SODIUM-STIMULATED TRANSPORT OF METABOLITES IN TWO MARINE BACTERIA

by

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SHORT TITLE

TRANSPORT IN MARINE BACTERIA

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ABSTRACT

M.Sc. RANDAL DRONIUK Microbiology

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The effects of Na^+ on the transport of a variety of metabolites were examined in the marine bacteria Alteromonas haloplanktis and Vibrio fischeri. A. haloplanktis required Na^+ for the uptake of all substrates tested. V. fischeri showed some ability to transport metabolites in the absence of Na^+. Na^+ was required by this organism for the optimal uptake of all the substrates tested except D-glucose, the accumulation of which was unaffected by the ion. Li^+, but not K^+, was able to replace Na^+ in promoting the uptake of some metabolites by both organisms. The Na^+ requirement for maximum rates of transport exceeded the Na^+ requirement for a maximum rate of respiration in both bacteria. The intracellular concentrations of transported metabolites were determined, and both organisms accumulated all of the substrates against their concentration gradients. Larger gradients were accumulated by the cells at high Na^+ concentrations than at low Na^+ concentrations, even though the external osmotic pressure was the same under both conditions. When they were accumulated, the size of the gradients increased with time. These results indicate that the metabolites were actively transported by the cells.

RESUME

M.Sc. RANDAL DRONIUK Microbiologie

STIMULATION DU TRANSPORT DES METABOLITES PAR L'ION SODIUM CHEZ DEUX BACTERIES MARINES

Les effets de l'ion sodium sur le transport de plusieurs métabolites ont été étudiés chez les bactéries marines Alteronomas haloplanktis et Vibrio fischeri. A. haloplanktis requiert le Na^+ pour le transport de tous les substrats examinés. L'ion Na^+ ne stimulait nullement le transport du glucose par V. fischeri. Cet organisme transportait les autres substrats en l'absence de Na^+, mais la présence de cet ion était nécessaire à l'obtention des taux maximaux de transport. Le Li^+, mais pas le K^+, pouvait remplacer le Na^+ comme stimulateur du transport de certains métabolites par les deux organismes. Chez les deux bactéries, l'exigence en Na^+ pour les taux maximaux de transport était plus grande que l'exigence en Na^+ pour un taux maximum de respiration. Les déterminations des concentrations intracellulaires des substrats transportés révélèrent, dans les deux cas, des concentrations supérieures aux celles trouvées dans le milieu. On trouva que l'accumulation des métabolites était plus efficace dans les milieux contenant de grandes concentrations de Na^+ que dans les milieux contenant de faibles concentrations de Na^+. Enfin, les concentrations intracellulaires des substrats augmentaient en fonction du temps. Ces résultats suggèrent que les métabolites étaient activement transportés par les cellules.

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GLOSSARY

AIB	:	α-aminoisobutyric acid
ChCl	:	choline chloride
CST	:	complete salts tris buffer
NADH	:	reduced nicotinamide adenine dinucleotide
PTS	:	phosphoenolpyruvate transferase system
P_i	:	inorganic phosphate
pmf	:	proton motive force

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INTRODUCTION

Investigations of the ionic requirements of marine bacteria have shown that these organisms have a specific sodium requirement for growth which distinguishes them from most of their terrestrial counterparts (MacLeod and Onofrey, 1957; Reichelt and Baumann, 1974). The physiological basis of this sodium dependence has been studied extensively only in Alteromonas haloplanktis, where it has been demonstrated that the need for sodium is related to the function of several critical cellular processes (MacLeod, 1980). Na^+ is specifically required for membrane transport (Drapeau and MacLeod, 1963) and is required less specifically for its osmotic action which affects the retention of intracellular solutes (Wong et al., 1969; MacLeod et al., 1978). Recent studies have defined the role of sodium in energy coupling to the transport of α-aminoisobutyric acid (AIB) (Niven and MacLeod, 1978; 1980). Sodium has also been shown to influence the respiratory activity of A. haloplanktis (G. Khanna, M.Sc. thesis, 1980).

This project was undertaken to expand our understanding of the role of Na^+ in membrane transport by marine bacteria. Most of the previous work on this topic was done using the non-metabolizable alanine analogue AIB as a substrate, therefore it was considered that it would be more relevant to examine the uptakes of metabolizable substrates which bacteria could actually use for growth. It would also be useful to study another marine bacterium, in addition to A. haloplanktis, to determine if Na^+ influences both organisms in the same way. Some of the techniques

have been refined since previous studies were done, and other modifications in experimental design were needed to account for osmotic effects on membrane permeability (MacLeod et al., 1978) and the effects of Na^+ on respiration (G. Khanna, M.Sc. thesis, 1980).

The effects of Na^+ on transport were studied in the marine bacteria Alteromonas haloplanktis and Vibrio fischeri. The aim of this research was to determine 1) if there is a Na^+ requirement for transport exceeding that for respiration, 2) if K^+ or Li^+ can spare the need for Na^+, and 3) if substrates are accumulated against their concentration gradients by an active transport mechanism.

The existence of an absolute Na^+ requirement for the uptake of nutrients would presumably be a major factor preventing marine bacteria from growing in the absence of Na^+.

LITERATURE REVIEW

1. The sodium requirement for growth

The sea is one of the most important environments on this planet. There is an enormous biological diversity within its boundaries ..... indeed, life is thought to have originated in the sea. Perhaps the single most important physical characteristic of the marine environment (besides an abundance of water) is its salinity, and most marine organisms have physiological features which allow them to accomodate to the problems associated with living in a saline environment. In most cases, these adaptations are associated with a dependence on salinity for survival. These creatures are therefore restricted to living in the sea and their marine nature often distinguishes them from similar organisms found in other habitats.

That marine bacteria have a special requirement for inorganic ions was first reported by Fischer (1894), who observed that the highest plate counts were obtained from seawater samples when the plating medium was prepared with seawater or supplemented with 3% NaCl. Harvey (1915) found that luminescent marine bacteria did not luminesce in fresh water. The presence of sucrose, as well as salts, permitted the organisms to luminesce. He concluded that salts were needed to provide a suitable extracellular osmotic pressure.

Richter (1928) showed that a marine bacterium had a specific Na^+ requirement for growth, and these results were confirmed by Mudrak

(1933) and Dianova and Voroshilova (1935). Other researchers reported, however, that the apparent Na^+ requirement for growth was an unstable characteristic that could be eliminated by "training" the organisms to survive on progressively lower amounts of Na^+ (see MacLeod, 1965 and 1968 for reviews).

Using chemically defined media, MacLeod and co-workers investigated the nutritional requirements of marine isolates for the purpose of resolving the controversy as to whether there were bacteria native to the marine environment with unique ionic requirements that distinguished them from terrestrial bacteria. Seawater could be replaced by an artificial salts mixture (MacLeod et al., 1954) and requirements for Mg^{+2}, PO_4^{-3}, SO_4^{-2}, K^+ and Na^+ were demonstrated (MacLeod and Onofrey, 1956). It was the requirement for Na^+, however, that distinguished these marine bacteria from non-marine forms. Further studies established the highly specific nature of this need for Na^+. Two hundred to 300 mM Na^+ was required for the optimal growth of three marine bacteria (MacLeod and Onofrey, 1957). Li^+, K^+, Rb^+ and sucrose could replace some, but never all, of the Na^+. It was concluded that the primary effect of Na^+ was not osmotic, since if this was the case, one should be able to replace it entirely with another solute.

MacLeod and Onofrey (1956, 1963) were unsuccessful in their attempts to train marine bacteria to grow in a Na^+-free chemically defined medium. A marine pseudomonad grew slowly in a complex medium with no added Na^+, but analysis of the medium revealed that there was

28 mM Na^+ present as contamination. Attempts to mutate marine bacteria to Na^+-independence have also been unsuccessful (Gow et al., 1981). Although they may grow in suboptimal levels of Na^+, it is evident that the Na^+ requirement for growth of marine bacteria is a very stable characteristic of these organisms.

The existence of a Na^+ requirement for the growth of marine bacteria has been well documented. Tyler et al. (1960) reported that a marine pseudomonad needed at least 60 mM Na^+ for growth. A marine Vibrio studied by Pratt and Austin (1963) grew only in the presence of Na^+, although K^+ or Mg^{+2} could partially spare this requirement. Four strains of Photobacterium fischeri examined by Srivastava and MacLeod (1971) required between 25 and 300 mM Na^+ for optimum growth. Reichelt and Baumann (1974) screened 700 strains of marine bacteria and found that they grew only in the presence of Na^+. Vibrio alginolyticus grew best in 500 mM Na^+ (Unemoto et al., 1977) as did Vibrio parahaemolyticus (Morishita and Takada, 1976). In the latter case it was found that Li^+ could replace most of the Na^+, but there was a minimum essential requirement for 3 mM Na^+. The authors concluded that there is a specific need for Na^+ plus a requirement for osmotic support which may be provided by Na^+ or other solutes. A psychrophilic marine Vibrio grew best in 200 to 300 mM Na^+, however at suboptimal Na^+ levels addition of K^+ or Mg^{+2} stimulated growth (Hayasaka and Morita, 1979).

Marine bacteria have a characteristically stable Na^+ requirement for growth, but it is now recognized that this feature is not unique

to these organisms. The rumen bacteria, Bacteroides succinogenes (Bryant et al., 1959) and Bacteroides amylophilus (Caldwell et al., 1973) have been shown to require Na^+, and in the latter case neither K^+, Li^+, Rb^+ nor Cs^+ was able to replace Na^+. Extreme halophiles grow best in media containing 20 to 30 percent NaCl (Larsen, 1967). The moderate halophiles Micrococcus halodenitrificans and Vibrio costicolus were shown to have a stable salt requirement (Forsyth and Kushner, 1970). Absolute Na^+ requirements have been reported for Rhodopseudomonas spheroides (Sistrom, 1960), Pseudomonas stutzeri (Kodama and Taniguchi, 1976) and an alkalophilic Bacillus (Kitada and Horikoshi, 1977).

Some organisms do not need Na^+ for growth, but may be halotolerant or are affected by Na^+ only under certain conditions of growth. Escherichia coli needed 3 mM Na^+ for optimal growth when glutamate was the sole carbon source (Frank and Hopkins, 1969). Aerobacter aerogenes was shown to have an irreplaceable Na^+ requirement for anaerobic, but not aerobic growth on citrate (O'Brien and Stern, 1969 a, b). Salmonella typhimurium needed 7 mM Na^+ for aerobic growth on citrate and this requirement was not spared by NH_4^+, K^+, Li^+, Rb^+ or Cs^+ (O'Brien et al., 1969).

2. The effect of Na^+ on marine bacteria

a. Transport

Studies on Na^+-dependent growth of marine bacteria indicated that the ion influenced more than just the osmotic pressure of the medium. The extreme stability of the requirement suggested that perhaps

Na^+ was associated with the function of some essential aspects of cellular physiology.

In order to determine the role of Na^+ in the growth of A. haloplanktis, the effect of Na^+ concentration on the metabolism of organic substrates was investigated (MacLeod et al., 1958; MacLeod and Hori, 1960). Fifty to 200 mM Na^+ was needed for the optimal oxidation of a variety of carboxylic acids by whole cells. When cell-free extracts were tested, neither Na^+ nor K^+ stimulated oxidation of the substrates. It was proposed that perhaps Na^+ was required by the cells to transport metabolites across the cytoplasmic membrane.

Payne (1960) showed that Na^+ stimulated uptake and oxidation of glucuronate in whole cells of a marine bacterium. He interpreted his results as indicating that Na^+ induced a glucuronate permease system, but that it was not needed for the actual uptake of substrates.

Drapeau and MacLeod (1963) found that α-aminoisobutyric acid (AIB), a non-metabolizable analogue of alanine, was rapidly accumulated by cells of A. haloplanktis in the presence of 200 mM Na^+. There was no uptake in the absence of Na^+, and the requirement for the ion was shown to be very specific in that neither K^+, Li^+, Rb^+, NH_4^+ nor sucrose was able to stimulate transport. Cells began to take up radioactivity immediately after addition of ^{14}C-AIB, and chloramphenicol did not inhibit transport. Payne's theory of a Na^+-induced permease was therefore discounted. The uptake of D-fucose, an analogue of galactose, was also shown to be Na^+-dependent.

Another marine organism, Photobacterium fischeri, was subsequently found to require Na^+ for the transport of AIB (Drapeau et al., 1966). Further studies showed that there was a relation between Na^+-dependent transport and growth of A. haloplanktis. The Na^+ requirement for oxidation of alanine and galactose was similar to the Na^+ requirement for transport of their non-metabolizable counterparts AIB and fucose. This suggested that the Na^+ requirement for oxidation represents a Na^+ requirement for transport. The 200 mM Na^+ requirement for optimum transport and oxidation of alanine corresponded well to the requirement for growth when alanine was the sole carbon source. Only 50 mM Na^+ was needed for the oxidation and transport of galactose as compared to 200 mM Na^+ for growth. It was suggested that the extra Na^+ needed for growth above the 50 mM level represents a non-specific need for a medium of suitable ionic strength (Drapeau et al., 1966).

Paul Wong (Ph.D. thesis, 1968) examined the effects of inorganic ions on the transport of metabolizable substrates by A. haloplanktis and P. fischeri. After ten minutes, both organisms had accumulated amino acids in a 200 mM NaCl buffer, but not in a 200 mM choline chloride buffer. The lack of uptake in the latter case was not due to inhibition by choline chloride (ChCl) since the uptake in 200 mM ChCl + 200 mM NaCl was similar to that in 200 mM NaCl alone. K^+, Li^+, NH_4^+ and sucrose did not stimulate transport. Na^+ stimulated the uptake of inorganic phosphate in both bacteria. A. haloplanktis took up D-galactose best at 100 mM Na^+, whereas 200 to 300 mM Na^+ was needed for the optimum uptake of citrate, succinate and malate.

Many aspects of membrane transport in A. haloplanktis have been characterized in subsequent studies. Kinetic analysis of AIB transport showed that, between 0 and 50 mM Na^+, the K_m decreased but V_{max} remained constant. Between 50 and 200 mM Na^+, the K_m was constant and V_{max} increased (Wong et al., 1969). DeVoe et al. (1970) showed that whole cells and protoplasts have the same capacity to transport AIB. Na^+ was required in both cases and these results suggested that Na^+ affected transport at the level of the cytoplasmic membrane. The essential role of intracellular K^+ in transport has been described (Thompson and MacLeod, 1971; 1973, 1974b) and Na^+-dependent K^+ transport was reported by Hassan and MacLeod (1975). Kinetic studies have revealed the existence of three neutral amino acid transport systems in this organism (Fein and MacLeod, 1975; Pearce et al., 1977).

There have been few studies on transport in other marine organisms. Baumann (1979) has recently reported a Na^+ requirement for the uptake of D-fructose by Pseudomonas doudoroffi. A psychrophilic marine Vibrio took up both glutamate and TMG (methyl-B-D-thiogalactoside) in the absence of Na^+. Transport was greatly enhanced by the addition of Na^+, but not by the addition of NH_4^+, Li^+ or Rb^+ (Hayasaka and Morita, 1979). The glucose: phosphotransferase system of Serratia marinorubra was unaffected by salinity (Hodson and Azam, 1979). Na^+-dependent uptake of nitrate and urea has been demonstrated in a marine diatom (Rees et al., 1980).

Much of the sea is characterized by low nutrient concentrations,

and recent studies have examined the ability of marine bacteria to take up substrates under these conditions. Akagi et al. (1980) have introduced the concept of "oligotrophic" and "heterotrophic" marine bacteria. Organisms of the former group appear to have a competitive advantage which enables them to grow at lower nutrient concentrations than organisms of the latter group. An oligotroph and a heterotroph were found to have K_m's of 0.2 uM and 1.8 uM respectively for proline transport, and K_m's of 13 uM and 3.2 uM for glucose transport. At low concentrations, both substrates were taken up more rapidly by the oligotroph than by the heterotroph, and substrate specificity of the oligotrophic proline transport system was much broader than that of the heterotrophic system (Akagi and Taga, 1980). A high affinity neutral amino acid uptake system with broad substrate specificity has also been detected in A. haloplanktis (Pearce et al., 1977). Geesey and Morita (1979) found that a marine psychrophile has a high affinity arginine uptake system with a K_m of 17 nM as well as a low affinity system with a K_m of 4.5 uM. In all three studies, the authors suggest that high affinity uptake mechanisms may be important adaptations which allow marine bacteria to survive in a nutrient-poor environment.

Although this concept may be valid, it seems doubtful that it would be applicable only to marine organisms. Bacteria, in many environments often have to cope with the extremes of "feast or famine".

b. Energy coupling to transport

Early studies of AIB transport by A. haloplanktis indicated that

the process is energy dependent. The cells' endogenous energy reserves could drive uptake, but addition of an exogenous electron donor such as D-galactose stimulated transport even further (Drapeau and MacLeod, 1963). Anaerobiosis and the metabolic inhibitors KCN and dinitrophenol inhibited AIB uptake (Drapeau et al., 1966; Sprott and MacLeod, 1972). Energy must be expended in order to concentrate a substrate against its gradient, and cells of A. haloplanktis were shown to accumulate AIB to an intracellular concentration of 600 mM from an initial extracellular concentration of 100 uM (Wong et al., 1969). Sprott and MacLeod (1972) reported that the transport of AIB and alanine into membrane vesicles could be energized by the oxidation of the exogenous electron donors NADH (reduced nicotinamide adenine dinucleotide) and ascorbate-reduced TMPD (N, N, N', N' - tetramethyl - p - phenylenediamine dihydrochloride). Cells of A. haloplanktis can also oxidize ethanol and use it as an energy source for transport (Sprott and MacLeod, 1974).

Thompson and MacLeod (1971) proposed a model explaining the effect of Na^+ on transport which was based on the observation that Na^+ decreases the K_m for AIB uptake (Wong et al., 1969). Na^+ would increase the affinity of the carrier for its substrate, and the ternary complex would be translocated to the inner face of the cytoplasmic membrane where the substrate would be released in response to a reduction in affinity of the carrier for its substrate. Further experiments seemed to discount the possibility that a Na^+ or K^+ gradient was the driving force for transport (Thompson and MacLeod, 1973). It was estimated that the intracellular Na^+ and K^+ concentrations in A. haloplanktis were 90 mM

and 440 mM respectively. Uptake of AIB was not inhibited, however, when the ion gradients were supposedly eliminated by suspending the cells in a buffer containing 90 mM Na^+ and 440 mM K^+. Studies on the ability of electron donors to stimulate transport (Sprott and MacLeod, 1974; Thompson and MacLeod, 1974) indicated that perhaps energy coupling could be explained by Kaback's theory (Kaback and Barnes, 1971) that transport carriers were redox proteins energized by the movement of electrons through the respiratory chain.

In 1971, Stock and Roseman presented evidence for a Na^+-sugar cotransport system in Salmonella typhimurium. Efforts at showing a similar mechanism in A. haloplanktis were unsuccessful (Sprott et al., 1975) since $^{22}Na^+$ could not be detected in the cells. This suggested that the organism actively excluded the ion and that perhaps the intracellular Na^+ concentration was negligible. Recent investigations have provided evidence favoring a chemiosmotic interpretation of energy coupling in A. haloplanktis. Cellular respiration establishes an inwardly directed transmembrane proton gradient, and a $Na^+ - H^+$ antiporter catalyzes an electroneutral exchange of protons for Na^+ ions (Niven and MacLeod, 1978). The Na^+ gradient can drive AIB uptake via a symport mechanism (Niven and MacLeod, 1980).

c. Respiration

An effect of Na^+ on bacterial respiration was first observed by Kodama and Taniguchi (1976) in their studies on Pseudomonas stutzeri. Although cells respired in the absence of Na^+ and K^+, addition of K^+

stimulated oxygen uptake slightly. Addition of Na^+ doubled the rate of respiration, but only in the presence of K^+.

Membrane preparations of the marine bacterium Vibrio alginolyticus and the halophile Vibrio costicolus were subsequently reported to require 300 mM and 500 mM Na^+ respectively for maximum NADH oxidase activity (Unemoto et al., 1977). Neither Li^+, K^+, Rb^+, Cs^+, Mg^{+2} nor Ca^{+2} was able to replace Na^+, although K^+ and Mg^{+2} appeared to cooperate with Na^+. Recent studies on V. alginolyticus have led the authors to conclude that Na^+ exerts its effect at the step of NADH: quinone oxidoreductase (Unemoto and Hayashi, 1979a).

In Photobacterium phosphoreum, Na^+ stimulated oxidation of NADH by acting at a point between NADH and cytochrome b in the electron transfer system (Watanabe et al., 1977). Both Na^+ and K^+ were shown to increase the affinity of NADH dehydrogenase for its substrate (Imagawa and Nakamura, 1978).

Recent investigations have established that Na^+ influences the respiratory activity of A. haloplanktis (G. Khanna, M.Sc. thesis, 1980). Two separate NADH dehydrogenase activities were detected. The external enzyme was unaffected by Na^+, but the internal enzyme required 100 mM Na^+ for maximal activity. It seemed paradoxical that cells which actively exclude Na^+ should have an internal enzyme with a high Na^+ requirement. It was suggested that perhaps this is a control mechanism for keeping Na^+ out of the cells. If Na^+ were to flood the cells, respiration would be

stimulated thus resulting in increased proton extrusion. Na^+ would then be pumped out by the Na^+ - H^+ antiporter.

The oxidation of ethanol by an external alcohol dehydrogenase peaked at 2 mM Na^+, whereas the intracellular activity was highest at 50 mM Na^+ and required the presence of NAD^+. Oxidation of ethanol would presumably generate NADH which in turn would be a source of reducing power for the respiratory chain. Oxidation of ascorbate-reduced TMPD required 5 mM Na^+. Since 250 mM Na^+ was required for a maximum rate of transport of AIB, these results indicated that there is a Na^+ requirement for transport which exceeds that for respiration.

The effect of Na^+ on endogenous respiration was less clear. In the presence of K^+, there was little variation in oxygen uptake between 0 and 250 mM Na^+, however in the absence of K^+, the rate doubled over the same range of Na^+ concentrations.

d. Retention of intracellular solutes and osmotic effects

Na^+ has been shown to be required for the retention of intracellular solutes by A. haloplanktis. Cells preloaded with ^{14}C-AIB in the presence of Na^+ lost most of the label when they were resuspended in a Na^+-free salts solution containing enough Mg^{+2} to prevent lysis (Drapeau and MacLeod, 1965; Wong et al., 1969). No AIB was lost when the cells were resuspended in a buffer containing Na^+. Li^+, and to a lesser extent K^+, were partially effective in preventing the release of AIB. Wong et al. (1969) discovered that there was a decrease in the viability

of the cells which was proportional to the amount of AIB lost. This effect may be a long-term consequence attributable to the loss of other essential intracellular solutes such as K^+.

Some of the early studies on A. haloplanktis suggested that Na^+ does not exert an osmotic effect on cells of this organism. Takacs et al. (1964) showed that Na^+ apparently equilibrated across the cytoplasmic membrane. Different ions also varied considerably in their ability to protect cells against lysis. Mg^{+2} was effective in preventing lysis at 1/10 to 1/100 of the concentration of monovalent cations needed for protection (MacLeod and Matula, 1962). Among monovalent cations, K^+ and NH_4^+ were only half as effective as Na^+ and Li^+. These results indicated that the ability of salts to protect against lysis was not due to their osmotic action, since if that was the case, it would be expected that isomolar concentrations of the various ions would be equally effective. Mg^{+2} has since been postulated to strengthen the cell wall by cross-linking the side chains of peptidoglycan (Rayman and MacLeod, 1975). Other evidence indicates that ions may contribute to the maintenance of cell wall integrity in other ways. At low salt concentrations, isolated cell envelopes released a non-dialyzable electronegative complex of lipid, protein and carbohydrate (Buckmire and MacLeod, 1965). It was postulated that cations protected the cell against lysis by screening the negative charges in the cell wall. Subsequent studies showed that the various cell envelope layers can be removed by multiple washings in 0.5 M sucrose (Forsberg et al., 1970). Na^+ was two to three times more effective than K^+ in protecting whole cells from being lysed by Triton-X-100 detergent, however neither ion was able to protect mureinoplasts or protoplasts

(Unemoto and MacLeod, 1975).

It is now known that cells of A. haloplanktis actively exclude Na^+ from the cytoplasm (Sprott and MacLeod, 1975; Niven and MacLeod, 1978). The osmotic effect of extracellular solutes was clarified in a recent study which showed that ions differ in their abilities to penetrate the cytoplasmic membrane (MacLeod et al., 1978). If the intracellular osmotic pressure is too great relative to the external pressure, the cell will swell and low molecular weight solutes could be expected to escape through the dilated pores in the stretched cytoplasmic membrane. At equiosmolal concentrations, Na^+, Li^+, Mg^{+2} and sucrose were more effective than K^+, Cs^+ and Rb^+ in preventing the loss of ^{14}C-AIB that had been previously accumulated by cells in the presence of Na^+. This phenomenon is best explained by the fact that K^+, Cs^+, and Rb^+ have smaller hydrated radii than Na^+, Li^+ and Mg^{+2}. Ions of the former group would be able to penetrate a stretched cytoplasmic membrane more readily than those of the latter group, and would therefore be less capable of providing osmotic support for the cell. Consequently, higher concentrations of small ions than large ions are required to achieve the same level of osmotic protection. If there is a sufficient difference between the internal and external osmotic pressures, ions with small hydrated radii will penetrate the stretched membrane better than those with large hydrated radii, and will therefore no longer be effective as osmotic solutes.

It was observed that AIB uptake increased as the Na^+ concentration

in the medium increased to 300 mM. At suboptimal Na^+ concentrations, addition of LiCl or sucrose increased the steady state level of AIB taken up. As an intracellular solute, accumulated AIB will contribute to the internal osmotic pressure. External solutes such as Li^+ or sucrose presumably act as a balance to this internal pressure and therefore allow more AIB to be transported before a steady state is achieved where uptake is balanced by leakage through the dilated pores of the cell membrane (MacLeod et al., 1978). These results also demonstrated that the osmotic action of certain cations can protect against cell lysis. If swelling precedes lysis, larger ions such as Na^+ or Li^+ will prevent lysis at lower concentrations than K^+ or NH_4^+ (MacLeod et al., 1978).

Hayasaka and Morita (1979) have reported that a marine Vibrio has a non-specific solute requirement for growth and transport. Their results can be interpreted as being due to the osmotic action of ions as described by MacLeod et al. (1978). At Na^+ levels suboptimal for growth, the addition of K^+ or Mg^{+2} was equally as effective in promoting growth as was the addition of Na^+. The organism has a galactose transport system that can be induced by galactose, fucose or TMG, but only in the presence of Na^+. Cells in a medium containing 110 mM Na^+ could not be induced to take up TMG. Addition of 330 mM Na^+ allowed induction to occur, as did addition of 330 mM K^+ or Mg^{+2}, although these latter ions were less effective than Na^+. The ability of induced cells to transport TMG was also influenced by the concentration of solutes in the assay medium. Cells suspended in a 110 mM Na^+ salts solution took up TMG, however addition of 110 mM Mg^{+2}, K^+, Na^+ or Li^+ increased the amount of substrate

transported by 75 to 150 percent. The authors concluded that the non-specific solute requirement has a major influence on the physiology of this organism. This requirement is defined as the additional solute needed to promote optimal growth and cellular function after all specific ion deficiencies have been satisfied. The existence of such non-specific solute requirements for the growth of marine bacteria has previously been alluded to by other authors (Pratt and Austin, 1963; Drapeau et al., 1966; Morishita and Takada, 1976).

3. Transport in non-marine bacteria

Na^+ has long been known to play an important role in eucaryotic membrane transport (Crane, 1965; Schultz and Curran, 1970). It is only in the last decade, however, that the influence of Na^+ on transport and membrane-related energy metabolism in many procaryotic systems has been clearly elucidated (Lanyi, 1979).

Na^+ affects the uptake of substrates in a wide variety of bacteria (Table 1), however many of the organisms listed here do not have an absolute Na^+ requirement for growth. Some of the transport systems are Na^+-dependent, whereas others are merely stimulated by the ion. In this latter group, it has been occasionally found that there are multiple uptake systems for a given substrate, only one of which is Na^+-dependent. Three glutamate transport systems have been resolved in E. coli, but only one requires Na^+ (Miner and Frank, 1974; Schellenberg and Furlong, 1977). Two uptake systems were found for leucine in Pseudomonas aeruginosa (Hoshino, 1979) and for glutamate in Bacillus subtilis (Kusaka and Kanai,

TABLE 1.a Effects of Na^+ on transport in non-marine bacteria

Organism	Substrate	Effect of Cations	Effect of Na^+ on Kinetics	Reference
Escherichia coli	Glutamate	Na^+ stimulates		Frank and Hopkins (1969)
Escherichia coli	Glutamate		K_m decreases	Halpern et al. (1973)
Escherichia coli	Succinate	Na^+, Li^+ stimulates		Rayman et al. (1972)
Escherichia coli	TMG	Na^+ or Li^+ required		Lopilato et al. (1978) Tsuchiya et al. (1977)
Escherichia coli	Melibiose	Na^+ stimulates	K_m decreases	Tanaka et al. (1980)
Escherichia coli	K^+	Na^+ stimulates TrKA system, Na^+ or Li^+ stimulates Kdp system		Sorensen and Rosen (1980)
Salmonella typhimurium	TMG	Na^+ required		Stock and Roseman (1971)
Salmonella typhimurium	TMG	Na^+ or Li^+ required	K_m decreases	Tokuda and Kaback (1977)
Salmonella typhimurium	Melibiose	Na^+ or Li^+ required	K_m decreases	Niiya et al. (1980)
Alkalophilic Bacillus	AIB	Na^+ required		Koyama et al. (1976)
Alkalophilic Bacillus	Glucose	Na^+ stimulates		Koyama et al. (1976)
Alkalophilic Bacillus	AIB		K_m decreases	Kitada and Horikoshi (1977,1980a)
Alkalophilic Bacillus	Serine	Na^+ required		Kitada and Horikoshi (1980b)

TABLE 1.b

Organism	Substrate	Effect of Cations	Effect of Na^+ on Kinetics	Reference
Halobacterium halobium	All amino acids	Na^+ required	V_{max} increases	MacDonald and Lanyi (1975) Lanyi *et al.* (1976a,b) MacDonald *et al.* (1977a)
Halobacterium salinarium	Glutamate	Na^+ required		Stevenson (1966)
Moderate halophile	Proline	Na^+ stimulates		Peleg *et al.* (1980)
Pseudomonas aeruginosa	Leucine Threonine	Na^+ stimulates Na^+ stimulates	K_m decreases	Hoshino and Kageyama (1979) Shiio *et al.* (1973)
Bacillus licheniformis	Glutamate	Na^+ or Li^+ stimulates		MacLeod *et al.* (1973)
Brevibacterium flavum	Threonine, serine	Na^+ or Li^+ stimulates		Shiio *et al.* (1973)
Micrococcus glutamicus	Threonine	Na^+ stimulates		Shiio *et al.* (1973)
Aerobacter cloacae	Threonine	Na^+ stimulates		Shiio *et al.* (1973)
Serratia marcescens	Threonine	Na^+ stimulates		Shiio *et al.* (1973)
Mycobacterium phlei	Proline	Na^+ or Li^+ required	V_{max} increases	Hirata *et al.* (1974)

TABLE 1.c

Organism	Substrate	Effect of Cations	Effect of Na^+ on Kinetics	Reference
Micrococcus lysodeikticus	Succinate Glucose, valine	Na^+ or Li^+ required Na^+ stimulates	K_m decreases	Ariel and Grossowicz (1974)
Pseudomonas stutzeri	K^+, P_i Glutamate	Na^+ required		Kodama and Taniguchi (1977)
Bacillus subtilis	Glutamate	Na^+ stimulates	K_m decreases	Kusaka and Kanai (1979)
Ectothiorhodospira shaposhnikovii	Succinate Malate Fumarate	Na^+ required		Karzanov and Ivanovsky (1980)

1979), but in both cases only one system actually depended on Na^+ for its activity.

Li^+ has been reported to stimulate transport in many cases. In E. coli, Li^+ was stimulatory, but less effective than Na^+ for succinate uptake (Rayman et al., 1972) and for K^+ transport via the Kdp system (Sorensen and Rosen, 1980). Similar effects have been observed in studies on Mycobacterium phlei (Hirata et al., 1974), Bacillus licheniformis (MacLeod et al., 1973), Brevibacterium flavum (Shiio et al., 1973) and Micrococcus lysodeikticus (Ariel and Grossowicz, 1974). Li^+ was more effective than Na^+ in stimulating the uptake of melibiose and TMG (thiomethyl-B-D-galactopyranoside) by Salmonella typhimurium (Niiya et al., 1980; Tokuda and Kaback, 1977). Li^+ promoted TMG uptake better than Na^+ in E. coli (Tsuchiya et al., 1977) but inhibited melibiose transport (Tanaka et al., 1980). Proline uptake into E. coli was stimulated by Li^+ but not by Na^+ (Kayama-Gonda and Kawasaki, 1979).

In an effort to determine the mechanism of Na^+-dependent transport, many researchers examined the kinetics of uptake, and in most cases found that Na^+ decreased the K_m (Table 1) without affecting V_{max}. Na^+ increased V_{max} without affecting K_m in Halobacterium halobium (Lanyi et al., 1976a,b) and Mycobacterium phlei (Hirata et al., 1974). Thompson and MacLeod (1971) theorized that Na^+ acts as a cofactor which increases the affinity of a carrier for its substrate, and other workers concluded that this model best explained their results (Ariel and Grossowicz, 1974; Kahane et al., 1975). Stock and Roseman (1971) reported that $^{22}Na^+$ was

cotransported with TMG into cells of S. typhimurium, but attempts at demonstrating a similar effect in A. haloplanktis (Sprott and MacLeod, 1975) and E. coli (Kahane et al., 1975) were unsuccessful. These latter results did not disprove the existence of a Na^+-substrate symporter however, since cotransport would be difficult to detect if the Na^+ were pumped out as quickly as it entered the cells. An efficient Na^+ pump would be required for maintenance of a Na^+ gradient if this was to be used as the driving force for transport.

The chemiosmotic hypothesis (Mitchell, 1966; 1973) has recently gained acceptance because of its ability to explain many experimental observations related to energy coupling and membrane transport. This theory suggests that protons are outwardly translocated across the cytoplasmic membrane by respiration, hydrolysis of ATP or by light-energized bacteriorhodopsin activity. Energy is therefore stored in the form of a transmembrane electrochemical proton gradient, or proton motive force (pmf), which consists of two parts: an inwardly directed proton gradient and an interior-negative membrane potential. The movement of protons down their concentration gradient can be directed through a membrane-bound ATPase in order to synthesize ATP, or can drive the uptake of a metabolite into the cell against its concentration gradient (i.e. active transport). Although proton circulation appears to be the primary form of membrane-linked energy conservation and transmission in bacteria, recent discoveries have illustrated the importance of Na^+ gradients in energy coupling. A proton gradient can be converted into a Na^+ gradient by means of a Na^+-H^+ antiporter. A low intracellular Na^+ concentration

is thus maintained by the exchange of extracellular protons for intracellular Na^+ ions.

Na^+-H^+ antiport activity has been demonstrated in E. coli (West and Mitchell, 1974), H. halobium (Lanyi and MacDonald, 1976), S. typhimurium (Tokuda and Kaback, 1977), A. haloplanktis (Niven and MacLeod, 1978), Azotobacter vinelandii (Bhattacharyya and Barnes, 1978) and in an alkalophilic Bacillus (Guffanti et al., 1980; Ando et al., 1980). Harold and Papineau (1972) reported the existence of a Na^+-H^+ antiporter in Streptococcus faecalis, but recent evidence suggests that, under certain conditions, the Na^+ pump may be energized by an ATP-linked process (Heefner and Harold, 1980). In addition to the proton pump bacteriorhodopsin, researchers have recently detected a second light-energized ion pump in H. halobium. This pigment, called halorhodopsin, translocates Na^+ out of the cell, however the primary Na^+ circulation appears to be catalyzed by the Na^+-H^+ antiporter (Lindley and MacDonald, 1979).

It has now been shown in several bacteria that a Na^+ gradient can drive the uptake of nutrients into cells. MacDonald and Lanyi (1975) demonstrated that, in the presence of an artificial Na^+ gradient, leucine was taken up into vesicles of H. halobium even though the pmf had been abolished by an uncoupler. Inhibitors of ATP and respiration had no effect, but transport was inhibited by the Na^+ ionophores gramicidin and monensin. Vesicles with an outwardly-directed Na^+ gradient did not take up glutamate until the Na^+ gradient was reversed by the activity of the Na^+-H^+ antiporter (Lanyi et al., 1976a). Evidence has also been presented

for the existence of Na^+-substrate symporters in E. coli (Hasan and Tsuchiya, 1977; MacDonald et al., 1977b; Lopilato et al., 1978), S. typhimurium (Tokuda and Kaback, 1977; Van Thienen et al., 1978), P. aeruginosa (Hoshino and Kageyama, 1979), B. subtilis (Kusaka and Kanai, 1979), A. haloplanktis (Niven and MacLeod, 1980) and E. shaposhnikovii (Karzanov and Ivanovsky, 1980).

4. Evolutionary and practical considerations of Na^+ dependence

It has been suggested that since life probably began in the sea, the Na^+-dependence of marine bacteria may have also been a characteristic of these original organisms. Perhaps marine bacteria were the common link between two divergent lines of evolution: salt-tolerant halophiles and terrestrial bacteria lacking a Na^+ requirement (MacLeod and Onofrey, 1957). Na^+-dependent transport in a terrestrial bacterium may be a vestigial remainder from a Na^+-requiring ancestor (MacLeod, 1980). Other authors have proposed that Na^+-stimulated transport in E. coli may indicate an evolutionary step towards acquiring Na^+-dependence (Niiya et al., 1980). Such theories are merely speculative, however, since little is known about the evolution of prokaryotes. Recent studies of bacterial phylogeny based on rRNA sequencing indicate that the archaebacteria (e.g. Halobacteria) and eubacteria diverged early in the evolutionary process (Fox et al., 1980). It is not inconceivable that Na^+-dependence could have arisen independently several times.

MacLeod (1965) has suggested that the Na^+ requirement of marine bacteria is related to their ability to survive and grow in the sea.

Wilson and Maloney (1976) have speculated that an "ATP"-driven proton pump was a feature of primitive cells. Osmoregulation could be facilitated by coupling a Na^+ pump to the circulation of protons. Energy stored in these transmembrane ion gradients could be used to drive energy-requiring processes such as the uptake of nutrients.

Skulachev (1978) has observed that conversion of a proton gradient to a Na^+ gradient may be advantageous to a cell because this increases the capacity of the transmembrane energy reservoir. The size of a pH gradient that a cell can establish is limited by the need to maintain a suitable cytoplasmic pH. Proton translocation results in alkalinization of the cytoplasm, however this obstacle can be overcome without affecting the membrane potential by exchanging Na^+ ions for protons. Brey et al. (1978, 1980) have taken the opposite approach by suggesting that maintenance of an optimal pH is the primary function of Na^+-H^+ antiport activity. A mutant alkalophilic Bacillus lacking the antiporter lost its ability to grow in an extremely alkaline medium (Krulwich et al., 1979). These results suggest that in this organism the antiporter is responsible for maintaining an acidic cytoplasm relative to the alkaline environment. A final consideration is that cells must maintain intracellular ionic conditions that are compatible with various metabolic processes. Protein synthesis in the halobacteria requires K^+ and is inhibited by Na^+ (Lanyi, 1974; 1979). Na^+ was also shown to inhibit the oxidation of carboxylic acids by cell-free extracts of A. haloplanktis (MacLeod et al., 1958).

MATERIALS AND METHODS

Organisms

Alteromonas haloplanktis, strain 214, variant 3, formerly known as marine pseudomonad B-16 (ATCC 19855), was maintained at 4°C by monthly transfers on slants of complex medium containing 0.8% (wt/vol) nutrient broth, 0.5% (wt/vol) yeast extract, 300 mM NaCl, 26 mM $MgSO_4$, 10 mM KCl and 1.5% (wt/vol) agar.

Vibrio fischeri, previously known as Photobacterium fischeri (Baumann et al., 1981), was the same organism used by Drapeau et al. (1966) and is kept in the culture collection (MAC 401) of this Department. Cultures were maintained at 20°C by bimonthly transfers on slants consisting of 0.8% (wt/vol) nutrient broth, 0.1% (wt/vol) yeast extract, 3.0% (wt/vol) NaCl, 0.3% (wt/vol) glycerol and 1.5% (wt/vol) agar (Srivastava and MacLeod, 1971).

Growth conditions

Alteromonas haloplanktis was grown in the same medium as used for culture maintenance except that the agar was omitted and the pH was adjusted to 7.2 with KOH. This medium was supplemented with 0.5% (wt/vol) citrate, succinate, L-malate or D-galactose to obtain cells for use in transport studies of these substrates (MacLeod et al., 1958).

Ten ml of medium contained in a 50 ml Erlenmeyer flask was inoculated from an agar slant and incubated at 25°C for twenty four hours

on a gyrotory shaker (200 rpm). A 2.5 ml portion of this starter culture was transferred to 250 ml of medium in a 2 l flask and incubated for sixteen hours under the same conditions as the starter. At sixteen hours, the culture pH was 7.8 to 8.0 (7.0 for galactose-induced cells) and the cells were in the stationary phase of growth.

Vibrio fischeri was grown in a medium containing 100 mM NaCl, 10 mM KCl, 2.5 mM $MgSO_4$, 5.5 mM NH_4Cl, 0.5 mM $CaCl_2$, 100 mM tris(hydroxymethyl)aminomethane (Tris) buffer, 0.5% (wt/vol) yeast extract and 0.3% (wt/vol) glycerol. The pH was adjusted to 7.5 with HCl (Srivastava and MacLeod, 1971). The growth conditions were the same as those used for A. haloplanktis except that the incubation times were only twelve hours. The pH of a twelve hour stationary phase culture was 7.6.

Preparation of cells

Twenty five ml portions of stationary phase cells were harvested by centrifugation at 40,000 x g (4°C) for ten minutes and washed three times with 25 ml volumes of complete salts-tris (CST) buffers. These complete salts solutions contained 50 mM tris(hydroxymethyl)aminomethane, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4 (omitted from $^{32}P_i$ experiments) and combinations of choline chloride, NaCl, KCl or LiCl totalling 300 mM, as required for the different experimental conditions. The pH was adjusted to 7.2 with HCl. The final volume of each stock of washed cells was 4 ml and these were stored in crushed ice.

Determination of cell dry weights

Aliquots from each stock of cells were diluted 50 to 200 times

with the same complete salts solution used in the washing procedure. The optical density of a suitably diluted sample cell suspension was measured at 660 nm on a Gilford 300-N microsample spectrophotometer. The dry weight of cells in the suspension was determined by referring to a previously calibrated curve relating optical density to dry weight. An optical density of 0.260 was equivalent to 100 ug dry wt/ml of cells of A. haloplanktis or 94 ug dry wt/ml of cells of V. fischeri. For transport studies, the densities of stock cell suspensions were adjusted with the appropriate complete salts solutions so that a 1/50 dilution gave 100 ug dry wt of cells/ml.

Measurement of transport

a. ^{14}C-labelled substrates

Stock cell suspensions were diluted with the appropriate complete salts solutions and preincubated for fifteen minutes at 25°C on a water bath shaker (250 rpm). Aliquots of the suspensions were then transferred to a reaction chamber maintained at 25°C by circulating water. The suspensions were constantly aerated by a magnetically stirred Teflon-coated bar. Chloramphenicol (100 ug/ml) was included for amino acid uptake experiments. Ethanol (25 mM) was provided as an exogenous electron donor for A. haloplanktis one minute before addition of the ^{14}C-labelled substrate. After all additions, the final reaction volume was 3.5 ml, and contained approximately 100 ug dry wt of cells/ml.

In all cases, the final concentration of the L-(^{14}C(U)) - amino acids and α-(1 - ^{14}C) - aminoisobutyric acid was 200 uM, while that of

L-(^{14}C(U)) - malic acid was 100 uM. The final concentration of D-(^{14}C(U))-galactose, D-(^{14}C(U)) - glucose, (1,5-^{14}C)-citric acid and (2,3-^{14}C) - succinic acid was 50 uM. The specific activities are indicated in the figure legends.

After addition of the substrate,0.5 ml samples were removed at thirty second intervals and filtered through 0.45 u HA Millipore filters. The cells retained on each filter were washed with 5 ml of the same complete salts buffer used for washing and incubation. Controls were done where 0.5 ml aliquots of the same reaction mixture containing no cells were filtered and washed as described above. The counts on these control filters represented non-specific binding of radioactivity to the filters. The net uptake of radioactivity into the cells was therefore equal to the difference between the control counts and those obtained on the filters used to filter the cell suspensions.

Uptakes were measured for three minutes after addition of the substrates. In those figures where uptakes are expressed as initial velocities (nmol/min/mg cell dry wt), the values used were equivalent to the amount of substrate accumulated by the cells at one minute.

b. Phosphate

The method was the same as that used for ^{14}C-labelled substrates with the following modifications. All complete salts buffers used for washing of cells, incubation and rinsing were the same as previously described except that 1 mM KH_2PO_4 was omitted. The membrane filters

(Amicon) were pretreated by boiling in 1 mM K_2HPO_4 (Grillo and Gibson, 1979); this resulted in greatly reduced $^{32}P_i$ counts on the control filters. The reaction mixtures were contained in 50 ml disposable polystyrene beakers (Fisher), and these were incubated at 25°C on a water bath shaker (250 rpm).

Inorganic phosphate, in the form of $K_2H^{32}PO_4$, was added to a final concentration of 25 uM and a specific activity of 50 uCi/umol. All glassware was cleaned with a phosphate free detergent and concentrated acid. The half life of the $^{32}P_i$ received from New England Nuclear was checked by counting the radioactivity in a set of standard vials at twenty four hour intervals.

Intracellular volumes

A value of 1.6 ul/mg dry wt of cells was used to calculate the intracellular metabolite concentrations in A. haloplanktis (Thompson and MacLeod, 1973).

The intracellular volume of V. fischeri was determined by measuring the extracellular fluid volume of a packed cell preparation. Fifty ml Erlenmeyer flasks, containing 12 ml of washed cells (11-14 mg cell dry wt/ml) in CST - 300 mM NaCl and 3 mM ^{14}C-(U)-sucrose (specific activity of 0.033 uCi/umol), were incubated at 25°C for fifteen minutes on a water bath shaker (Thompson and MacLeod, 1973). Ten ml volumes were transferred to preweighed ultracentrifuge tubes and the cells spun down for forty five minutes at 50,000 x g (4°C) in a Beckman L5-65 ultracentrifuge (Buckmire

and MacLeod, 1970). The supernatants (S1) were poured off and the tubes plus pellets were weighed. The cells were resuspended in CST-300 mM NaCl (final volume 10 ml) but the ^{14}C-sucrose was omitted. The cells were spun down as before and the supernatants (S2) were recovered. The volumes of extracellular fluid trapped in the first pellets were determined by comparing the radioactivity per unit volume of S1 to that of S2, as described by Buckmire and MacLeod (1970). The total available fluid space in a pellet was equal to the difference between the weight of the packed wet pellet and the total dry weight of cells in the pellet.

The dry weight of cells was determined by adding 3 ml of CST-300 mM NaCl, or 3 ml aliquots of the same cell suspensions used above, to acid cleaned 10 ml beakers that had been previously dried to constant weight. The solutions were dried at 80°C until constant weight was achieved on three consecutive days. The residues in the beakers were ashed at 500°C for twenty four hours and the constant weights were determined as above. The dry weight of cells was the difference between the weight of the preashed material, the weight of the ash and the weight of entrapped water lost from the complete salts aliquots during ashing. (Forsberg et al., 1970).

Extraction of intracellular substrate pools after transport

a. ^{14}C-labelled substrates

The procedure was similar to that used by Sprott and MacLeod (1974). Transport was measured as previously described, except that the final reaction volume was 1.1 ml. Each reaction mixture at each Na^+

concentration was sampled twice (at 0.5 and 3 minutes). After filtering the samples and rinsing with 5 ml CST, each filter for each time and Na^+ concentration was transferred as quickly as possible to a separate centrifuge tube containing 20 ml water and maintained at 90°C in a water bath. Each uptake experiment was repeated six times using cells from the same stock suspension. Thus, a total of six filters with 300 ug dry weight of adhering cells were collected at each Na^+ concentration at 0.5 and 3 minutes. The tubes containing the filters were incubated at 90°C for twenty minutes after addition of the last filter and then were centrifuged for fifteen minutes at 40,000 x g (4°C). The supernatants were collected. A further 20 ml of water was added to each tube containing the filters, and these were heated for twenty minutes at 90°C and centrifuged as before. Each supernatant was pooled with its corresponding fraction from the first treatment, thus giving a total of 40 ml of extract for each time and Na^+ concentration. The extracts were freeze dried and each was resuspended in 1 ml water. If a more concentrated extract was needed, these 1 ml volumes were dried and resuspended in 0.2 ml water.

b. Extraction of $^{32}P_i$

Intracellular $^{32}P_i$ pools were extracted by soaking individual filters, containing 50 ug dry wt of cells, for one hour in 2 ml of 5% (wt/vol) trichloroacetic acid (0°C) (Wilkins, 1972; Grillo and Gibson, 1979). Filters from control runs (no cells) were also extracted, as significant amounts of radioactivity were recovered which had to be subtracted from the counts obtained from the cellular extracts. The extracts were centrifuged for fifteen minutes in a clinical centrifuge (4,800 rpm) and

the supernatants were recovered for analysis.

Analysis of cell extracts

a. Thin layer chromatography of amino acids

A micropipette was used to spot 5 ul of concentrated radioactive extract on a thin layer plate (0.1 mm cellulose, MN300). To facilitate detection, 1 ul of a 50 mM solution of the amino acid to be isolated (or of any other reference amino acids desired) was also spotted. The spot was dried in a stream of warm air. The method of Jones and Heathcote (1966) was used to develop the plates and detect the isolated amino acids. The first dimension solvent (80 ml propan-2-ol, 20 ml water and 4 ml formic acid) was run 12 to 13 cm from the origin and the plates were then dried in a stream of warm air. The second dimension solvent (50 ml tertbutanol, 30 ml methyl ethyl ketone, 10 ml NH_4OH and 10 ml water) ran 12 to 13 cm and the plates were dried as before. The spots were detected by spraying the plates with ninhydrin reagent (0.3 g ninhydrin, 20 ml glacial acetic acid, 5 ml 2, 4, 6 - trimethylpyridine (~~S-collidine~~) and 75 ml ethanol) and warming them in a stream of hot air. The spots were scraped into scintillation vials and the radioactivity was measured. See addendum for further information on this procedure.

b. Thin layer chromatography of citric acid cycle compounds

The plates were spotted as above, dried in a stream of cool air and developed 15 cm from the origin in both dimensions using the method of Myers and Huang (1969). The first dimension solvent contained 70 ml ethyl ether, 20 ml formic acid and 10 ml water. The second dimension solvent was composed of 83 ml liquid phenol, 17 ml water and 1 ml formic

acid. The plates were dried and the spots detected by spraying with bromocresol green indicator. This reagent was prepared by dissolving 0.04 g bromocresol green in 100 ml ethanol and adding 0.1N NaOH until a blue coloration appeared. The spots were scraped into vials for measurement of radioactivity.

c. Paper chromatography of sugars

D-galactose was isolated from extracts by descending chromatography on 40 cm strips of Whatman no. 1 chromatography paper (Hais and Macek, 1963; Zweig and Sherma, 1972). Ten ul of 10 mM D-galactose and 5 ul of radioactive extract were spotted and dried under a stream of warm air. The solvent (160 ml isopropanol and 40 ml water, or 80 ml N-butanol, 80 ml pyridine and 40 ml water) was allowed to run at least 30 cm from the origin. The isolated galactose spot was detected by spraying the dried chromatogram with phthalate-aniline reagent (6.25 ml butanol, 93.75 ml water, 0.91 ml aniline and 1.66 g potassium hydrogen phthalate) and heating for five minutes at 100°C. The galactose spot was cut out and placed in a scintillation vial and the radioactivity was measured.

d. $^{32}P_i$ analysis

The $^{32}P_i$ in extracts was precipitated in the form of phosphomolybdate salt (Sugino and Miyoshi, 1964). A 0.1 ml volume of 4 mM K_2HPO_4 and 0.35 ml of a reagent containing one part 0.2 M triethylamine, two parts 0.08 M ammonium molybdate and four parts water, were added to 1 ml samples of trichloracetic acid extract. The mixtures were allowed to stand for thirty minutes and the resulting precipitates were filtered on

0.45 u membrane filters and rinsed with 5 ml water. The filters were transferred to vials and the Cerenkov radiation was measured.

Radioactivity measurements

Filters with ^{14}C-labelled material were placed in plastic screw cap vials and dried under a heat lamp for fifteen to thirty minutes. A 10 ml volume of scintillation fluid (Aquasol) was added to each vial and these were counted for ten minutes each on program 2 of a Nuclear Chicago Isocap/300 liquid scintillation spectrometer. The counts were corrected for quench using the channels ratios method (Wang and Willis, 1965).

For the measurement of ^{32}P, 10 ml of water was added to each vial and the Cerenkov light emission was measured on program 1 of a Beckman LS 7500 liquid scintillation counter. The counts were corrected by taking the efficiency of counting and the decay rate of ^{32}P into consideration.

Estimation of intracellular substrate pools

After having analyzed a known volume of cell extract, one can extrapolate back and determine the amount of unmodified radioactive substrate in the entire extract. Since this extract was obtained from a known quantity of cells, the amount of substrate in these cells can be determined. The intracellular volume was used to convert the nmol extracted substrate/mg cell dry wt to an intracellular concentration.

Respiration

Respiration was measured polarographically in an oxygen electrode apparatus (Rank Bros., Bottisham, Cambridge, England) where a complete salts solution (25°C) was aerated by a magnetically stirred Teflon-coated bar. About 1 mg dry weight of cells was added to bring the final volume of the suspension to 3 ml. The electrode was connected to a chart recorder (model 83860-40, Cole Parmer Instruments Co., Chicago, Ill.). One hundred percent air saturation of the 3 ml suspension equalled 1410 natoms O (Chappell, 1964) and the rate of oxygen consumption was measured as natoms O/min/mg cell dry wt.

Flame spectrophotometric analysis of Na^+

Na^+ contamination in buffers was determined by flame emission spectrophotometry using a Unicam SP90A atomic absorption spectrophotometer (Pye Unicam Ltd., Cambridge, England).

Chemicals

Nutrient broth, yeast extract and agar were purchased from Difco Laboratories (Detroit, Mich.). L-^{14}C-malic acid was obtained from Amersham/Searle Corp. (Arlington Heights, Ill.). All other radioactive materials, scintillation counting vials and Aquasol were products of New England Nuclear (Boston, Mass.). Membrane filters were supplied by Millipore Corp. (Bedford, Mass.) and Amicon Co. (Lexington, Mass). Tris(hydroxymethyl)-aminomethane and chloramphenicol were obtained from Sigma Chemical Co. (St. Louis, Mo.). Ninhydrin and 2, 4, 6 - trimethylpyridine were from Baker Chemical Co. (Phillipsburg, N.J.) and choline chloride was purchased

from Eastman Kodak Co. (Rochester, N.Y.). Bromocresol green and potassium hydrogen phthalate were products of Fisher Scientific Co. (Fair Lawn, N.J.). Thin layer chromatography plates were supplied by Brinkman Instruments (Canada) Ltd. (Rexdale, Ont.). All other chemicals were of analytical quality.

RESULTS

I Effect of Na^+ on transport

a. Alteromonas haloplanktis

The effect of Na^+ on the transport of metabolizable substrates by A. haloplanktis was first examined by Paul Wong (Ph.D. thesis, 1968). The ability to take up ^{14}C-labelled amino acids was evaluated by measuring the radioactivity accumulated in ten minutes by cells suspended in a buffer containing either 200 mM NaCl or 200 mM choline chloride. Wong also determined the effect of a range of Na^+ concentrations on the uptakes of D-galactose, P_i (inorganic phosphate) and three carboxylic acids, however no choline chloride was used as an osmotic balance when suboptimal Na^+ concentrations were tested. It has since been shown that the addition or removal of Na^+ has a direct influence on transport, as well as an effect on the retention of solutes by cells that can be attributed to changes in the osmotic pressure of the medium (MacLeod et al., 1978). It is important to distinguish between these two effects, therefore throughout this study, choline chloride was used as an osmotic balance to compensate for the absence of Na^+. When the concentration of Na^+ was less than 300 mM, sufficient choline chloride was added to bring the total concentration of NaCl and choline chloride to 300 mM.

Sprott and MacLeod (1972; 1974) have demonstrated that electron donors such as ethanol can promote transport in A. haloplanktis by stimulating respiration. Na^+ has also been shown to affect the respiratory activity of this organism (G. Khanna, M.Sc. thesis, 1980), therefore it

was possible that any addition of Na^+ resulting in increased transport ability of the cells might have been due to a stimulation of respiration. The use of ethanol as an energy source for A. haloplanktis provided a means for distinguishing between the effects of Na^+ on transport and respiration. Two mM Na^+ is required for a maximal ethanol-energized respiratory rate (G. Khanna, M.Sc. thesis, 1980), therefore any enhancement of transport by Na^+ above the 2 mM level should be attributable to a direct effect of the ion on the uptake system rather than to a stimulation of respiration.

The effect of Na^+ on the uptake of a variety of metabolites by A. haloplanktis is shown in Figures 1, 2 and 3. The initial rates of uptake of each substrate were determined on the basis of the radioactivity accumulated by the cells after one minute of incubation in the presence of the substrate. None of the eleven substrates were transported in the absence of Na^+, and in all cases, uptake was stimulated by the addition of Na^+. The sensitivity to Na^+ of the different transport systems varied considerably. Cells suspended in a 10 mM NaCl buffer were capable of taking up substantial quantities of P_i, L-lysine and D-galactose. Under the same conditions, citrate, succinate and L-leucine were not taken up at all. The maximum rates of transport of D-galactose and P_i were reached at 50 mM Na^+. The rates of uptake of succinate, L-lysine, L-arginine, L-glutamate and L-malate peaked in the range of 200 to 300 mM Na^+. The maximum rate of uptake of L-alanine and L-leucine was only achieved at 400 to 500 mM Na^+. The rate of citrate accumulation appeared to level off between 200 and 300 mM Na^+, but increased again beyond this point.

High Na^+ concentrations inhibited the uptake of D-galactose, as well as the uptakes of the basic and acidic amino acids.

b. Vibrio fischeri

Previous studies on V. fischeri have demonstrated that Na^+ stimulates the uptake of AIB (Drapeau et al., 1966), amino acids and P_i (P. Wong, Ph.D. thesis, 1968). No exogenous energy source was provided for the cells in these experiments. The cells clearly had adequate endogenous energy reserves to drive uptake in the presence of a sufficient concentration of Na^+. It was not known, however, if Na^+ influenced respiration. Perhaps the inability of cells to transport substrates in the absence of Na^+ actually represented a requirement by transport for Na^+-dependent respiration.

The effect of Na^+ on endogenous respiration and AIB transport was examined (Figure 4). The uptake of AIB was Na^+-dependent, however there was no effect of the ion on respiration. If the respiratory activity was the same at all Na^+ concentrations, the cells should have been equally capable of generating a pmf (proton motive force). These results suggest that a pmf alone was not able to drive the uptake of AIB. Na^+ was required, perhaps in the form of an electrochemical Na^+ gradient, as has been demonstrated for other Na^+-dependent ransport systems (MacDonald and Lanyi, 1975; Hasan and Tsuchiya, 1977; Niven and MacLeod, 1980).

As was the case with A. haloplanktis, Na^+ had varying effects on the transport of different metabolizable substrates by V. fischeri

(Figures 5 and 6). D-glucose was unique in that its uptake was unaffected by Na^+. The transport of all other substrates was stimulated by the addition of Na^+. Except for L-alanine, all the substrates were taken up in the absence of added Na^+, but at rates which were poor in comparison to the maximum rates obtained in the presence of Na^+. The rates of uptake of L-alanine, succinate and L-arginine peaked at 50, 100 and 200 mM Na^+ respectively, and then decreased at higher concentrations. The transport of L-glutamate levelled off between 50 and 200 mM Na^+ and that of P_i did the same between 200 and 300 mM Na^+. In both cases, uptake was further stimulated at higher Na^+ concentrations.

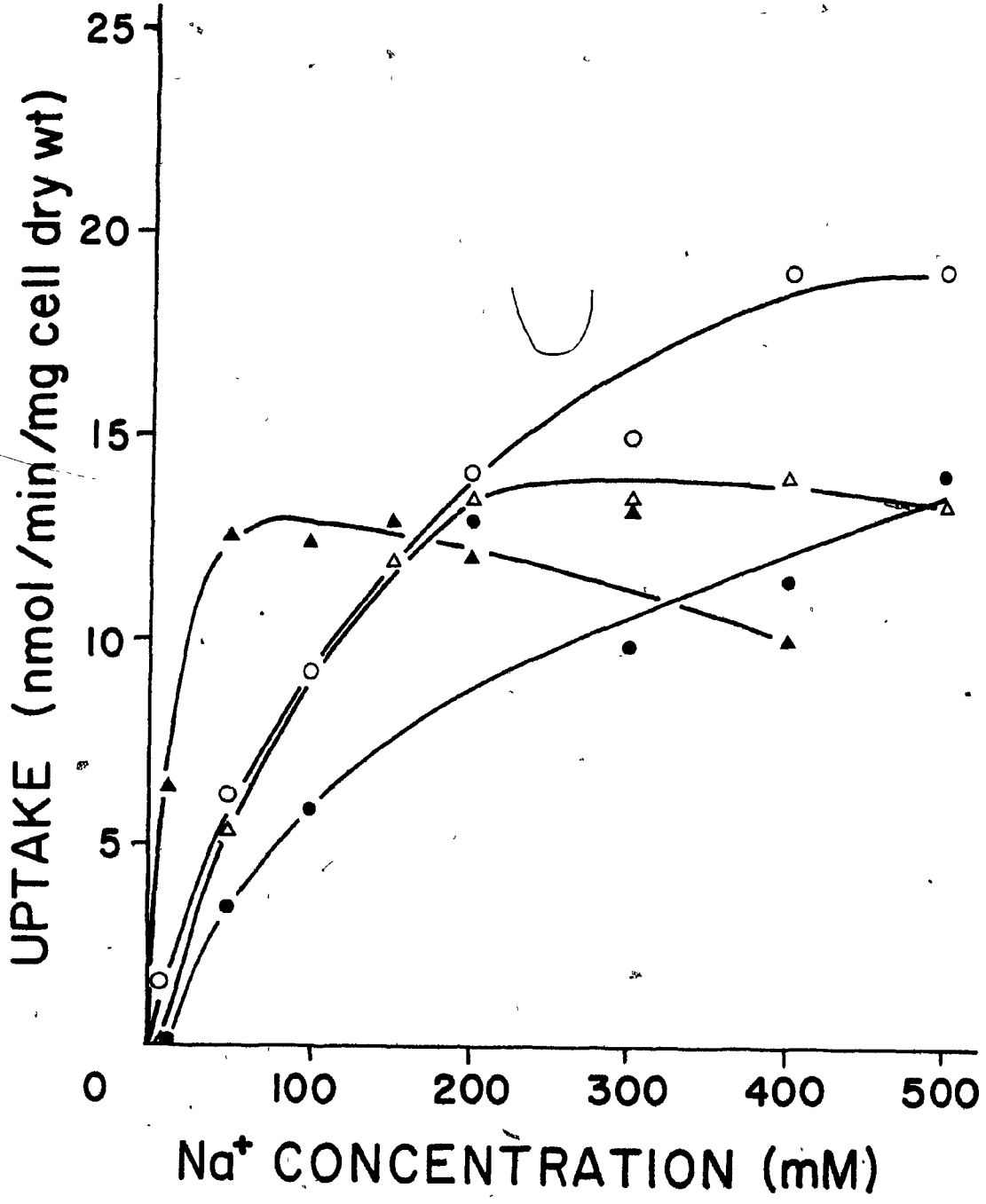
These results illustrate that, except for the transport of D-glucose by V. fischeri, Na^+ is required for the optimal uptake of metabolites by these two marine bacteria.

Figure 1. Effect of Na^+ concentration on the rate of uptake of alanine, leucine, succinate and galactose by A. haloplanktis.

Cells were suspended at a density of about 100 ug dry wt/ml in complete salts buffer (50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4, pH 7.2 plus NaCl and choline chloride as required). At Na^+ concentrations of less than 300 mM, choline chloride was added, as an osmotic balance, such that the total concentration of NaCl and choline chloride was 300 mM. Ethanol was added to a final concentration of 25 mM. The rates of uptake of the following radioactively-labelled substrates were determined at each of the various Na^+ concentrations:

- ○ L-alanine
- ● L-leucine
- △ Succinic acid
- ▲ D-galactose

The specific activity of the substrates was 0.5 uCi/umol in all cases. Other conditions were as described in the Materials and Methods.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400
500
UPTAKE (nmol/min/mg cell dry wt)
Na+ CONCENTRATION (mM)

Figure 2. Effect of Na^+ concentration on the rate of uptake of glutamate, aspartate, arginine and lysine by A. haloplanktis.

○ L-lysine
● L-arginine
△ L-aspartic acid
▲ L-glutamic acid

The specific activity of the substrates was 0.5 uCi/umol in all cases. Other conditions were as described in Figure 1.

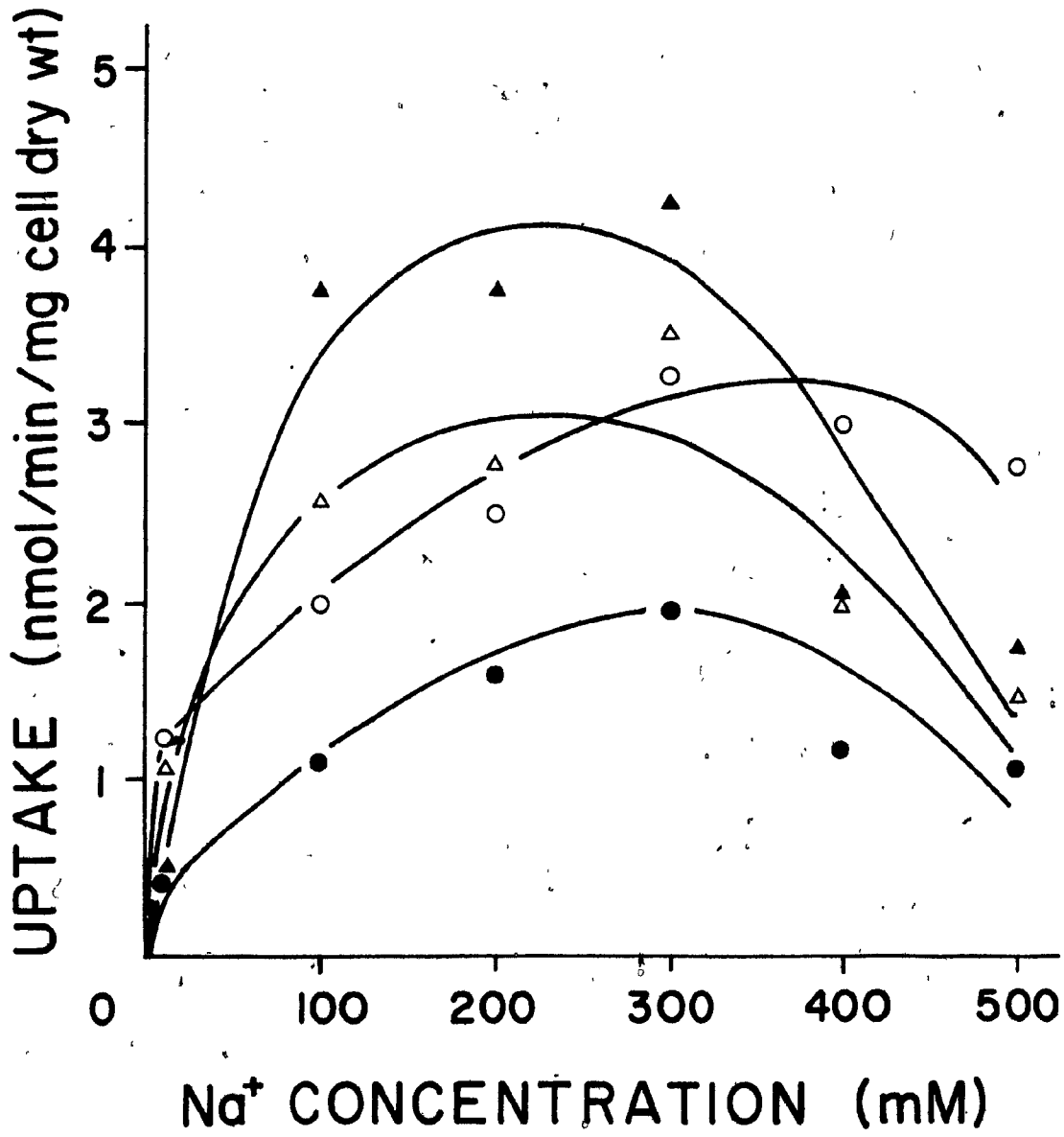

UPTAKE (nmol/min/mg cell dry wt)
5
4
3
2
1
0
100
200
300
400
500
Na+ CONCENTRATION (mM)

Figure 3. Effect of Na^+ concentration on the rate of uptake of citrate, malate and $^{32}P_i$ by A. haloplanktis.

○ Citric acid

● L-malic acid

△ $^{32}P_i$

The specific activity of citrate and malate was 2.5 uCi/umol, and that of $^{32}P_i$ was 50 uCi/umol. Other conditions were as described in Figure 1.

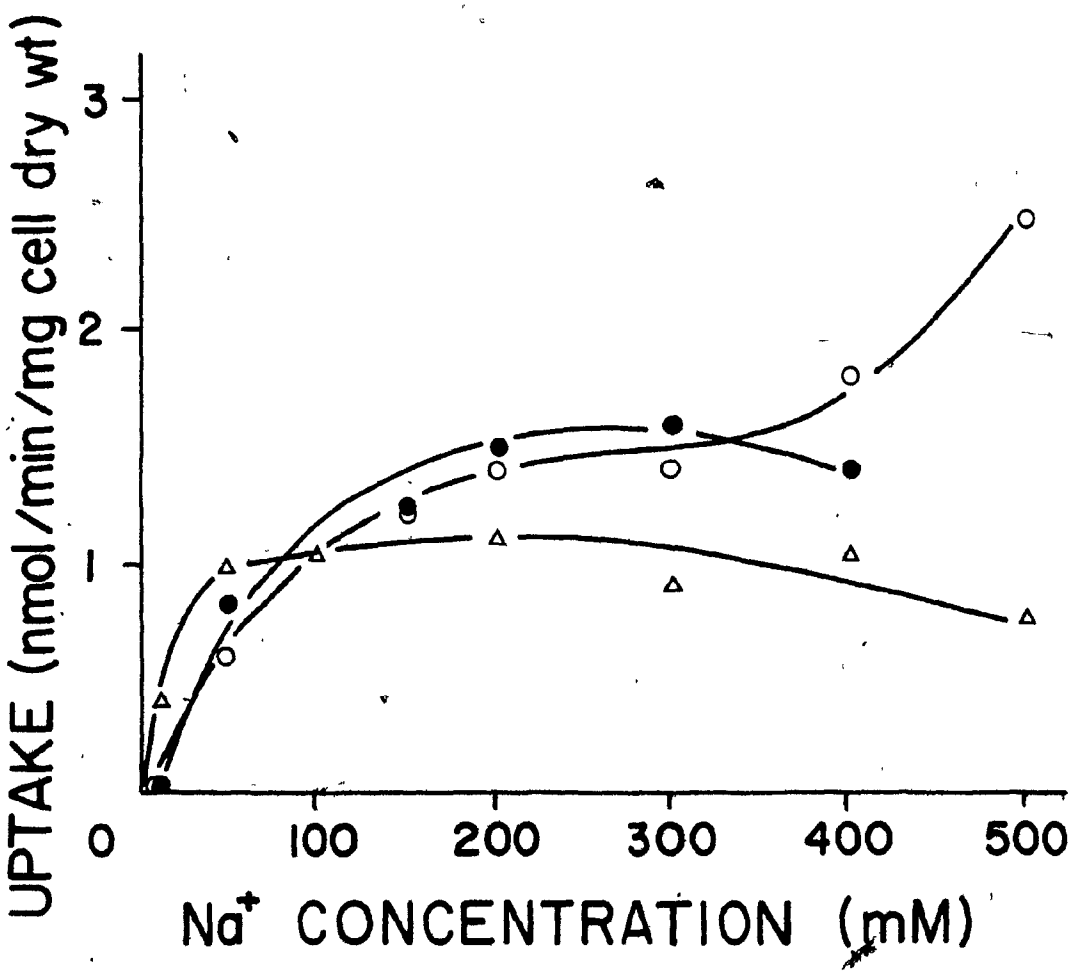

UPTAKE (nmol/min/mg cell dry wt)
0
1
2
3
100
200
300
400
500
Na+ CONCENTRATION (mM)

Figure 4. Effect of Na^+ concentration on endogenous respiration and the rate of uptake of AIB (α-aminoisobutyric acid) in *V. fischeri*.

Respiration (O) was measured as described in the Materials and Methods. The rate of uptake of AIB (●) was measured as described in Figure 1, except that the ethanol was omitted from the incubation medium. The specific activity of the AIB was 0.5 uCi/umol.

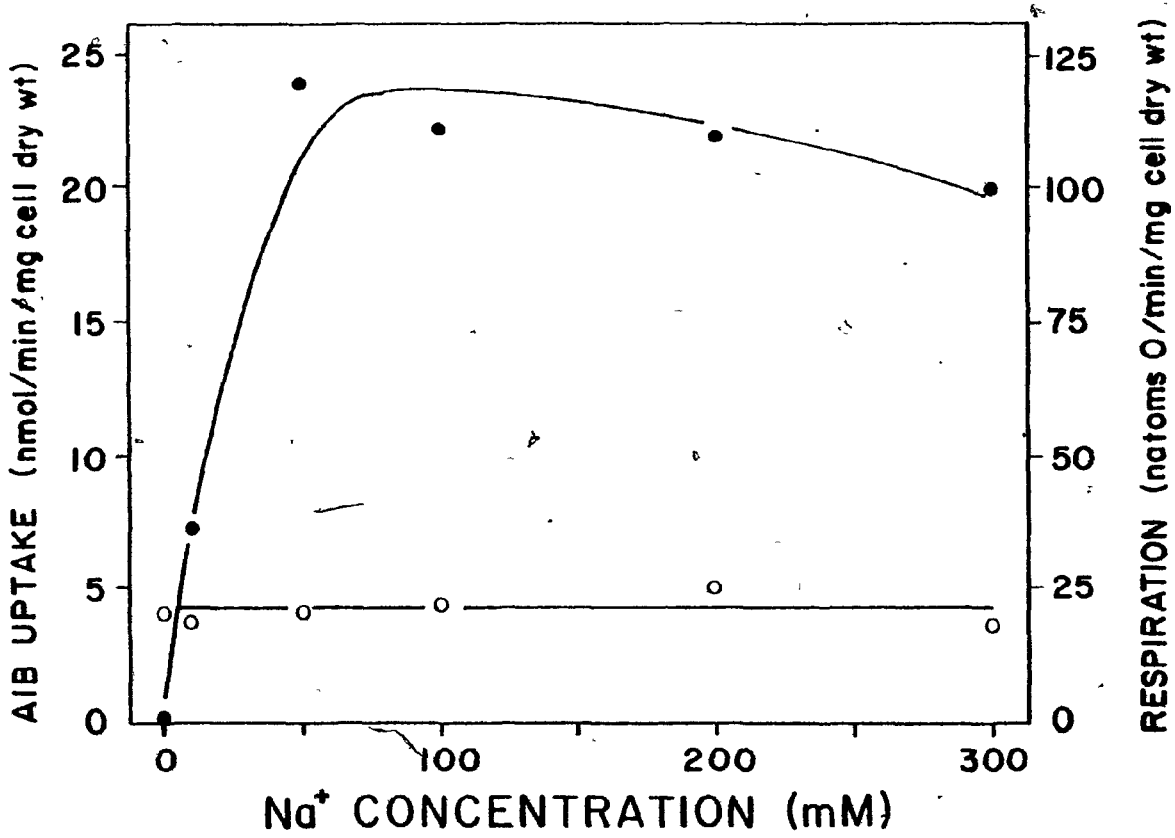

AIB UPTAKE (nmol/min/mg cell dry wt)
RESPIRATION (natoms O/min/mg cell dry wt)
Na+ CONCENTRATION (mM)
0
5
10
15
20
25
0
25
50
75
100
125
0
100
200
300

Figure 5. Effect of Na^+ concentration on the rate of uptake of glucose, alanine and succinate by V. fischeri.

○ D-glucose
△ Succinic acid
● L-alanine

The specific activity of alanine and succinate was 0.5 uCi/umol, and that of glucose was 5 uCi/umol. Other conditions were as described in Figure 1, except that the ethanol was omitted from the incubation medium.

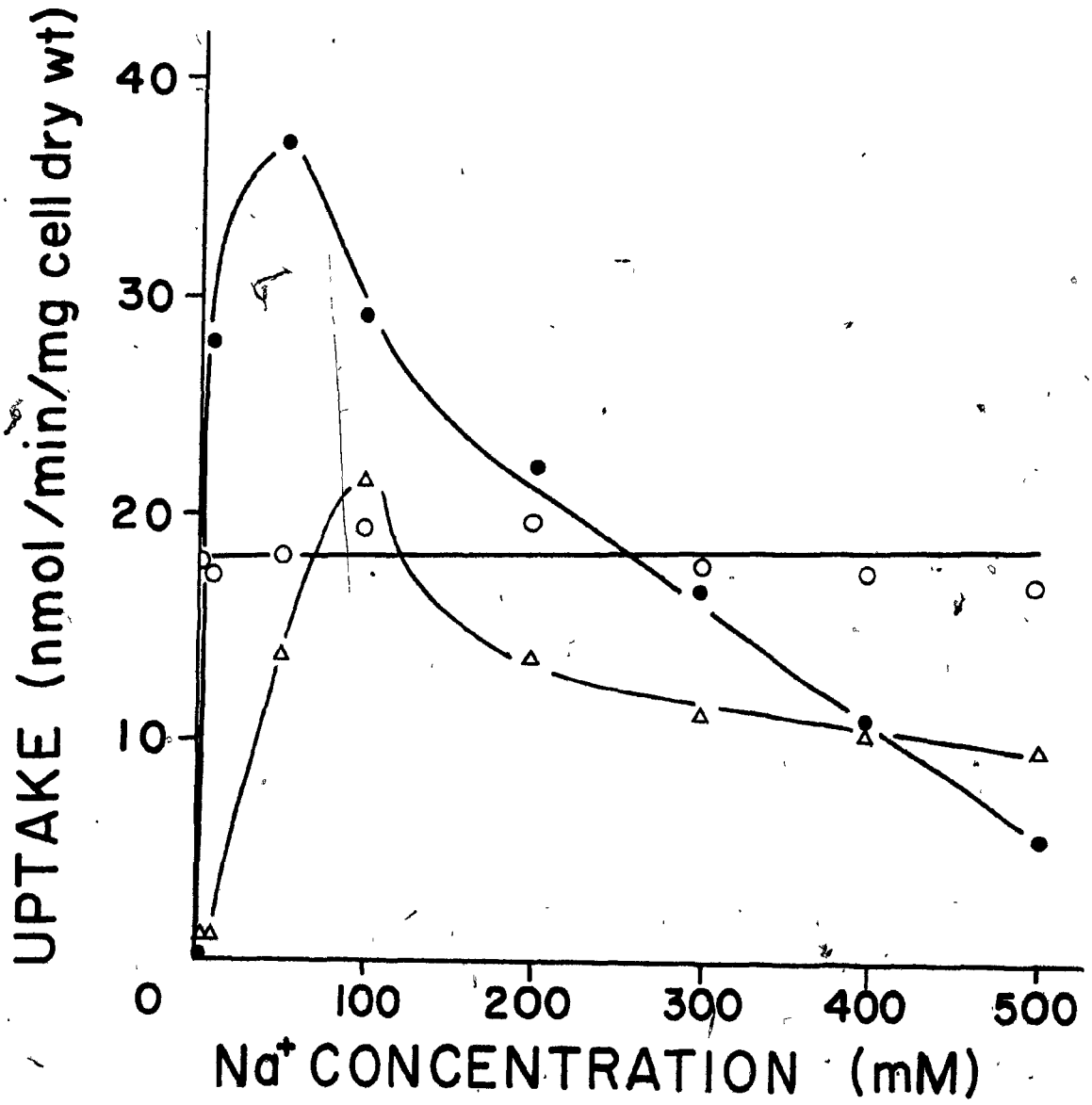

UPTAKE (nmol/min/mg cell dry wt)
40
30
20
10
0
100
200
300
400
500
Na+ CONCENTRATION (mM)

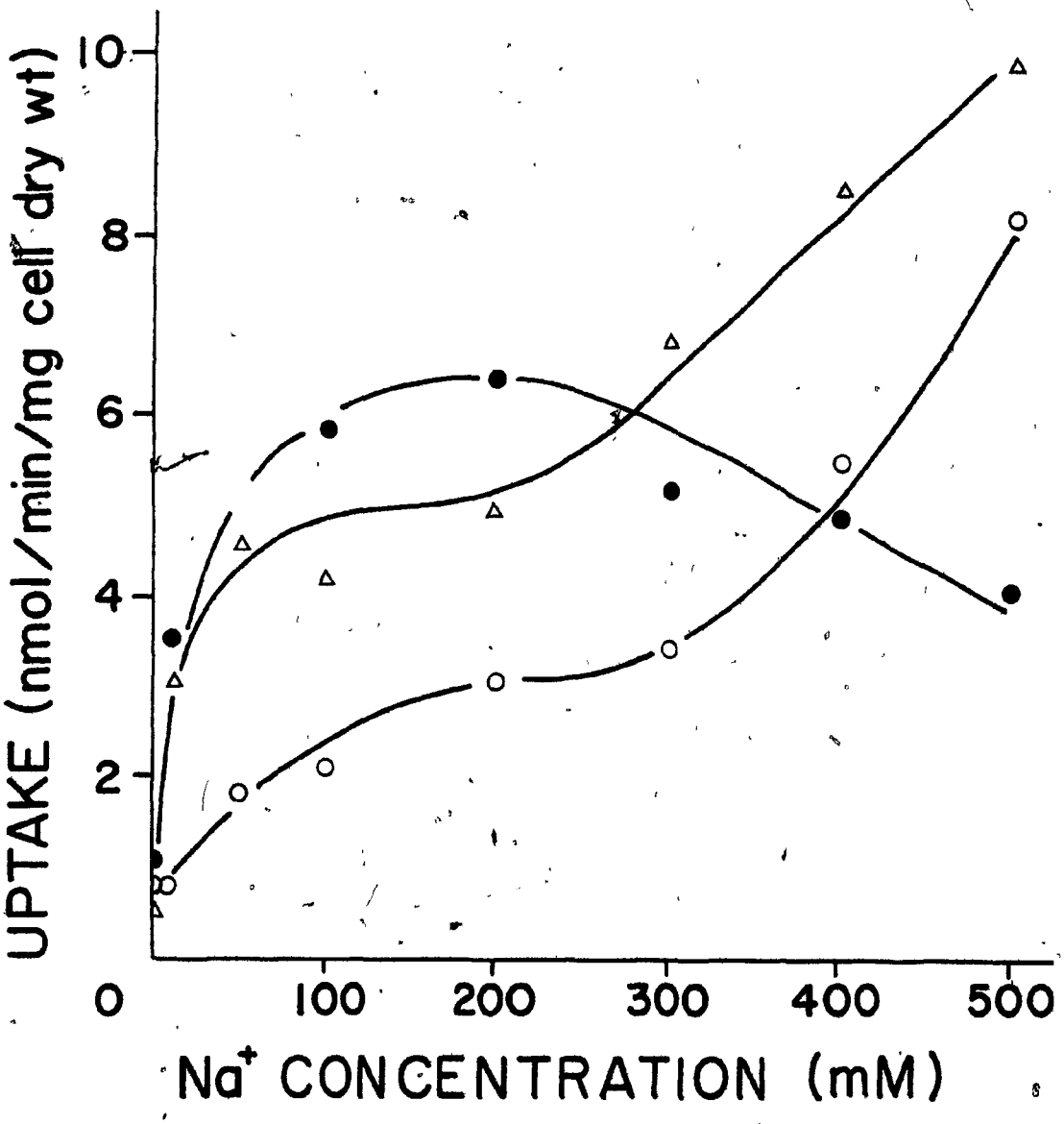
Figure 6. Effect of Na^+ concentration on the rate of uptake of glutamate, arginine and $^{32}P_i$ by V. fischeri.

● L-arginine

△ L-glutamic acid

○ $^{32}P_i$

The specific activity of arginine and glutamate was 0.5 uCi/umol, and that of $^{32}P_i$ was 50 uCi/umol. Other conditions were as described in Figure 1, except that the ethanol was omitted from the incubation medium.


UPTAKE (nmol/min/mg cell dry wt)
0
2
4
6
8
10
100
200
300
400
500
Na+ CONCENTRATION (mM)

II Effects of Na^+, K^+ and Li^+ on transport

a. Alteromonas haloplanktis

Previous studies have indicated that there is a specific Na^+ requirement for the transport of AIB, L-alanine, L-serine, L-glutamate and L-tyrosine by A. haloplanktis (Drapeau et al., 1966; P. Wong, Ph.D. thesis, 1968). In the absence of Na^+, neither NH_4^+, K^+, Li^+ nor sucrose could promote uptake. The ineffectiveness of these solutes did not appear to be due to inhibitory effects, since addition of Na^+ restored the ability of the cells to transport the substrates.

The specificity of the Na^+ requirement for transport was examined in the present study by comparing the ability of cells of A. haloplanktis to take up metabolizable substrates under five different sets of conditions. The total concentration of the cations being tested was always kept at 300 mM in order to minimize the possible influence of osmotic effects on transport.

Under the conditions chosen, the cells were suspended in:

1) 300 mM ChCl (Choline chloride). This served as the Na^+-free control.
2) 10 mM NaCl + 290 mM ChCl. This provided more than sufficient Na^+ for a maximum rate of ethanol-energized respiration.
3) 300 mM NaCl. This provided the level of Na^+ required for optimum growth of the organism. A rate of uptake in 300 mM Na^+ higher than that in 10 mM Na^+ should be due to a specific effect of Na^+ on the transport system, since both concentrations of Na^+ permit a maximum rate of ethanol-energized respiration.

4) 10 mM NaCl + 290 mM KCl.

5) 10 mM NaCl + 290 mM LiCl. These latter two conditions allow us to determine if K^+ or Li^+ can replace the Na^+ required for transport beyond the level needed for maximum respiration.

Uptake was followed for three minutes after addition of the radioactively labelled substrates to cells suspended in CST buffers. In the absence of Na^+, the cells did not actively accumulate any of the eleven metabolites tested (Figure 7 to 17). Significant quantities of some substrates, such as L-lysine (Figure 12), became associated with the cells under these conditions. This was considered to represent binding, rather than transport, since the process was completed by the time the first sample was taken at thirty seconds. After this point, there was no change in the rate of substrate "accumulation".

All of the substrates, except for L-leucine, succinate and citrate (Figures 8, 13, 16) were taken up by cells in CST 10 mM NaCl + 290 mM ChCl. In all cases, the rates of transport in 300 mM NaCl were superior to those in 10 mM NaCl + 290 mM ChCl. The rates of uptake in 10 mM NaCl + 290 mM KCl never exceeded those in 10 mM NaCl + 290 mM ChCl.

Upon comparison of the uptakes in 10 mM NaCl + 290 mM LiCl with those in 10 mM NaCl + 290 mM ChCl, it was seen that Li^+ had varying effects on metabolite transport by A. haloplanktis. Li^+ had no effect on the uptakes of L-alanine and L-leucine, whereas the ion inhibited the uptakes of L-glutamate and L-aspartate (Figures 7 to 10). The transport of

L-arginine, L-lysine, L-malate and citrate appeared to be slightly stimulated by the ion (Figures 11, 12, 15, 16).

Cells of A. haloplanktis in 10 mM NaCl + 290 mM ChCl did not take up succinate, but there clearly was accumulation of this substrate when the ChCl was replaced with LiCl (Figure 13). This rate of uptake was approximately equivalent to that in 25 mM NaCl + 275 mM ChCl. This effect was so prominent, that the stimulatory ability of Li^+ was tested in the absence of Na^+, however no succinate was transported by cells in the 300 mM LiCl buffer.

The transport of D-galactose was also found to be significantly influenced by Li^+ (Figure 14). Ten mM NaCl + 290 mM LiCl was much more effective in promoting uptake than 10 mM NaCl + 290 mM ChCl, but was less effective than 300 mM NaCl. Unlike succinate, D-galactose was transported by cells suspended in 300 mM LiCl. This rate of uptake was the same as that observed in 10 mM NaCl + 290 mM ChCl.

In those cases where Li^+ promoted transport, it was less effective than an equal amount of Na^+, except in the case of P_i transport by A. haloplanktis (Figure 17). The uptake in 10 mM Na^+ + 290 mM Li^+ slightly exceeded that in 300 mM Na^+. In 10 mM Na^+ + 290 mM Li^+, the rate of P_i uptake was approximately equivalent to that in 200 mM Na^+ (Figure 3). At Na^+ concentrations higher than 200 mM, the uptake of P_i was slightly less than the maximum rate, and this may account for the differences observed in Figure 17. The rate of transport in 300 mM LiCl

was similar to that in 10 mM NaCl + 290 mM ChCl.

The possibility had to be considered that the apparent stimulatory effect of Li^+ was actually due to the presence of significant amounts of contaminating Na^+ in the stock of LiCl used in the buffers. Flame spectrophotometric analysis of CST buffers indicated that this was not the case. Solutions of 600 mM ChCl and 600 mM LiCl both contained approximately 15 uM Na^+. A double strength CST buffer containing either 600 mM ChCl or 600 mM LiCl contained approximately 20 to 25 uM Na^+.

b. Vibrio fischeri

The effects of Na^+, K^+ and Li^+ on the transport of five metabolizable substrates by V. fischeri (Figures 18 to 22) were examined under conditions similar to those used for A. haloplanktis. The maximum concentration of Na^+ tested was 100 mM, which corresponded to the level of Na^+ in the medium used for growth of the cells. The suspending buffers were balanced to 300 mM with choline chloride, as was done in Figures 4 to 6 where it was desirable to examine transport ability over a wide range of Na^+ concentrations without variations in osmotic activity. Ethanol was not added as an energy source because cells of V. fischeri do not oxidize this substrate. Furthermore, endogenous respiration alone can drive transport, and this former activity is not affected by Na^+ (Figure 4).

All of the substrates, except L-alanine, were taken up to some extent by cells in the 300 mM ChCl buffer. P_i in particular was taken up

at a very appreciable rate by cells in the absence of Na^+, as compared to the uptake in 100 mM Na^+ (Figure 22). It should be noted that this latter rate is much less than the maximum observed in the presence of higher concentrations of Na^+ (Figure 6). Transport of succinate and P_i (Figures 21, 22) was not enhanced by the addition of 10 mM NaCl. The uptake of L-alanine, L-glutamate and L-arginine (Figures 18, 19, 20) by cells in 10 mM NaCl + 290 mM ChCl exceeded that in 300 mM ChCl. In all cases, there was greater uptake of the metabolites in 100 mM NaCl than in 10 mM NaCl. The ability of cells to transport substrates in 10 mM NaCl + 90 mM KCl + 200 mM ChCl never exceeded that in 10 mM NaCl + 290 mM ChCl.

The Na^+-sparing ability of Li^+ was examined by comparing the rates of transport by cells of V. fischeri in 10 mM NaCl + 90 mM LiCl + 200 mM ChCl with the rates in 10 mM NaCl + 290 mM ChCl. The uptake of L-arginine was similar under both conditions (Figure 20), whereas L-alanine transport was slightly inhibited by Li^+ (Figure 18). In the presence of 10 mM NaCl, 90 mM Li^+ clearly had a positive effect on the accumulation of L-glutamate, however this combination was less effective than 100 mM Na^+ (Figure 19). The transport of glutamate in 100 mM LiCl + 200 mM ChCl was negligible and similar to that in 300 mM ChCl. As was observed with A. haloplanktis, the uptake of succinate by V. fischeri was stimulated by Li^+ in the presence of 10 mM Na^+ (Figure 21). The rate in 100 mM LiCl + 200 mM ChCl was very low but slightly greater than that in 300 mM ChCl. The rate of P_i transport (Figure 22) in 10 mM NaCl + 90 mM LiCl + 200 mM ChCl was similar to the rates observed in 10 mM NaCl + 290 mM ChCl and in 300 mM ChCl. These rates were greater than the rate in 100 mM LiCl + 200

mM ChCl. Thus, in the absence of Na^+, Li^+ appeared to have an inhibitory effect on P_i transport by V. fischeri.

Figure 7. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-alanine by A. haloplanktis.

Cells were suspended at a density of about 100 ug dry wt/ml in the various complete salts buffers. The final concentration of ethanol was 25 mM, and that of L-alanine (0.5 uCi/umol) was 200 uM. Uptake was measured in the following CST buffers:

○ 300 mM ChCl (Choline chloride)
● 300 mM NaCl
△ 10 mM NaCl + 290 mM KCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 290 mM LiCl

Other conditions were as described in the Materials and Methods.

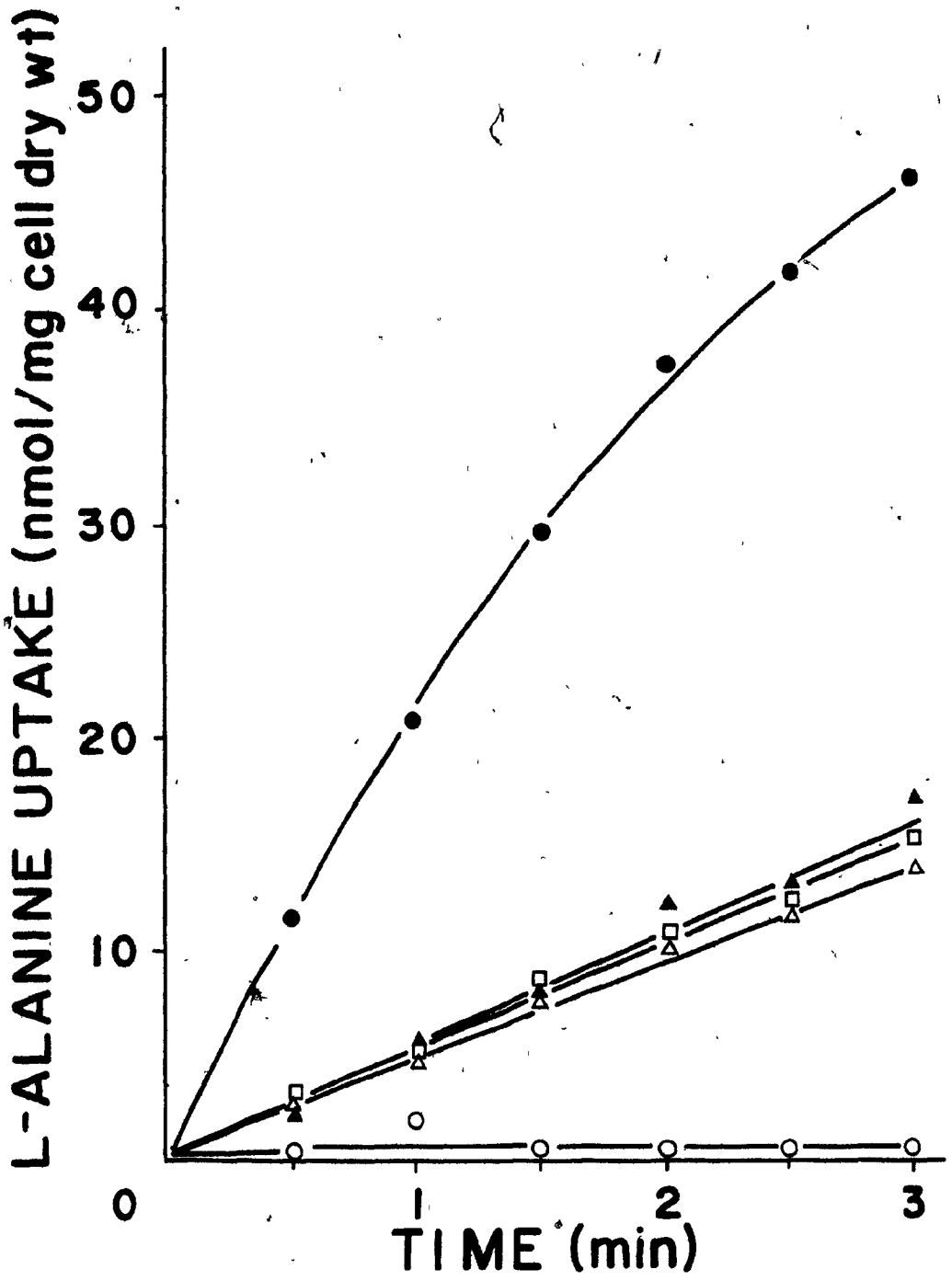

L-ALANINE UPTAKE (nmol/mg cell dry wt)
50
40
30
20
10
0
1
2
3
TIME (min)

Figure 8. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-leucine by A. haloplanktis.

The uptake of L-leucine (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
▲ 10 mM NaCl + 290 mM ChCl (actual data plotted)
▲ 10 mM NaCl + 290 mM KCl (a)
▲ 10 mM NaCl + 290 mM LiCl (a)

Conditions were as described in Figure 7.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 10 mM NaCl + 290 mM ChCl (actual data plotted).

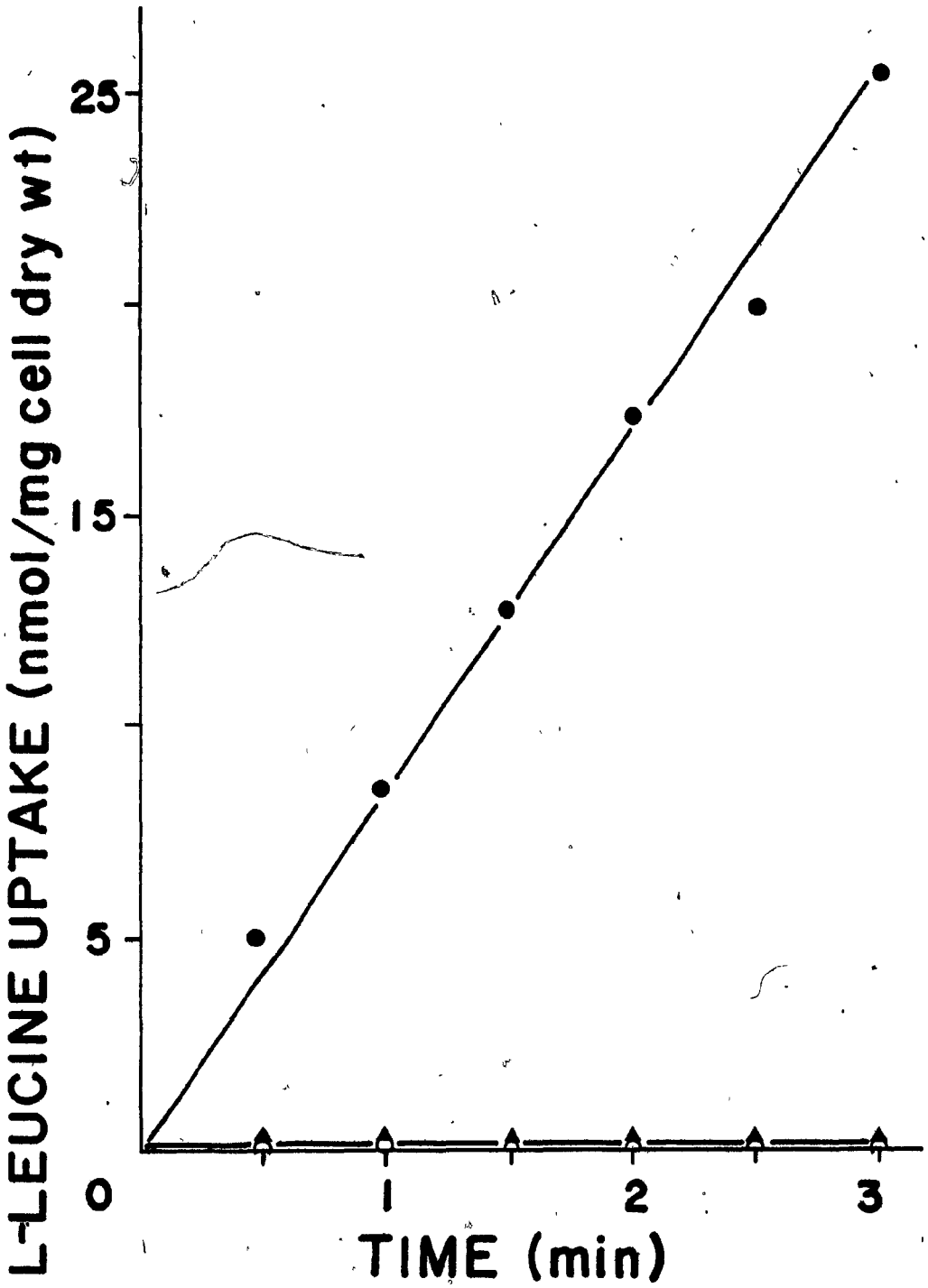

L-LEUCINE UPTAKE (nmol/mg cell dry wt)
25
15
5
0
1
2
3
TIME (min)

Figure 9. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-glutamic acid by A. haloplanktis

The uptake of L-glutamate (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
△ 10 mM NaCl + 290 mM KCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 290 mM LiCl

Conditions were as described in Figure 7.

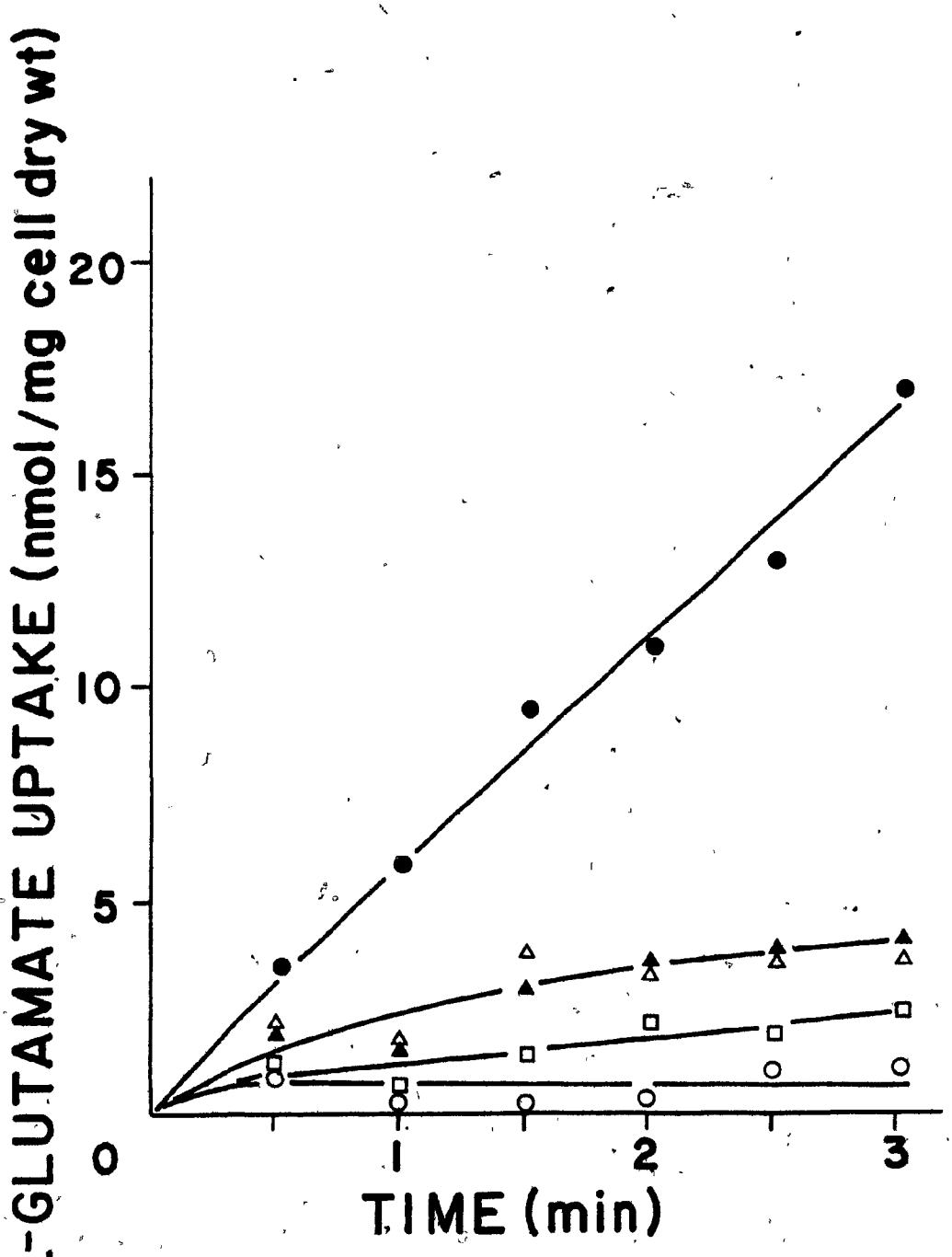

L-GLUTAMATE UPTAKE (nmol/mg cell dry wt)
0
5
10
15
20
1
2
3
TIME (min)

Figure 10. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-aspartic acid by A. haloplanktis.

The uptake of L-aspartate (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

- ○ 300 mM ChCl (actual data plotted)
- ● 300 mM NaCl
- ▲ 10 mM NaCl + 290 mM ChCl
- ○ 10 mM NaCl + 290 mM KCl (a)
- ○ 10 mM NaCl + 290 mM LiCl (a)

Conditions were as described in Figure 7.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 300 mM ChCl (actual data plotted).

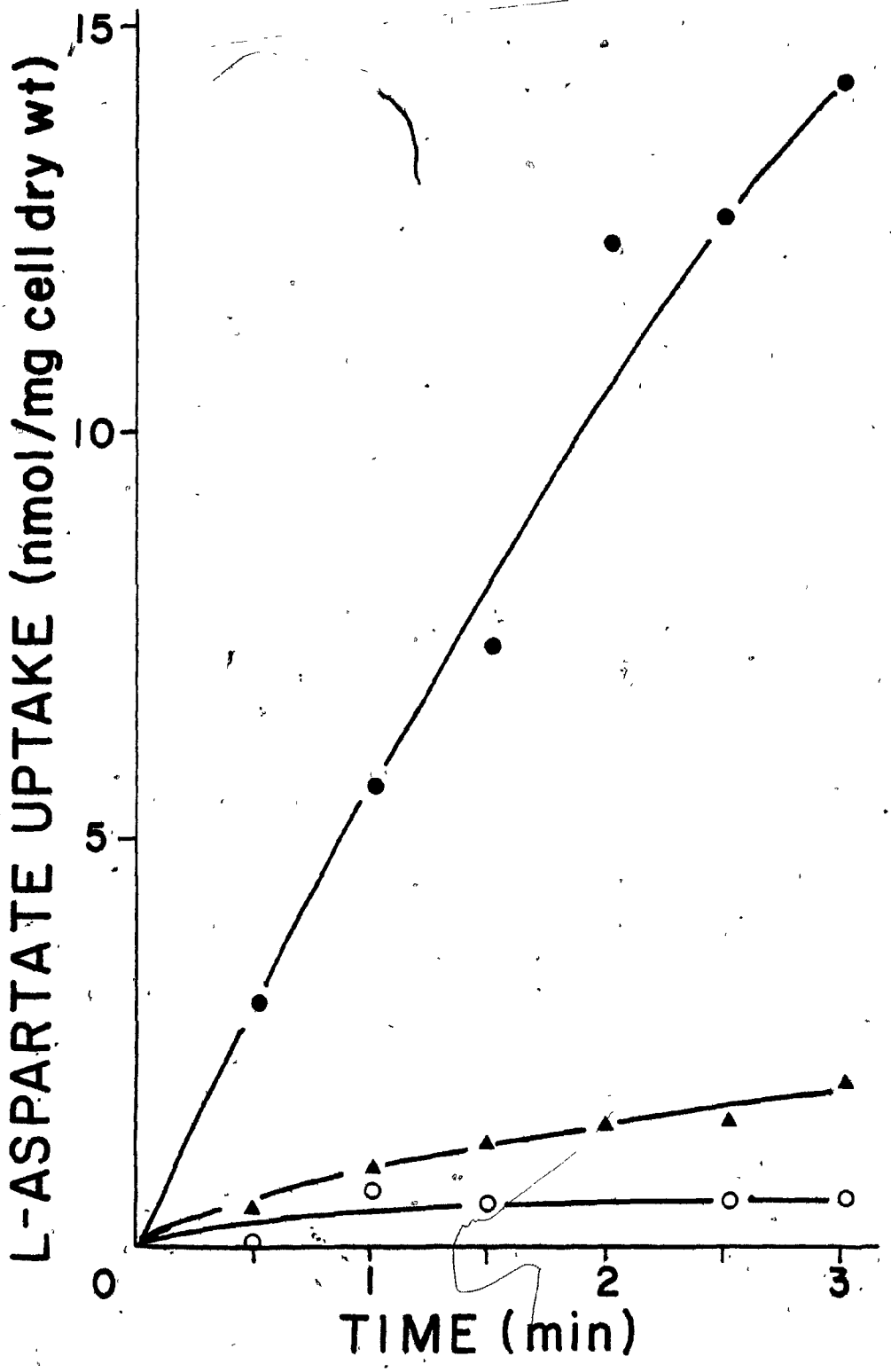

L-ASPARTATE UPTAKE (nmol/mg cell dry wt)
15
10
5
0
1
2
3
TIME (min)

Figure 11. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-arginine by A. haloplanktis.

The uptake of L-arginine (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
△ 10 mM NaCl + 290 mM KCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 290 mM LiCl

Other conditions were as described in Figure 7.

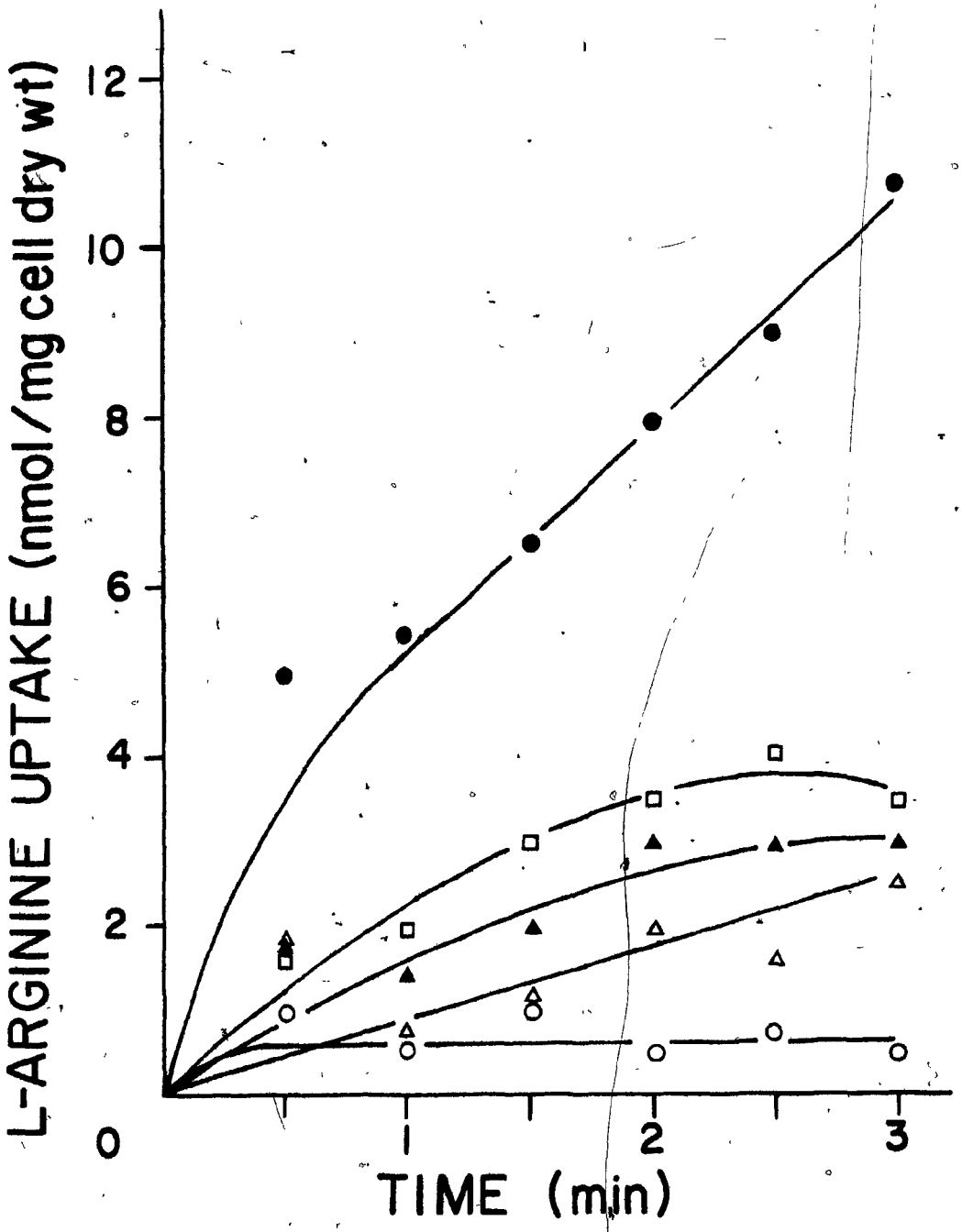

L-ARGININE UPTAKE (nmol/mg cell dry wt)
0
2
4
6
8
10
12
1
2
3
TIME (min)

Figure 12. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-lysine by A. haloplanktis.

The uptake of L-lysine (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
△ 10 mM NaCl + 290 mM KCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 290 mM LiCl

Other conditions were as described in Figure 7.

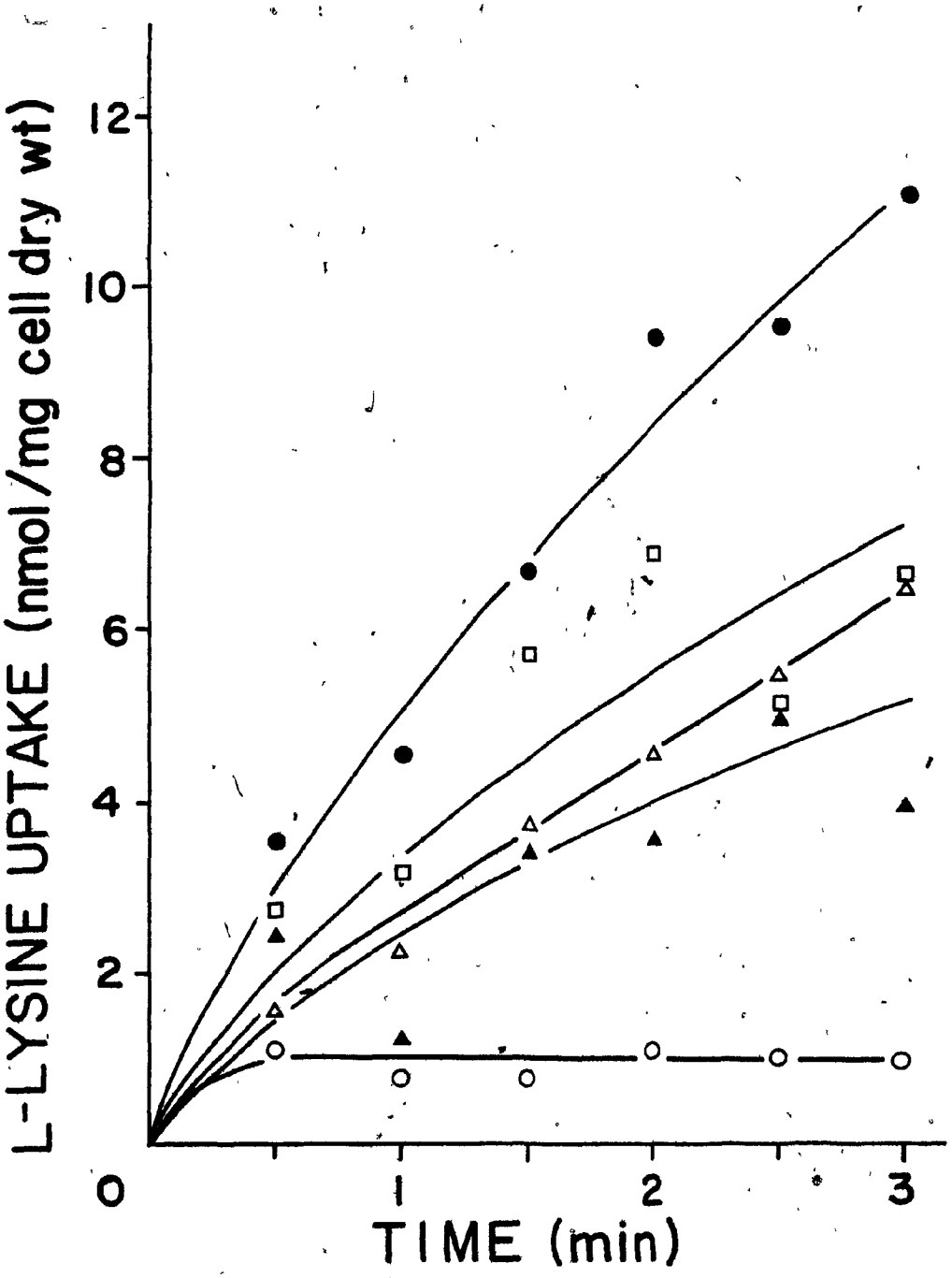

L-LYSINE UPTAKE (nmol/mg cell dry wt)
0
2
4
6
8
10
12
1
2
3
TIME (min)

Figure 13. Effects of Na^+, K^+ and Li^+ on the rate of uptake of succinic acid by A. haloplanktis.

The uptake of succinate (final concentration 50 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl (actual data plotted)
● 300 mM NaCl
□ 10 mM NaCl + 290 mM LiCl
○ 10 mM NaCl + 290 mM ChCl (a)
○ 10 mM NaCl + 290 mM KCl (a)
○ 300 mM LiCl (a)
△ 25 mM NaCl + 275 mM ChCl

Other conditions were as described in Figure 7.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 300 mM ChCl (actual data plotted).

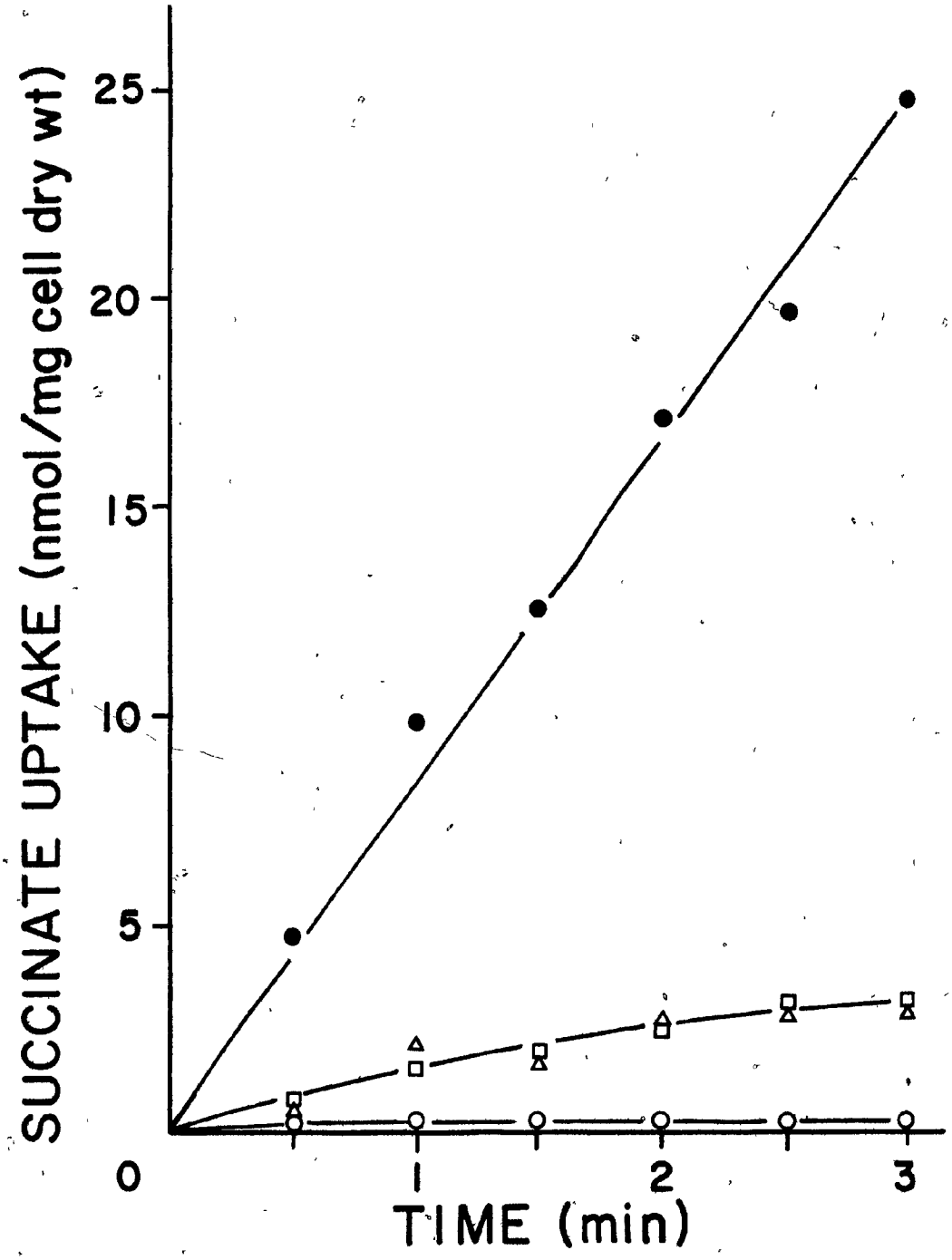

SUCCINATE UPTAKE (nmol/mg cell dry wt)
25
20
15
10
5
0
1
2
3
TIME (min)

Figure 14. Effects of Na^+, K^+ and Li^+ on the rate of uptake of D-galactose by A. haloplanktis.

The uptake of D-galactose (final concentration 50 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
▲ 10 mM NaCl + 290 mM ChCl (actual data plotted)
□ 10 mM NaCl + 290 mM LiCl
▲ 10 mM NaCl + 290 mM KCl (a)
▲ 300 mM LiCl (a)

Other conditions were as described in Figure 7.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 10 mM NaCl + 290 mM ChCl (actual data plotted).

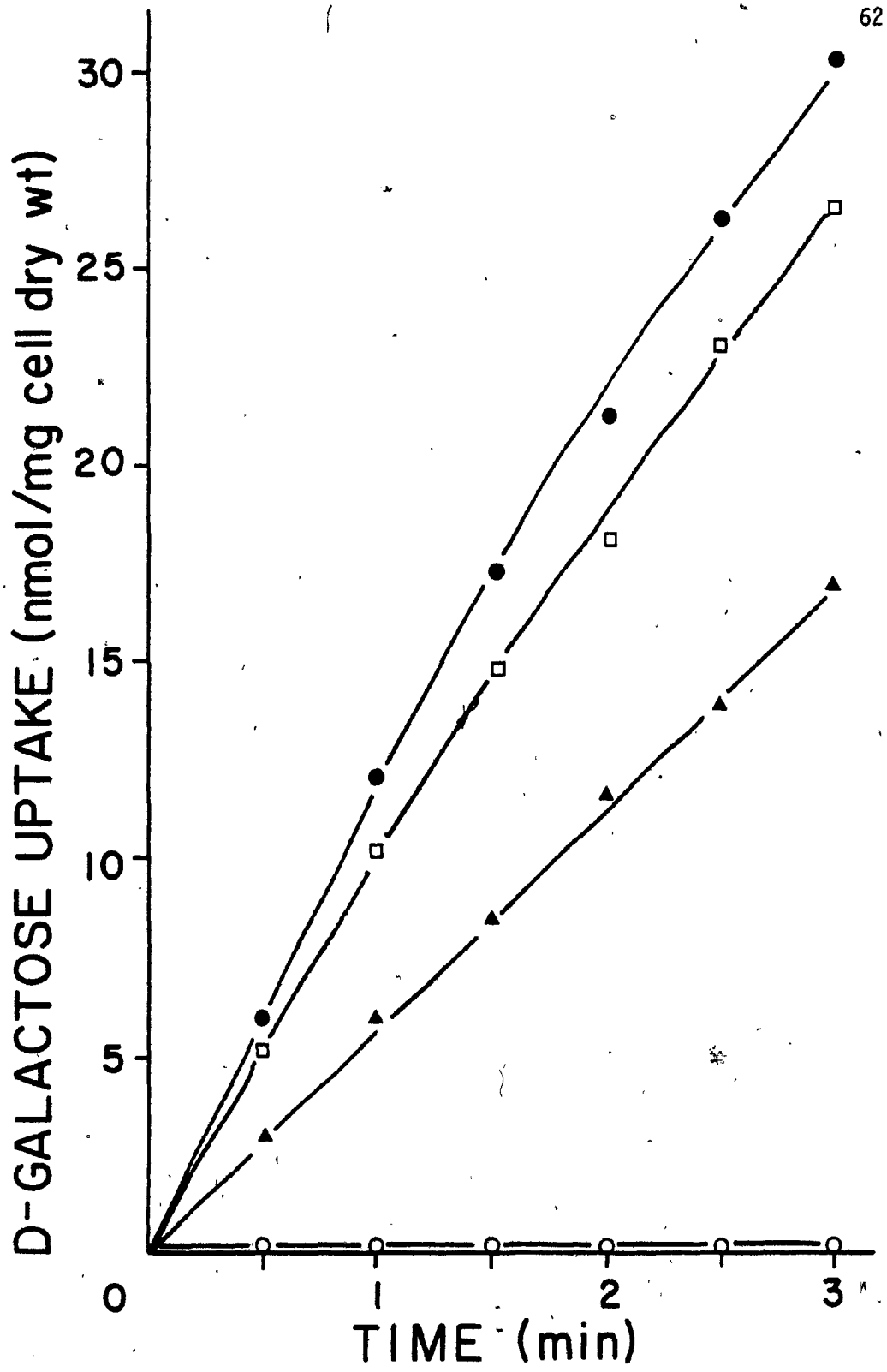

D-GALACTOSE UPTAKE (nmol/mg cell dry wt)
30
25
20
15
10
5
0
1
2
3
TIME (min)

Figure 15. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-malic acid by A. haloplanktis.

The uptake of L-malate (final concentration 100 uM, specific activity 2.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
△ 10 mM NaCl + 290 mM KCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 290 mM LiCl

Other conditions were as described in Figure 7.

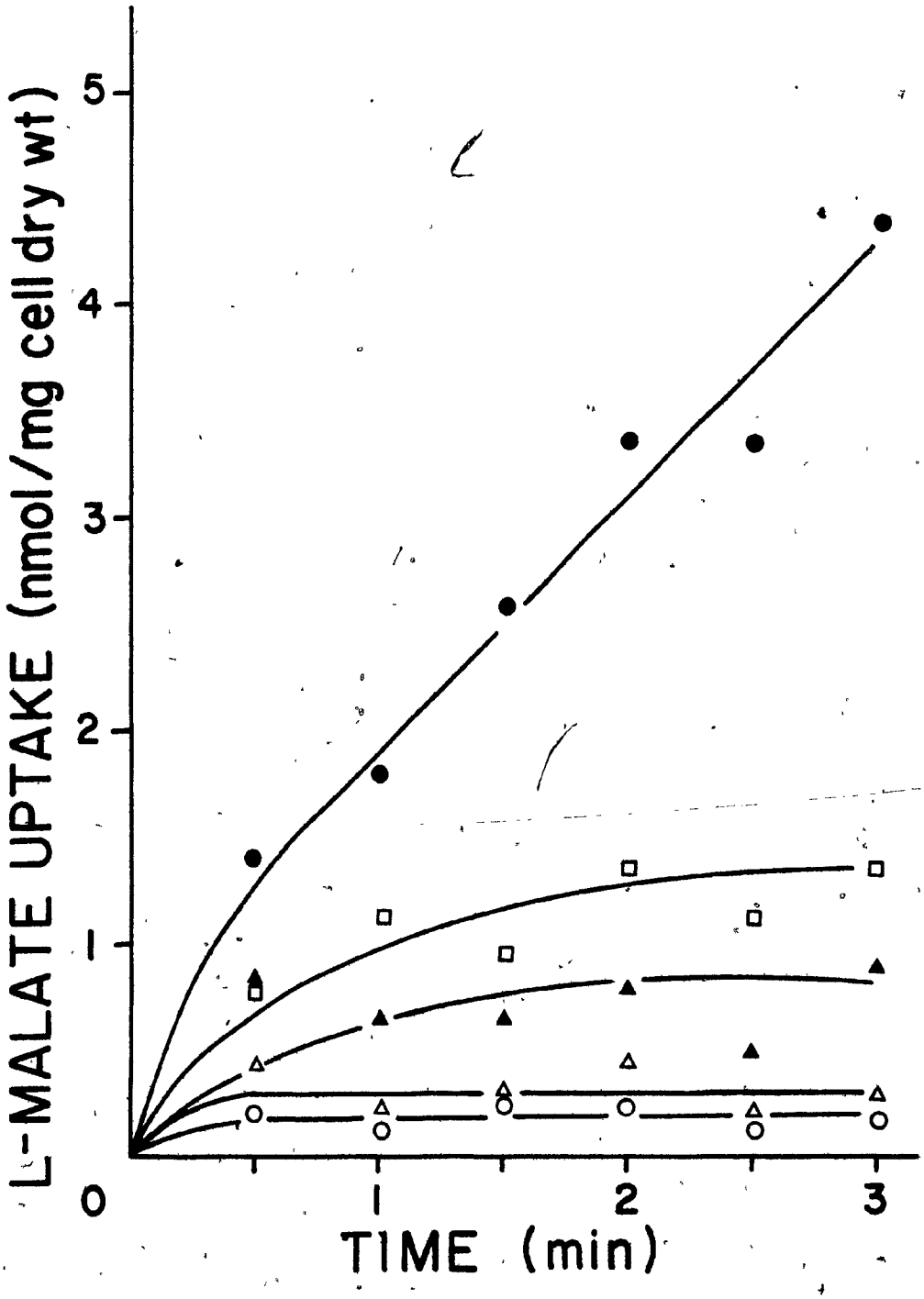

L-MALATE UPTAKE (nmol/mg cell dry wt)
0
1
2
3
4
5
1
2
3
TIME (min)

Figure 16. Effects of Na^+, K^+ and Li^+ on the rate of uptake of citric acid by A. haloplanktis.

The uptake of citrate (final concentration 50 uM, specific activity 2.5 uCi/umol) was measured in the following CST buffers:

- ○ 300 mM ChCl (actual data plotted)
- ● 300 mM NaCl
- □ 10 mM NaCl + 290 mM LiCl
- ○ 10 mM NaCl + 290 mM ChCl (a)
- ○ 10 mM NaCl + 290 mM KCl (a)

Other conditions were as described in Figure 7.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 300 mM ChCl (actual data plotted).

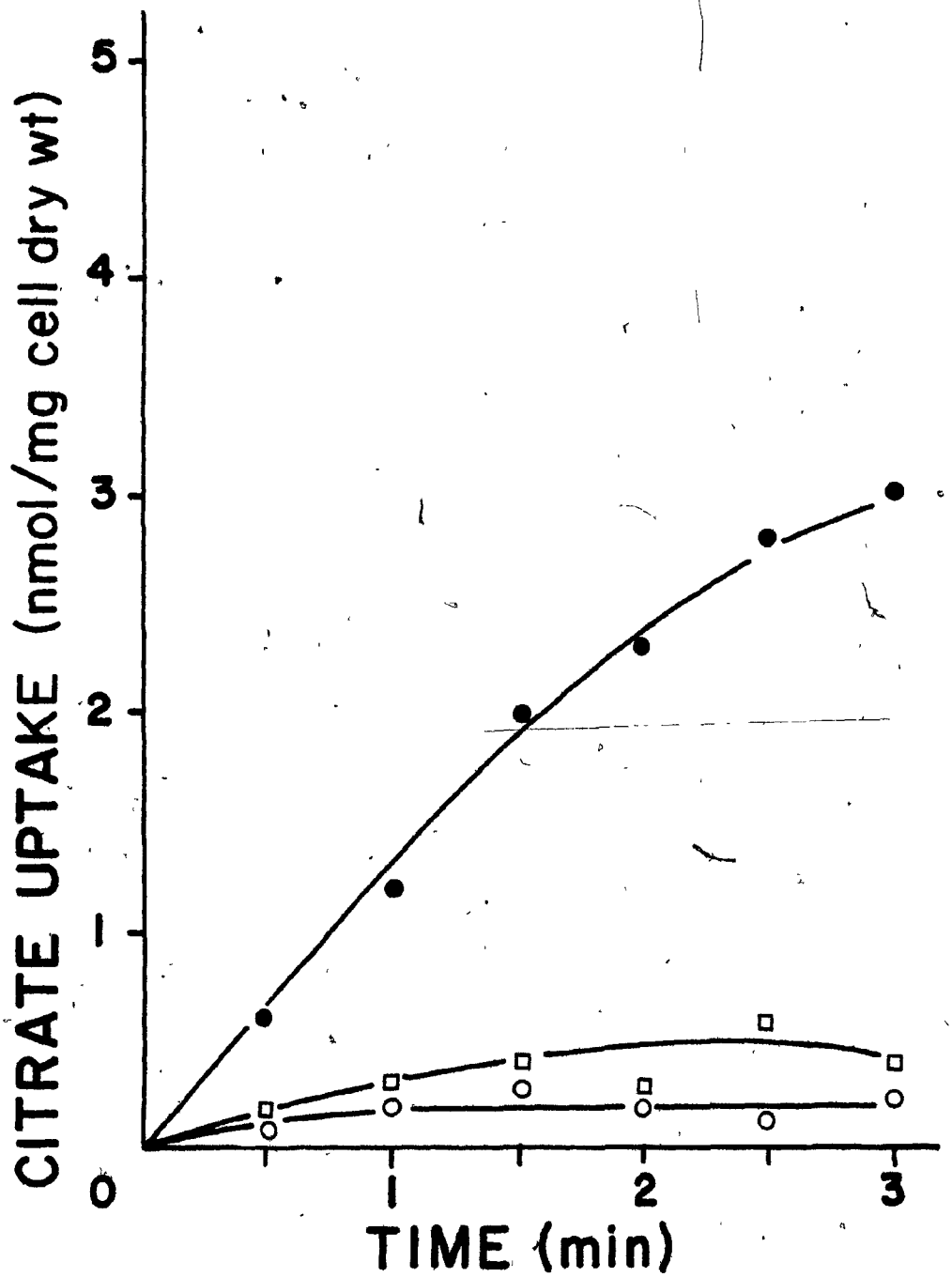

CITRATE UPTAKE (nmol/mg cell dry wt)
0
1
2
3
4
5
TIME (min)
1
2
3

Figure 17. Effects of Na^+, K^+ and Li^+ on the rate of uptake of $^{32}P_i$ by A. haloplanktis.

The uptake of $^{32}P_i$ (final concentration 25 uM, specific activity 50 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 300 mM NaCl
△ 10 mM NaCl + 290 mM KCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 290 mM LiCl
■ 300 mM LiCl

Other conditions were as described in Figure 7.

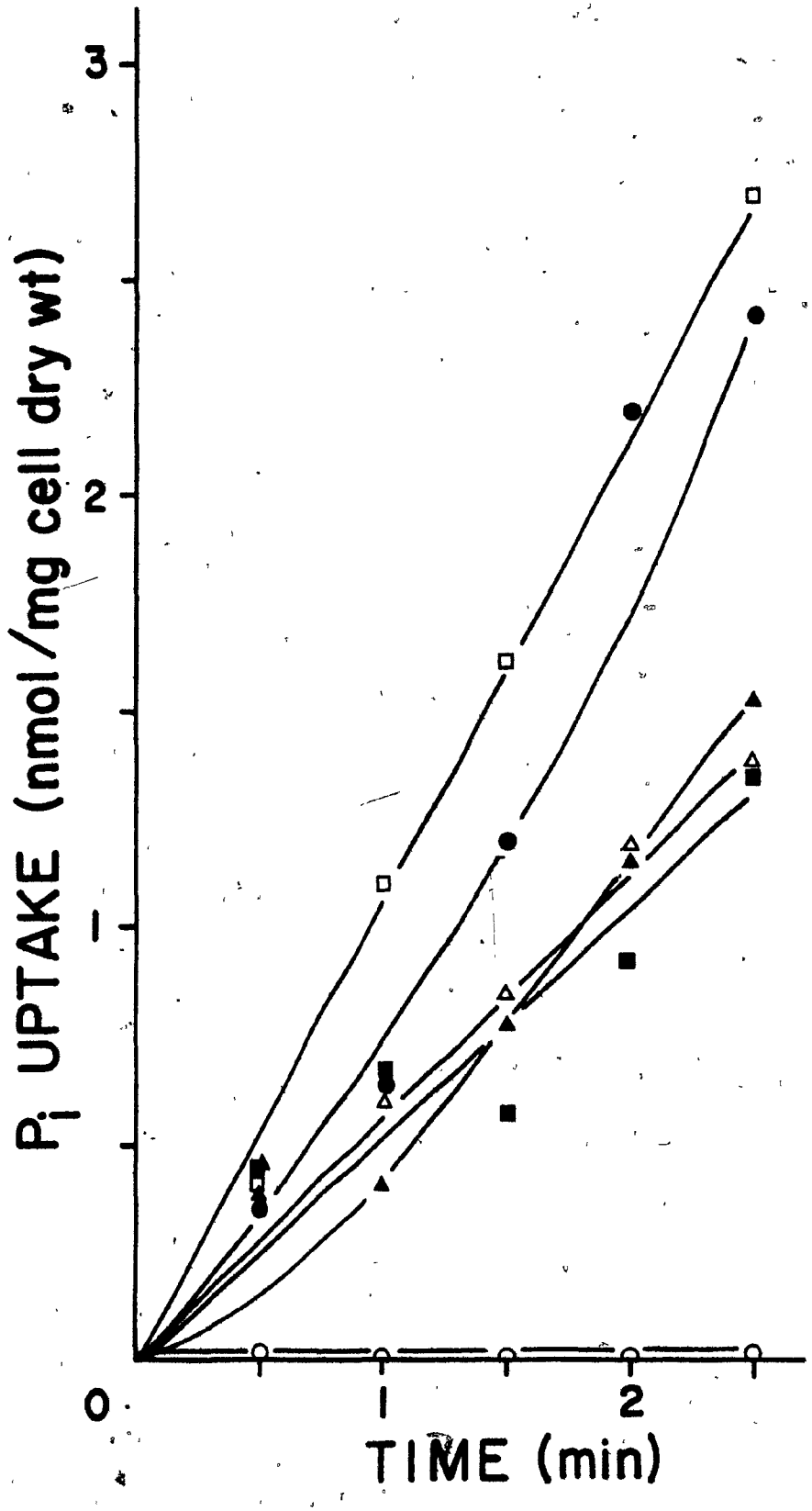

P_i UPTAKE (nmol/mg cell dry wt)
3
2
1
0
1
2
TIME (min)

Figure 18. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-alanine by V. fischeri.

The uptake of L-alanine (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 100 mM NaCl + 200 mM ChCl
▲ 10 mM NaCl + 290 mM ChCl (actual data plotted)
▲ 10 mM NaCl + 90 mM KCl + 200 mM ChCl (a)
□ 10 mM NaCl + 90 mM LiCl + 200 mM ChCl

Other conditions were as in Figure 7, except that the ethanol was omitted from the incubation medium.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 10 mM NaCl + 290 mM ChCl (actual data plotted).

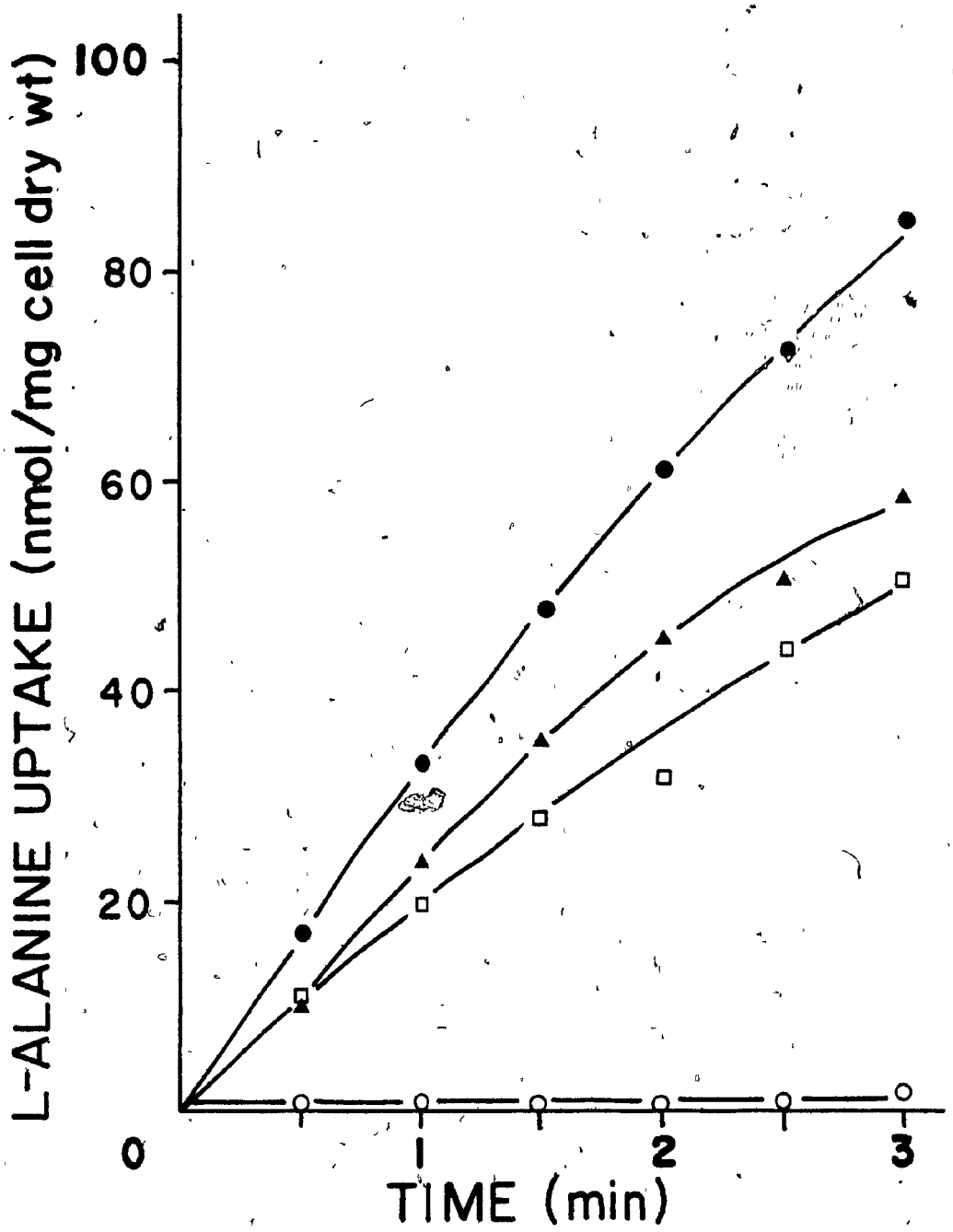

L-ALANINE UPTAKE (nmol/mg cell dry wt)
100
80
60
40
20
0
1
2
3
TIME (min)

Figure 19. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-glutamic acid by V. fischeri.

The uptake of L-glutamate (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 100 mM NaCl + 200 mM ChCl
△ 10 mM NaCl + 90 mM KCl + 200 mM ChCl
▲ 10 mM NaCl + 290 mM ChCl
□ 10 mM NaCl + 90 mM LiCl + 200 mM ChCl
■ 100 mM LiCl + 200 mM ChCl

Other conditions were as described in Figure 7, except that the ethanol was omitted from the incubation medium.

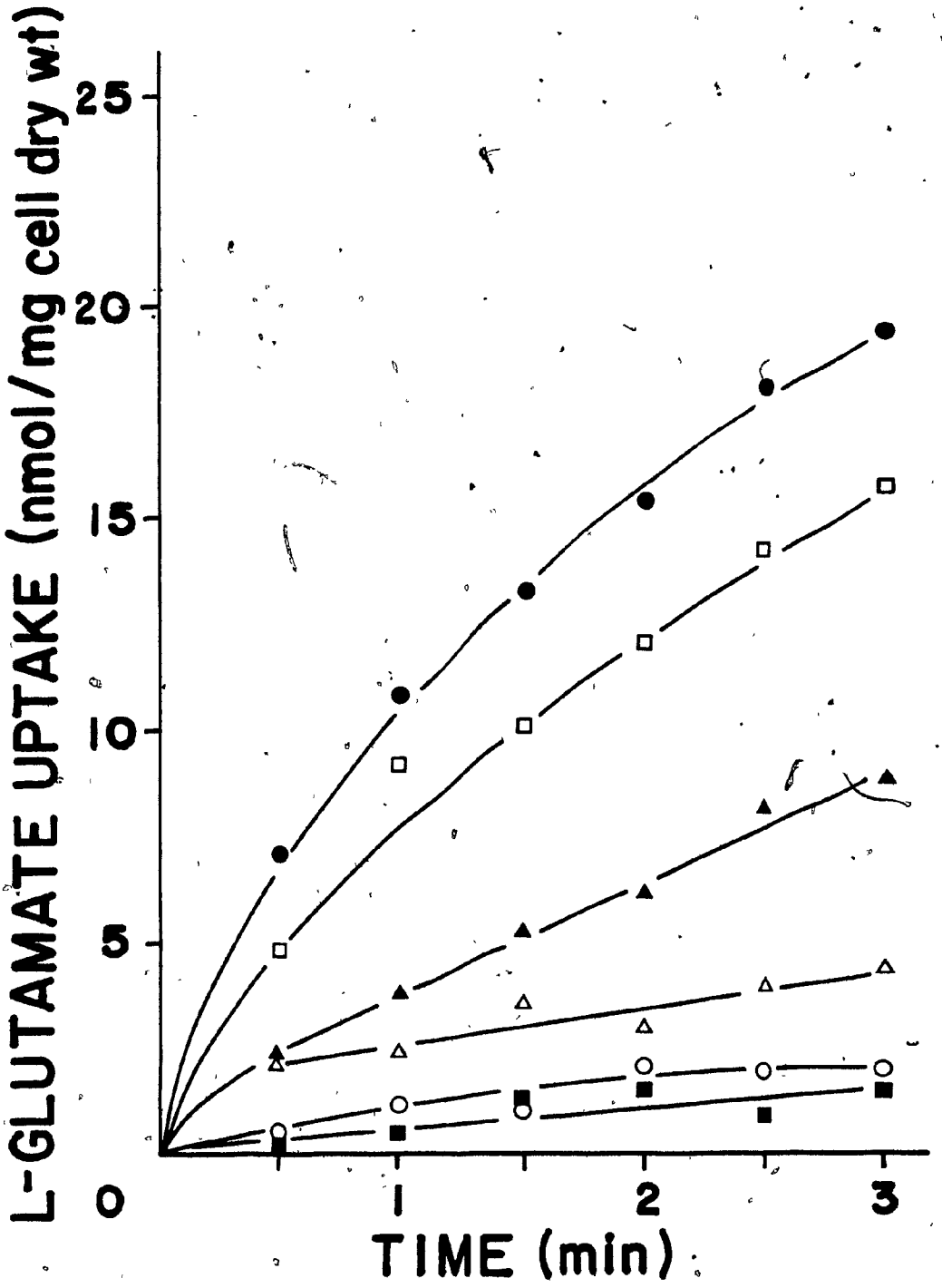

L-GLUTAMATE UPTAKE (nmol/mg cell dry wt)
0
5
10
15
20
25
1
2
3
TIME (min)

Figure 20. Effects of Na^+, K^+ and Li^+ on the rate of uptake of L-arginine by V. fischeri.

The uptake of L-arginine (final concentration 200 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

- ○ 300 mM ChCl
- ● 100 mM NaCl + 200 mM ChCl
- △ 10 mM NaCl + 90 mM KCl + 200 mM ChCl
- ▲ 10 mM NaCl + 290 mM ChCl
- □ 10 mM NaCl + 90 mM LiCl + 200 mM ChCl

Other conditions were as described in Figure 7, except that the ethanol was omitted from the incubation medium.

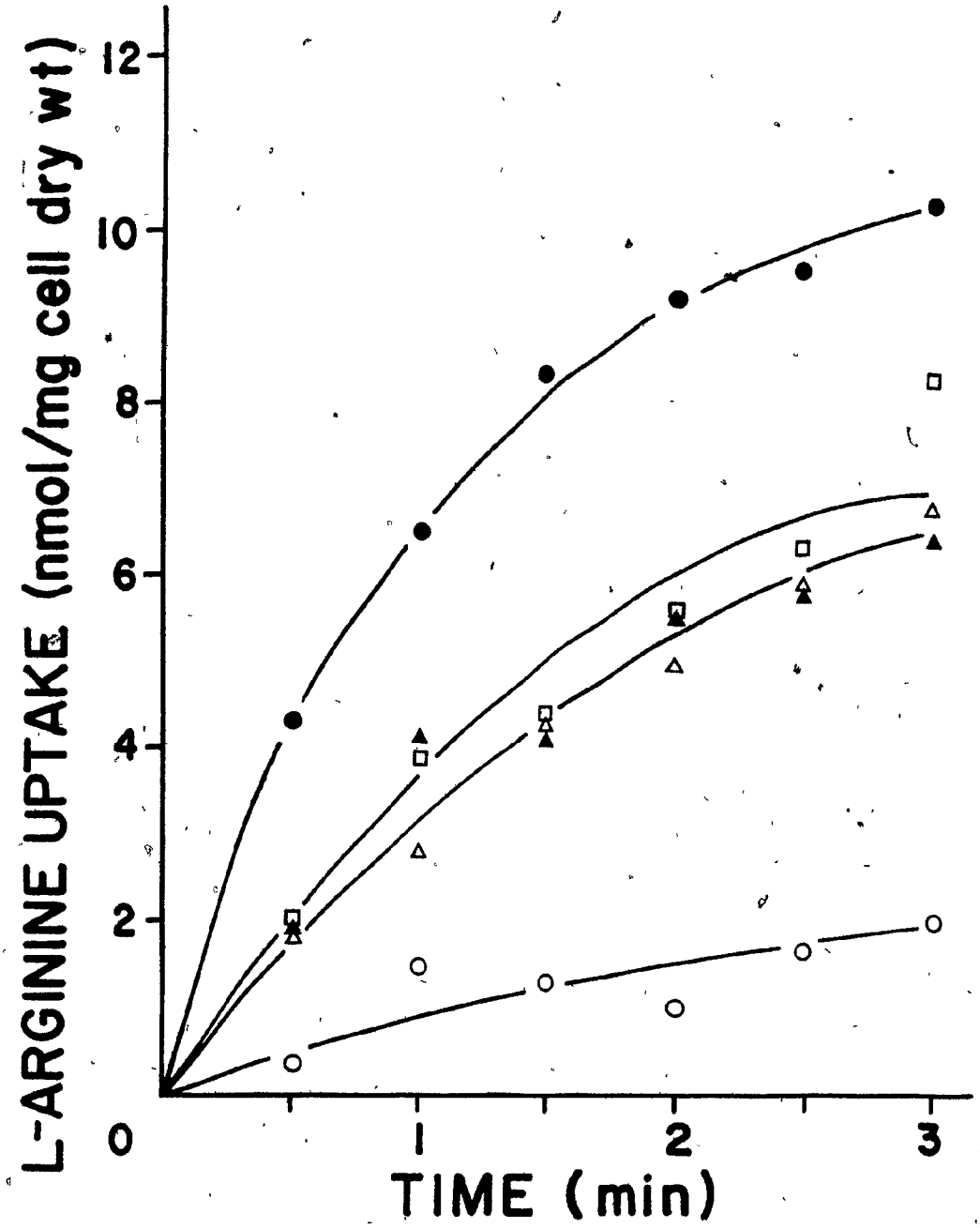

L-ARGININE UPTAKE (nmol/mg cell dry wt)
0
2
4
6
8
10
12
1
2
3
TIME (min)

Figure 21. Effects of Na^+, K^+ and Li^+ on the rate of uptake of succinic acid by V. fischeri.

The uptake of succinate (final concentration 50 uM, specific activity 0.5 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl (actual data plotted)
● 100 mM NaCl + 200 mM ChCl
□ 10 mM NaCl + 90 mM LiCl + 200 mM ChCl
■ 100 mM LiCl + 200 mM ChCl
○ 10 mM NaCl + 90 mM KCl + 200 mM ChCl (a)
○ 10 mM NaCl + 290 mM ChCl (a)

Other conditions were as described in Figure 7, except that the ethanol was omitted from the incubation medium.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 300 mM ChCl (actual data plotted).

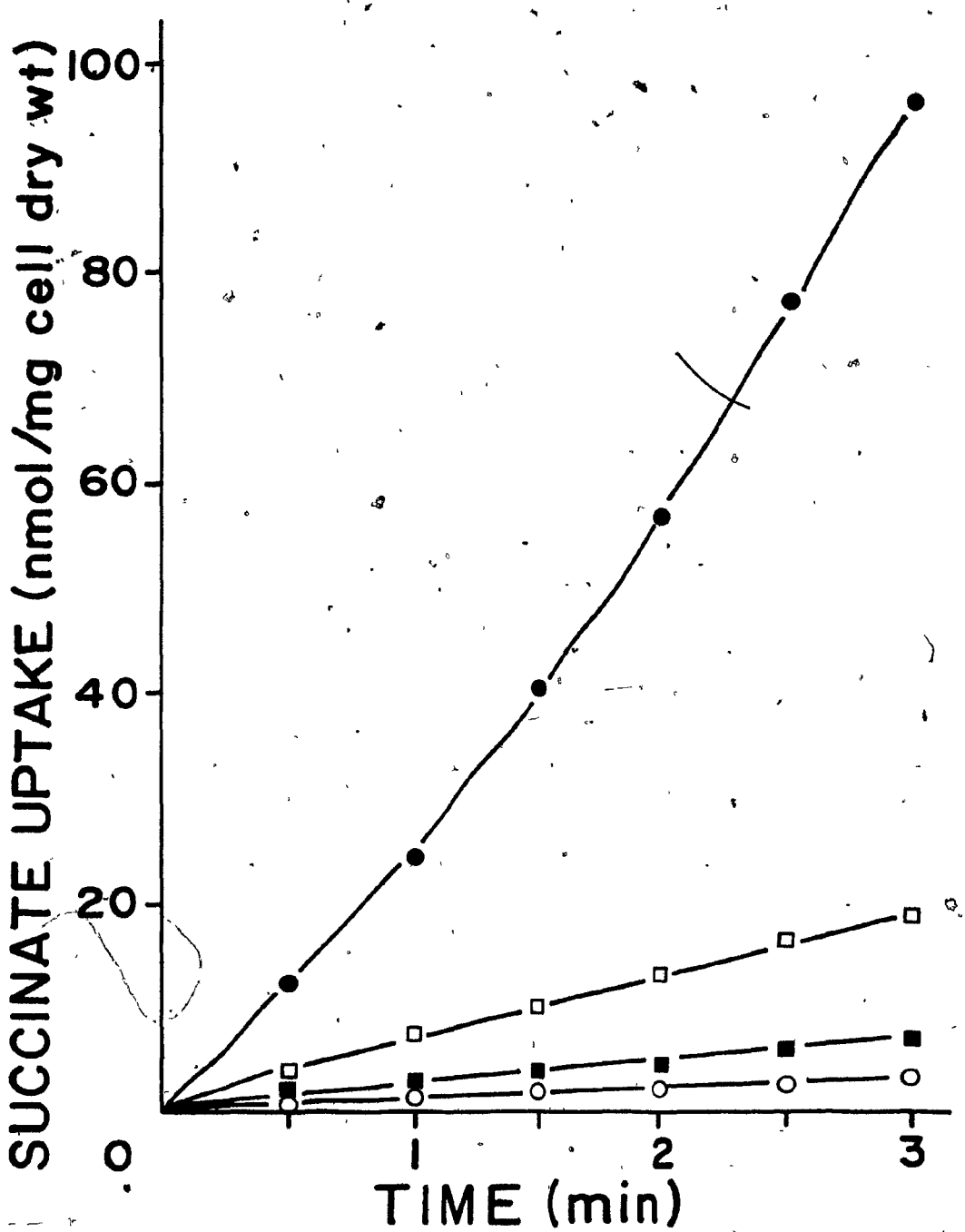

SUCCINATE UPTAKE (nmol/mg cell dry wt)
100
80
60
40
20
0
1
2
3
TIME (min)

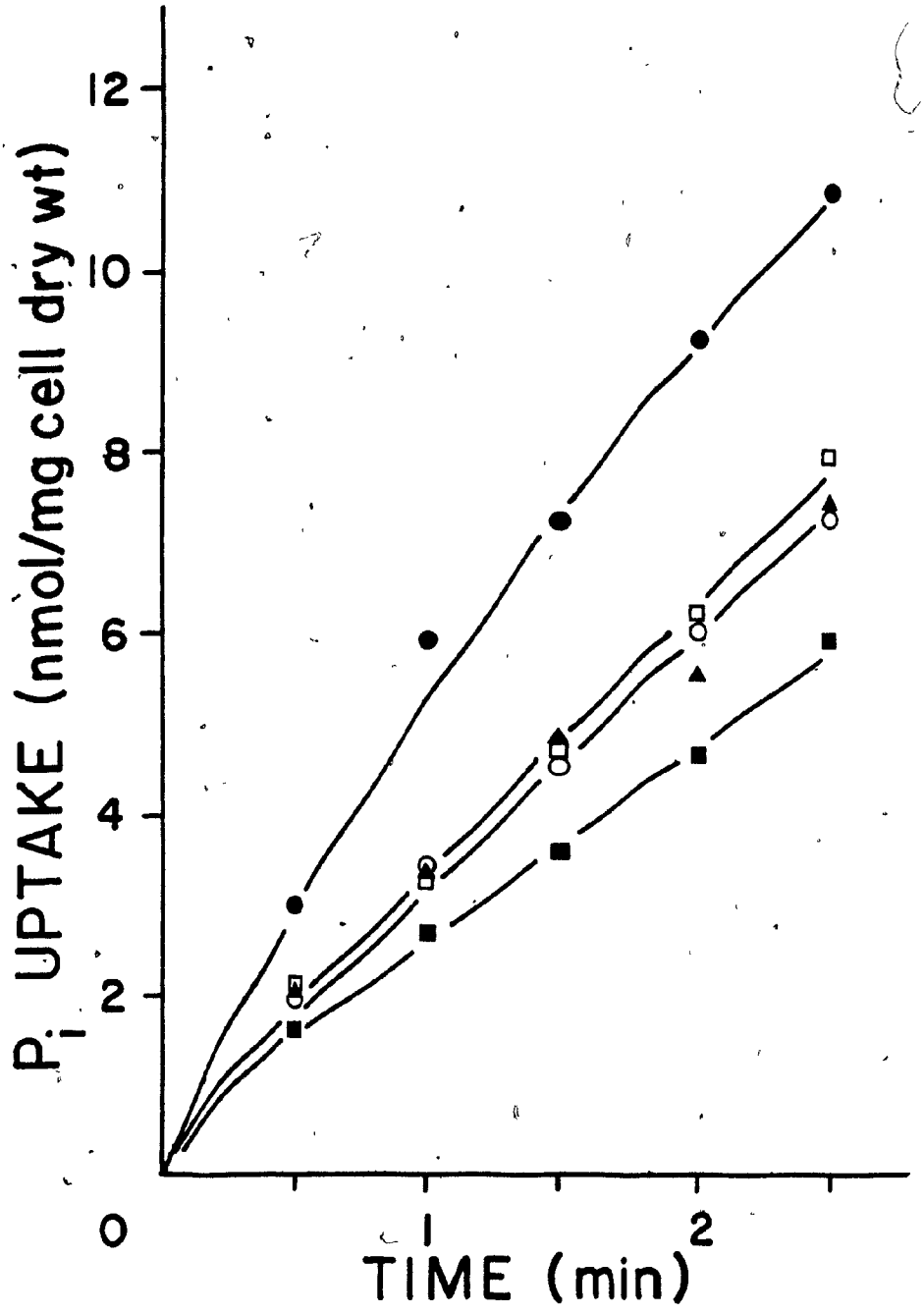
Figure 22. Effects of Na^+, K^+ and Li^+ on the rate of uptake of $^{32}P_i$ by V. fischeri.

The uptake of $^{32}P_i$ (final concentration 25 uM, specific activity 50 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
● 100 mM NaCl + 200 mM ChCl
▲ 10 mM NaCl + 290 mM ChCl (actual data plotted)
□ 10 mM NaCl + 90 mM LiCl + 200 mM ChCl
■ 100 mM LiCl + 200 mM ChCl
▲ 10 mM NaCl + 90 mM KCl + 200 mM ChCl (a)

Other conditions were as described in Figure 7, except that the ethanol was omitted from the incubation medium.

a - Under these conditions, the results were not significantly different from those obtained when uptake was measured in CST 10 mM NaCl + 290 mM ChCl (actual data plotted).


P_i UPTAKE (nmol/mg cell dry wt)
0
2
4
6
8
10
12
1
2
TIME (min)

III Evidence for active transport of metabolizable substrates

The metabolic activity of cells is a factor that must be taken into consideration when interpreting data obtained in transport studies. Radioactivity detected in cells does not necessarily represent the original form of the substrate added to the medium. Without information about the identity of the radioactive material in the cells, one would be unable to determine if the substrate had entered by facilitated diffusion or active transport. For example, a substrate may enter the cells by a facilitated diffusion mechanism and then be metabolized into other products. One way of circumventing this problem is to use non-metabolizable substrate analogues such as AIB, fucose or TMG. Accumulation of these substrates could not be influenced by subsequent metabolic activity. A second approach is to use membrane vesicles which transport substrates without metabolizing them. These methods do have disadvantages however. Not all substrates of interest have transportable analogues. The use of analogues may also be criticized on the grounds that these are artificial substrates, the transport characteristics of which do not represent reality. Vesicles are artificial systems which may differ from whole cells. The transport ability of these membrane preparations may be altered due to the inactivation or loss of essential components of the uptake systems, such as binding proteins.

The non-metabolizable substrate AIB has been a useful tool for examining transport in A. haloplanktis, however vesicles of this organism have not been satisfactory for such studies. Vesicles did not take up K^+, and their ability to take up L-alanine was quite poor in comparison to that

of whole cells (Sprott and MacLeod, 1974).

Active transport implies the accumulation of a chemically unmodified substrate to an intracellular concentration which is greater than that in the extracellular medium (Hamilton, 1975). The ability to concentrate a substrate against its gradient is dependent upon the expenditure of metabolic energy. Facilitated diffusion merely results in equilibration of the solute across the cell membrane and does not require any input of energy. In order to distinguish between active transport and facilitated diffusion, it is useful to express data in such a way that intracellular and extracelluar substrate concentrations can be compared (Maloney et al., 1975).

In this study, the possibility had to be considered that what appeared to be Na^+-stimulated transport of metabolizable substrates was in fact a Na^+-independent facilitated diffusion process followed by Na^+-dependent metabolism. The ability of cells of A. haloplanktis and V. fischeri to accumulate substrates against their gradients was evaluated at high and low concentrations of Na^+. After a substrate was transported, the contents of the cells were extracted and the unmodified substrate was recovered and quantitated. Since the approximate intracellular volume of the cells was known, the amount of substrate could be expressed as a molarity and compared to the original extracellular concentration. An intracellular substrate concentration greater than that in the medium would indicate that uptake had occured by an active transport mechanism.

a. Intracellular volume of Vibrio fischeri

The intracellular volume of A. haloplanktis has previously been estimated to be 1.6 ul/mg (Thompson and MacLeod, 1973), however no such determination has been made for cells of V. fischeri. Radioactive inulin, which is excluded by the outer membrane of cells, is commonly used as a marker for extracellular water (Maloney et al., 1975). In A. haloplanktis, sucrose is thought to penetrate to the level of the cytoplasmic membrane (Thompson and MacLeod, 1971), and would therefore not take into account the volume of the periplasmic space when used for measuring cell volumes. If this method is to be used for measuring the intracellular volume of V. fischeri, it is necessary that the cells do not metabolize sucrose. The ability of sucrose to stimulate respiration in cells of this organism was examined. As was the case in A. haloplanktis (Buckmire and MacLeod, 1970), sucrose did not stimulate respiration in V. fischeri under conditions where D-glucose and succinate greatly enhanced respiratory activity.

^{14}C-sucrose was used as a marker of extracellular water in packed cell preparations of V. fischeri. The total available fluid space, extracellular fluid and cell dry weight in the pellet were measured in two separate experiments (Table 2). The intracellular volume of the cells was determined and an average value of 2.8 ul/mg was used for subsequent calculations of intracellular substrate concentrations. This estimate of cell volume is comparable to similar values that have been determined for other bacteria (Maloney et al., 1975).

Table 2. Data for the determination of the intracellular volume of *V. fischeri*.

The data was obtained and used to determine the intracellular volume, as described in the Materials and Methods. Triplicate determinations were done in two separate experiments, and the average of these two results was used.

Intracellular Volume of *Vibrio fischeri*

	Experiment 1	Experiment 2
Weight of Wet Pellet (g)	0.568 ± 0.003	0.756 ± 0.030
Dry Weight of Cells (g)	0.110 ± 0.001	0.147 ± 0.001
Total Available Fluid in Pellet (a,b) (ml)	0.458 ± 0.003	0.609 ± 0.030
Extracellular Fluid Trapped in Pellet (c) (ml)	0.133 ± 0.008	0.225 ± 0.018
Intracellular Fluid Trapped in Pellet (d) (ml)	0.325 ± 0.010	0.384 ± 0.047
Intracellular Volume (e) (ul/mg)	2.95 ± 0.09	2.61 ± 0.31

a - (Weight of wet pellet) -(Dry weight of cells)

b - Density of complete salts buffer = 1 g/ml

c - Determined as described in materials and methods

d - (Total available fluid) - (Extracellular fluid)

e - Intracellular fluid/dry weight of cells

b. Effect of Na^+ on active transport of metabolizable substrates

Substrates were extracted and recovered from cells at the beginning and end of each transport run. The rates of uptake by cells of A. haloplanktis in 300 mM NaCl were compared with the rates in 10 mM NaCl + 290 mM ChCl (Figures 23 to 26). The rates of transport of all the substrates tested were greater in the high concentration of Na^+ than those in the low concentration of Na^+. The unmodified substrates were accumulated to intracellular concentrations that exceeded the original extracellular concentrations, and these outwardly-directed gradients increased with time.

Cells of V. fischeri also transported substrates against their concentration-gradients (Figures 27, 28). The uptake of L-alanine by cells in 10 mM NaCl + 290 mM ChCl was similar to that in 100 mM NaCl + 200 mM ChCl, however the substrate was not accumulated by cells in 300 mM ChCl. The similar rates of uptake of L-alanine at 10 mM and 100 mM Na^+ are also seen in Figure 5. The rates of transport of L-glutamate, L-arginine, succinate and $^{32}P_i$ were greater in 100 mM NaCl + 200 mM ChCl than in 10 mM NaCl + 290 mM ChCl. L-glutamate was accumulated against its concentration gradient even in the absence of Na^+.

The ability of these two marine organisms to concentrate substrates against their gradients indicates that the uptake is indeed an energy-requiring active transport process.

Figure 23. Effect of Na^+ on the ability of A. haloplanktis to accumulate L-alanine, L-leucine and L-glutamate against their concentration gradients.

The substrates (final concentration 200 uM) were taken up by cells suspended in either CST 10 mM NaCl + 290 mM ChCl (▲) or CST 300 mM NaCl (●). The conditions of uptake were as described in Figure 7, except that the specific activity of the substrates was 10 uCi/umol. After samples of the reaction mixture were filtered at 0.5 and three minutes, the cell contents were extracted with hot water. The extracts were concentrated, and the radioactive substrates were reisolated by chromatography as described in the Materials and Methods section.

The nmol substrate/mg cell dry wt extracted and recovered was converted to molarity as described in the Materials and Methods (nmol/mg ÷ ul/mg = nmol/ul = mM).

The horizontal dotted line represents the extracellular substrate concentration at zero time. The concentration gradient is the ratio of the intracellular substrate concentration to the extracellular substrate concentration.

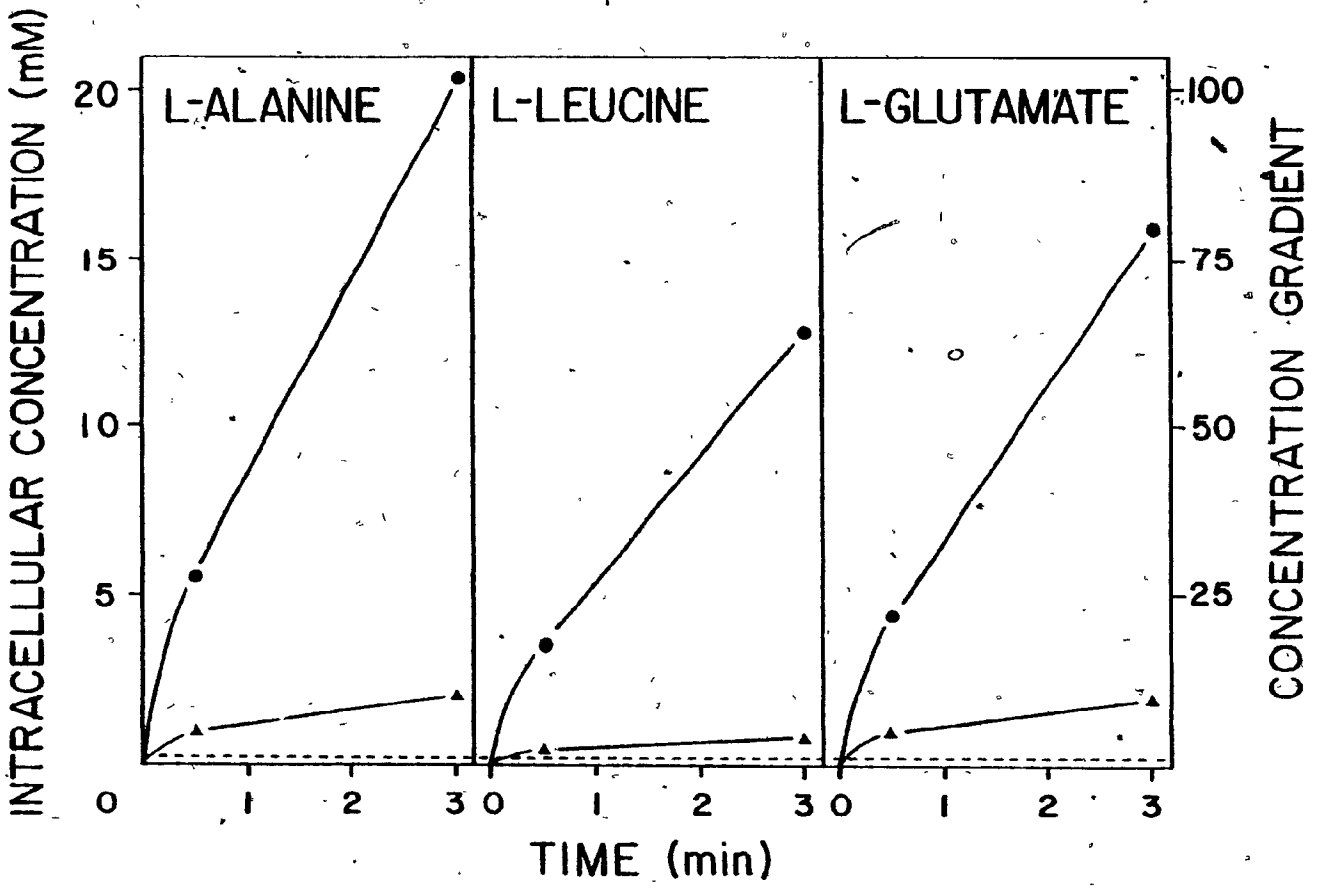

L-ALANINE
L-LEUCINE
L-GLUTAMATE
INTRACELLULAR CONCENTRATION (mM)
CONCENTRATION GRADIENT
TIME (min)
0
5
10
15
20
25
50
75
100
1
2
3

Figure 24. Effect of Na^+ on the ability of A. haloplanktis to accumulate L-aspartic acid; L-arginine and L-lysine against their concentration gradients.

The uptake of the substrates (final concentration 200 uM) was measured in the following CST buffers:

▲ 10 mM NaCl + 290 mM ChCl
● 300 mM NaCl

The specific activity of aspartate was 10 uCi/umol, and that of arginine and lysine was 20 uCi/umol. Other conditions were as described in Figure 23.

The horizontal dotted line represents the extracellular substrate concentration at zero time. The concentration gradient is the ratio of the intracellular substrate concentration to the extracellular substrate concentration.

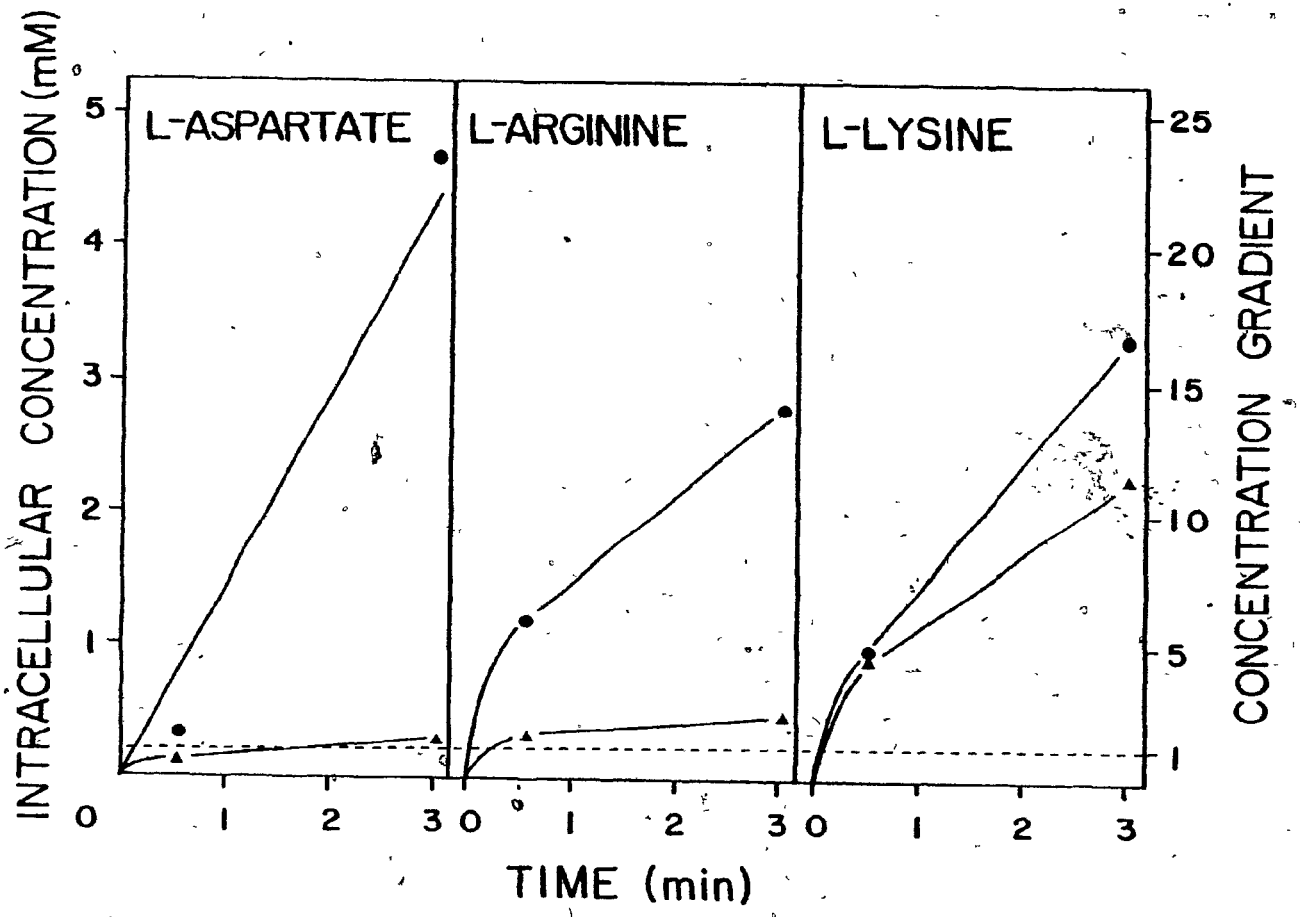

L-ASPARTATE
L-ARGININE
L-LYSINE
INTRACELLULAR CONCENTRATION (mM)
CONCENTRATION GRADIENT
TIME (min)
0
1
2
3
4
5
10
15
20
25

Figure 25. Effect of Na^+ on the ability of A. haloplanktis to accumulate citric acid, succinic acid and D-galactose against their concentration gradients.

The uptake of the substrates (final concentration 50 uM) was measured in the following CST buffers:

▲ 10 mM NaCl + 290 mM ChCl
● 300 mM NaCl

The specific activity of citrate was 20 uCi/umole, that of succinate was 22.7 uCi/umol, and that of D-galactose was 10 uCi/umol. Other conditions were as described in Figure 23.

The horizontal dotted line represents the extracellular substrate concentration at zero time. The concentration gradient is the ratio of the intracellular substrate concentration to the extracellular substrate concentration.

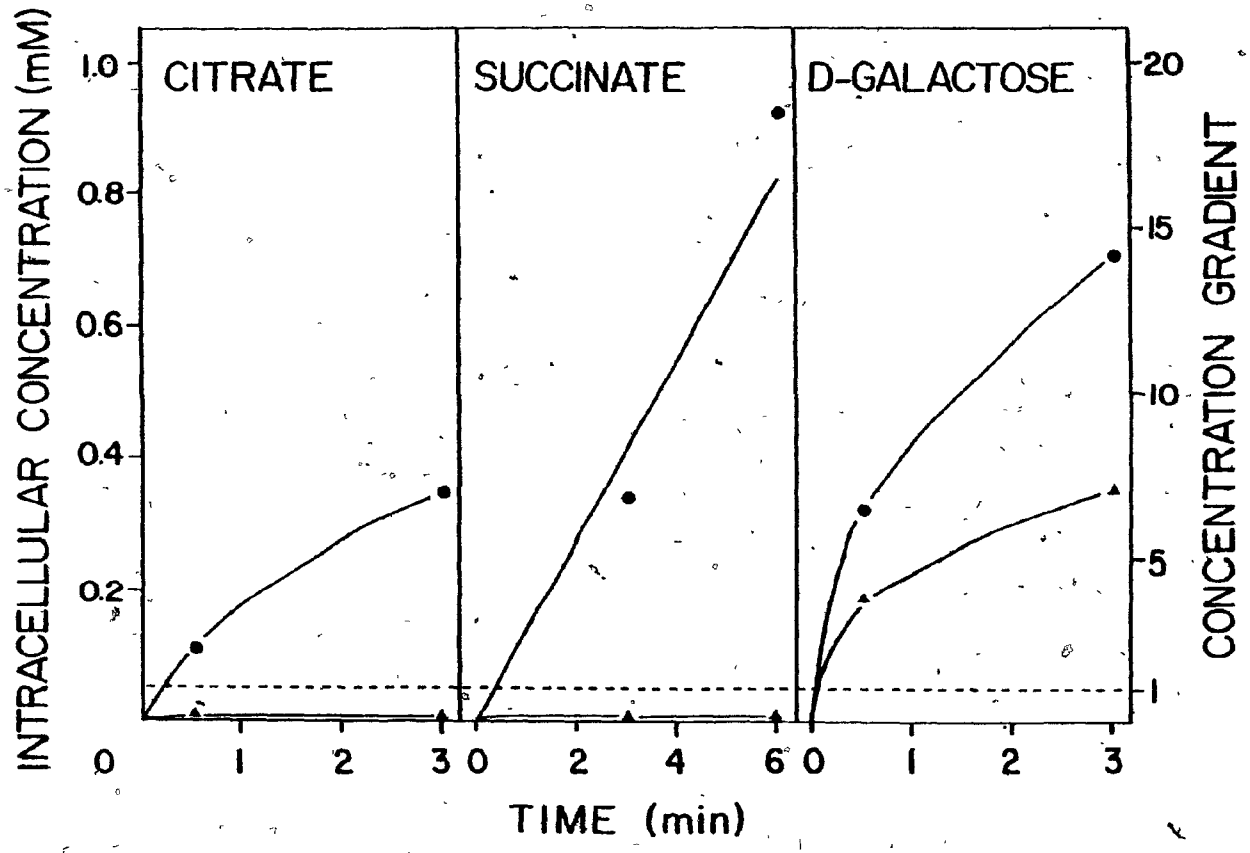

CITRATE
SUCCINATE
D-GALACTOSE
INTRACELLULAR CONCENTRATION (mM)
CONCENTRATION GRADIENT
TIME (min)
1.0
0.8
0.6
0.4
0.2
0
20
15
10
5
1
0 1 2 3
0 2 4 6
0 1 2 3

Figure 26. Effect of Na^+ on the ability of A. haloplanktis to accumulate L-malic acid and $^{32}P_i$ against their concentration gradients.

The uptake of malate and $^{32}P_i$ (final concentration 100 uM and 25 uM respectively) was measured in the following CST buffers:

▲ 10 mM NaCl + 290 mM ChCl
● 300 mM NaCl

The specific activity of malate was 20 uCi/umol, and that of $^{32}P_i$ was 50 uCi/umol. Other conditions were as described in Figure 23. The $^{32}P_i$ was extracted into five percent trichloroacetic acid, and recovered by precipitation of phosphomolybdate as described in Materials and Methods.

The horizontal dotted line represents the extracellular substrate concentration at zero time.

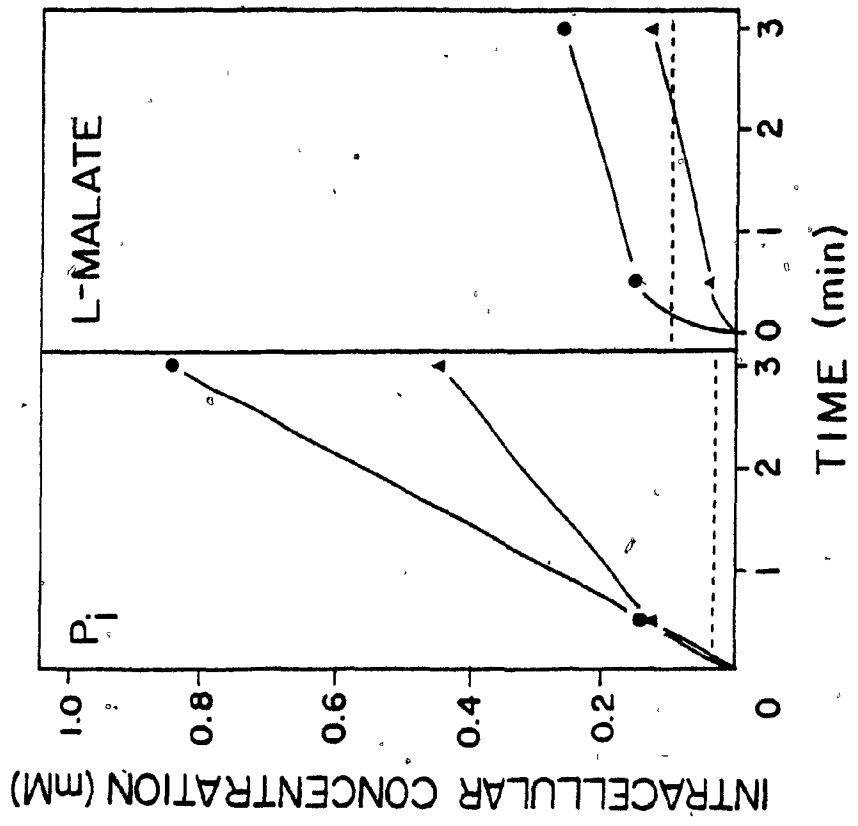

INTRACELLULAR CONCENTRATION (mM)
1.0
0.8
0.6
0.4
0.2
0
P_i
L-MALATE
1
2
3
0
1
2
3
TIME (min)

Figure 27. Effect of Na^+ on the ability of V. fischeri to accumulate L-alanine, L-glutamic acid and L-arginine against their concentration gradients.

The uptake of the substrates (final concentration 200 uM, specific activity 10 uCi/umol) was measured in the following CST buffers:

○ 300 mM ChCl
▲ 10 mM NaCl + 290 mM ChCl
● 100 mM NaCl + 200 mM ChCl

Other conditions were as described in Figure 23, except that the ethanol was omitted from the incubation medium.

The horizontal dotted line represents the extracellular substrate concentration at zero time. The concentration gradient is the ratio of the intracellular substrate concentration to the extracellular substrate concentration.

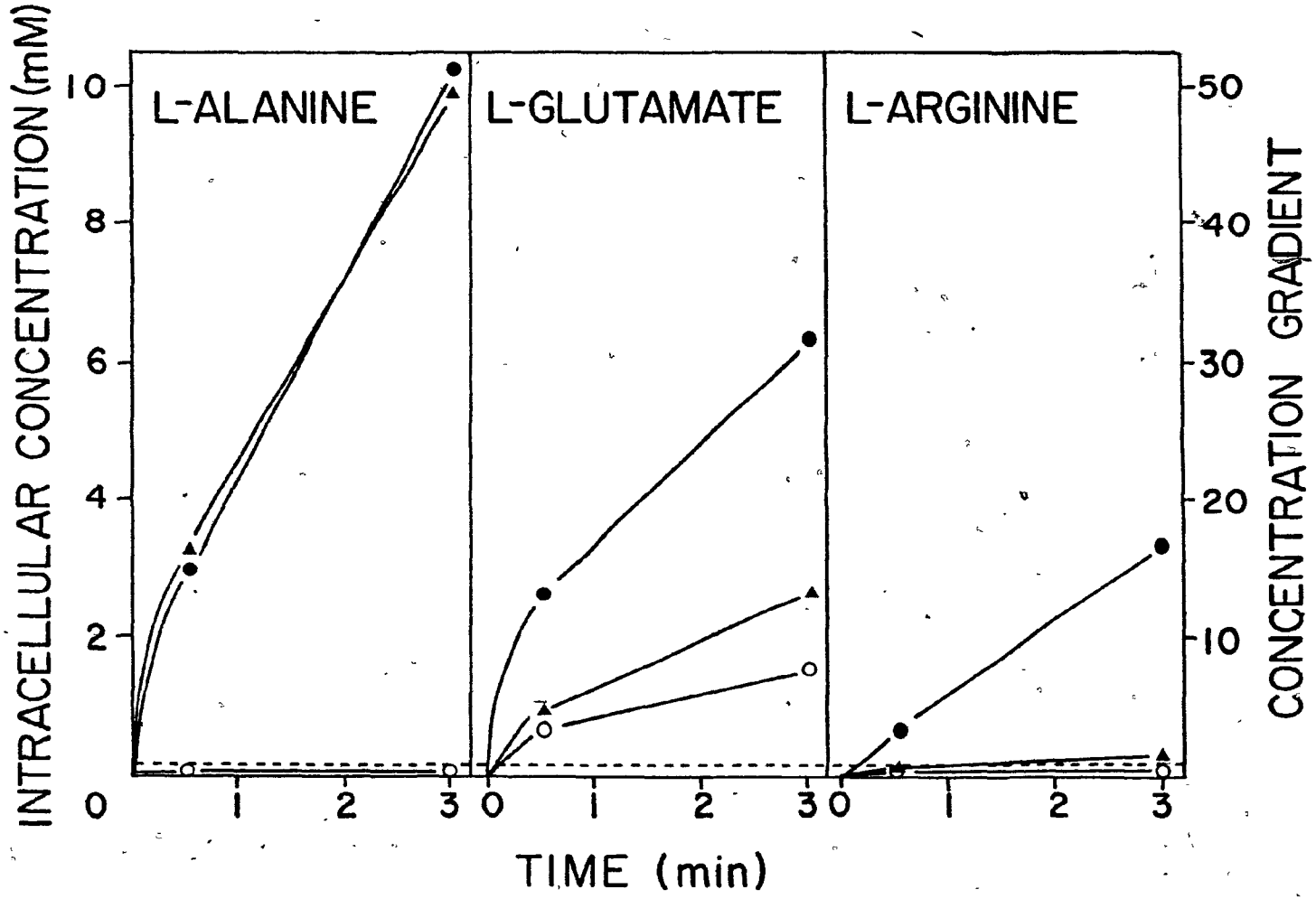

L-ALANINE
L-GLUTAMATE
L-ARGININE
INTRACELLULAR CONCENTRATION (mM)
CONCENTRATION GRADIENT
TIME (min)
0
2
4
6
8
10
10
20
30
40
50
0
1
2
3

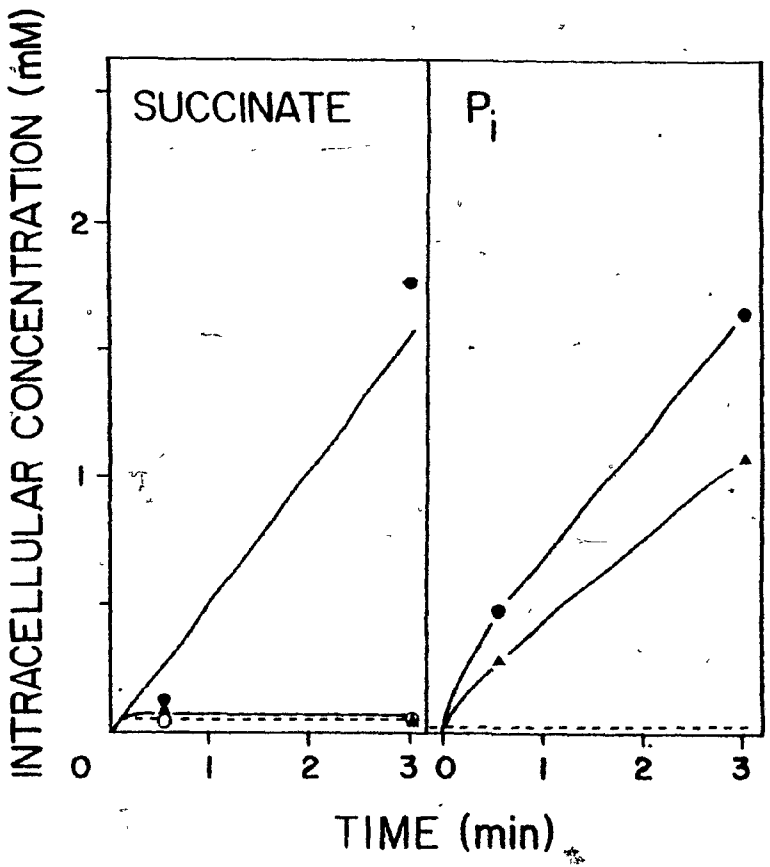
Figure 28. Effect of Na^+ on the ability of V. fischeri to accumulate succinic acid and $^{32}P_i$ against their concentration gradients.

The uptake of succinate and $^{32}P_i$ (final concentration 50 uM and 25 uM respectively) was measured in the following GST buffers:

○ 300 mM ChCl (data for succinate only)
▲ 10 mM NaCl + 290 mM ChCl
● 100 mM NaCl + 200 mM ChCl

The specific activity of succinate was 22.7 uCi/umol, and that of $^{32}P_i$ was 50 uCi/umol. Other conditions were as described in Figure 23, except that ethanol was omitted from the incubation medium. The $^{32}P_i$ was extracted into five percent trichloroacetic acid, and recovered by precipitation of phosphomolybdate as described in Materials and Methods.

The horizontal dotted line represents the extracellular substrate concentration at zero time.


SUCCINATE
P_i
INTRACELLULAR CONCENTRATION (mM)
2
1
0
1
2
3
0
1
2
3
TIME (min)

DISCUSSION

The results of this study illustrate the important influence of Na^+ on the membrane transport of metabolites in the two marine bacteria examined. This aspect of the physiology of marine bacteria is of interest as it relates to the Na^+ requirement for growth of these organisms. Such information may provide insights into the mechanisms that allow marine bacteria to survive in the sea.

Cells of A. haloplanktis required Na^+ for the transport of all substrates tested. V. fischeri needed Na^+ for the optimal uptake of all substrates except D-glucose, the accumulation of which was unaffected by Na^+. The various uptake systems differed in their sensitivities to Na^+, as has been reported by other researchers in this laboratory. Although there are similarities in the trends observed in this study, as compared with previous investigations, the results may not be entirely comparable because of differences in the conditions and methods of assay. For example, in Wong's study (1968) the buffers were not osmotically balanced, nor was any exogenous energy source provided for the cells, as was done here. In my experiments, the amount of substrate taken up at one minute was used as a measure of initial velocity. Wong determined the uptakes of P_i and AIB only after sixty minutes, and those of D-galactose and the carboxylic acids after ten minutes.

Wong (1968) reported that the maximum rates of uptake of D-galactose and D-fucose by cells of A. haloplanktis were attained at 100 mM Na^+, while those of L-malate and P_i peaked at 200 mM Na^+. Citrate and

succinate transport was optimal at 250 mM Na^+. Similar results were obtained in this survey, with the exception of citrate. The rate of uptake of this substrate leveled off between 200 and 300 mM Na^+, but then increased at higher Na^+ concentrations (Figure 3).

Three neutral amino acid transport systems have been resolved in A. haloplanktis. Fein and MacLeod (1975) determined the effect of Na^+ on the DAG and LIV-I systems, which take up L-alanine, by measuring AIB transport. It was found that uptake was optimal at 300 to 400 mM Na^+. No ethanol was provided as an energy source, and LiCl was used as the osmotic balance. It should be noted that LiCl is not suitable for this purpose since it has several effects on the physiology of A. haloplanktis, as will be discussed later. In this study, the uptake of L-alanine peaked at 400 mM Na^+. Using an osmotically unbalanced medium, Sprott et al., (1975) found that the maximum rate of L-alanine uptake by whole cells was reached at 200 mM Na^+, while the maximum rate in vesicles occurred at 75 mM Na^+. L-leucine is taken up by the LIV-I and LIV-II systems (Pearce et al., 1977), and the pattern of uptake reported by these workers was the same as that observed in this study.

The effect of Na^+ on AIB uptake by V. fischeri (Figure 4) was similar to that observed by Drapeau et al. (1966). P_i was taken up by V. fischeri in the absence of Na^+, however the ion clearly stimulated transport (Figure 6). The rate of P_i uptake leveled off between 200 and 300 mM Na^+ and then increased at higher Na^+ concentrations. Using carrier-free $^{32}P_i$, Wong (1968) also demonstrated uptake by this organism

in a Na^+-free buffer. The maximum rate of transport was attained at 200 mM Na^+, however, and then decreased at Na^+ concentrations greater than this.

It has previously been established that the osmotic activity of the medium may have a significant influence on the transport of substrates by cells of A. haloplanktis (MacLeod et al., 1978; Pearce et al., 1977), and by cells of a marine Vibrio (Hayasaka and Morita, 1979). In my experiments (Figures 1 to 6), the buffers were osmotically balanced only to the 300 mM level. When the rates of substrate uptake were measured in 400 and 500 mM Na^+, the total salt concentration of the suspending solutions was increased by 100 and 200 mM respectively. This increase in osmotic pressure may have had an effect on the nature of the response obtained with certain substrates.

As the Na^+ concentration increased, discontinuous curves were obtained for the uptakes of citrate by A. haloplanktis (Figure 3) and P_i and L-glutamate by V. fischeri (Figure 6). It would be interesting to do further studies to determine if this phenomenon can be attributed to an increase in osmotic pressure at Na^+ concentrations exceeding 300 mM. If this is the case, the addition of appropriate solutes such as choline, Li^+, Mg^{+2} or sucrose, would be expected to give results similar to those obtained with additional Na^+, if 300 mM Na^+ is present in the medium.

It is interesting, however, that discontinuous curves were obtained for only a few of the metabolites tested. Perhaps these substrates,

whose accumulation is stimulated by high salt concentrations, are used by the cells as internal solutes which serve to prevent plasmolysis at high osmotic pressures. A cell usually maintains its cytoplasm hypertonic in relation to the environment. Certain amino acids have been shown to contribute to the regulation of intracellular osmotic pressure. The marine bacterium Vibrio alginolyticus was found to increase its total internal solute concentration as the amount of NaCl in the medium increased from 0.2 to 1.5 M. The concentration of glutamate increased from 40 to 254 mM, and that of proline increased from 6.6 to 72 mM (Unemoto and Hayashi, 1979b). A variety of non-halophiles also responded to changes in Na^+ concentration by altering their glutamate and proline pools (Measures, 1975).

Another possibility is that the discontinuous curves are the results of combined uptakes mediated by more than one transport system. It is not uncommon that a substrate can be accumulated by two or more separate systems which may respond differently to Na^+ (Schellenberg and Furlong, 1977; Hoshino, 1979). Studies on the kinetics of P_i transport in V. fischeri indicated that this organism may have high and low affinity uptake systems, however the effects of Na^+ were not investigated (MacLeod and DeVoe, unpublished data). It is thus evident that the interpretation of results such as these can be complicated unless the individual transport systems for a given substrate are resolved and their properties determined.

The transport of D-glucose by V. fischeri was unique in that Na^+

did not affect its activity. This may be because energy coupling is achieved by a phosphoenolpyruvate: sugar phosphotransferase (PTS) system. Hodson and Azam (1979) have described such a Na^+-independent transport system in the marine bacterium Serratia marinorubra. They also report having found PTS systems in V. fischeri and Beneckea harveyi, as well as in marine representatives of the genera Flavobacterium, Pseudomonas and Bacillus. Studies in this laboratory on Photobacterium phosphoreum, Vibrio natriegens and Vibrio parahaemolyticus indicate that these marine bacteria may also take up D-glucose by a Na^+-independent mechanism. In the absence of Na^+, addition of D-glucose to cells of these organisms produced a significant stimulation of respiration. Presumably, the sugar had to be transported for this to occur. Cells of A. haloplanktis were not stimulated under similar conditions (MacLeod and DeVoe, unpublished data). More detailed investigations of the mechanism of sugar uptake would be required to ascertain the existence of PTS systems in these bacteria. Transport via a PTS system does not require an energized membrane state and is therefore unaffected by uncouplers, inhibitors of electron transport and inhibitors of ATP synthesis. The accumulated sugar must also be recovered from the cells in its phosphorylated form (Hodson and Azam, 1979).

Cells of V. fischeri took up succinate, L-arginine, L-glutamate and P_i in the absence of Na^+, however Na^+ stimulated transport significantly and was required for the optimal uptake of these substrates. Recent studies have illustrated that the transport of some substrates by other marine bacteria might best be described as Na^+-stimulated rather than Na^+-

dependent. In the absence of Na^+, a marine Vibrio took up the galactose analogue TMG and L-glutamate (Hayasaka and Morita, 1979). Cells of P. phosphoreum were also able to accumulate P_i under similar conditions (MacLeod and DeVoe, unpublished data). As is the case with V. fischeri, substrate transport by both of these organisms was greatly enhanced by Na^+.

There are several possible explanations for the ability of cells of V. fischeri to take up substrates in the absence of added Na^+. Under these conditions, L-arginine and succinate (Figures 27 and 28) were not accumulated against their concentration gradients. These results are consistent with a facilitated diffusion mechanism of uptake. L-glutamate, however, was concentrated by the cells (Figure 27), which suggests that this substrate was actively transported. The P_i pools were not extracted after uptake in CST - 300 mM ChCl, however the rate of uptake in this buffer was equivalent to that in 10 mM NaCl (Figure 22). Since P_i was actively transported in the latter case (Figure 28), this was presumably also true in the former.

A second possibility is that Na^+ is not involved in energy coupling, but stimulates transport by promoting optimal function of the carriers. Wong et al. (1969) demonstrated that Na^+ decreases the K_m for AIB transport in A. haloplanktis, and similar results have been observed in other Na^+-dependent microbial systems (Table 1). There is evidence which indicates that, in Salmonella typhimurium, both Na^+ and the membrane potential can increase the affinity of the melibiose transport system for its substrates (Tokuda and Kaback, 1978; Cohn and Kaback, 1980). Lanyi and Silverman (1979) have suggested that transmembrane ion

gradients may regulate the activity of transport carriers in Halobacterium halobium. In all of these organisms, however, the energy for transport is provided by an electrochemical Na^+ gradient. Thus, Na^+ may be involved in both energy transduction and regulation of the carriers.

A more viable alternative is that V. fischeri possesses both Na^+-dependent and Na^+-independent systems for the uptake of some substrates. Another consideration is that the level of Na^+ contamination in the "Na^+-free" buffer is sufficient to allow for some degree of Na^+-dependent uptake. If this is the case, the uptake systems must be very sensitive to Na^+. Flame spectrophotometry of a double strength CST buffer containing 600 mM choline chloride showed that there was only about 25 uM Na^+ in this buffer.

The fact remains that although some of these bacteria are apparently capable of transporting metabolites in the presence of little or no Na^+, they clearly do have a specific requirement for significant quantities of Na^+ for growth. V. fischeri requires Na^+ for growth in a chemically defined medium with D-glucose as the sole source of carbon (Srivastava and MacLeod, 1971), despite the fact that Na^+ is not required for the uptake of this sugar or P_i. What then is the basis of the Na^+ requirement for growth? Perhaps there is an absolute Na^+ requirement for the uptake of K^+, as has been reported for A. haloplanktis (Hassan and MacLeod, 1975). Similarly, Na^+ may be needed for the accumulation of other essential ions such as Mg^{+2} or Fe^{+3}.

That Na^+ enhances the respiratory activity of some marine bacteria (Watanabe et al., 1977; Unemoto et al., 1977; Khanna, 1980) indicates that this may be an important physiological basis for Na^+-dependent growth. Unfortunately, it is not clear if these bacteria have an absolute dependence on Na^+ for respiration. Under the conditions of assay, the effect of Na^+ was merely stimulatory, however this does not preclude an absolute Na^+ requirement for respiration. Cell suspensions of A. haloplanktis, washed three times in choline chloride buffer, contained less than 50 uM Na^+ (Niven and MacLeod, 1978). The washing procedure may be ineffective in removing Na^+ from the micro-environment where the Na^+-activated respiratory components are located. The amount of contaminating Na^+ in the system may be sufficient to provide for the partial or complete function of Na^+-dependent enzymes.

The results of the present study indicate that the Na^+ requirement for transport exceeds that for respiration in both of the organisms examined. After sufficient Na^+ (2 mM) was provided to allow for a maximum rate of ethanol-energized respiration by cells of A. haloplanktis, addition of more Na^+ continued to increase the rates of substrate transport. Khanna (1980) has demonstrated that the electron donors ethanol, NADH, ascorbate-reduced TMPD and succinate can stimulate the uptake of AIB by cells of A. haloplanktis. The organism was also able to transport AIB in the absence of an exogenous energy source. The enhancement of AIB uptake by Na^+ was not attributable to a stimulation of respiration. In the absence of Na^+, the endogenous rate of oxygen uptake by intact cells was 46 n atoms O/mg/min, whereas the addition of ethanol or NADH resulted

in rates of 120 and 210 n atoms O/mg/min respectively. The cells did not take up AIB under any of these conditions. In 250 mM Na^+, the endogenous rate of respiration was 47 n atoms O/mg/min, yet the cells rapidly accumulated the substrate.

Na^+ did not affect the endogenous respiratory activity of V. fischeri, however the uptake of AIB was Na^+-dependent (Figure 4). It should be emphasized that these results do not show that respiration is not influenced by Na^+. More detailed studies on the ability of cells to oxidize specific substrates must be carried out in order to clarify the role of Na^+ in the respiratory activity of V. fischeri. Under the conditions used to measure transport, cells of V. fischeri clearly have sufficient endogenous energy reserves to drive the uptake of AIB, but only in the presence of Na^+. This suggests that Na^+ may be required to effect energy coupling. If this is the case, respiratory activity presumably maintains an inwardly-directed electrochemical Na^+ gradient which is the immediate energy source for active transport. This interpretation is only speculative, however, since the organism has not been shown to have a Na^+-H^+ antiporter, and the experiment does not attempt to distinguish between the possible mechanisms of energy coupling. As previously mentioned, the ability of cells of V. fischeri to take up some substrates in the apparent absence of Na^+ might be explained if Na^+ increased the affinity of the transport carriers for their substrates (i.e. decreased the K_m) while energy is provided in a form other than a Na^+ gradient. There also might be two uptake systems: one powered by A Na^+ gradient, and the other by a proton gradient (pmf). It would be

useful to study the energetics and kinetics of transport in V. fischeri in order to clarify the role of Na^+ ions in this process.

Some substrates are known to be transported by ATP-dependent systems which may or may not require the presence of a pmf (Rosen and Kashket, 1978). Other metabolites are accumulated by group translocation mechanisms such as the Na^+-independent PTS system already discussed. This latter method of uptake does not fit our criterion for active transport, however, because the substrates are accumulated within a cell in a chemically modified form. As will be discussed later, the evidence obtained in this study suggests that all of the substrates tested, with the exception of D-glucose, were "actively" transported by cells of V. fischeri.

The specificity of the Na^+ requirement for transport was investigated by replacing NaCl with isomolal amounts of choline chloride, KCl or LiCl. K^+ was unable to stimulate transport, but Li^+ clearly had a positive effect on the uptake of certain substrates by cells of both organisms. In those cases where Li^+ promoted transport, it was generally less effective than an equal quantity of Na^+. In some cases, Li^+ appeared to have an inhibitory effect on transport.

Previous studies have indicated that there is an absolute Na^+ requirement for the transport of certain substrates by A. haloplanktis. Other cations were unable to spare the need for Na^+ for AIB uptake (Drapeau et al., 1966; Khanna, 1980) or for the accumulation of some amino

acids (Wong, 1968). The substrates whose uptakes were found to be promoted by Li^+ in this study had not previously been tested in this regard. That Li^+ can partially replace the effect of Na^+ on some functions in A. haloplanktis has been documented in other studies. Stimulation of D-galactose uptake by Li^+ in the absence of Na^+ has previously been observed by Gerson (1980). Li^+ also promotes the retention of intracellular solutes, although it is less effective than Na^+ (MacLeod et al., 1978). The Na^+-H^+ antiporter recognizes Li^+. This ion actually appears to be bound more tightly by the antiporter than is Na^+, thus resulting in an inhibition of antiporter activity (Niven and MacLeod, 1978). Li^+ stimulates the oxidation of NADH and ethanol by cells of A. haloplanktis, albeit less effectively than Na^+ (Khanna, 1980). The ability of Li^+ to replace Na^+ in promoting transport is commonly observed in many other bacteria (see Literature Review).

There are several ways in which Li^+ might affect transport. A positive influence may be due to the fact that Li^+ is a better external osmotic solute than K^+ or choline. Other evidence suggests, however, that Li^+ has a specific stimulatory effect on A. haloplanktis similar to that of Na^+. The cells used in the respiration studies done by Khanna (1980) were toluene-treated to permit free passage of solutes across the cytoplasmic membrane, therefore Li^+ would not be expected to have an osmotic effect on cellular activity. The fact that the ion stimulated the oxidation of substrates indicates that it has a specific action similar to that of Na^+ on certain components of the respiratory chain. Li^+ catalyzes antiporter activity (a non-osmotic function), therefore it is

conceivable that an electrochemical Li^+ gradient might drive transport in the same way that a Na^+ gradient does. Because the antiporter is inhibited by Li^+, less energy would be stored in a Li^+ gradient than a Na^+ gradient, thus accounting for the lower efficacy of the former in promoting transport.

In addition to possible contributions to the energy state of the cytoplasmic membrane, Li^+ may also influence the regulation of transport carriers. Tokuda and Kaback (1978) showed that Li^+, as well as Na^+, could promote substrate binding in S. typhimurium. Li^+ has been reported to decrease the K_m for melibiose transport in E. coli (Tanaka et al., 1980) and in S. typhimurium (Niiya et al., 1980).

One of the most striking features about transport in A. haloplanktis and V. fischeri is the variation in sensitivity to Na^+ and Li^+ of the rates of uptake of the various metabolites examined. The maximum rate of transport of different substrates occur at different Na^+ concentrations, and a variety of responses to Li^+ are detected. This suggests that there may be a variety of independent uptake systems for the different substrates, and that these systems differ from one another in their properties. The affinity of some carriers for their substrates may be stimulated by both Li^+ and Na^+ to varying degrees. Other carriers may be inhibited by Li^+, or be unaffected by the ion entirely. For example, the fact that Li^+ stimulates the uptake of L-glutamate by cells of V. fischeri only in the presence of Na^+, implies that Na^+ may have two effects on transport. One function, such as binding of the substrate, may specifically require Na^+,

whereas both Na^+ and Li^+ may be capable of contributing to the electrochemical ion gradient. In those cases where Li^+ alone promotes uptake, the ion would be capable of increasing the affinity of the carrier for its substrate. Substrate binding may be inhibited by Li^+ in another uptake system.

It would appear that, among the alkalai metal ions, Li^+ and Na^+ may have similar effects on these marine bacteria because of the large size of their hydrated radii (MacLeod et al., 1978).

It should be noted that, in a sense, the effects of Li^+ can be regarded as experimental artifacts. The concentration of Li^+ in 35°/oo seawater is only 26 uM (Brewer, 1975), therefore this ion would not be expected to have a significant influence on the physiology of marine bacteria in their natural environment.

Determinations of the intracellular concentrations of the substrates transported by cells of A. haloplanktis and V. fischeri showed that, at high concentrations of Na^+, the metabolites were generally accumulated better than at suboptimal concentrations of Na^+. The concentration gradients also increased with time. The ability of a cell to concentrate a solute against its gradient is taken as evidence for uptake by an energy-requiring active transport process.

Previous studies on transport in A. haloplanktis have demonstrated the ability of cells of this organism to accumulate substrates against

their concentration gradients. Transported AIB and D-fucose were extracted from cells and chromatographically recovered by Drapeau et al. (1966). Wong et al. (1969) estimated that 50 uM AIB in the medium was concentrated by cells to an intracellular concentration of 600 mM. After L-alanine was transported by vesicles, 98% was recovered in its original form, whereas only 27% was recovered from cells (Sprott and MacLeod, 1974). It has also been reported that whole cells of A. haloplanktis accumulated an intracellular concentration of 15 mM L-leucine in three minutes from the 5 uM concentration provided in the medium (Pearce et al., 1977). This agrees very favorably with the results in Figure 23 that show an intracellular L-leucine concentration of 13 mM at three minutes.

Methods similar to those used in this study have been employed by other researchers in their studies on Na^+-dependent transport. As is the case here, it has been found that a large proportion of the metabolizable substrates taken up by cells or vesicles of other organisms can be recovered in their original form, thus demonstrating active transport. Stevenson (1966) reisolated 80% of transported glutamate, representing a gradient of 50/1, from cells of Halobacterium salinarium. Cells of B. licheniformis were found to contain 90% of the glutamate, 58% of the alanine and 52% of the aspartate that had been accumulated. Between 82 and 85% of the same substrates were reisolated from vesicles (MacLeod et al., 1973). After ten minutes of uptake, 50% of transported glutamate was recovered from cells of E. coli (Frank and Hopkins, 1969), and most of the proline taken up in a Na^+-dependent manner by vesicles of M. phlei was recovered (Hirata et al., 1974). MacDonald et al. (1977a) concluded that most, if not all, amino acids were accumulated in an

unaltered form by vesicles of H. halobium. Over half of the P_i taken up by cells of P. stutzeri in fifteen minutes was found not to have been incorporated (Kodama and Taniguchi, 1977). Studies on P_i transport in E. coli (Rosenberg et al., 1977) and Synechococcus (Grillo and Gibson, 1979) showed that 63 to 72%, and 93% respectively, of the P_i was recoverable in the first one to two minutes of uptake.

Some of the substrates taken up by cells of A. haloplanktis are quickly metabolized in various ways. D-galactose is rapidly incorporated into lipopolysaccharide (Bilous and MacLeod, unpublished data) and succinate is oxidized by the cells (Khanna, 1980). The radioactivity in the succinate and D-galactose pools represents only two to three percent of that taken into the cells, however these quantities of unaltered substrates still represent outwardly-directed concentration gradients.

Na^+ is required for metabolites to cross the cytoplasmic membrane and be concentrated within the cell. As previously discussed, the exact nature of the association of Na^+ with transport is not elucidated in these experiments. There is much evidence, however, that Na^+ promotes transport by virtue of its effect(s) on the cytoplasmic membrane, rather than by stimulating other metabolic processes. In cells of A. haloplanktis, the Na^+ requirement for transport exceeds that for respiration, as demonstrated here and elsewhere (Khanna, 1980). Na^+ is actively pumped out of the cytoplasm by the Na^+-H^+ antiporter (Sprott et al., 1975; Niven and MacLeod, 1978), and would therefore not normally be found in the cell at concentrations as high as those required for maximum rates of transport. Na^+ is required for the oxidation of succinate by whole cells of A. halo-

planktis. Since toluene-treated cells and cell-free extracts did not need the ion for similar activity, the requirement by intact cells most likely represents a requirement for transport (Khanna, 1980). The oxidation of a variety of substrates by cell-free extracts of A. haloplanktis was in fact inhibited by those concentrations of Na^+ required for maximum rates of oxidation by whole cells, thus providing evidence for an effect of Na^+ on membrane transport (MacLeod et al., 1958; MacLeod and Hori, 1960). Membrane vesicles of A. haloplanktis, devoid of cytoplasmic metabolic activity and endogenous energy reserves, needed both Na^+ and an exogenous electron donor to drive the uptake of L-alanine (Sprott and MacLeod, 1974). Thus, while the energy for active transport was derived from the activity of the electron transport chain, Na^+ was needed to complete the coupling. This illustrates clearly that Na^+ must affect transport primarily because of its association with the cytoplasmic membrane.

The authors of other studies have interpreted their results as indicating that Na^+ acts on the cell membrane. As was the case with A. haloplanktis, the transport of amino acids by vesicles of an alkalophilic Bacillus was dependent upon Na^+ and the respiratory activity generated by electron donors. The Na^+ requirement for transport exceeded that for respiration (Kitada and Horikoshi, 1980b). Although cells of P. stutzeri respired in the absence of Na^+, the ion was required for the uptake of P_i and glutamate (Kodama and Taniguchi, 1976; 1977). It was proposed that the Na^+ requirement for growth of this organism reflected the critical role of Na^+ in coupling respiration to energy-requiring membrane-linked processes. The active transport of substrates driven by a transmembrane electro-chemical Na^+ gradient has been demonstrated in a variety of

bacteria, including A. haloplanktis (Niven and MacLeod, 1980). The ability of Na^+ to promote the accumulation of substrates against their gradients, as observed in this survey, is best explained by the Na^+ gradient mechanism of transport. In contrast, it is interesting that the PTS-mediated uptake of glucose does not require Na^+. This form of transport is not dependent upon an energized membrane, as the energy is supplied by phosphoenolpyrurate.

It is evident that there are no unique qualities which absolutely define marine bacteria as being "marine" other than the fact that they are found in the sea, and have the ability to grow and survive in that environment (MacLeod, 1965). These marine organisms are taxonomically distinct species, which apparently share the common feature of requiring Na^+ for growth (Baumann, 1979). Baumann has suggested that there is an ecological separation of gram-negative marine and terrestrial bacteria resulting from specific adaptations to their respective habitats. Organisms usually adopt a strategy which allows them to exploit a specific environmental niche. Perhaps the Na^+ requirement for growth in some way facilitates the survival and competitive ability of marine bacteria in the sea. As was discussed in the Literature Review, Na^+ in the sea represents a large energy reservoir that a cell can maintain without the problem of internal alkalinization (Skulachev, 1978). The ability to store energy in the form of a Na^+ gradient may be advantageous under certain conditions in a nutrient-poor environment such as the sea.

In A. haloplanktis, the transport of all nutrients so far examined is dependent upon Na^+. This requirement appears primarily to reflect the association of Na^+ with the energy state of the cytoplasmic membrane. The pumping of Na^+ out of the cell via the antiporter is dependent upon the expenditure of energy in the form of a pmf generated by respiration (Niven and MacLeod, 1978). Respiration itself is influenced by Na^+, and it has been postulated that the Na^+-stimulated internal NADH dehydrogenase of this organism may be involved in the regulation of respiratory activity which ensures that Na^+ is kept out of the cell (Khanna, 1980).

The quesion arises as to how representative A. haloplanktis is of marine bacteria in general. Unfortunately there have been few studies on the physiology of Na^+-dependence in other marine prokaryotes. A survey of the effects of ions on the maintenance of the structural integrity of the cell envelope of a variety of gram-negative marine bacteria showed that there was a wide spectrum of responses among the organisms to salts. Furthermore, there was no clear cut distinction between marine and terrestrial bacteria in this regard (Laddaga and MacLeod, submitted for publication). Considering the diverse spectrum of organisms comprising the marine bacteria, it should not be expected that A. haloplanktis, or any other organism, could serve as a universal model. V. fischeri, as seen in this study, and a psychrophilic marine Vibrio (Hayasaka and Morita, 1979) have some ability to take up substrates in the absence of Na^+. There is clearly a need for more detailed studies on the effects of Na^+ on membrane-related energy functions in these bacteria.

There is a potentially significant difference between A. haloplanktis and V. fischeri which may account for the differences between these two organisms: the former is a strict aerobe, whereas the latter is a facultative anaerobe. If the ability to generate a Na^+ gradient is dependent upon Na^+-activated respiration, how would Na^+ be used by V. fischeri under anaerobic conditions? Even though cells of this organism could presumably generate a pmf by hydrolysing ATP, the ability to maintain a substantial Na^+ gradient might be limited as compared to that under aerobic conditions. If this is true, the transport systems in V. fischeri may be sensitive to much smaller Na^+ gradients than those of A. haloplanktis. Under the conditions used in this investigation, the levels of contaminating Na^+ may have been sufficient to permit some transport to occur. Alternatively, there may be separate Na^+-dependent and Na^+-independent uptake systems. If the Na^+ requirement for growth reflects a need for Na^+ for respiration, perhaps a facultative organism would not require Na^+ at all under anaerobic conditions. Baumann (1979) has screened the Na^+ requirements of his isolates only under aerobic conditions.

In conclusion, the marine bacterium A. haloplanktis needed Na^+ for the active transport of a variety of metabolizable substrates. The Na^+ requirement for transport exceeded that needed for maximum respiration, and would therefore appear to be a major factor accounting for the dependency of this organism on Na^+ for growth. The situation is less clear in V. fischeri, where substrates were taken up in the absence of Na^+,

although the ion was required for optimal rates of transport. Na^+ did not affect the transport of D-glucose by cells of this organism, which may indicate that this substrate is taken up by a PTS system. That the transport systems of the facultative anaerobe V. fischeri differ somewhat from those of A. haloplanktis in their sensitivities to Na^+, may reflect a requirement in the former organism for transport systems capable of functioning under anaerobic conditions.

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ADDENDUM

Since the ninhydrin reaction results in the removal of carboxyl groups (as CO_2) from amino acids, some ^{14}C label will be lost when extracted amino acids are reisolated by TLC and detected with ninhydrin. An experiment was conducted to estimate the reduction in radioactivity attributable to this procedure.

Aliquots of ^{14}C-amino acids were treated in the following ways: a) 5 ul was dispensed directly into a scintillation vial (control), b) 5 ul was spotted on a TLC plate and then scraped into a vial, c) 5 ul was spotted on a TLC plate, sprayed with ninhydrin and then scraped into a vial after development of the colour, d) 5 ul was spotted, the plate was developed with the appropriate solvent systems, sprayed with ninhydrin, and the detected spot was scraped into a scintillation vial. Scintillation cocktail was added to the vials and the radioactivity was measured.

The radioactive counts obtained after treatments b, c and d were compared to those obtained from the controls (a). The counts obtained under condition b averaged 90 percent of the control counts, and those of conditions c and d averaged 80 percent of the control counts. Thus, of the radioactivity spotted on a TLC plate, about 10 percent is not recovered when the spot is scraped into a vial, and a further 10 percent is lost via the ninhydrin reaction.