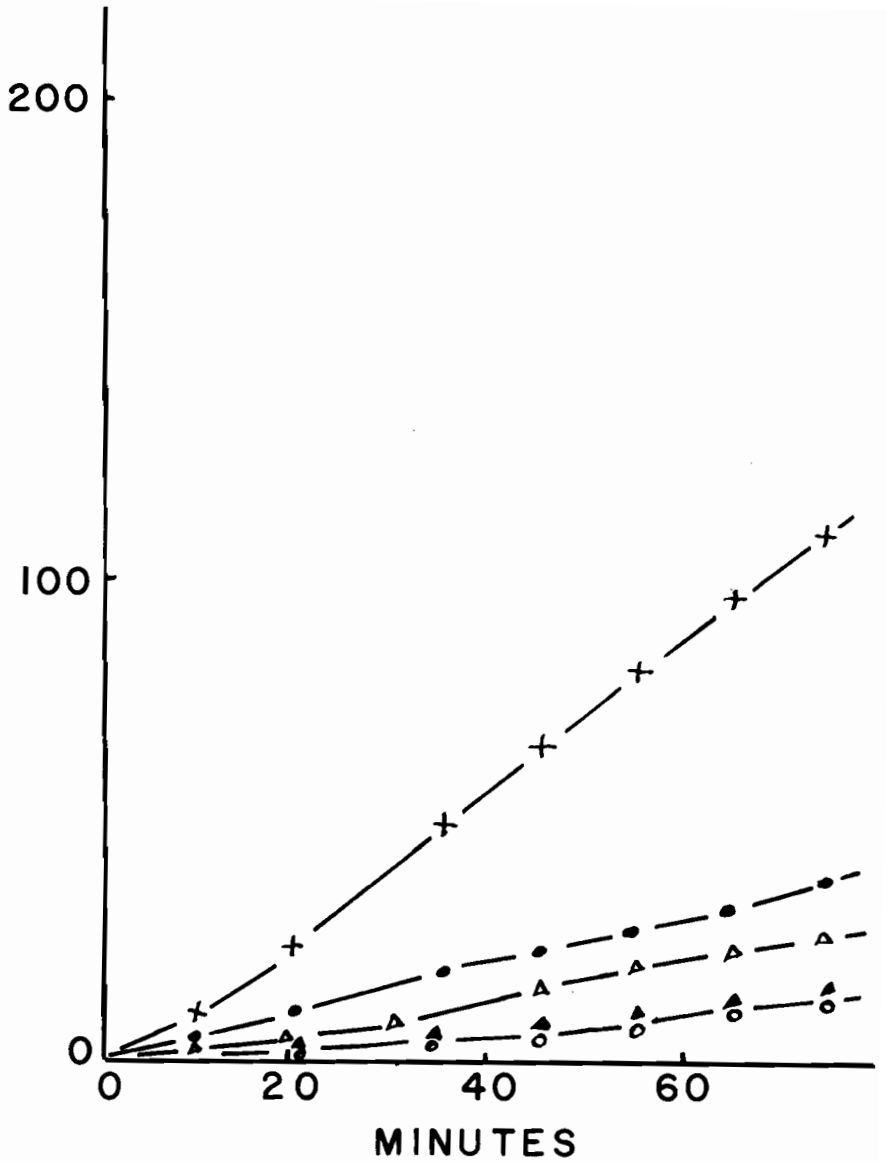


Figure 8 B

It had been observed previously in growth experiments that although the mutants did not require Na^+ for growth, the addition of 0.02 M NaCl to the medium stimulated early growth of the cultures (MacLeod and Onofrey, 1961). To see if Na^+ at this low level might stimulate oxidation of substrates by cell suspensions of the mutants, the effect of adding 0.02 M NaCl to the Warburg vessels was determined using mutant C-2. From the results presented in Table I it is evident that no significant degree of stimulation was obtained in the presence of any of the compounds and in the case of propionate oxidation considerable inhibition resulted from the presence of the Na^+.

Table I. Effect of 0.02 M NaCl on the oxidation of various substrates by washed cell suspensions of mutant C-2.

Substrate	No NaCl	0.02 M NaCl
	O_2 Uptake (ul) x	
Acetate	297.5	298
Butyrate	392.5	385
Propionate	156.5	52
Succinate	72.2	70.3
Citrate	30.9	36.3

x After 70 minutes (corrected for endogenous).

Spectrophotometric Determinations of Fumarase and Malic Dehydrogenase

The fact that the mutants like the parent could oxidize acetate, butyrate, propionate, succinate and citrate but unlike the parent could not oxidize fumarate or malate indicated that a difference had developed in the metabolism of the mutant cells. It was of interest to know if this difference was due to a loss of the enzymes involved in the metabolism of the compounds or to a change in the permeability of the cells. To distinguish between these possibilities, cell-free extracts of the mutants were tested for the presence of fumarase and malic dehydrogenase.

Spectrophotometric studies showed that both enzymes were present in the mutant cells. In the case of malic dehydrogenase, the specific activity of the enzyme in the mutant preparations was much lower than that found in the parent (Table II). Whereas the malic dehydrogenase of B-16 required K^+ for activity, the presence of K^+ at a level optimum for maximum activity of the enzyme in the parent cells inhibited the action of the enzyme in the mutant. Further investigation revealed also that the malic dehydrogenase of the mutants like that of the parent was TPN

Table II. Comparison of the malic dehydrogenase activity in the presence and absence of K^+ of cell-free extracts of marine bacterium B-16 and of three mutants derived from it.

Organism	K^+ Present	K^+ Absent
	Specific Activity ⁂	
B-16	221.8	0
A-1	5.2	21.2
B-1	6.4	52.0
C-2	11.3	53.6

⁂ One unit of activity is the amount of enzyme required to change the optical density 0.001 per minute. Specific activity is the number of these units per mg of protein in the cell-free preparation.

rather than DPN specific.

Table III shows that the reverse situation applied in the case of fumarase activity. Here the mutant extracts contained the more active enzyme. Since the malic dehydrogenase of the mutant was inhibited rather than stimulated by levels of K^+ required to activate the malic dehydrogenase of the parent, it was of interest to know if the amount of K^+ present as a contaminant in the mutant extracts would be sufficiently high to mask the effect of K^+ for this enzyme.

Determination of K^+ in the reaction systems used to determine enzyme activity (Table IV) showed that those prepared with extracts from the mutants did indeed contain more K^+ than those prepared with extracts of B-16. The concentration in the reaction mixtures, however, was far less than the 167 mM concentration of K^+ required for maximum activation of the enzyme from organism B-16. It is of interest though that reaction mixtures prepared with extracts of the mutants contained more K^+. This extra K^+ must have come from the extracts and hence from the cells of the mutant organisms.

Table III. Comparison of fumarase activity in cell-free extracts of B-16 and three mutants derived from it.

Organism	Specific Activity *
B-16	131.4
A-1	245
B-1	240
C-2	427

* See footnote, Table II.

Table IV. Amounts of K^+ present as a contaminant in reaction mixtures used for the determination of the malic dehydrogenase activity of cell-free extracts of B-16 and three mutants derived from it.

Organism used to prepare extract	K^+ conc. (mM)
B-16	.071
A-1	2.0
B-1	3.0
C-2	4.7

DISCUSSION

Washed cell suspension of the three mutants A-1, B-1, and C-2 were found to be capable of oxidizing acetate, butyrate, propionate, succinate and citrate in the absence of Na^+. It is possible that fumarate and malate are oxidized also though a very low rate. Oxygen uptake was always higher in the presence than in the absence of these substrates. No noticeable differences in the rate of oxidation of the substrates were observed among the three mutants. Concentrations of Na^+ optimum for oxidation of the substrates by the parent cells were inhibitory for the mutants. Even low concentrations of NaCl (0.02M) did not stimulate oxidation of the substrates and in one case (propionate) inhibited it. It is thus obvious that Na^+ is no longer required by these organisms for the oxidation of these substrates.

When the oxidative capacity of these mutants towards the substrates examined is compared with the parent culture, it is apparent that some differences exist here also. Whereas the parent culture could oxidize both fumarate and malate rapidly, the mutants failed to do so even after the substrates had been included in the growth medium in an effort to adapt

the cells. Another difference was shown by the fact that the mutants oxidized the monocarboxylic acids more readily than the parent, with the exception of propionate while, on the other hand, the di- and tricarboxylic acids were not so rapidly attacked. When the cells of B-16 and the mutants were washed under the same conditions, extracts of the latter were found to contain more K^+ than the former. Moreover, the enzyme malic dehydrogenase behaved differently from the enzyme isolated from B-16 in that it did not require K^+ for maximal activity.

This brings to four the total number of differences between the mutants and the parent in the present study. These four differences should be added to the differences observed by MacLeod and Onofrey (1961). These were, firstly, that the mutants did not lyse after growth had ceased or when suspended in distilled water; secondly, they had lost the capacity to liquify gelatin; and thirdly, two of the mutants, B-1 and C-2 had acquired the ability to reduce nitrates.

The possibility is inconceivable that these seven differences between the parent and the mutant could have occurred as a result of seven genetic changes occurring in the course of one irradiation. For these

changes to have arisen as a result of a one-step mutation it is necessary to assume that those properties which underwent change on irradiation were maintained in the parent culture by a sequence of steps having in common a Na^+ dependent step which could be eliminated by mutation.

Although these observations do not establish the function of Na^+ in marine bacteria, it is possible to conclude from these observations that the function of Na^+ probably can be localized in the bacterial envelope. Indeed, most of the changes that have occurred by mutation, that is by losing the Na^+ requirement, can be explained in terms of a change in the composition of the cell wall with a resultant modification of the permeability of the cell membrane.

For instance, the mutant cells do not appear to be as permeable to substrates as the parent cell. The monocarboxylic acids are oxidized by B-16 at about the same rate as the di- and tricarboxylic acids. In the mutants, the monocarboxylic acids are oxidized more readily. In B-16, adaptation to malate and citrate involves induction of a transport mechanism since unadapted cells contain the TCA cycle enzymes (MacLeod and Hori, 1960). Since the mutant also contains

fumarase and malic dehydrogenase, failure to oxidize fumarate and malate would appear to be due to the lack of an inducible transport mechanism in these cells. It is true that malic dehydrogenase was one quarter as active in the mutant as in B-16. If this were the factor limiting malate oxidation, oxidation of the compound should have been reduced by only seventy-five per cent. Actually, it was completely eliminated. Hence the relative activity of the enzymes alone cannot account for the lack of oxidation of the substrate. It has been shown also that cell-free extracts of the mutants contain more K^+ than the cell-free extracts of B-16. It appears that this too might be due to a difference in permeability of the cells.

Unlike the malic dehydrogenase of B-16, this enzyme in the mutants was found to be less active and did not show any stimulation by K^+. These two observations cannot be directly explained by a change of permeability of the cell envelope. However, it is known that the tricarboxylic acid cycle enzymes of bacterial cells are localized in the membrane (Marr, 1960). Any mechanical modification of the cell envelope might have hampered or modified the enzyme action. This could also explain the reason why the mutants grow very much more slowly than organism B-16. Finally, the fact

that the mutant cells do not autolyse detectably in the medium after growth has ceased, a phenomenon which occurs in B-16, and the fact that they do not lyse when washed in distilled water are obvious indications that the wall-membrane complex of these organisms has been modified.

Two of the differences between the parent and the mutants are less easy to explain on the basis of a modified cell wall composition. One is the failure to liquify gelatin, the other is the acquisition of a capacity to reduce nitrate.

It is interesting to note that the incorporation of amino acids into the protein of isolated thymus nuclei is sodium-dependent (Allfrey et al. 1961). This dependence reflected the operation of sodium specific "transport" or "gate" mechanisms which admit amino acids into the intranuclear "pool". It will be interesting to determine if a similar mechanism is present in marine bacterium B-16.

PART II

EFFORTS TO TRANSFORM NON-Na^+ REQUIRING MUTANTS OF A MARINE BACTERIUM TO Na^+ DEPENDENCE

INTRODUCTION

It has been postulated that both terrestrial bacteria and halophilic bacteria have evolved from marine bacteria, the former by developing an ability to grow without Na^+ and the latter by becoming adapted to growth in the presence of high concentrations of salt (MacLeod *et al*. 1957).

The fact that mutants capable of growing in a Na^+ deficient medium could be produced by ultraviolet irradiation of a Na^+ requiring strain gave good support to the suggestion that terrestrial bacteria could have been derived from marine bacteria. As a definite proof that these mutants are immediately derived from the parent B-16 it was also of interest to attempt to transform these mutants into genotypes requiring Na^+ for growth.

For this purpose the non-Na^+ requiring mutants were treated with DNA preparations from the parent Na^+ requiring cells. Conditions were then chosen which were designed to permit the selection of any transformant which could be produced. Various methods for deoxyribonucleic acid isolation and purification have been tried. Environmental conditions which have been

shown to favor transformation in terrestrial organisms have been selected in an effort to obtain transformants.

REVIEW OF LITERATURE

A transformation reaction may be defined as "an hereditary alteration in a susceptible cell resulting from the acquisition from its environment, by other than sexual means, of a genetically active unit directing the inheritable change" (Austrian, 1952).

The nature of transforming principle

Bacterial colonial forms designated by smooth and rough colonies indicating the presence or absence of capsular materials in pneumococci were reported by Griffith (1923). In 1928, he noted bacterial transformation of this organism from a type which produced a smooth colony to one which produced a rough one in a study of the conditions responsible for the acquisition of a capsule by an uncapsulated strain.

In 1944, Avery et al. showed the activity of the transforming principle of pneumococcus to be associated intimately with a desoxyribonucleic acid fraction of that organism. Subsequent studies by McCarty (1946) and by McCarty and Avery (1946) on the purification and action of deoxyribonuclease upon pneumococcal transforming principle gave added support

to the idea that the biologic specificity of transforming principle resided in nucleic acids of the deoxyribose type.

Cellular Competence in Bacteria

In addition to those of the pneumococci, transformation reactions have now been described employing strains of Hemophilus influensae (Alexander and Leidy, 1950), Escherichia coli (Boivin, 1947), Shigella paradysenteriae (Weil and Binder, 1947), Neisseria meningitidis (Catlin, 1960), Bacillus subtilis (Spizizen, 1958), Xanthomonas phaeseoli (Corey and Starr, 1957), Agrobacterium tumefaciens (Klein and Klein, 1956) and a Rhizobium strain (Szende et al. 1961). In all species, transformation has been restricted to certain strains, other seemingly similar strains failing to participate in the reaction under comparable conditions. In each instance, the transformation principle has been derived from cells of a heterologous type within the same species. Little is known concerning the basis in bacterial cells upon which competence in transformation reaction rests, though a few data relevant to this subject are available.

It has been found (Neufeld and Levinthal,

1928), for example, that of two strains of pneumococcus tested for ability to be transformed the one which was more susceptible to bile solubility participated readily in the reaction, whereas the more difficultly soluble one did not.

A second factor related to the competence of an organism with regard to a specific transformation may reside in its genetic constitution. Some genetic control of the attainment of the competent state has been suggested by recent studies (Young & Spizizen, 1961) which indicate that sensitivity to transformation occurs at a stage of growth in which presporulating events occur. Strains of B.subtilis unable to undergo sporulation did not become competent. It was suggested that some alterations in the structure of the cell wall associated with presporulation physiology are the basis for competency in these strains.

Another factor which may have a bearing upon the ability of a bacterial cell to undergo transformation is the capacity of the cell to produce extracellular desoxyribonuclease. It is not unreasonable to anticipate that difficulty may be encountered in effecting transformations within such bacterial species as group A- hemolytic steptococci which introduce into the

environment large amounts of desoxyribonuclease. Similarly, DNA added to a culture filtrate of E.coli was found to be depolymerized (Sinai and Yudkin, 1959). Hotchkiss (1954) observed a transformable cycle in pneumococci. On synchronization of the culture more cells were found to be transformed at one particular stage of the growth cycle than at any other.

Similarly it had been found (Anagnostopoulos and Spizizen, 1961) that the development of sensitivity to transformation in a B.subtilis strain occurred toward the end of the logarithmic phase. In addition, it was established that a metal chelating agent such as L-hisdidine was required. The presence of relatively large amounts of amino acids, such as are present in acid hydrolyzed casein, reduced the sensitivity to transformation, presumably by permitting the synthesis of wall components which may have prevented DNA uptake.

It has been shown (Young and Spizizen, 1961) (Lerman and Tolmach, 1957) that when cells become sensitive to transformation they are capable of taking up highly polymerized DNA irreversibly. The ability to take up DNA may involve some structural changes in the cell wall which permit the penetration of the macromolecules. In addition, some enzymatic mechanism for

the active uptake of DNA may be developed, as suggested by Anagnostopoulos (unpublished data) which showed that certain metabolic poisons inhibited uptake.

Environmental Conditions for Bacterial transformation

The environmental conditions under which transformation reactions will take place vary among bacterial species. Those permitting transformation of pneumococci are complex. Reactions carried out "in vitro" require, in addition to a nutrient medium, certain factors present in serum or in serous fluids (Avery, MacLeod & McCarty, 1944).

It has been shown that the transformation of H.influenzae (Alexander and Leidy, 1951) will take place when competent cells are placed in contact with transformation principle for 15 minutes in an environment which fails to support multiplication.

MATERIALS AND METHODS

Organisms

The organisms used in this study were organism B-16, a Na^+ requiring organism and three non-Na^+ requiring mutants derived from it, A-1, B-1 and C-2. These organisms were described in some detail in Part I.

Isolation of DNA

Three methods for the isolation of highly polymerized DNA from organism B-16 were used.

The first method was one described by Hotchkiss (1957). The cells were harvested by centrifugation from one liter of nutrient broth-yeast extract medium and resuspended without washing in about 10 ml of 0.05% Na citrate. Lysis was initiated by adding 2 ml of 5% sodium deoxycholate and the mixture was then left at room temperature with occasional shaking. Changes were noted within one to two minutes. The culture became progressively more ropy and then viscous. After five minutes, no further change was apparent, and 50 ml of chlorophorm and 1 ml of isoamyl alcohol were added. Vigorous mechanical shaking in a well-stoppered

centrifuge bottle for 15 to 20 minutes led to protein denaturation as indicated by flocculation. Upon centrifugation three layers were formed, a bottom layer of chloroform, an intermediate layer of denatured protein-chloroform emulsion, and a cloudy yellowish, viscous supernatant solution containing DNA on top. The top layer was removed with the aid of a pipette and treated with chloroform and iso-amyl alcohol twice more. This was sufficient to remove all of the protein.

The layer containing the DNA was removed and 25 ml of 95% alcohol were added. This caused the DNA to precipitate. The stringy precipitate was collected promptly by winding on a clean glass rod. It was then dispersed in 0.85% saline with shaking. Two further alcohol precipitations of the DNA were used to remove the chloroform and iso-amyl alcohol, and any low molecular weight impurities remaining. The DNA fibers were then suspended in 0.85% NaCl, made 70% with respect to ethanol and held for 18 hours at 4° C. to destroy any living cells that might be present. The strands were collected from the suspension by filtration on a sterile filter, washed with a few ml of saline and finally solubilized in saline. The strands solubilized with great difficulty.

The second method for the isolation of transforming principle was the one devised by Mayers and Spizizen (1954) and modified by Sinai and Yudkin (1959). An 18 hr culture of the organism was centrifuged and washed once with saline containing 0.2 M sodium citrate. To the packed organisms was added 6% (W/V) sodium dodecylsulphate (Duponol). Lysis began immediately and was completed after stirring at 500 revolutions per minute for 45 minutes with a magnetic stirrer. 120 ml of a 3 M solution of Na acetate at pH 7 was added, the mixture warmed to 60° C. and stirred for 15 minutes. It was then cooled to 5° C. and centrifuged.

The supernatant fluid was added to 2.8 volumes of acidified alcohol (95%, V/V ethanol, 5%, V/V methanol, with 1 part concentrated HCl to 175 parts mixture). The strings of DNA were removed with a glass rod and then washed with 100 ml ethanol in a Buckner funnel. The DNA was placed in a centrifuge tube with 90% ethanol and left overnight at 4° C. for sterilization. The DNA was centrifuged from suspension and resuspended and dissolved in an arbitrary volume of saline.

A third method developed by Huppert and Rebeyrotte (1960) which was claimed to be an improvement over other methods in use, was also investigated.

Cells of the donor organism were washed and resuspended in a volume of 0.01 M phosphate buffer pH 7.2 containing 0.02 M EDTA equal to the volume of the growth medium. Sodium laurylsulfate was added to a final concentration of 0.4%. Instead of using the Mickle apparatus employed by the original investigators, a Waring blendor was used at low speed for 10 minutes to facilitate cell destruction. An equal volume of water-saturated phenol was added to the lysate and stirring was continued for 10 additional minutes. After centrifugation the aqueous layer was treated twice more with water-saturated phenol. The nucleic acids were rapidly precipitated from the final suspension with 2 volumes of cold ethanol in the presence of 2% Na acetate. The precipitate was removed immediately by centrifugation, and washed 4 times by resuspension and centrifugation from a solution of 2 volumes of ethanol and one volume of 0.14 M NaCl. After sterilization in a 0.85% saline solution made 70% with respect to ethanol the DNA preparation was finally dissolved in a solution of 0.14 M NaCl, 0.01 M glycine.

Selective Media

One of the important technical problems in transformation is the devising of a way to select

transformants from a much larger population of untransformed cells. In the case of transformation to Na^+ dependence, selection could not be based merely on a requirement for Na^+ since both parent and mutant cells grew at levels of Na^+ required by the parent for growth.

In order to prevent the growth of non-transformed cells and encourage the growth of any transformed mutants three different media have been used. The first one was a nutrient broth-yeast extract agar medium containing 0.5 M NaCl. This medium was chosen because the parent Na^+ requiring cells were known to be able to grow at this concentration of NaCl while the mutant non-Na^+ requiring cells were not. Use of this selective medium was based on the assumption that non-Na^+ requiring mutants transformed to Na^+ dependence would also acquire the capacity to resist the high concentration of NaCl.

A second selective medium was used containing fumarate as sole carbon source. The use of this medium was based on the fact that the parent culture but not the non-Na^+ requiring mutant could use fumarate as a sole carbon source (cf:Part I). Again it was assumed that transformation of the non-Na^+ requiring mutant to Na^+ dependence would at the same time confer on the

mutant the parent's capacity to grow on fumarate.

A third selective medium was also devised to increase the possibility of selecting transformants. Since the parent culture could liquify gelatin it could grow profusely on a medium containing gelatin as a sole source of carbon and nitrogen. The Na^+ independent mutant did not liquify gelatin and did not use it as a carbon source. It was concluded that transformation of the mutant to Na^+ dependence might also restore the capacity to liquify gelatin. Thus a Na^+ containing gelatin medium was used in an effort to select Na^+ requiring transformants from a population of non-Na^+ requiring mutants.

In principle, the best method of selecting transformants from the original untransformed cells would be to apply the penicillin technique of Davis (1948) and Lederberg and Zinder (1948). This technique is based on the fact that penicillin destroys multiplying but not resting cells. In the present case, a cell suspension treated with transforming principle could be plated on a medium containing penicillin but no added Na^+. Untransformed cells would multiply in the absence of Na^+ and be destroyed by the penicillin. Overlaying this medium with one containing Na^+ would then permit

the isolation of transformants. Unfortunately, the mutant cultures were all resistant to the action of penicillin. At a concentration of penicillin as high as 8000 ugm per ml the mutant cells were not converted to protoplasts after 12 hours in the growth medium. Because of the resistance of untransformed cells to the antibiotic, the penicillin technique could not be employed.

Growth Media

Three different growth media were used, a synthetic medium, a trypticase medium, and a nutrient broth-yeast extract medium. The same salt solution as was described in Part I served as the diluent for the three media. The synthetic medium contained: L-alanine 0.027 M; aspartic acid, 0.0072 M; and glutamic acid, 0.0073 M dissolved in the salt solution. Trypticase medium was prepared by making the salt solution 1% with respect to B.B.L. trypticase. The nutrient broth-yeast extract medium contained 0.8% Difco nutrient broth and 0.5% yeast extract dissolved in the salt solution.

Procedure

Cells of the organism to be transformed were inoculated into 10 ml of one of the three above-mentioned

media and incubated with shaking for 18 to 24 hours. Five ml of the culture were then transferred aseptically to a sterile 25 ml flask containing 5 ml of the same medium. 0.5 ml of the DNA solution isolated from parent cell was added and the flasks were incubated for periods varying from 30 minutes to 18 hours at 25° C. At the end of each incubation period 0.5 ml volumes were withdrawn and spread over the selective media. The plates were incubated at 20° C. for 48 hours.

Various Modifications.

The addition of 0.05% Na^+ citrate to the medium for transformation studies was found to enhance transformation in Bacillus subtilis (Spizizen 1958). This addition was made in various experiments. Duponol (sodium lauryl sulfate), found to increase the number of transformants in E.coli, presumably by altering the cell wall (Sinai and Yudkin, 1959), was also tried at 0.1% concentration in the transforming medium.

Some bacterial species liberate DNA in the culture medium and culture filtrate have been used for the transformation of virulence in Agrobacterium tumefaciens (Klein and Klein, 1956). In one experiment the mutant cells were incubated with the filtrate of an

18 hour old broth culture of B-16.

Hotchkiss (1954) showed that transformation of pneumococci occurred only during a short period of the division cycle. Synchronization was attempted by cooling the organisms at 4° C. for 18 hours, before adding the transforming principle. It is not known, however, if the cells were dividing synchronously after this treatment.

RESULTS

DNA preparations from organism B-16 were prepared in the three ways described. Each preparation was used in an attempt to transform each of the 3 mutants to Na^+ dependence. Transformations were attempted in each case in each of the three growth media. No colonies appeared on any of the selective media on plating the transforming systems.

None of the additional modifications described gave rise to Na^+ dependent transformants.

DISCUSSION

The phenomenon of transformation is a very complex one involving a great number of variables. Furthermore, there is no report in the literature concerning transformations involving any species of pseudomonads. Since negative results are not usually reported, it is not known if any attempts have been made to bring about such transformations. In view of the failure to obtain transformants, it would be desirable to see if other factors known to be transformable in other bacteria, such as drug resistance, could be transferred in these organisms. Even if a drug resistance character could be transferred, it would still be unknown if the character for the Na^+ requirement would be taken up by the cell.

Another difficulty in this work lay in the selective media that had to be used. The use of salt concentrations high enough to inhibit the growth of untransformed cells, or the use of nutrients that can be utilized by the prototrophs but not the auxotrophs may not be suitable for selecting transformants. Use of these media was based on the assumption which may be unwarranted, that by reacquiring the Na^+ requirement for growth, other characters such as the ability to

grow on gelatin, fumarate or 0.5 M NaCl would be acquired also.

PART III

STUDIES ON AN ADENOSINETRIPHOSPHATASE IN MARINE BACTERIUM B-16

INTRODUCTION

A sodium-potassium-activated adenosinetriphosphatase, possibly related to the active, linked transport of sodium and potassium across the cell membrane has been shown to be present in the plasma membrane of various animal cells.

Since various marine bacterial species have been shown to resemble animal cells in requiring both sodium and potassium for growth and metabolism it was of interest to determine if a membrane adenosinetriphosphatase might be involved in the transport of sodium and potassium in these cells.

REVIEW OF LITERATURE

Skou (1957, 1961) described a sodium activated adenosinetriphosphatase (ATPase) in the submicroscopic particles of crab nerve (*Carcinus maenas*). This enzyme requires magnesium ions for activity, is stimulated by sodium and potassium ions, and is inhibited by calcium ions and high concentrations of potassium ions. His observations led him to the conclusion that this enzyme "may be involved in the active extrusion of sodium from nerve fiber". Jarnefelt (1960, 1961) reported a sodium-activated, magnesium requiring ATPase in the submicroscopic particle fraction of rat brain, which was however not stimulated by potassium ions. More recent experiments, (Jarnefelt, 1961a) have shown that the stimulation of the microsomal ATPase by sodium ions could be considerably increased by the presence of small amounts of potassium. Nevertheless, potassium alone has no effect. These findings abolished the main discrepancy between the results obtained by other workers and Jarnefelt.

A similar enzyme was described by Post *et al.* (1960) and by Dunham and Glynn (1961) in the human erythrocyte membrane. Their studies indicated a role of the erythrocyte enzyme in the simultaneous extrusion of sodium ions and the accumulation of potassium ions by

active transport, across the cell membrane. Identification of the ATPase with Na^+ and K^+ transport was based by Post et al. on the following observations. Both the ATPase activity and transport require sodium and potassium ions together, not separately. Both are inhibited by ouabain, both accept ammonium ion in place of potassium ion; and both show a competitive inhibition of potassium ion activation by high concentrations of sodium ion. Furthermore, both were located in the membrane and both utilized adenosine triphosphate in contrast to inosine triphosphate.

Besides its presence in crab nerve, rat brain, and erythrocyte ghosts, a Na^+-K^+ activated ATPase has also been reported in guinea pig kidney (Post et al. 1960), in various tissues of the cat, and in human retina, ciliary body, and erythrocytes (Bonting et al. 1961). Highest activities were noted in nervous tissues and in tissues concerned with secretory function.

Adenosinetriphosphatases have also been studied in bacteria although no roles have yet been suggested for these enzymes. A heat stable ATPase was first described by Marsh and Militzer (1956) in Bacillus stearothermophilus. This enzyme was further located in a red fraction which was considered to be the cell

membrane (Georgi et al. 1955). The pH optimum for maximal enzyme activity was found to be about 8.5. Activity of the enzyme reached a maximum when the ratio of ATP to Mg^{++} was 2.0 but fell off rapidly to zero with increasing ATP concentration.

An ATPase has also been found in preparations of Streptococcus feacalis cell membranes (Abrams et al. 1960). These membranes contained an adenosinetriphosphatase which catalysed the reaction ATP-ADP - inorganic phosphate. The enzyme required Mg^{++} for activity at an optimum Mg^{++}/ATP ratio of 1:1. The authors did not report on the effects of monovalent ions on the activity of the enzyme.

Weibull et al. (1962) reported the presence of adenosinetriphosphatase activity in cell fractions of Bacillus megaterium. In the presence of calcium or magnesium ions, all cellular fractions (soluble protoplasm and ghosts) were able to release inorganic phosphate from adenosine triphosphate. No activation of the enzyme by Na^{+} and K^{+} could be demonstrated. Greenawalt et al. (1962) presented evidence which suggested that activation of adenosinetriphosphatases by calcium and magnesium ions in the cellular fractions studied reflected the presence of at least two enzymic

systems which contain one or more common steps or intermediates. No roles were suggested for the ATPases of Bacillus megaterium.

Since most terrestrial bacteria which have been examined have been found not to require Na^+ for growth although they do require K^+, one would not expect to find a transport system in the membranes of these cells which was dependent on the presence of both Na^+ and K^+. Marine bacterial cells, on the other hand, have been found to require both Na^+ and K^+ for growth and metabolism and since their quantitative requirements for these ions are similar to those found by Eagle (1956) for animal cells in tissue culture one might expect that they would transport these ions by a mechanism similar to that operating in animal cells.

With methods available for the formation of protoplasts and for the isolation of the protoplast membranes of Gram-positive bacteria, it has been possible to come to some conclusions about the localization of certain enzymes in the major surface structure of these cells. The biosynthetic capabilities of the bacterial protoplast are so similar to those of intact cells that it appears unlikely that the cell walls of Gram-positive bacteria contributes much more than mechanical stability

to the bacterial cell (Weibull, 1958) (McQuillen, 1960).

The peripheral structures of Gram-negative bacteria are much more complex than those of Gram-positive bacteria and a clear division into cell wall and protoplast membrane is not possible. However, there have been several studies of the distribution of enzymes in soluble, particulate, and "envelope" or "hull" fractions of Gram-negative bacteria. For example, a number of the enzymes of the electron transport system have been found in the "envelope" fractions of Gram-negative bacteria (Cota-Robles et al. 1958). Hunt et al. (1959) isolated a "cell wall membrane" fraction from mechanically disintegrated cells of a strain of Pseudomonas fluorescens. From their study they concluded that the nicotinic acid hydroxylase and succinic acid dehydrogenase systems were located in this complex structure.

In the present study, cells have been converted to spherical forms with versene and lysosyme. These spherical forms were then disrupted by osmotic shock and their membranes isolated. These have been tested for adenosinetriphosphatase activity.

MATERIALS AND METHODS

Organisms

Two marine microorganisms were used in this study. One is the marine pseudomonad B-16 whose morphological and cultural characteristics have been described in Part I. The other organism used was an agar-digesting bacterium belonging to the genus Cytophaga and designated as B-9. It is a Gram-negative rod form which exhibits gliding motility on the surface of agar. It has also agar-digesting capacity. It forms yellow pigmented colonies. The organic nutritional requirements have been studied and its requirements for inorganic ions for growth and metabolism investigated. It can grow on glucose provided that one amino acid is added. The amino acid needed could be either arginine, glutamic acid, proline, histidine, or aspartic acid. The metal ions required are Na^{+}, K^{+}, Mg^{++} and Ca^{++} (MacLeod et al. 1954).

Preparation of Cell Membranes

The organism under investigation was inoculated into 250 ml of the nutrient broth-yeast extract medium described in Part I contained in two-liter

erlenmeyer flasks. Organism B-16 was incubated for 15 hours, organism B-9 for 48 hours on a rotary shaker at 25° C. The cells were harvested by centrifugation and washed three times by resuspension and sedimentation in amounts of 1.0 M NaCl equal in volume to that of the growth medium. Cells harvested from a total of one liter of medium were resuspended in 250 ml of 0.5 M TrisCl buffer pH 8.0 and 1.257 mg per ml of neutral versene (MacLeod et al. 1960). Lysosyme was added at a concentration of 100 ugm per ml and the mixture was stirred slowly for one hour. At the end of this time lysosyme was again added at a level of 100 ug per ml. Usually by the end of the second hour all of the cells could be observed by phase contrast microscopy to have changed from rod forms to spheres. Occasionally, the cells had to be treated for longer periods of time to obtain maximum spheroplast formation.

When all the cells had been converted to spheres, they were centrifuged from the suspending salt solution and resuspended in 200 ml of cold 0.1 M TrisCl, pH 8.0. Lysis was instantaneous. The disrupted membranes or ghosts were separated from the soluble "cytoplasmic" fraction by centrifugation at 50,000 x G for 20 minutes in a Spinco ultracentrifuge. The particulate material which was collected was

washed five times with 100 ml portions of cold 0.05 M $MgSO_4$ or 0.2 M NaCl by repeated centrifugation and and resuspension. Only trace amounts of protein could be detected in the supernatant liquid after the fifth washing. The membrane fraction was finally suspended in 15 to 20 ml of either 0.05 M $MgSO_4$ or 0.2 M NaCl. This suspension was divided into a series of 2 ml volumes which were then frozen at -20° C.

In order to have a homogeneous suspension of the membrane for enzyme studies, the preparations were treated for two minutes in a sonic occillator after thawing.

Measurement of ATPase Activity

ATPase activity was determined by measuring the rate of release of inorganic P from ATP. The reaction mixture contained TrisCl 0.1 M, pH 9.0, 5×10^{-3} M ATP, 5×10^{-3} M $MgSO_4$ and 0.1 ml of the enzyme suspension (containing about 0.50 mg protein) in a final volume of 1.0 ml. ✱ In experiments where

---

✱ Throughout this test the following abbreviations have been used:

ATP: Adenosine triphosphate
ADP: Adenosine diphosphate
AMP: Adenosine monophosphate
ITP: Inosine triphosphate

the effects of Na^+ and K^+ were not measured, a 300 mM concentration of K^+ as KCl was added to the reaction tube.

All experiments were performed at 25° C. After a 5 minute temperature equilibration period the reaction was started by the addition of enzyme, and the mixture was incubated for 30 minutes. The reaction was stopped by the addition of 1.0 ml of cold 20% trichloroacetic acid for B-16 or 1.0 ml of ice cold 5% perchloric acid for B-9. After centrifugation, aliquots of 0.4 ml were removed for determination of inorganic phosphate by the method of Fiske and Subbarow (1925). Corrections were applied for blanks containing substrate without enzyme and enzyme without substrate.

In all the diagrams, except where otherwise stated, the enzyme activity is expressed as micromoles of inorganic P split off from the substrate in 30 minutes.

Protein determinations were made in some experiments. It was estimated by the biuret method (Gornall et al. 1949) using a crystalline pepsin (Worthington Biochemical Corporation) as a standard.

Effect of Ouabain on Na^+ Transport by Whole Cells

Cells of B-16 were washed three times in 0.05 M $MgSO_4$. Aliquots consisting of 2.5 grams wet weight of cells in 7.5 ml of suspension were added to each of the three Spinco ultracentrifuge tubes containing sufficient ouabain to give final concentrations of 3×10^{-3}, 3×10^{-4}, 3×10^{-5} M respectively. After thorough mixing, the suspensions were incubated for 10 minutes. At the end of the incubation period a solution of salts was added to the suspension to give the following concentrations: NaCl, 200 mM; KCl, 10 mM; $MgSO_4$, 50 mM; and Na^{22}, 0.1 microcurie in a final volume of 10 ml. After standing for 30 minutes at 25° C. the cells were centrifuged at 50,000 x G for 20 minutes and the supernatant liquid discarded. The cells were resuspended in water and the volume of the suspension adjusted to 5 ml. The tubes were put directly in a scintillation well counter and counted on a Picker scaler. The results were compared with another aliquot of cells treated in the same way except that ouabain was omitted.

Chemicals

The Na^+ salts of ATP, ADP, AMP, and ITP were obtained from Sigma Chemical Company. ATP was rendered

sodium-free by passage through a Dowex 50 W-X8 cation exchange column in the tris form. This treatment removed 99.9% of the sodium as established by flame photometry. Tris (Tris - (hydroxymethyl) aminomethane) and 2 amino- 2 methyl- 3 propanediol buffers were purchased at Sigma Chemical Company and Nutritional Biochemical Company respectively. The Na^+, K^+ and Mg^{++} salts were of reagent grade. Radioactive Na^{22} was obtained from the Canadian Atomic Energy Commission. Lysozyme came from the Nutritional Biochemical Company.

ATP was determined spectrophotometrically (Kalckar, 1947). Na^+ and K^+ were determined with a Zeiss Ikon flame spectrophotometer. pH was measured with a Coleman pH meter model G.

Photographic Materials

For photomicrographic purposes carboxymethylcellulose, Type 7 H obtained from Hercules Powder Company,[*] was added to the cell suspension to stop motility and Brownian movement in wet mounts of the organism, a technique described by Abram and Gibbons (1960).

---

[*] We are indebted to the Hercules Powder Company for generously making this sample available.

The film used for photomicrography was Kodak High Contrast Copy Film M 402 (Microfile). The film was processed in Kodak D-27 developer, and the negatives printed on extra hard glossy bromide paper, Ilford B4-1P.

RESULTS

General Properties of the ATPase Preparation

The first figure (Figure 1) is a photomicrograph showing whole cells, spheroplasts * and spheroplast membranes of organism B-16 as seen with a Zeiss Jena phase contrast microscope. It can be seen that the membranes show very little contrast as compared with the spheroplasts and original cells. Very few membranes appeared whole in the preparations. Presumably they were fragmented by the osmotic shock treatment.

Localization of ATPase

When the membrane and cytoplasmic fractions of the bacterial cells were tested for ATPase both were found to possess activity (Table I). It was therefore

---

* The term spheroplast has been used to describe a spherical form of the cell which has some of the cell wall still attached. In the present study it is not known to what extent the cell wall of this organism has been modified or removed when spherical forms were produced from rods.

Figure 1.

Whole cells (left), spheroplasts (center) and spheroplast membranes (right) of marine bacterium B-16. (2000 x)

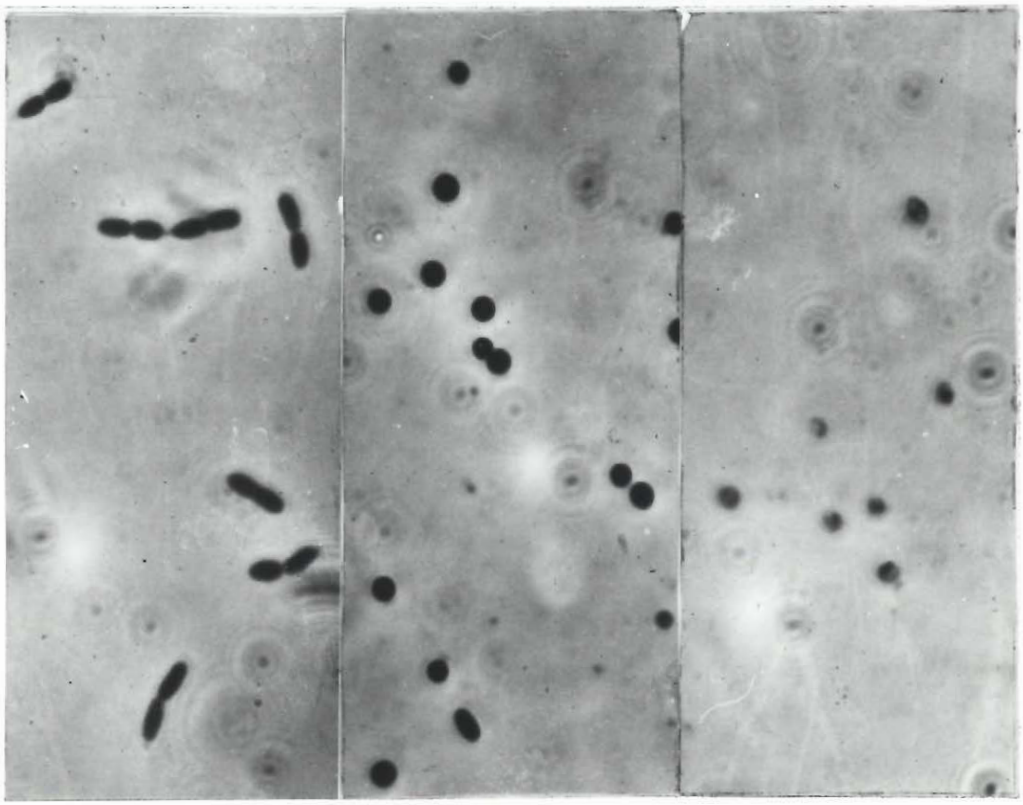


Figure 1.

Table I. Relative capacity of spheroplast membranes and cytoplasm to liberate inorganic phosphate from ATP.

Preparation	Pi Liberated umoles *
Cell membranes	2.71
Soluble "cytoplasmic" fraction	3.46

* per mg protein.

necessary to ascertain whether the membrane fraction contained an intrinsic ATPase or merely contaminating ATPase from the cytoplasm. To distinguish between these possibilities, 10 ml samples of a suspension of membranes after osmotic treatment were added to each of five Spinco ultracentrifuge tubes. After the first centrifugation the supernatant liquid was decanted in each case. One tube was then removed and the membranes from this tube were resuspended in 2 ml of 0.20 M NaCl and stored for determination of ATPase activity. The membranes in the remaining four tubes were resuspended in 0.2 M NaCl and recentrifuged. Again a tube was removed and the remainder resuspended and centrifuged. This operation was repeated until a total of five samples had been collected representing membranes which had received from one to five washings.

The ATPase activity of the preparations was then determined. The results are shown in Table II. It can be seen that washings did not affect the ATPase activity of the membrane. These findings indicate that the cell membrane contains an ATPase which appears to be an intrinsic part of its structure.

Table II. Effect of the number of washings on the ATPase activity of spheroplast membranes prepared from marine bacterium B-16.

No. of washings	Pi liberated umoles *
1	2.20
2	2.31
3	2.75
4	2.47
5	2.71

* per mg protein.

Effect of pH

Preliminary experiments on the pH optimum for enzyme activity indicated that it lay close to pH 9.0. Since this is the upper limit of the buffer range of Tris, another buffer, Ammediol (2 amino-2 methyl- 3 propanediol) pH range 8.4-10.4 was chosen to obtain a pH activity curve. However, due to its small inhibitory effect, it was not used in subsequent experiments. The pH optimum for the ATPase activity was found to be 8.8 (Figure 2).

For routine experiments, the use of TrisCl buffer pH 9.0 at a concentration of 0.1 M in the reaction vessel was found to be quite satisfactory. With this buffer, the final pH in the reaction tube lay between 8.4 and 8.6 when taken at the beginning of the reaction. Since the pH range is rather wide (Figure 2) it did not affect the enzymatic activity sensibly.

Stimulation of Cell Membrane ATPase by Mg^{++}

Figure 3 shows that a 5 mM concentration of Mg^{++} was required to give maximum phosphate liberation in the presence of 5 mM ATP. When the ATP concentration was 10 mM, the optimum concentration of Mg^{++} was

Figure 2.

Effect of pH on the ATPase activity of spheroplast membranes prepared from marine bacterium B-16.

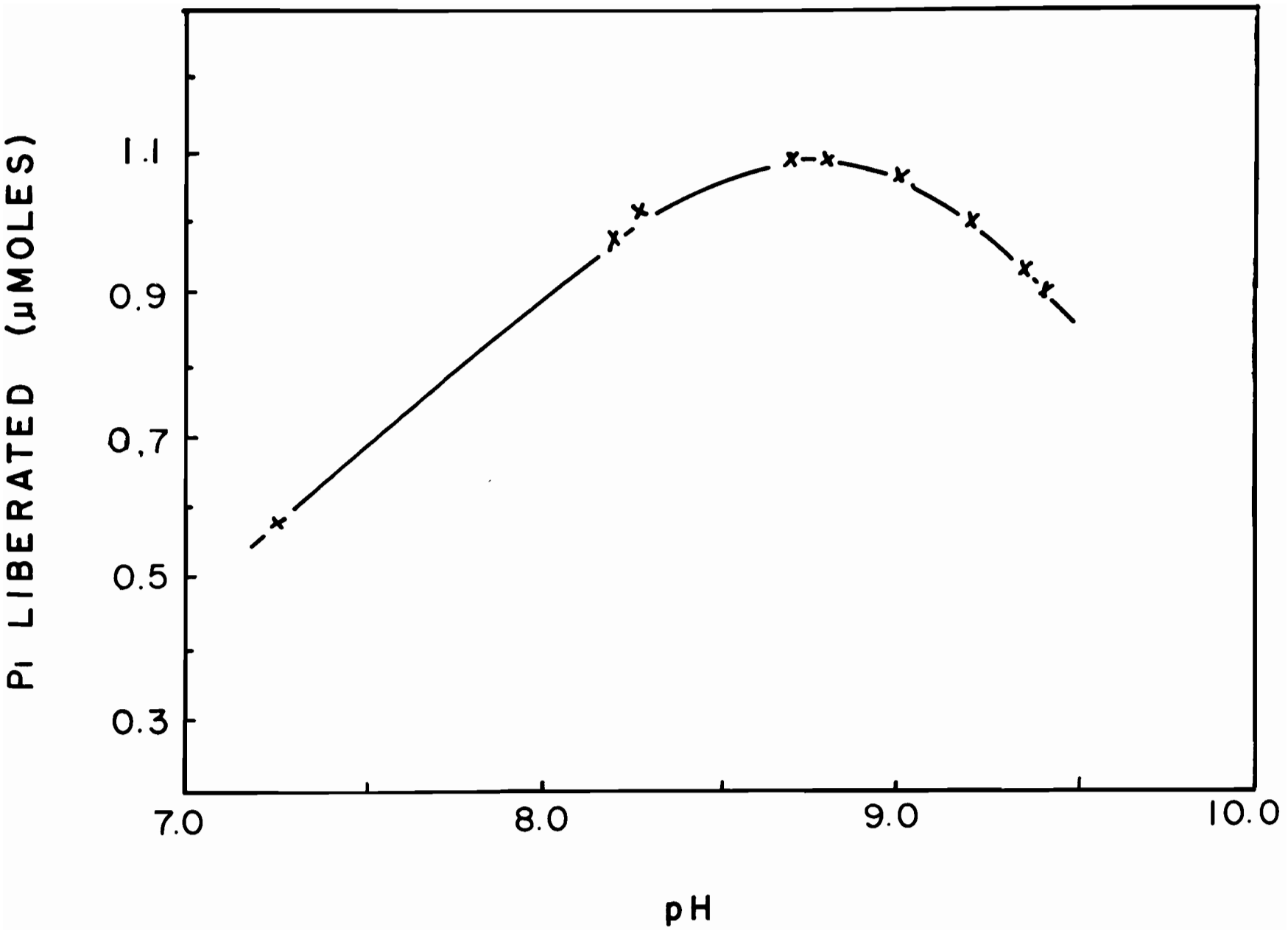


Figure 2.

Figure 3.

Stimulation of spheroplast membrane ATPase of marine bacterium B-16 by Mg^{++}.

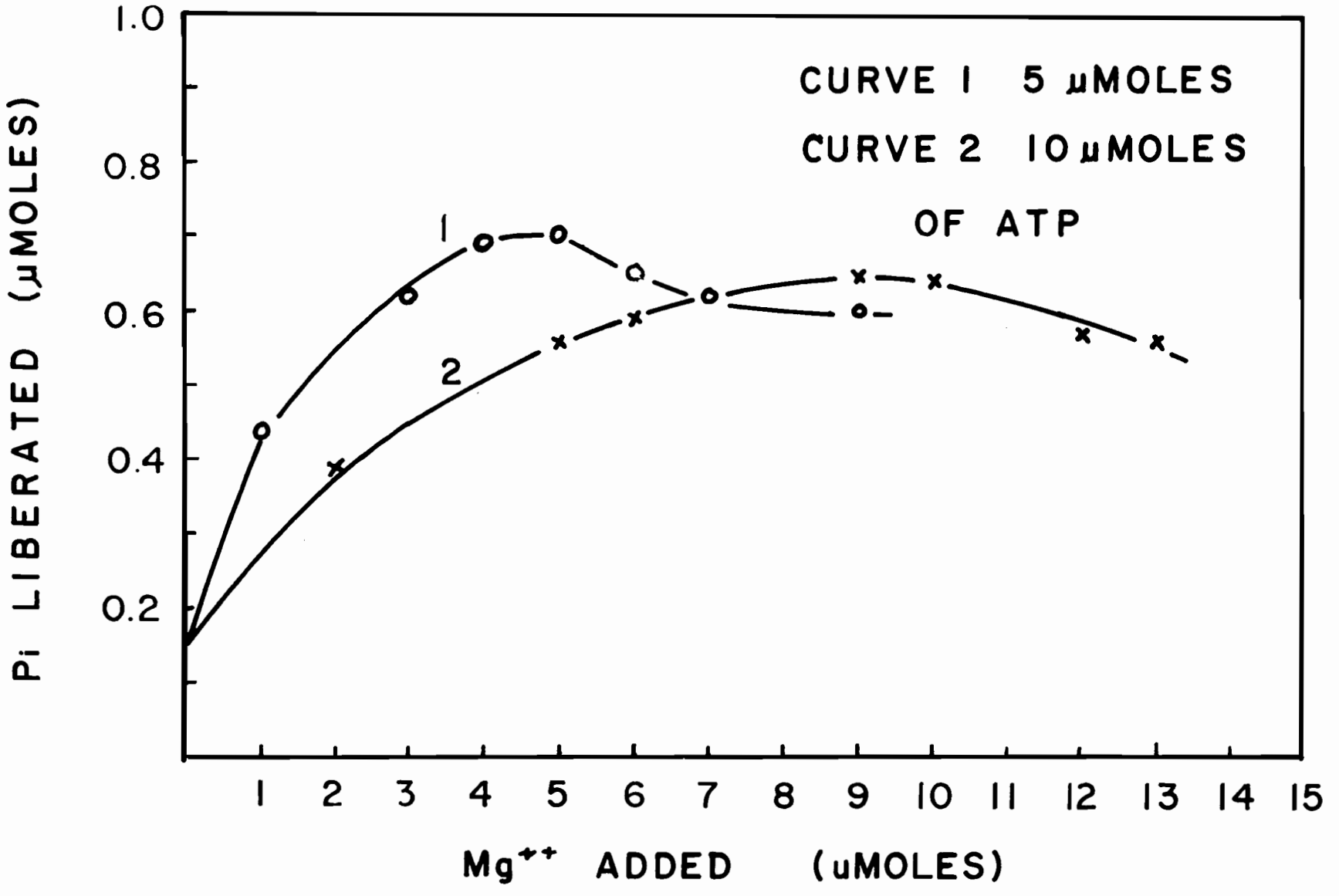

CURVE 1 5 µMOLES
CURVE 2 10 µMOLES
OF ATP
Pi LIBERATED (µMOLES)
1.0
0.8
0.6
0.4
0.2
1
2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Mg++ ADDED (uMOLES)

Figure 3.

found to be around 10 mM. This indicates that the optimum Mg^{++}/ATP ratio is 1:1 for this enzyme. Calcium could not be substituted for Mg^{++}.

Rate of Liberation of Inorganic Phosphate

The liberation of inorganic phosphate (Pi) with time upon incubation of ATP with a cell membrane preparation is shown in Figure 4. It can be seen that the enzyme liberated nearly three micromoles of Pi from one micromole of ATP, indicating that ATP, ADP, and AMP must all have been hydrolysed. This was confirmed by showing that each of the compounds could serve as a substrate for the enzyme, (Table III). In addition inosine triphosphate was also hydrolysed but not inorganic pyrophosphate.

Velocity of the Reaction

The velocity of the reaction did not increase quite linearly with an increase in membrane concentration (Figure 5). This suggests that an inhibitor is present, the effect of which is evident at higher concentrations of the enzyme preparation.

Figure 4.

Rate of liberation of inorganic phosphate by spheroplast membrane ATPase of marine bacterium B-16.

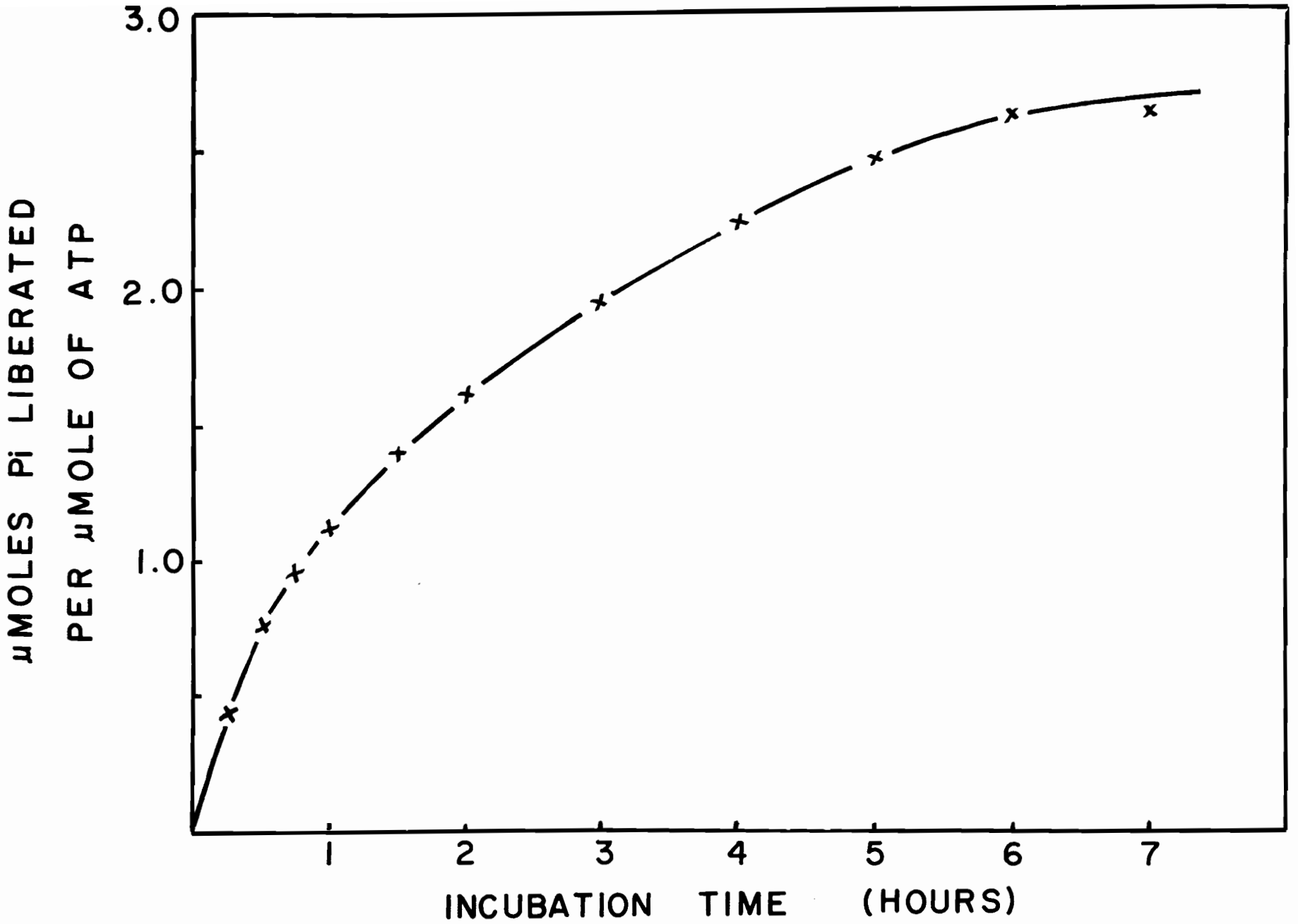

3.0
2.0
1.0
µMOLES Pi LIBERATED
PER µMOLE OF ATP
1
2
3
4
5
6
7
INCUBATION TIME (HOURS)

Figure 4.

Table III. Relative capacity of spheroplast membranes of marine bacterium B-16 to release inorganic phosphate from various substrates.

Substrate tested 5 umoles	Pi liberated umoles
ATP	2.38
ADP	1.10
AMP	0.86
ITP	1.68
Pyrophosphate	.00

Figure 5.

Velocity of the reaction by spheroplast membrane ATPase of marine bacterium B-16.

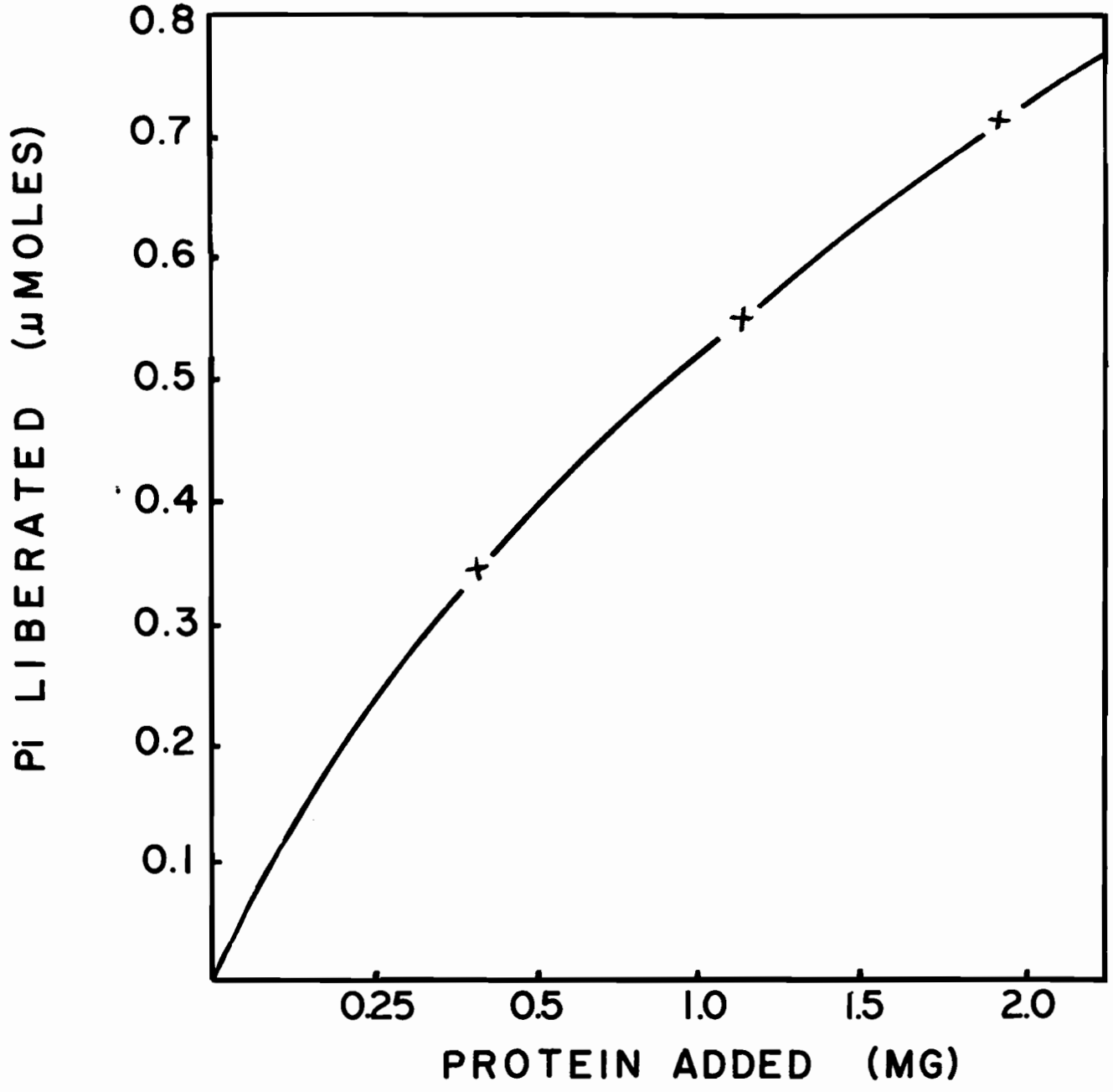

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Pi LIBERATED (μMOLES)
0.25
0.5
1.0
1.5
2.0
PROTEIN ADDED (MG)

Figure 5.

Response of ATPase to Monovalent Salts

When the response of the enzyme to various inorganic salts was investigated, it was found that the ATPase was nonspecifically activated by all those which were tested. The greatest stimulation was obtained with KCl (Figure 6). The sulfate salts of Na^+ and K^+ were only slightly less effective (Figure 7). This non-specific capacity of a number of different salts to stimulate enzyme action suggested an ionic strength effect. Maximal activity was obtained with an ionic strength due to added salt of between 0.2 and 0.3. The ionic strength of the substrate, buffer, and Mg^{++} salt in the system was 0.14 M.

Activation of ATPase by a Combination of Na^+ and K^+

The effect of adding NaCl to a system already containing KCl was then examined. In Table IV we see that adding sufficient KCl to increase the ionic strength to 0.2 produced the expected increase in enzyme activity. Adding more KCl had little further effect. Similarly, the addition of KCl to the system already activated by NaCl produced no more effect than one would expect from the increase in ionic strength. Flame photometric analysis of the enzyme system without

Figure 6.

Response of spheroplast membrane ATPase of marine bacterium B-16 to monovalent salts.

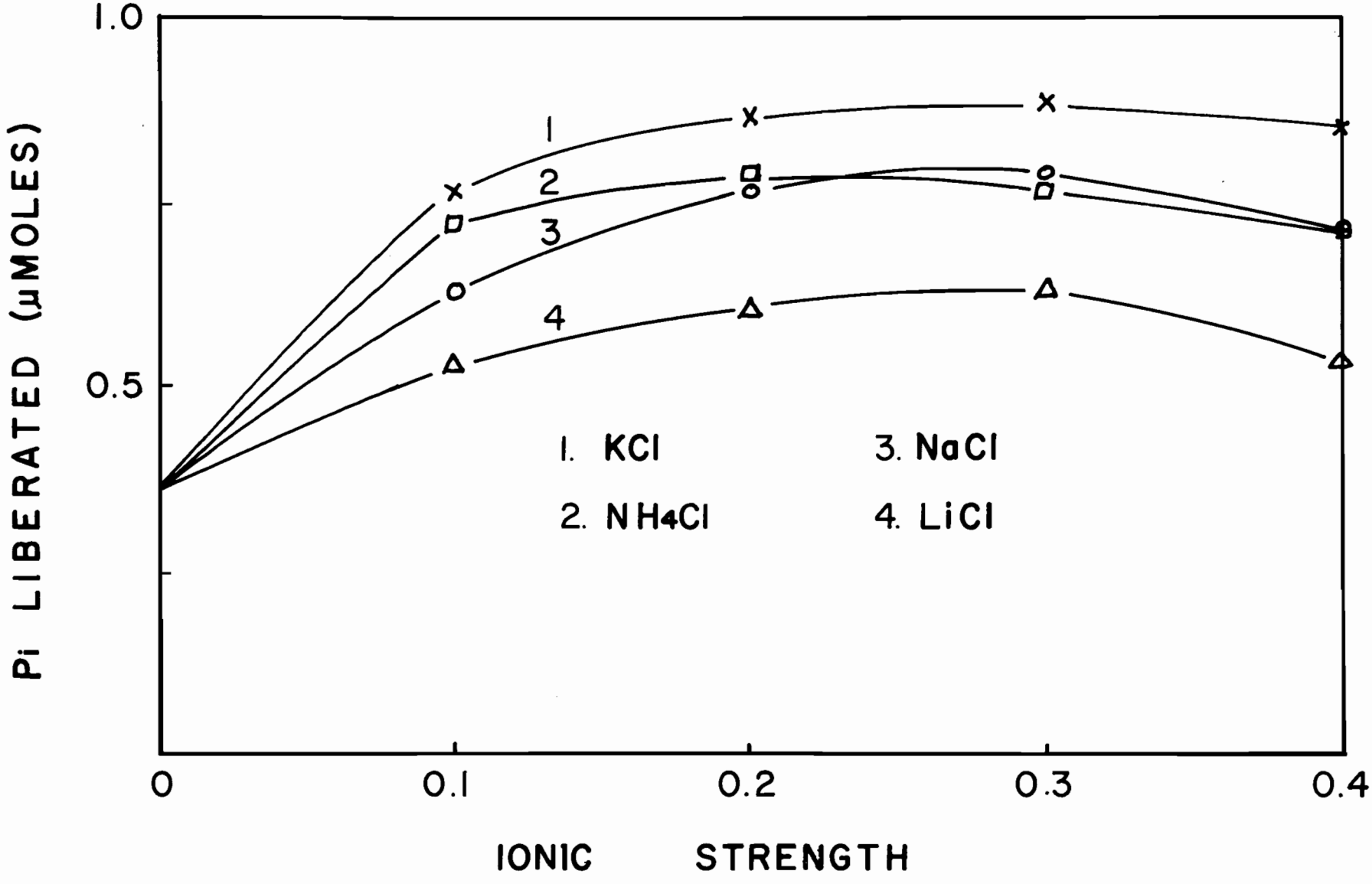

1.0
0.5
Pi LIBERATED (μMOLES)
0
0.1
0.2
0.3
0.4
IONIC STRENGTH
1
2
3
4
1. KCl
2. NH4Cl
3. NaCl
4. LiCl

Figure 6.

Figure 7.

Response of spheroplast membrane ATPase of marine bacterium B-16 to chloride and sulfate salts of Na^+ and K^+.

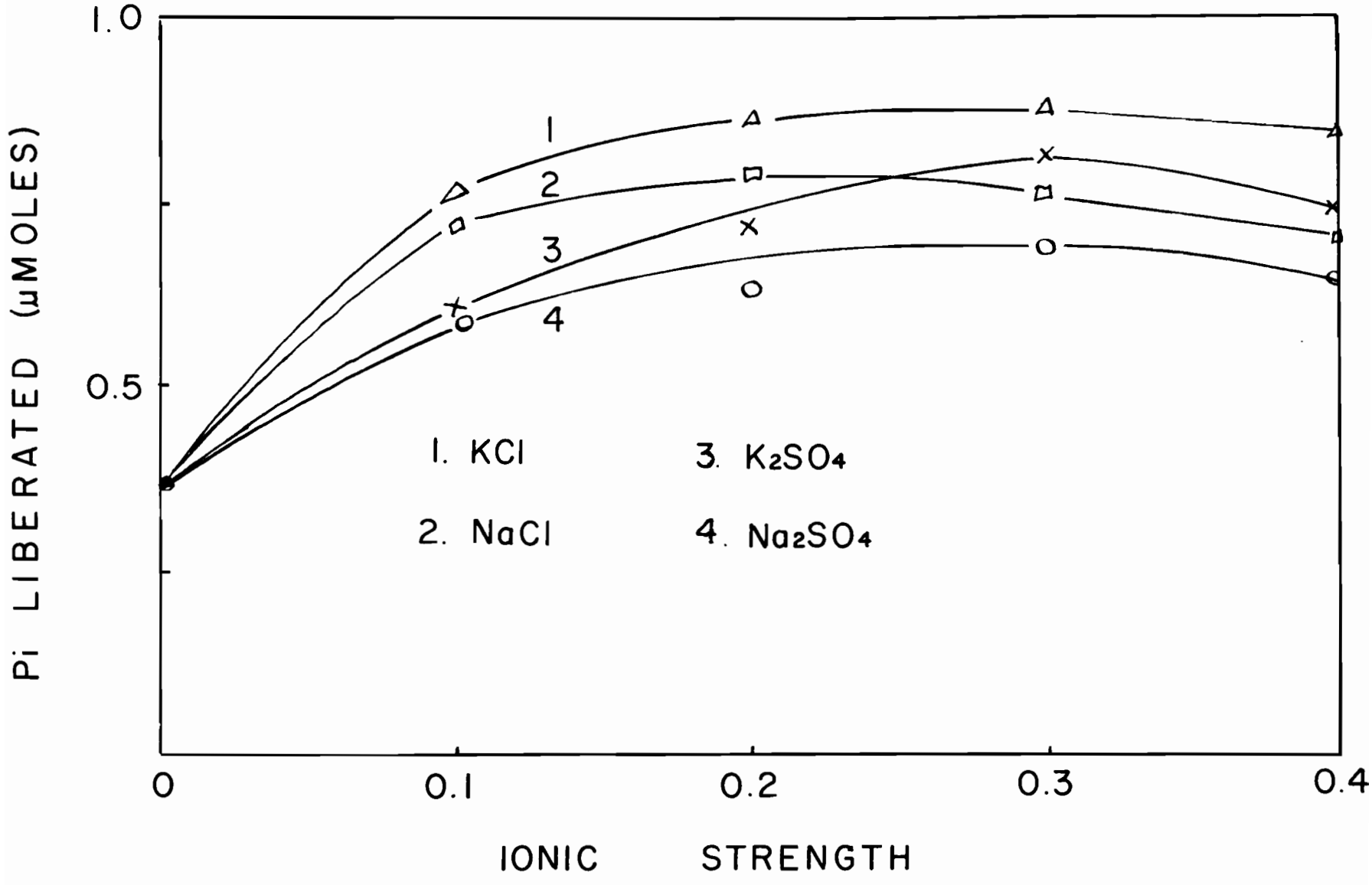

1.0
0.5
Pi LIBERATED (μMOLES)
1
2
3
4
1. KCl
2. NaCl
3. K2SO4
4. Na2SO4
0
0.1
0.2
0.3
0.4
IONIC STRENGTH

Figure 7.

Table IV. Effect of Na^+ and K^+ alone and in combinations on the ATPase activity of membranes of marine bacterium B-16.

KCl u	NaCl u	Pi Liberated umoles
0.0	0.0	0.64
0.2	0.0	1.22
0.3	0.0	1.27
0.2	0.01	1.22
0.2	0.05	1.22
0.2	0.10	1.26
0.2	0.20	1.26
0.0	0.2	1.06
0.0	0.3	1.18
0.01	0.2	1.18
0.05	0.2	1.21
0.10	0.2	1.23
0.20	0.2	1.22

added NaCl showed that only 2.9×10^{-5} M Na^+ was present as a contaminant. Thus no evidence was obtained of an ATPase in the membrane specifically activated by a combination of Na^+ and K^+.

ATPase in whole Cell Extracts

The possibility was considered that a Na^+-K^+ activated ATPase might be labile and hence inactivated during isolation and washing of the membranes. Accordingly, a suspension of cells which had been washed five times in 0.05 M $MgSO_4$ was disrupted by sonic treatment for five minutes. The complete mixture of membranes and cytoplasm was tested for ATPase activity (Table V). As one can see, no component specifically activated by a combination of Na^+ and K^+ could be demonstrated in this system either.

Effect of Ouabain

Ouabain, a cardiac glycoside, has been known to inhibit active transport in a number of biological systems (Schatzmann, 1953). The effect of this compound was studied on both the enzyme preparation and on the whole cells of marine bacterium B-16. Table VI shows that ouabain did not cause any inhibition of the

Table V. Effect of Na^+ and K^+ alone and in combinations on the ATPase activity of whole extracts of marine bacterium B-16.

KCl u	NaCl u	Pi Liberated umoles
0	0	0.65
0.2	0	1.53
0.3	0	1.62
0.2	0.01	1.40
0.2	0.05	1.54
0.2	0.10	1.53
0.2	0.20	1.40
0	0.2	1.32
0	0.3	1.36
0.01	0.2	1.18
0.05	0.2	1.41
0.10	0.2	1.44
0.20	0.2	1.44

Table VI. Effect of ouabain on the ATPase activity of spheroplast membranes of marine bacterium B-16. *

Ouabain added M	Pi liberated umoles
0	1.84
3×10^{-4}	1.79
3×10^{-5}	1.84
3×10^{-6}	1.84

* In addition to ATP and Mg^{++}, the reaction tubes contained: NaCl, 200 mM; and KCl, 100 mM.

membrane ATPase when tested at concentrations considerably higher than those found to inhibit the enzyme in animal cells (Skou, 1960) (Post et al. 1960).

The effects of ouabain on the capacity of whole cells of the marine bacterium to take up and maintain a level of Na^{22} in the cells was also investigated. Cells of the marine bacterium were suspended in a solution containing Na^{22} under conditions known from other experiments (Takacs and MacLeod, 1962) to permit Na^{22} uptake and release from the cells. The procedure used in this experiment has been described under Methods. It is evident from the results in Table VII that ouabain when tested at levels far in excess of those required to inhibit Na^{+} transport in animal cells, had no significant effect on the level of Na^{22} attained by the marine bacterial cells.

ATPase activity of spheroplast membranes prepared from marine bacterium B-9.

Spheroplast membranes prepared from organism B-9, the Cytophaga species, were also found to contain an active ATPase. Like the membrane preparation of B-16, that of B-9 hydrolysed ATP, ADP, AMP, and ITP. Pyrophosphate was barely attacked (Table VIII).

Table VII. Effect of ouabain on the level of Na^{22} in cells of marine bacterium B-16.

Ouabain added M	Counts *
0	11,272 ± 171
3×10^{-3}	11,273 ± 35
3×10^{-4}	11,407 ± 73
3×10^{-5}	11,418 ± 85

* per 2 minutes per gram of wet cells.

Table VIII. Relative capacity of spheroplast membranes of marine bacterium B-9 to release inorganic phosphate from various substrates.

Substrate tested 5 umoles	Pi liberated umoles
ATP	1.99
ADP	2.65
AMP	2.12
ITP	1.18
Pyrophosphate	0.26

Activation of the ATPase of B-9 by a combination of Na^+ and K^+

When NaCl and KCl were tested separately for their capacity to activate the spheroplast ATPase of B-9, both salts were found to be able to increase the enzyme activity. As in the case of B-16, however, combinations of the salts had no more effect than one would expect from an increase in ionic strength (Table IX).

Effect of ouabain on the ATPase of B-9.

As in the case of B-16, ouabain did not inhibit the action of the membrane ATPase of organism B-9 (Table X).

Table IX. Effect of NaCl and KCl alone and in combinations on the ATPase activity of membranes of marine bacterium B-9.

KCl u	NaCl u	Pi liberated umoles
0	0	0.35
0.2	0	0.75
0.3	0	0.92
0.2	0.01	0.71
0.2	0.05	0.89
0.2	0.10	0.83
0.2	0.20	0.98
0	0.2	0.82
0	0.3	0.94
0.01	0.2	0.78
0.05	0.2	0.83
0.10	0.2	0.92
0.20	0.2	0.93

Table X. Effect of ouabain on the ATPase activity of spheroplast membranes of marine bacterium B-9. *

Ouabain added M	Pi liberated umoles
0	1.20
3×10^{-4}	1.19
3×10^{-5}	1.19
3×10^{-6}	1.21

* In addition to ATP and Mg^{++}, the reaction tubes contained: NaCl, 200 mM; and KCl, 100 mM.

DISCUSSION

Evidence has been obtained that one or more enzymes capable of releasing inorganic phosphate from ATP are present in the spheroplast membranes of the two marine bacterial cells examined. No indication has been obtained that this ATPase activity is due, even in part to an enzyme specifically activated by a combination of Na^+ and K^+. Furthermore, no effect of ouabain either on membrane ATPase activity or on Na^+ transport by the whole cells could be detected. There is thus no evidence in the marine bacterial cells examined of a link between Na^+ transport and membrane ATPase activity. If the Na^+, K^+ activated ouabain sensitive ATPase in the membrane of animal cells is indeed involved in ion transport then the results reported here indicate that significant differences in the mechanism of ion regulation exist between marine bacterial cells and animal cells.

Besides ion activation and ouabain sensitivity there are other differences in the characteristics of the membrane ATPase of marine bacterial cells and animal cells.

1. The enzyme action in B-16 has a high pH optimum, 8.8. In animal cells, it

is 7.2 for nerve cells (Skou, 1957) and 7.5 for red blood cells and brain microsomes (Post, 1960; Dunham and Glynn and Jarnefelt, 1961).

2. Substrate specificity is different in the animal and bacterial systems. The crab nerve enzyme preparation hydrolyzes ATP and to a limited extent ITP, but not ADP, AMP or pyrophosphate (Skou, 1957). The red blood cell system attacks ATP and ADP but not AMP, ITP or pyrophosphate (Post et al. 1960). The significance of these differences in substrate specificity is difficult to assess, since none of the enzyme preparations has been shown to contain only a single enzyme.

Since an ATPase specifically activated by Na^+ has not been demonstrated in marine bacterial cells, a specific function of Na^+ for these cells still remains to be established.

SUMMARY

In an effort to learn more about the function of Na^+ in the metabolism of a marine bacterium, the capacity of three non-Na^+ requiring mutants to oxidize various substrates was compared with that of the Na^+ requiring parent from which the mutants were derived. The mutants all oxidized acetate, propionate and butyrate at about the same rate as the parent. In the case of both parent and mutant cells citrate was oxidized only if the cells were first adapted by including citrate in the growth medium. Whereas the parent cells, after adaptation oxidized citrate more rapidly than the monocarboxylic acids, the mutants oxidized it more slowly. The parent cells also needed malate in the growth medium to oxidize malate but attacked fumarate without adaptation. The mutant cells could not be induced to attack either malate or fumarate even when these compounds had been present in the growth medium.

Na^+ at the level required for maximum rate of oxidation of the various compounds by the parent cells inhibited to some extent oxidation of the substrates by the mutant cells. When Na^+ was tested at a level which stimulated early growth of the

mutant cells (0.02 M) it failed to stimulate oxidation of substrates by the mutant cells. In the case of propionate oxidation, Na^+ proved to be inhibitory even at this low concentration.

To determine if failure of the mutant cells to oxidize malate and fumarate was due to a lack of intracellular enzymes capable of attacking these compounds, cell-free extracts of the mutants were prepared and tested for malic dehydrogenase and fumarase activity. Malic dehydrogenase was present in extracts of the mutant cells, but at a lower level of activity than in the parent. The mutant enzyme, like that of the parent was TPN specific, but unlike the parent did not require K^+. Although the mutant extracts could be shown to contain more K^+ than those of the parent, the amount present was far below the level required to activate the malic dehydrogenase obtained from the parent cells. Fumarase was also present in the mutant extract and at a higher level of activity than in extracts of the parent cells.

It was concluded that the differences between the properties of the parent and mutant cells could best be explained on the basis of a difference in cell wall composition which would bring about a

modification of cell wall permeability and the capacity to form inducible permeases.

In an effort to establish more clearly the genetic relation between parent and mutant cells, attempts were made to transform the non-Na^+ requiring mutants to Na^+ requiring strains using DNA preparation prepared from the Na^+ requiring parent cells. Three different methods for the isolation and purification of DNA have been tried. Three selective media devised to enhance growth of transformants and to inhibit growth of untransformed cells have been used. Various modifications of a transforming medium have been employed. All attempts to isolate Na^+ requiring transformants, however, were unsuccessful.

Since the membranes of animal cells contain a ouabain sensitive ATPase specifically activated by a combination of Na^+ and K^+ which appears to be linked with ion transport, an effort was made to determine if a similar enzyme could be located in the membranes of marine bacterial cells. Cells of the marine bacterium were converted to spheroplasts by treatment with versene and lysozyme. Spheroplast membranes were isolated after subjection of the spheroplasts to osmotic shock. Both the membranes

and cytoplasm could be shown to possess ATPase activity. The membrane ATPase had a pH of 8.8 and was activated by Mg^{++}. The optimum Mg^{++}/ATP ratio for enzyme activity was found to be 1:1. The membrane preparation liberated inorganic phosphate from ATP, ADP, AMP and ITP but not from pyrophosphate. Unlike the enzyme preparation from the membranes of animal cells, the bacterial preparation was non-specifically activated by a number of different salts and was not inhibited by ouabain. No evidence of an ATPase specifically activated by a combination of Na^{+} and K^{+} was obtained. In addition Na^{22} uptake by whole cells was not affected by ouabain. It was concluded that ion transport in marine bacterial cells must be mediated by a mechanism different from that believed to operate in animal cells.

REFERENCES

Abram,D. and Gibbons,N.E.

1960 Turbidity of suspension and morphology of red halophilic bacteria as influenced by sodium chloride concentration. Can.J.Microbiol. 6:535-543.

Abrams,A., McNamara,P. and Johnson,F.B.

1960 Adenosine triphosphatase in isolated bacterial cell membranes. J.Biol.Chem. 235:3659-3662.

Alexander,H.E. and Leidy,G.

1950 Transformation type specificity of H.influenzae. Proc.Soc.Exp.Biol.Med. 73:485-487.

Alexander,H.E. and Leidy,G.

1951 Determination of inherited traits of H.influenzae by desoxyribonucleic acid fractions from type-specific cells. J.Exp.Med. 93:345-359.

Allfrey,V.G., Meudt,R. Hopkins,J.W. and Mirsky,A.E.

1961 Sodium-dependent "transport" reactions in the cell nucleus and their role in protein and nucleic acid synthesis. Proc.Natl.Acad.Sci. 47:907-932.

Anagnostopoulos,C. and Spizizen,J.

1961 Requirements for transformation in Bacillus subtilis. J.Bacteriol. 81:741-746.

Austrian,R.

1952 Bacterial transformation reactions. Bact.Rev. 16:31-50.

Avery,O.T., MacLeod,C.M. and McCarty,M.

1944 Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a deoxyribonucleic acid fraction isolated from Pneumococcus Type III.
J.Exp.Med. 79:137-158.

Berkeley,C.

1919 A study of marine bacteria, Strait of Georgia, B.C.
Trans.Roy.Soc.Canada, Ser.3, Sec.5.
13:15-43.

Boivin,A.

1947 Directed mutation in colon bacilli, by an inducing principle of desoxyribonucleic nature: its meaning for the general biochemistry of heredity.
Cold Spring Harbor Symposia on Quant. Biol. 12:7-17.

Bonting,S.L., Simon,K.A. and Hawkins,N.M.

1961 Studies on sodium-potassium-activated adenosine triphosphatase. I. Quantitative distribution in several tissues of the cat.
Arch.Biochem.and Biophys. 95:416-423.

Boring,J.P. and Pratt,D.B.

1961 Spheroplasts of marine bacteria induced by the action of penicillin.
Nature. 192:773-774.

Butkevich,V.S.

1928 Die Bildung der Eisenmangan-Ablagerungen am Meeresboden und die daran beteiligten Mekroorganismen.
Ber.wiss.Meeresinst., Moscow.
3 (3):5, 1928.

Catlin,B.W.

1960 Transformation of Neisseria meningitidis by deoxyribonucleates from cells and from culture slime. J.Bact. 79:570-590.

Chabereck,S. and Bersworth,F.C.

1955 Chelating agents as metal buffers in biological systems. I. Principles and use of ethylenediaminetetraacetic acid. Arch.Biochem.and Biophys. 55:321-327.

Cohn,F.

1865 Zwei neue Beggiatoen Hedwigia. 4:81-84.

Corey,R.C. and Starr,M.P.

1957 Genetic transformation of colony type in Xantkomonas phaeseoli. J.Bact. 74:141-145.

Cota-Robles,E.H., Marr,A.G. and Nilson,E.H.

1958 Submicroscopic particles in extracts of Azotobacter agilis. J.Bact. 75:243-252.

Davis,B.D.

1948 Isolation of biochemically deficient mutants of bacteria by penicillin. J.Am.Chem.Soc. 70:4267.

Drew,G.H.

1912 Report of investigations on marine bacteria carried on at Andros Island, Bahamas, British West Indies, in May, 1912. Carnegie Inst.Wash.Yearbook, No.11, p.136.

Dunham,E.T. and Glynn,I.M.

1961 Adenosinetriphosphatase activity and the active movements of alkali metal ions.
J.Physiol. 156:274-293.

Eagle,H.

1956 The salt requirements of mammalian cells in tissue culture.
Arch.Biochem.and Biophys. 61:356-366.

Ehrenberg,C.G.

1838 Die Infusionsthierchen als vollkommene Organismen. Ein Blick in das tiefere organische Leben der Natur.
Folio Leipzig; 547 pp.

Fischer,B.

1894 Erqebnisse einiger auf der Plankton-expedition ausgefuhrten bakteriologishen Untersuchungen der Luft uber dem Meere.
Zeitschr.f.Hyg. 17:185-194.

Fiske and Subbarow.

1925 The colorimetric determination of phosphorus.
J.Biol.Chem. 66:375-400.

Georgi,C.E., Militzer,W.E. and Decker,L.S.

1955 The organelle nature of a particle isolated from Bacillus stearothermophilus.
J.Bacteriol. 70:716-725.

Gornall,A.G., Bardowill,C.J. and David,M.M.

1949 Determination of serum protein by means of the biuret reaction.
J.Biol.Chem. 177:751-766.

Greenawalt,J.W., Weibull,C. and Low,H.

1962 The hydrolysis of adenosine triphosphate by cell fraction of Bacillus megaterium. II. Stimulation and inhibition of the enzymic activities. B.Biol.Chem. 237:853-858.

Griffith,F.

1923 The influence of immune serum on the biological properties of pneumococci. Great Britain Rep.on Public Health and Med.Subjects, Ministry of Health. 18:1-13.

Griffith,F.

1928 The significance of pneumococcal types. J.Hyg. 27:113-159.

Harvey,E.N.

1915 The effect of certain organic and inorganic substances upon light production by luminous bacteria. Biol.Bull. 29:308-311.

Hotchkiss,R.D.

1954 Cyclical behavior in pneumococcal growth and transformability by environmental changes. Proc.Natl.Acad.Sci. U.S. 40:49-55.

Hotchkiss,R.D.

1957 Isolation of sodium deoxyribonucleate in biologically active form from bacteria. Book III, pp.692-696. In "Methods in Enzymology". Ed.by S.P.Colowick and N.O.Kaplan. Academic Press Inc. New York.

Hunt,A.L., Rodgers,A. and Hughes,D.E.

1959 Sub-cellular particles and the nicotinic acid hydroxylase system in extracts of Pseudomonas fluorescens K B 1. Biochim.et Biophys.Acta. 34:354-372.

Huppert,J. and Rebeyrotte,N.

1960 Extraction d'un acide déoxyribonucléique transformant par le dodécyl sulfate de sodium et le phénol. Biochim.Biophys.Acta. 45:189-191.

Jarnefelt,J.

1961 Sodium-stimulated adenosinetriphosphatase in microsomes from rat brain. Biochim.Biophys.Acta. 48:104-110.

Jarnefelt,J.

1961 Inhibition of the brain microsomal adenosinetriphosphatase by depolarizing agents. Biochim.Biophys.Acta. 48:111-116.

Jarnefelt,J.

1961a Mechanism of sodium transport in cellular membranes. Nature. 190:694-697.

Jardetzsky,O. and Wertz,J.E.

1956 The complexing of sodium ion with some common metabolites. Arch.Biochem.and Biophys. 65:569-572.

Kalckar,M.H. and Bessman,A.N.

1947 The determination of adenine compounds. J.Biol.Chem. 167:445-459.

Klein,D.T. and Klein,R.M.

1956 Quantitative aspects of transformation of virulence in Agrobacterium tumefaciens. J.Bact. 72:308-313.

Korinek,J.

1927 Veber Susswasserbakterien im Meere. Centralbl.f.Bakt., Abt. 66:500-505.

Langvad-Nielson,A.

1944 Change of capsule in the pneumococcus. Acta Path.Microbiol.Scand. 21:362-369.

Lederburg,J. and Zinder,N.D.

1948 Concentration of biochemical mutants of bacteria with penicillin. J.Am.Chem.Soc. 70:4267.

Lerman,L.S. and Tolmach,L.J.

1957 Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in pneumococcus. Biochim.Biophys.Acta. 26:68-82.

Lipman,C.B.

1926 The concentration of sea water as affecting its bacterial population. J.Bact. 12:311-313.

MacLeod,R.A., Claridge,C.A., Hori,A. and Murray,J.F.

1958 Observations on the function of sodium in the metabolism of a marine bacterium. J.Biol.Chem. 232:829-834.

MacLeod,R.A. and Hori,A.

1960 Nutrition and metabolism of marine bacteria. VIII. Tricarboxylic acid cycle enzymes in a marine bacterium and their response to inorganic salts. J.Bacteriol. 80:464-471.

MacLeod,R.A., Hori,A. and Fox,S.M.

1960 Nutrition and metabolism of marine bacteria. IX. Ion requirements for obtaining and stabilizing isocitric dehydrogenase from a marine bacterium. Can.J.Biochem.Physiol. 38:693-701.

MacLeod,R.A. and Matula,T.

1961 Solute requirements for preventing lysis of some marine bacteria. Nature. 192:1209-1210.

MacLeod,R.A. and Matula,T.

1961 Personal communication.

MacLeod,R.A., Onofrey,E. and Norris,M.E.

1954 Nutrition and metabolism of marine bacteria. I. Survey of nutritional requirements. J.Bacteriol. 68:680-686.

MacLeod,R.A. and Onofrey,E.

1956 Nutrition and metabolism of marine bacteria. II. Observations on the relation of sea water to the growth of marine bacteria. J.Bacteriol. 71:661-667.

MacLeod,R.A. and Onofrey,E.

1957 Nutrition and metabolism of marine bacteria. VI. Quantitative requirements for halides, magnesium, calcium and iron. Can.J.Microbiol. 3:753-759.

MacLeod,R.A. and Onofrey,E.

1957 Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth. J.Cell.Comp.Phys. 50:389-401.

MacLeod,R.A. and Onofrey,E.

1961 Studies on the stability of the Na^+ requirement of marine bacteria. Bact.Proc. 14:44.

Marr,A.G.

1960 Enzyme localization in bacteria. Ann.Rev.Microbiol. 14:241-260.

Marsh,C. and Militzer.

1956 Thermal enzymes. VII. Further data on an adenosinetriphosphatase. Arch.Biochem.Biophys. 60:433-438.

Mayers,V.L. and Spizizen,J.

1954 The isolation of deoxyribonucleic acid from bacteriophage by an improved method. J.Biol.Chem. 210:877-884.

McCarty,M.

1946 Purification and properties of desoxyribonuclease isolated from beef pancreas. J.Gen.Physiol. 29:123-139.

McCarty,M. and Avery,O.T.

1946 Studies on the chemical nature of the substance inducing transformation of pneumococcal types. II. Effect of desoxyribonuclease on the biological activity of the transforming substance. J.Exp.Med. 83:89-96.

McQuillen,K.

1960 Bacterial protoplasts. In The Bacteria, Vol.1:pp.249-359. Edited by I.C.Gunsalus and R.Y.Stanier. Academic Press, Inc. New York.

Neufeld,F. and Levinthal,W.

1928 Beitrage zur Variabilitat der Pneumokokken. Z.Immunitatsforsch. 55:324-340.

Ochoa,S., Mehler,A.H. and Kornberg,A.

1948 Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalysing the reversible oxidative decarboxylation of l-malic acid. J.Biol.Chem. 174:979-1000.

Payne, W.J.

1958 Studies on bacterial utilization of uronic acids. III. Induction of oxidative enzymes in a marine isolate. J.Bact. 76:301-307.

Payne,W.J.

1960 Effects of sodium and potassium ions on growth and substrate penetration of a marine pseudomonad. J.Bact. 80:696-700.

Post,R.L., Merritt,C.R., Kinsolving,C.R. and Albright,C.D.

1960 Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J.Biol.Chem. 235:1796-1802.

Pratt,D.B. and Riley,W.H.

1955 Lysis of a marine bacterium in salt solutions. Bact.Proc. 55:26.

Pratt,D.B. and Waddell,G.H.

1959 Adaptation of marine bacteria to growth in media lacking sodium chloride. Nature 183:1208-1209.

Racker,E.

1950 Spectrophotometric measurements of the enzymatic formation of fumaric and cis-aconitic acids. Biochim.et Biophys.Acta. 4:211-214.

Schatzmann,H.J.

1953 Cardioactive glycosides as inhibitors for the active transport of potassium and sodium through the erythrocyte membrane. Helv.Physiol.et Pharmacol.Acta. 11:346-354.

Sinai,J. and Yudkin,J.

1959 The origin of bacterial resistance to proflavine. V. Transformation of proflavine resistance in E.coli. J.Gen.Micro. 20:400-413.

Skou,J.C.

1957 The influence of some cations on an adenosine triphosphatase from peripheral nerves.
Biochim.Biophys.Acta. 23:394-401.

Skou,J.C.

1960 Further investigations on a Mg^{++} + Na^{+}-activated adenosintriphosphatase, possibly related to the active, linked transport of Na^{+} and K^{+} across the nerve membrane.
Biochim.Biophys.Acta. 42:6-23.

Spizizen,J.

1958 Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate.
Proc.Natl.Acad.Sci. 44:1072-1078.

Stanier,R.Y.

1941 Studies on marine agar-digesting bacteria.
J.Bact. 42:527-559.

Szende,K., Sik,T., Ordogh,F. and Gyorffy,B.

1961 Transfer of immunity by nucleic acids of a lysogenic Rhizobium strain.
Biochim.Biophys.Acta. 47:215-217.

Takacs,F.P. and MacLeod,R.A.

1962 Personal communication.

Tomlinson,N. and MacLeod,R.A.

1957 Nutrition and metabolism of marine bacteria. IV. The participation of Na^{+} K^{+}, and Mg^{++} salts in the oxidation of exogenous substrates by a marine bacterium.
Can.J.Microbiol. 3:627-638.

Tyler,M.E., Bielling,M.C. and Pratt,D.B.

1960 Mineral requirements and other characters of selected marine bacteria. J.Gen.Microbiol. 23:158-161.

Umbreit,W.W., Burris,R.H. and Stauffer,J.F.

1959 Manometric Techniques, Third Edition. Burgess Publishing Co., Minneapolis, Minn.

Voroschilova,A. and Dianova,E.

1935 The role of plancton in the multiplication of bacteria in isolated samples of sea water. Mikrobiologiia. 6:741-753.

Waksman,S.A.

1934 The role of bacteria in the cycle of life in the sea. Sci.Monthly. 38:35.

Warming,E.

1875 Om nogle ved Danmarks Kyster levende Bakterien. Medd.Naturhist.For.Kjobenhavn. 7:356-376.

Warming,E.

1876 Om nogle ved Danmarks Kyster levende Bakterien. Medd.Naturhist.For.Kjobenhavn. 8:21-42.

Weibull,C.

1958 Bacterial protoplasts. Ann.Rev.Microbiol. 12:1-26.

Weibull,C., Greenawalt,J.W. and Low,H.

1962 The hydrolysis of adenosinetriphosphate by cell fractions of Bacillus megaterium. I. Localization and general characteristics of the enzymic activities. J.Biol.Chem. 237:847-852.

Weil,A.J. and Binder,M.

1947 Experimental type transformation of Shigella paradysenteriae (Flexner). Proc.Soc.Exp.Biol.Med. 66:349-352.

Wood,E.J.F.

1958 The significance of marine microbiology. Bact.Rev. 22:1-19.

Young,F.E. and Spizizen,J.

1961 Physiological and genetic factors affecting transformation of Bacillus subtilis. J.Bacteriol. 81:823-829.

ZoBell,C.E.

1946 Marine Microbiology. Waltham, Mass., The Chronica Botanica Co.

ZoBell,C.E.

1952 Part played by bacteria in petroleum formation. J.Sediment.Petrol. 22:42.

ZoBell,C.E. and Conn,J.E.

1940 Studies on the thermal sensitivity of marine bacteria. J.Bact. 40:223-238.

ZoBell,C.E. and Upham,H.C.

1944 A list of marine bacteria including descriptions of sixty new species. Bull.Scripps Inst.Oceanogr. 5: 239-292.