NA^+-DEPENDENT ACTIVATION OF RESPIRATION AND MEMBRANE TRANSPORT IN A MARINE BACTERIUM

by

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SHORT TITLE

NA^+ REQUIREMENT OF A MARINE BACTERIUM

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ABSTRACT

M.Sc. GITA KHANNA Microbiology

NA^+-DEPENDENT ACTIVATION OF RESPIRATION AND MEMBRANE TRANSPORT IN A MARINE BACTERIUM

The quantitative Na^+ requirement for maximum respiratory activity was examined and compared with that for the maximum rate of transport in the marine organism, Alteromonas haloplanktis. Na^+-dependent activation of respiration was examined using several exogenously-added oxidizable substrates. NADH was actively oxidized by intact cells but Na^+ had no stimulatory effect. However, oxidation of NADH or ethanol by toluene-treated cells required 100 mM or 50 mM Na^+ respectively for maximum activity. Succinate and membrane-bound ascorbate/TMPD oxidases showed no specific requirement for Na^+, whereas soluble ascorbate/TMPD oxidase required 5 mM NaCl for maximum activity. Ethanol oxidation by intact cells was stimulated by 2 mM NaCl. Maximum rates of α-aminoisobutyric acid (AIB) transport were shown to require 250 mM Na^+. Since, with ethanol as an energy source, only 2 mM Na^+ is required by whole cells for maximum rate of respiration and 250 mM Na^+ is needed for maximum rate of AIB transport into the cells, there is clearly a Na^+ requirement for transport of AIB which is separate from the Na^+ requirement for respiration.

RESUME

M.Sc. GITA KHANNA Microbiologie

DEPENDANCE DE L'ION Na^+ POUR L'ACTIVATION DE LA RESPIRATION ET DU TRANSPORT CHEZ UNE BACTERIE MARINE

Les besoins en ion Na^+ pour une activité respiratoire maximale ont été quantifiés et comparés avec ceux requis pour un taux maximal de transport chez l'organisme marin, Alteromonas haloplanktis. On a examiné l'effet stimulateur de l'ion Na^+ sur l'activité respiratoire des cellules entières au moyen de plusieurs substrats oxydables exogènes. Le NADH était activement oxydé par les cellules entières sans aucun effet stimulateur de l'ion Na^+. Cependant, l'oxydation des substrats NADH et éthanol par des cellules traitées au toluène exigeait 100 mM et 50 mM respectivement pour atteindre une activité maximale. Les oxydases liées au succinate et à l'ascorbate/TMPD de la membrane n'ont pas montré de dépendance spécifique pour le Na^+ alors que l'oxydase soluble de l'ascorbate/TMPD exigeait 5 mM NaCl pour atteindre une activité maximale. L'oxydation de l'éthanol par les cellules entières était stimulée par 2 mM NaCl. Le taux maximal de transport pour l'acide α-amino-isobutyrique (AIB) exigeait la présence de 250 mM Na^+. Comme l'activité respiratoire des cellules entières avec l'éthanol requiert 2 mM Na^+ et que le transport actif de l'AIB requiert 250 mM Na^+, on peut donc déduire que l'exigence en Na^+ pour le transport de l'AIB est bien différente et séparée de l'exigence en Na^+ pour la respiration.

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GLOSSARY

AIB	:	α-aminoisobutyric acid
CoQ	:	coenzyme Q
NADH	:	reduced nicotinamide adenine dinucleotide
TMPD	:	N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride
mM Na^+	:	mg ions/l Na^+

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INTRODUCTION

The feature which distinguishes gram negative marine bacteria most clearly from their terrestrial counterparts is the strict dependence of the marine species upon the presence of Na^+ in the environment for optimum growth and metabolism (MacLeod, 1965). Drapeau and MacLeod (1966) demonstrated that the Na^+ requirement for the growth of a marine bacterium, Alteromonas haloplanktis, reflects a requirement for Na^+ to transport metabolites into the cells. In addition, Na^+ was also found to have a function in preventing the loss of intracellular solutes from the cells (Wong et al., 1969). Thus, it is apparent that marine bacteria require Na^+ for the maintenance of cell integrity and for active transport functions in the cell membrane. Recent studies by Unemoto et al. (1977) demonstrated that Na^+ was required for maximum activity of the membrane-bound NADH oxidase of the marine organism Vibrio alginolyticus; similar results were obtained with the moderate halophile Vibrio costicola. As the NADH oxidase from non-halophilic Escherichia coli showed no specific requirement for Na^+ (Unemoto et al., 1977), the Na^+-dependent activation of NADH oxidase was considered to be a characteristic feature of halophilic bacteria.

Since Na^+ has been shown to have a function in the transport of metabolites (Drapeau and MacLeod, 1963, 1966), as well as a specific involvement in the respiration of marine organisms (Unemoto et al., 1977), it seems necessary to differentiate the quantitative Na^+ requirement for the maximum activity of each of these processes. The objective of the present research was to determine the quantitative Na^+ requirement for transport and respiration in the marine bacterium, Alteromonas haloplanktis. The Na^+ requirement for the maximum oxidation of several exogenous substrates was examined and compared quantitatively with the concentration of Na^+ needed for maximum uptake of the non-metabolizable analogue, α-aminoisobutyric acid, into the cell. Based on the quantitative Na^+ requirement for the maximum respiration of different oxidizable substrates, an additional purpose of this investigation was to identify the possible site of Na^+-dependent activation in the electron transfer chain of A. haloplanktis.

LITERATURE REVIEW

1. Role of Na^+ in marine bacteria

A large proportion of the bacterial flora of the oceans consists of gram negative organisms which have a specific requirement for sodium ion (MacLeod, 1965, 1968; Baumann and Baumann, 1977). Richter (1928) was the first to establish that a marine luminous bacterium had a specific requirement for Na^+. Later, Mudrak (1933) confirmed and extended Richter's findings by demonstrating that ten additional strains of marine luminous bacteria also required Na^+ for growth. Dianova and Voroshilova (1935) subsequently found that Na^+ salts were required for the growth of a number of marine isolates and could not be replaced by equimolar concentrations of K^+ salts. Using a chemically defined medium, MacLeod *et al.* (1954) investigated the requirement for inorganic ions for the growth of six marine bacteria and found that all of them had a requirement for Na^+, K^+, Mg^{+2}, PO_4^{-2} and SO_4^{-2} for growth. Several of the organisms also required Ca^{+2} and Cl^{-1}. When the quantitative requirements of three marine bacteria for Na^+ and K^+ were further investigated, MacLeod and Onofrey (1957) found that the maximum rate and extent of growth was achieved with 0.2 to 0.3 M Na^+. Li^+, Rb^+ and Cs^+ had no capacity to replace Na^+, although K^+ exhibited some sparing action at sub-optimal concentrations of Na^+. The specificity of the Na^+ requirement

for marine bacteria was also shown by Payne (1960) in his study of the growth and metabolism of a marine pseudomonad. Recently, some 700 strains and species of marine bacteria were examined and shown to grow only if Na^+ was present in the medium (Reichelt and Baumann, 1974).

A Na^+ requirement for growth was initially believed to be a characteristic unique to marine bacteria; however, studies conducted on some terrestrial bacteria have revealed that several species of the latter also require Na^+ for growth. Halophilic bacteria have been isolated from several salty non-marine environments. As described in numerous reviews (Larsen, 1962, 1967; Dundas, 1977), these organisms collectively labelled as "extreme halophiles" are unique in that they not only tolerate but require NaCl concentrations above 10-15% for survival, and often need 20% NaCl for optimal growth. Among the non-halophiles, however, Escherichia coli was found to require 3 mM sodium for an optimal growth rate on glutamate (Frank and Hopkins, 1969). Salmonella typhimurium requires about 8 mM Na^+ for an optimal yield in the fermentation of citrate (O'Brien and Stern, 1969). The rumen inhabitant Bacteriodes amylophilus has also been shown to require 60 mM sodium for an optimal growth rate and yield (Caldwell et al., 1973). With the exception of some ecologically specialized organisms that inhabit environments with relatively constant Na^+ concentrations, most terrestrial organisms do not appear to have a specific requirement for sodium. Where a requirement has been demonstrated, it has usually been found to be considerably

lower than observed in marine bacteria and is often dependent on the conditions of cultivation.

Studies on Na^+-dependent growth and the physiological basis for this feature had been confined to two marine species, Alteromonas haloplanktis and Photobacterium fischeri. MacLeod and his co-workers have shown that Na^+ serves two functions in these organisms. One is a specific involvement of Na^+ in metabolite transport (Drapeau and MacLeod, 1963, 1966; Hassan and MacLeod, 1975) and the other, a less specific effect in that Na^+ prevents the loss of intracellular solutes from the cells (Drapeau and MacLeod, 1965; Wong et al., 1969; MacLeod et al., 1978). Recent studies on the marine bacteria Photobacterium phosphoreum and Vibrio alginolyticus have revealed that Na^+ has also a function in the respiratory activities of these organisms (Watanabe et al., 1977; Unemoto et al., 1977).

a. Na^+-dependent transport in marine bacteria

Earlier studies by MacLeod et al. (1958) revealed that Na^+ is required for the oxidation of exogenous substrates by cell suspensions of A. haloplanktis. For the oxidation of monobasic acids and hexose sugars, 0.05 M Na^+ was required; for di- and tri-basic acids, however, two to three times this level of Na^+ were needed. When cell-free extracts of the organism were examined, none of the enzymes of the tricarboxylic acid cycle could be shown to require Na^+ specifically for activity (MacLeod and Hori, 1960). That the requirement for Na^+

by intact cells of A. haloplanktis might represent a Na^+ requirement for the transport of metabolites into the cells was first indicated by Drapeau and MacLeod (1963). They showed that the active transport of the non-metabolizable amino acid analogue, α-aminoisobutyric acid (AIB) into cells of A. haloplanktis and P. fischeri is Na^+-dependent. More detailed studies with the marine bacteria (Drapeau and MacLeod, 1966) revealed that the amount of Na^+ required for transport varied with the compound transported and corresponded to the Na^+ requirement for the oxidation by whole cells of the corresponding metabolizable substrate. This led to the conclusion that the Na^+ requirement for oxidation represents a Na^+ requirement for transport. This conclusion was substantiated by the evidence that the quantitative Na^+ requirement for the uptake of D-fucose, a non-metabolizable analogue of D-galactose, was similar to the amount of Na^+ needed for maximum rate of oxidation of D-galactose (Drapeau and MacLeod, 1966).

Recent studies by Wong et al. (1969) have shown that the uptake of inorganic orthophosphate by the marine bacterium A. haloplanktis is also a Na^+-dependent process. Subsequent studies conducted on K^+-depleted cells of the marine bacterium (Hassan and MacLeod, 1975; Thompson et al., 1970) revealed that K^+ transport is a saturable process and requires Na^+.

Hence, the results obtained from transport studies have led to the conclusion that the primary function of Na^+ in A. haloplanktis is to permit the transport of essential metabolites into the cell. Since other alkali metal ions could not satisfy the Na^+ requirement

for transport, it was concluded that the role of Na^+ in transport was a specific one.

b. Na^+ requirement for the retention of intracellular solutes

In the course of studies on AIB uptake in A. haloplanktis, Drapeau and MacLeod (1965) found that the retention of this non-metabolizable compound by the cells was also markedly affected by the ion composition and concentration of the suspending medium. Cells were allowed to accumulate ^{14}C-labelled AIB, separated from the medium and then resuspended in different salt solutions. When the preloaded cells were resuspended in a solution of NaCl, KCl and $MgSO_4$, the labelled compound was retained by the cells even after one hour of incubation. However, when the suspending medium contained only 0.05 M $MgSO_4$, the label in the cells was rapidly lost. It was also observed that in the absence of a sufficient concentration of cations, the cells lost their viability (Wong et al., 1969). Since the release of intracellular AIB occurred prior to the loss of viability, loss of viability is probably a long-term consequence of changes associated with the loss of intracellular solutes (Wong et al., 1969). In these experiments, sufficient Mg^{+2} was present to prevent lysis of the cells. LiCl and, to a lesser extent, KCl were both partially effective in preventing the loss of ^{14}C-AIB from the cells. Since prevention of release of AIB from the cells could be effected, at least partially, by other ions, the role of Na^+ in this function is

a less specific one. Somewhat similar observations were made by Srivastava (1965) with the marine bacterium Photobacterium fischeri.

In considering the mechanism by which salts could prevent the release of intracellular solutes from the cells, it was hypothesized that Na^+ was required to maintain cytoplasmic membrane proteins in the proper configuration to permit tight packing of membrane sub-units (Buckmire and MacLeod, 1965). The possible osmotic effect of Na^+ in preventing loss of intracellular solutes was not considered since individual salts were shown to differ markedly in their ability to prevent loss of solutes from the cells. Lower concentrations of NaCl and LiCl than KCl or NH_4Cl were effective in this function (Drapeau and MacLeod, 1965). Furthermore, it was observed that the concentration of NaCl in the cells was the same as that prevailing in the medium (Takacs et al., 1964). In view of the recent findings in A. haloplanktis that internal Na^+ concentration is lower than the external concentration (Thompson and MacLeod, 1973), the role of Na^+ in preventing loss of intracellular solutes from A. haloplanktis cells as a result of an osmotic effect was re-examined. Recent studies by MacLeod et al. (1978) indicated that the porosity of the cytoplasmic membrane in this marine bacterium was determined by the difference between the osmotic pressure of the cytoplasm and the suspending medium. The lesser effectiveness of K^+, Rb^+ and Cs^+ than Na^+, Li^+ or Mg^{+2} in permitting the retention of solutes by the cell was attributed to the greater penetrability of the hydrated ions of the former group through the dilated pores of a stretched cytoplasmic membrane.

2. Role of Na^+ in respiration

It is now firmly established that all aerobic bacteria possess membrane-bound electron transport chains that result in net proton translocation across a membrane of limited ion permeability during oxidation-reduction reactions. The diversity of the individual membrane-bound redox components found in bacteria, as well as the great variations in the physiological reductants and oxidants utilized by bacteria, has been well documented in many reviews (Haddock and Jones, 1977; Jones, 1978; Jones et al., 1975; Bartsch, 1968; Harold, 1972). However, comparatively little attention has been directed towards the composition and properties of the respiratory chain of bacteria known to require Na^+ for growth.

Recent studies on the electron transport systems of several halophiles belonging to the Halobacterium group which grow best in a medium containing 25-30% NaCl, suggest that these bacteria have more than one oxidase during their logarithmic phase of growth (Larsen, 1967). The halobacteria examined were shown to contain cytochromes of the b- and c-types as well as an o-type cytochrome oxidase (Cheah, 1969, 1970a; Lieberman and Lanyi, 1971). In addition, these bacteria were shown to have respiratory-linked NADH dehydrogenase activity (Hochstein, 1975; Hochstein and Dalton, 1973; Lanyi, 1969a). Enzymes of extremely halophilic bacteria have been found to require high concentrations of salts for maximum activity and stability. Extensive reviews on the studies of these salt effects on halophilic enzymes have been written (Larsen, 1962, 1967; Brown, 1964; Dundas, 1977;

Lanyi, 1974). The NADH oxidases of H. cutirubrum and halophile AR-1 have been shown to be inactive without added salt and to require 2-3 M NaCl for full activity (Lanyi, 1969a, Hochstein and Dalton, 1968a). At low salt concentrations the enzymes were found to be irreversibly inactivated (Lanyi, 1968; Hochstein and Dalton, 1968b). The salt requirement of NADH oxidase was found to be non-specific since large numbers of salts such as KCl, RbCl and CsCl could replace NaCl with little or no loss of activity (Lanyi, 1969a). In addition to salts of monovalent cations, other substances such as $MgCl_2$, $CaCl_2$, spermine and polyamine have been shown to activate halophilic respiratory enzymes (Lanyi, 1969b). Hence, on the basis of these studies, and other similar observations, it has been suggested that high concentrations of salts are essential for the maintenance of the respiratory systems of halophiles (Lanyi, 1974).

Some general features and properties of marine respiratory systems have also been recently documented. Studies conducted on the marine bacterium Beneckea natriegens have shown that this organism contains a complex respiratory system with four or possibly five CO-binding cytochromes, including an unusual high potential soluble c-type cytochrome, each of which could be acting as a terminal oxidase (Weston et al., 1973, 1974). The presence of a periplasmic soluble CO-binding c-type cytochrome has also been shown in Alteromonas haloplanktis (Knowles et al., 1974). More recent studies on the extracts of B. natriegens showed that this organism also contains NADH, succinate and ascorbate/TMPD oxidase activities (Weston et al., 1974).

The effect of Na^+ on the respiratory chains of marine bacteria was first documented by Unemoto et al. (1977). Using cell-free extracts, they showed that the membrane-bound NADH oxidase activity of slightly halophilic marine Vibrio alginolyticus required 0.3 M Na^+ for maximum activity. Other cations such as Li^+, K^+, Rb^+, Cs^+, Mg^{+2} and Ca^{+2} were relatively ineffective as replacements for Na^+; however, K^+ and Mg^{+2} cooperated with Na^+ for activation. In a subsequent study, the respiratory chain of V. alginolyticus was examined to identify the components conferring the Na^+ activation (Unemoto and Hayashi, 1979). The respiratory chain in this marine organism was found to contain ubiquinone (Q), menaquinone (MK), cytochromes of the b-, c-, d- and o-type. The membrane-bound and partially purified NADH dehydrogenase was stimulated two- to three-fold by the addition of 0.2 M Na^+ or K^+. No specific requirement for Na^+ was observed in this reaction step. The cytochrome oxidase showed no requirement for monovalent cations. The respiratory activity of the membrane was lost on removal of the quinones and the reincorporation of authentic Q-10 and MK-4 restored the activity. The rate of MK-4 reduction by NADH was activated by Na^+, but only slightly by K^+. Likewise, the ubiquinone reductase was more effectively activated by Na^+ than by K^+. These results strongly suggested that the site of Na^+-dependent activation in the respiratory chain of marine V. alginolyticus was at the step of NADH-quinone oxidoreductase.

Na^+-dependent activation of NADH oxidase has also been observed in another marine bacterium, Photobacterium phosphoreum. Based on the results obtained, it was suggested that Na^+ activated the cytochrome electron transfer system in this organism at a point between NADH and cytochrome b (Watanabe et al., 1977). Recent studies on the membrane bound NADH dehydrogenase of P. phosphoreum have shown that this enzyme, which contains FAD as a prosthetic group, is activated by monovalent cations. The cation-dependence of NADH dehydrogenase activity, however, was not specific since various monovalent cations produced almost the same degree of activation as Na^+ (Imagawa and Nakamura, 1978).

The effect of Na^+ on respiration has also been studied in the non-halophilic bacterium, Pseudomonas stutzeri. Respiration in this organism proceeded at an appreciable rate in the absence of K^+ and Na^+. However, the oxygen uptake rate was enhanced about 1.5 times by K^+ and doubled when Na^+ was added subsequently. No enhancement was observed when Na^+ was added without simultaneous addition of K^+. Hence, K^+ is necessary for the enhancing function of Na^+. Other alkali metals, added as chlorides in place of NaCl, did not stimulate respiration (Kodama and Taniguchi, 1976).

3. Na^+-dependent transport

Sodium-dependent transport in bacteria has been recognized for many years. Drapeau and MacLeod (1963) and Drapeau et al. (1966) presented the first definitive experiments showing Na^+ and K^+ to be

required for the transport of aminoisobutyric acid and fucose into the marine bacterium Alteromonas haloplanktis. Recent studies have shown that all amino acid transport systems in Halobacterium halobium membrane vesicles were Na^+-dependent. Glutamate uptake into the cells and membrane vesicles of Escherichia coli was shown to be stimulated by Na^+ (Frank and Hopkins, 1969; MacDonald et al., 1977b; Hasan et al., 1977). In a study of the melibiose permease system of Salmonella typhimurium, Stock and Roseman (1971) showed that Na^+ was required for the uptake of thiomethylgalactoside. Other examples of Na^+-dependent transport in terrestrial procaryotes include succinate transport in Micrococcus lysodeikticus (Ariel and Grossowicz, 1974) and the transport of proline into Mycobacterium phlei (Hirata et al., 1974).

Other cations were found to have less specific or more elusive effects. K^+ was found to be required for the accumulation of aminoisobutyric acid in a marine pseudomonad (Drapeau and MacLeod, 1966) and for the maximum accumulation of glutamate in E. coli (Halpern et al., 1973). Li^+ in some systems exhibited effects similar to those of Na^+, as in thiomethyl galactoside uptake in S. typhimurium (Stock and Roseman, 1971), but appeared ineffective in other systems, as in aminoisobutyric acid transport in A. haloplanktis (Drapeau et al., 1963) and glutamate transport in E. coli (Kahane et al., 1975; Hasan et al., 1977).

That sodium is required for transport suggests that Na^+ acts as a cofactor influencing the binding of the substrate to the

transport carrier and/or the conformation of that carrier. Na^+ need not necessarily be bound to the carrier protein but if it is and the Na^+ is translocated at the same time as the substrate, then the electrochemical Na^+ gradient could serve as a driving force for active transport of the substrate in question.

Early studies by Thompson and MacLeod (1973) appeared to rule out the involvement of a Na^+ gradient in the accumulation of amino-isobutyric acid by A. haloplanktis. When Na^+ and K^+ gradients were seemingly abolished (extra-cellular ion concentrations were adjusted to that estimated to be present intracellularly), there was no effect on the uptake of the substrate. This conclusion was substantiated by studies conducted by Kahane et al. (1975) in E. coli, where it was not possible to demonstrate substrate-dependent Na^+ influx during glutamate transport. The importance of a concentration gradient of Na^+ in the Na^+-stimulated transport systems had been rejected on the basis of other experimental results as well. Results obtained on the effect of Na^+ on the kinetic parameters, Km and Vmax, revealed that Na^+ acts by either decreasing the Km value or by increasing Vmax. Examples of bacterial systems where the Km for the transported metabolite was decreased (i.e., the affinity for the transport substrate was raised) in the presence of Na^+ and the Vmax was unaffected include glutamate transport in E. coli (Halpern et al., 1973), succinate uptake in M. lysodeikticus (Ariel and Grossowicz, 1974) and AIB transport in A. haloplanktis (Wong et al., 1969). The amino acid transport systems in H. halobium (MacDonald et al., 1977)

exemplified a case where the Vmax for transport was increased in the presence of Na^+, whereas the Km remained unaffected. This suggested that the carrier traversed the membrane more rapidly in the presence of both substrates. The observation that both the parameters, Km and Vmax, did not change with Na^+ concentration has been used to argue against cotranslocation of the cation with the substrate and in favor of a cofactor effect.

Recent evidence for a H^+/Na^+ antiporter in E. coli (West and Mitchell, 1974) cells has stimulated a large number of new studies and the re-evaluation of a number of the earlier ones on the role of Na^+ gradient in transport in accordance with Mitchell's chemiosmotic hypothesis.

According to the chemiosmotic hypothesis, the transmembrane proton gradient effected by respiration, ATPase or bacteriorhodopsin activity is used to extrude Na^+ from the bacterial cells by a Na^+-H^+ antiporter resulting in the creation of an inwardly directed transmembrane Na^+ gradient. The chemical and electrical components of this gradient, either separately or in combination can be used to drive the intracellular accumulation of nutrients (Mitchell, 1966, 1973; Harold, 1972).

Evidence for the existence of a H^+/Na^+ antiporter in E. coli was provided by studies conducted by West and Mitchell (1974). Anaerobic cells were incubated in the presence of K^+ and the permeant anion SCN^-, which compensated for any charge imbalance created by

ionic movement across the membrane. Addition of Na^+ caused acidification of the cell suspension which was reversed in the presence of the uncoupler FCCP. The authors concluded that the Na^+ influx caused coupled H^+ efflux from the cells. When a pulse of oxygen was introduced, the H^+ was expelled and returned slowly into the cell in the absence of Na^+; however, in the presence of Na^+, the H^+ returned rapidly.

The presence of H^+/Na^+ antiporters has since been shown in a number of microbial systems. Lanyi and MacDonald (1976) provided evidence for a H^+/Na^+ antiporter in H. halobium cell envelope vesicles in which the source of the proton gradient was the light-induced extrusion of protons by bacteriorhodopsin. Evidence for H^+/Na^+ antiporters has also been shown in S. typhimurium (Tokuda and Kaback, 1977) and marine bacterium A. haloplanktis (Niven and MacLeod, 1978).

The observation that Na^+ is required for the transport of a number of substrates suggests that these substrates may be symported with Na^+, that the electrochemical potential inherent in the Na^+ gradient provides the energy required for uphill transport of metabolites. Cell membrane vesicles of H. halobium, by virtue of their light-dependent proton pump, bacteriorhodopsin, have provided a valuable tool for the study of the energetics of Na^+-dependent transport.

A detailed study by MacDonald and Lanyi (1975) on the uptake of leucine in H. halobium vesicles suggested that the leucine transport

system is not coupled to ATP hydrolysis and responds to membrane potential rather than a pH gradient. The Na^+-dependence of the transport and the observation that a NaCl pulse caused transient leucine influx in the dark in KCl-loaded vesicles, even in the presence of FCCP, suggested that the uphill transport of leucine could be effected by symport with sodium which moves down a chemical or electrical gradient. The idea that Na^+ gradients drive amino acid transport in H. halobium was reinforced by results obtained with glutamate (Lanyi et al., 1976a; Lanyi et al., 1976b). The amino acid was rapidly taken up into vesicles, but only when the external sodium concentration was appreciably higher than the internal concentration. When NaCl was included in the vesicles in increasing amounts, light induced accumulation of glutamate occurred only after lags of increasing lengths. Moreover, if vesicles were illuminated for varying periods of time, and then the light turned off, the ability to accumulate glutamate remained, but slowly decayed with time. This Na^+ gradient was generated by the light-induced proton motive force through the H^+/Na^+ antiport.

Subsequent studies by MacDonald et al. (1977a) with H. halobium showed that, with the exception of cysteine, all amino acids are transported in a Na^+-gradient dependent manner.

The role of Na^+ in several other bacterial transport systems has been clarified recently. MacDonald et al. (1977b) demonstrated the presence of a Na^+/glutamate symporter in E. coli membrane vesicles.

The results showed that the vesicles could accumulate glutamate in the presence of an uncoupler when a concentration gradient of Na^+ was provided. Transport under these conditions was sensitive to monensin, a sodium ionophore, but not to nigericin, a K^+ and H^+ ionophore. Further evidence for the electrochemical gradient of Na^+ as the source of energy for active transport of substrates has also been shown in S. typhimurium membranes for the transport of thiomethyl galactoside (Tokuda and Kaback, 1977). Recent studies in A. haloplanktis (Niven and MacLeod, 1979, unpublished data) have demonstrated that the uptake of aminoisobutyric acid is driven by the electrochemical gradient of Na^+. An earlier study by Thompson and MacLeod (1973) had demonstrated that the inwardly directed Na^+ gradient and an outwardly directed K^+ gradient were not required for the accumulation of AIB by A. haloplanktis. In these experiments, the extracellular Na^+ and K^+ concentrations were adjusted to those determined to be present intracellularly and this adjustment was found to have no effect on the rate of AIB transport. However, it is now known that A. haloplanktis exhibits respiration-driven proton translocation and possesses an extremely active Na^+/H^+ antiporter (Niven and MacLeod, 1978) and under optimal respiratory conditions, the intracellular Na^+ concentration is lower than originally estimated. Thus, under the conditions above, which were designed to abolish transmembrane Na^+ and K^+ gradients, the cells were still capable of re-establishing such ion gradients.

Hence, at present, the evidence is overwhelming that many of the Na^+-stimulated metabolite transport systems are driven by the Na^+ gradient through a symport mechanism.

MATERIALS AND METHODS

Organism

Alteromonas haloplanktis, strain 214, variant 3, previously known as a marine pseudomonad B-16 (ATCC 19855) was the organism used in this study. The culture was maintained by monthly transfer to slants containing 0.8% (w/v) nutrient broth (Difco), 0.5% yeast extract, 0.22 M NaCl, 0.026 M $MgSO_4$, 0.01 M KCl and 1.5% ~~nutrient~~ agar. (Difco)

Growth conditions

Cells were grown in a complex medium containing 0.8% nutrient broth (Difco), 0.5% yeast extract (Difco), 0.3 M NaCl, 0.026 M $MgSO_4$, 0.01 M KCl and KOH to pH 7.2. The inoculum was obtained by transferring a loopful of cells from an agar slant to a 50 ml Erlenmeyer flask containing 10 ml of complex medium. After 24 hours incubation at 25°C on a gyratory shaker (200 rpm), 0.3 ml (0.1% inoculum) of the starter culture was transferred to 300 ml of medium in a 2-liter flask. Incubation was for 16 hours at 25°C, 200 rpm to give cells at the stationary phase of growth (pH 7.6, E_{660}-6.5).

Preparation of bacterial suspension

Following 16 hours of growth, each 50 ml volume of culture used was harvested by centrifugation (40,000 x g, 10 min. 4°C), washed twice with 50 ml volumes and once with a 25 ml volume of complete salt solution (50 mM Tris (hydroxy methyl) aminomethane-HCl, 300 mM choline chloride, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4 - pH 7.2). The washed cells were then resuspended in approximately 4 ml of fresh buffer. This stock cell suspension (about 20 mg dry wt/ml) was stored on ice.

In experiments concerning the effect of K^+ on O_2 uptake, the cells were washed and resuspended in a complete salt solution without K^+ (50 mM Tris/HCl, 310 mM choline chloride, 50 mM $MgSO_4$, 0.006% (v/v) 85% concentrated phosphoric acid - pH 7.2).

Preparation of toluene-treated cells

Stationary phase cells were harvested, washed and resuspended as described previously. To the cell suspension toluene was added to a final concentration of 1% (v/v) and was subsequently vortexed for about 1 minute at room temperature. The cells were then incubated for 30 minutes on ice before use. These toluenized cells could be stored on ice for up to 8 hours with no detectable change in the activities being monitored.

Preparation of cell fractions

Washed cells were suspended in complete salt solution to a concentration of 20-25 mg dry weight/ml, and the cell suspension was then disrupted in a French Pressure Cell operated at 14000 lb per sq.in. Debris was removed by centrifugation at 27000 x g for 15 minutes at 4°C. The supernatant fluid was designated the cell-free extract (CFE). This extract was subsequently separated into particulate and soluble fractions by ultracentrifugation at 150,000 x g for 3 hours at 4°C (Beckman Model L5-65 Ultracentrifuge). The top transparent half of the resulting supernatant fraction was retained as the soluble fraction, the protein content of which was between 5 and 10 mg/ml. The portion which sedimented, the particulate fraction, was rinsed once with the complete salt solution and resuspended in the buffer to a protein concentration of 8-12 mg/ml. Both fractions were then kept on ice.

Cell dry weight and protein determination

The cell density of a given suspension was determined by turbidity. The cell sample was diluted 200 x with complete salt solution and the optical density read at 660 nm on a Gilford spectrophotometer model 300-N. The cell dry weight content was then determined using a previously calibrated curve relating turbidity (E_{660} nm) to dry weight of the cells. An E_{660} of 0.26 was equivalent to a cell density of 20 mg dry wt/ml in the original suspension.

Protein was determined on various cell fractions by the Biuret method (Gornall et al., 1949) using 0.5% (w/v) Lysozyme (Sigma) as standard. The cell fraction was appropriately diluted with complete salt solution. Some component of the salt solution reacted with the protein solution to form a precipitate. To clarify the mixture, the sample was centrifuged on a clinical centrifuge (International Equipment Co. Model CL) for 5 minutes at reading 7. The clear supernatant was then read at 540 nm. Appropriate blanks were treated under the same conditions.

Preparation of the oxidative substrates

The substrates used in this study, as electron donors, were NADH, ethanol, succinate and Ascorbate/TMPD; NAD^+ was used as an oxidized substrate. All the substrates were made up in the appropriate salt solution; where the substrate rendered the solution acidic, small aliquots of 2 M Tris were added until the pH of the solution returned to 7.2. β-NADH (Sigma) and β-NAD^+ (Sigma) were made up to a concentration of 150 mM just prior to addition to the reaction mixture. Ascorbate (Fisher Scientific) was made up to a concentration of 600 mM and TMPD (Eastman) was made up to the concentration of 20 mM. Some difficulty was encountered in preparing a more concentrated solution of TMPD since at a concentration greater than 20 mM, raising the pH to 7.2 would cause the TMPD to come out of solution. Ascorbate and TMPD were each prepared fresh for each set of experiments, since they were prone to increasing autoxidation with time. Succinate and

and ethanol were made up to concentrations of 0.5 M and 1 M, respectively. In the course of the experiment, all solutions of substrates were kept on ice.

Experimental conditions for O_2 uptake

Respiration was measured polarographically at 25°C using an O_2 electrode (Rank Bros. Bottisham, Cambridge, England). The reaction mixture contained different volumes of complete salt solution and NaCl-salt solution (50 mM Tris/HCl, 300 mM NaCl, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4 - pH 7.2) so that the final concentrations of salts were always 50 mM Tris/HCl, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4 - pH 7.2 and the concentrations of Na^+ were those required under the conditions of the assay. Where low concentrations of Na^+ were examined, larger volumes of a more dilute NaCl salt solution (50 mM Tris/HCl, 30 mM NaCl, 270 mM choline chloride, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4 - pH 7.2) were added to the reaction vessel. In experiments concerning the effect of either K^+ or Li^+ on the O_2 uptake, different volumes of KCl- and LiCl-salt solutions were added to give the suitable final concentration in the reaction mixture.

To the aerobic salt solution, different volumes of bacterial suspension and cell fractions were introduced by means of a Hamilton microliter syringe and the endogenous respiration subsequently measured. To the aerobic cell suspension was then added substrates to final concentrations of 10 mM for either succinate or ethanol,

2.5 mM for either NADH or NAD^+ or 10 mM Ascorbate plus 0.5 mM TMPD. For each assay of Ascorbate/TMPD oxidase at different cation concentration, the non-enzymatic autoxidation rate was also recorded and this rate was then subtracted from the rate obtained after the addition of Ascorbate/TMPD to the aerobic cell suspension. Although the autoxidation rate increased with time, since the Ascorbate/TMPD oxidase activity was always corrected for the autoxidation rate, consistent results were obtained with respect to the cation concentration.

In all cases, the respiratory rate obtained with each substrate was corrected for the endogenous rate under identical conditions of assay.

Recording O_2-uptake rates

The O_2 electrode was connected to a chart recorder (model 8373-20, Cole Parmer Instruments Co., Chicago, Ill.) to measure the dissolved O_2 tension present in the reaction vessel. 100% air saturation of the buffered solution was equivalent to 1410 natoms O/3 ml of reaction volume (Chappell et al., 1964). Any percentage change with time due to the activity of a known concentration of cell suspension was then converted to natoms O utilized/min/mg dry wt of cells. In case of cell fractions, the respiratory rates were recorded as natoms O/min/mg protein.

Measurement of ^{14}C-AIB uptake

Washed cells were suspended at a final concentration of 100 µg dry weight per ml in a reaction mixture containing complete salt solution and different volumes of NaCl-salt solution. The reaction volume was 5 ml and the incubation temperature was 25°C. To the cell suspension was added an oxidizable substrate and the cells were allowed to equilibrate for 1 minute. The oxidizable substrates were added at the concentrations used in the oxidative assays with the exception of ascorbate/TMPD which was found to energize maximally ^{14}C-AIB uptake at concentrations of 10 mM ascorbate/1.0 mM TMPD (Figure 24). The reaction was started by the addition of the ^{14}C-AIB; the final concentration of ^{14}C-AIB in the reaction mixture was 200 µmolar with a specific activity of 0.5µ Curie/µmole. Every 30 seconds a 0.5 ml aliquot of the cell suspension was filtered through a 0.45µ HA millipore filter. The cells retained on the filters were washed quickly by drawing through the filter 5 ml of complete salt solution.

The amount of ^{14}C-AIB taken up by the cells during the first 3 minutes of incubation was measured. Over this period uptake was linear with time, and thus the initial rate of uptake was expressed as µmoles ^{14}C-AIB taken up/min/mg dry wt of cells.

Radioactive counting

For ^{14}C measurement, the millipore filters with their adhering washed cells were transferred to screw cap vials and dried slowly under

an infra-red lamp. Then 8 ml of scintillation fluid (0.5 g POPOP, 16.5 g PPO, 2 ℓ toluene, 1 ℓ Triton-X-100) were added to the vials and the samples counted in a Nuclear Chicago Isocap/300 liquid scintillation spectrophotometer. All counts were corrected for quench against a prepared quench curve and the efficiency of counting was about 75% for ^{14}C.

Chemicals

NADH, NAD^+, lysozyme, unlabelled α-AIB and Trizma base (Tris (hydroxy-methyl) aminomethane) were obtained from Sigma Chemical Co. Ascorbic acid and lithium chloride were products of Fisher Scientific Company. Choline chloride and N,N,N', N'-tetramethyl p-phenylene diamine dihydrochloride (TMPD) were obtained from Eastman Company. α-[1-^{14}C]-aminoisobutyric acid was obtained from the New England Nuclear Corporation. Millipore filters (HA 0.45 μ pore size) were from the Millipore Corporation. Succinic acid was from Baker Chemical Co. All other chemicals used were of analytical grade.

RESULTS

1. Respiratory studies

A. Endogenous respiration

The first indication that endogenous substrates are actively oxidized by intact cells of marine Pseudomonad B-16 was provided by Tomlinson and MacLeod (1957). It was later shown, with the same organism, that these endogenous reserves were oxidized at a sufficient rate to drive the uptake of AIB (Sprott and MacLeod, 1974). These findings have been extended to determine the rate at which the endogenous substrates are oxidized in the presence of increasing NaCl concentration.

Table 1 shows the endogenous respiratory rates obtained when intact cells were added to an aerobic salt solution containing 10 mM K^+ and varying concentrations of NaCl. There was a small increase in the endogenous oxidation rates in response to Na^+, reaching a maximum at 50 mM Na^+. Although the increment in the oxidation rates with increasing Na^+ concentrations was small, the difference between 0 mM and 50 mM Na^+ was found to be statistically significant, suggesting that the oxidation of endogenous substrates is slightly stimulated by externally added Na^+. When whole cells were added to a reaction medium without added potassium, there was a 50% reduction in the

TABLE 1. Effect of Na^+ on the endogenous respiratory rate in intact cells and toluenized cells in the presence and absence of potassium

Na^+ concentration (mM)	Intact cells		Toluenized cells	
	+ K^+ [b]	- K^+ [c]	+ K^+ [b]	- K^+ [c]
	Respiratory rate [a]			
0	45.77 ± 8.59	22.18 ± 3.05	5.79 ± 1.23	5.90 ± 1.13
0.5	40.85 ± 6.07	-	-	-
1.0	42.42 ± 4.18	21.98 ± 1.95	-	-
2.0	41.21 ± 6.00	24.54 ± 2.96	7.05 ± 1.49	7.87 ± 2.77
5.0	48.55 ± 7.89	26.85 ± 5.28	5.57 ± 1.75	7.18 ± 0.61
10.0	50.19 ± 6.59	28.38 ± 6.09	5.46 ± 0.55	5.33 ± 2.47
50.0	56.82 ± 6.81	-	4.71 ± 0.84	4.81 ± 1.48
100.0	55.86 ± 9.82	-	5.55 ± 2.46	5.05 ± 1.23
150.0	46.05 ± 7.91	-	-	-
200.0	-	-	-	-
250.0	46.69 ± 9.70	42.42 ± 6.02	4.45 ± 1.74	6.43 ± 0.81

[a] Respiratory rate expressed as natoms O/min/mg dry wt.

[b] The salt solution containing K^+ consisted of 50 mM Tris/HCl, 300 mM choline chloride, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4 - pH 7.2.

[c] The salt solution without K^+ consisted of 50 mM Tris/HCl, 310 mM choline chloride, 50 mM $MgSO_4$, 0.006% (v/v) 85% concentrated phosphoric acid - pH 7.2.

endogenous respiration (Table 1). Under these conditions, the oxygen uptake increased with Na^+ concentration, with the highest rate being obtained at 250 mM Na^+. This rate, in the absence of K^+, was comparable to that obtained in the presence of 10 mM K^+ and 250 mM Na^+. These results indicate that at a high enough Na^+ concentration there is little or no response of the endogenous respiratory rate to K^+, but that at lower Na^+ concentrations an effect of K^+ can be demonstrated. It is possible that the endogenous respiration of this organism has an absolute requirement for K^+ which, at high Na^+ concentrations, may be satisfied by small amounts of contaminating K^+ present in the NaCl used. It is also possible that Na^+, at high concentration, can substitute for K^+. The data do not permit a distinction between these possibilities. Table 1 further demonstrates that the endogenous respiration by toluenized cells was both Na^+ as well as K^+ independent. It is worth noting that the oxidation rates are considerably lower in the toluenized cells than in whole cells. Since treatment of whole cells with toluene renders the cell "leaky," allowing free passage of small molecules across the membrane (Jackson and DeMoss, 1965), this treatment would cause a loss of the endogenous substrates and hence the lower oxidation rates.

When intact cells were disrupted to produce cell-free extracts and subsequently partitioned to produce particulate and soluble fractions, the endogenous respiratory activities as shown in Table 2 were obtained. As is evident from the data, the oxygen uptake by

TABLE 2. Effect of Na^+ on the respiratory rate[a] in cell-free extract and particulate and soluble fractions[b]

Na^+ concentration (mM)	Cell-free extract	Soluble fraction	Particulate fraction
0	3.82 ± 0.78	1.57 ± 0.42	3.79 ± 0.90
0.5	-	1.50 ± 0.06	2.68 ± 1.81
1.0	3.75 ± 0.93	1.12 ± 1.05	2.70 ± 1.53
2.0	4.30 ± 1.28	0.46 ± 0.75	-
5.0	-	0.47 ± 1.05	3.15
10.0	4.41 ± 0.90	0.84 ± 1.09	2.51 ± 1.28
50.0	4.63 ± 0.86	-	-
100.0	4.93 ± 1.30	-	-
150.0	4.76 ± 0.78	-	-
250.0	5.20 ± 0.77	-	-

[a] The respiratory rate is expressed as natoms O/min/mg protein.

[b] All the fractions were suspended in a salt solution containing 50 mM Tris/Hcl, 300 mM choline chloride, 50 mM $MgSO_4$, 9 mM KCl and 1 mM KH_2PO_4 - pH 7.2.

the soluble fraction was almost negligible, whereas the particulate fraction gave rates comparable to those obtained by the cell free extracts. Both the particulate as well as the soluble fractions of the cell free extracts showed no response to Na^+.

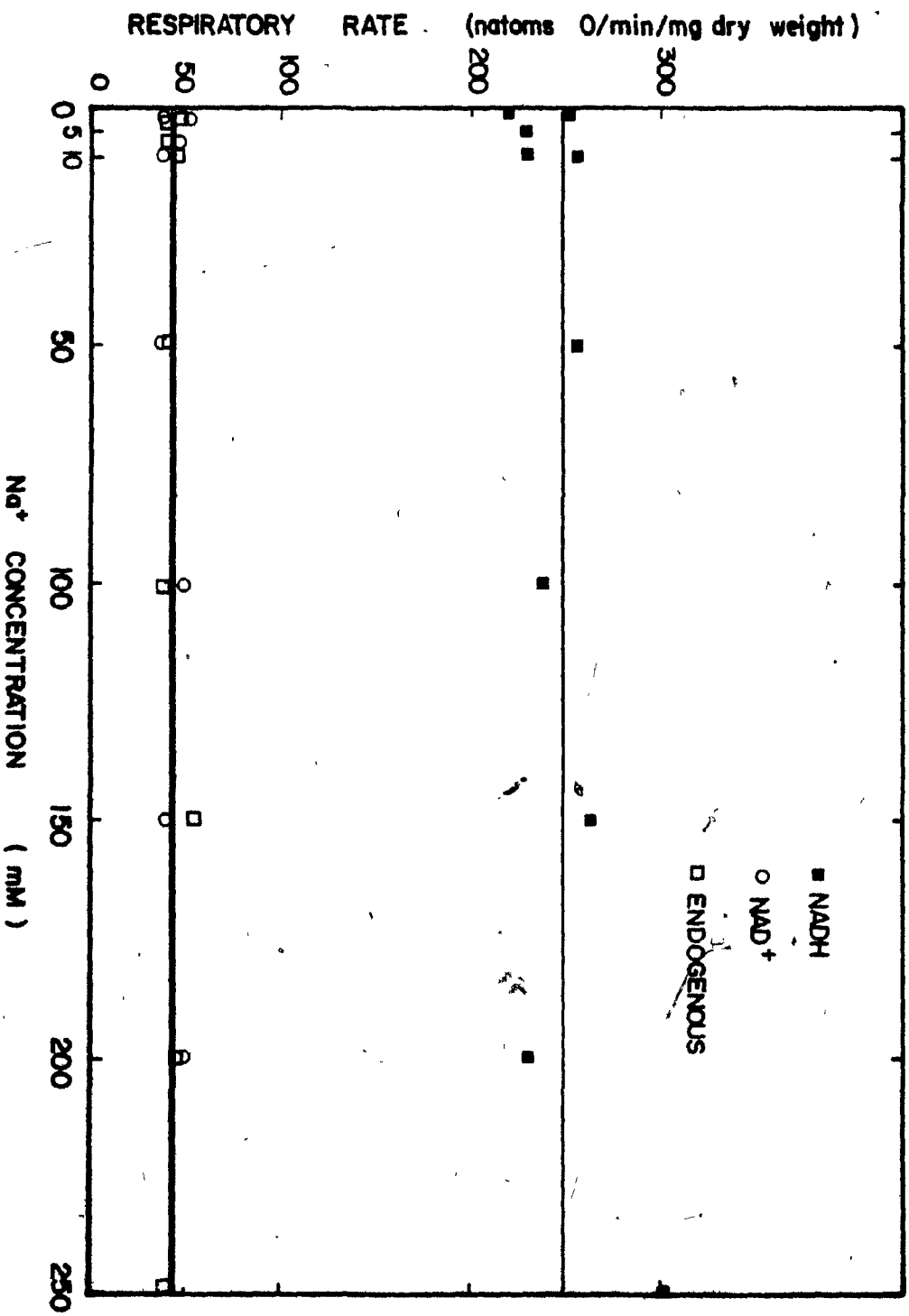
B. Oxidation of reduced nicotinamide adenine dinucleotide (NADH)

a. Oxidation of NADH by intact cells

Most membranes are impermeable towards pyridine nucleotides. However, previous results obtained in this laboratory (Calcott and MacLeod, unpublished data) have demonstrated that NADH is oxidized by whole cell suspensions of A. haloplanktis. Figure 1 shows the respiratory rate obtained with exogenously added NADH as substrate in the presence of various concentrations of NaCl. Na^+ had no stimulatory effect. That NADH, and not some breakdown product, was the substrate being oxidized was investigated by repeating the experiment with NAD^+ as substrate. If NADH was being catabolised by some periplasmic enzyme and the product of this reaction transported and oxidized intracellularly, it would seem reasonable to assume that NAD^+ would be treated similarly. However, both in the presence and absence of Na^+, the presence of exogenous NAD^+ did not stimulate the rate of endogenous respiration, suggesting that exogenously added NADH was in fact being oxidized by an NADH dehydrogenase located on the outer surface of the cytoplasmic membrane.

Figure 1. Effect of Na^+ on the respiratory activity of intact cells in the presence and absence of NADH and NAD^+.

Cells were suspended at a cell density of about 1 mg dry wt per ml in a reaction medium containing complete salt solution (50 mM Tris/HCl, 300 mM choline chloride, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4, pH 7.2) and volumes of NaCl-salt solution (50 mM Tris/HCl, 300 mM NaCl, 50 mM $MgSO_4$, 9 mM KCl, 1 mM KH_2PO_4, pH 7.2), such that the final concentration of Na^+ in each reaction mixture was that which was required under the conditions of the assay, keeping the concentrations of other salts constant. Endogenous respiration (□) was determined prior to addition of the substrates. Substrates were added to a final concentration of 2.5 mM NAD(O) and 2.5 mM NADH (■) in the reaction medium.


RESPIRATORY RATE (natoms O/min/mg dry weight)
0
50
100
200
300
Na+ CONCENTRATION (mM)
0.5
10
50
100
150
200
250
■ NADH
○ NAD+
□ ENDOGENOUS

Since the experiment concerning the effect of Na^+ on NADH oxidation was conducted in a complete salt solution containing 10 mM K^+, the possibility that perhaps K^+ was substituting for a Na^+ effect had to be examined. Figure 2 reveals that, in the absence of K^+, NADH oxidase was stimulated by 2 mM Na^+. However, this activation by Na^+ was no longer apparent in the presence of 10 mM K^+. Conversely, oxidation of NADH was stimulated by 2 mM K^+, irrespective of the concentration of Na^+ present in the reaction medium (Figure 3). The effect of another cation, Li^+, on the respiration of NADH, is shown in Figure 4; even in the absence of K^+, Li^+ had no effect on the oxidation of the substrate. It is interesting to note that considerable NADH oxidase activity existed even in the absence of any added cation.

b. Oxidation of NADH by toluenized cells

Toluene-treatment of E. coli has been shown to render both sides of the cytoplasmic membrane accessible to exogenous substrates (Jackson and DeMoss, 1965). Previous studies on A. haloplanktis have shown the presence of a second NADH dehydrogenase located on the inside surface of the cytoplasmic membrane (Knowles, Calcott and MacLeod, 1975, unpublished data; Calcott and MacLeod, 1977, unpublished data). In view of these results, it was of considerable interest to investigate the effect of Na^+ on the activity of the inner NADH dehydrogenase. To examine the response of this enzyme to Na^+, washed whole cells were treated with 1% toluene. The effect

Figure 2. Effect of Na^+ on the oxidation of NADH by intact cells in the presence and absence of potassium.

In the determination of the Na^+-dependent activation of NADH oxidase in the presence of 10 mM K^+ (□), experimental conditions were as described in Figure 1. For the determination of the Na^+ effect on NADH oxidation in the absence of K^+ (■), cells were washed and resuspended in a salt solution without K^+ (50 mM Tris/HCl, 310 mM choline chloride, 50 mM $MgSO_4$, 0.006% v/v phosphoric acid, pH 7.2) and Na^+ was added to each reaction mixture as a NaCl-salt solution without K^+ (50 mM Tris/HCl, 300 mM NaCl, 10 mM choline chloride, 50 mM $MgSO_4$, 0.006% v/v phosphoric acid, pH 7.2) to give the desired final concentration of NaCl in each incubation medium. The respiratory rates recorded were corrected for endogenous oxidation at each concentration of Na^+ tested.

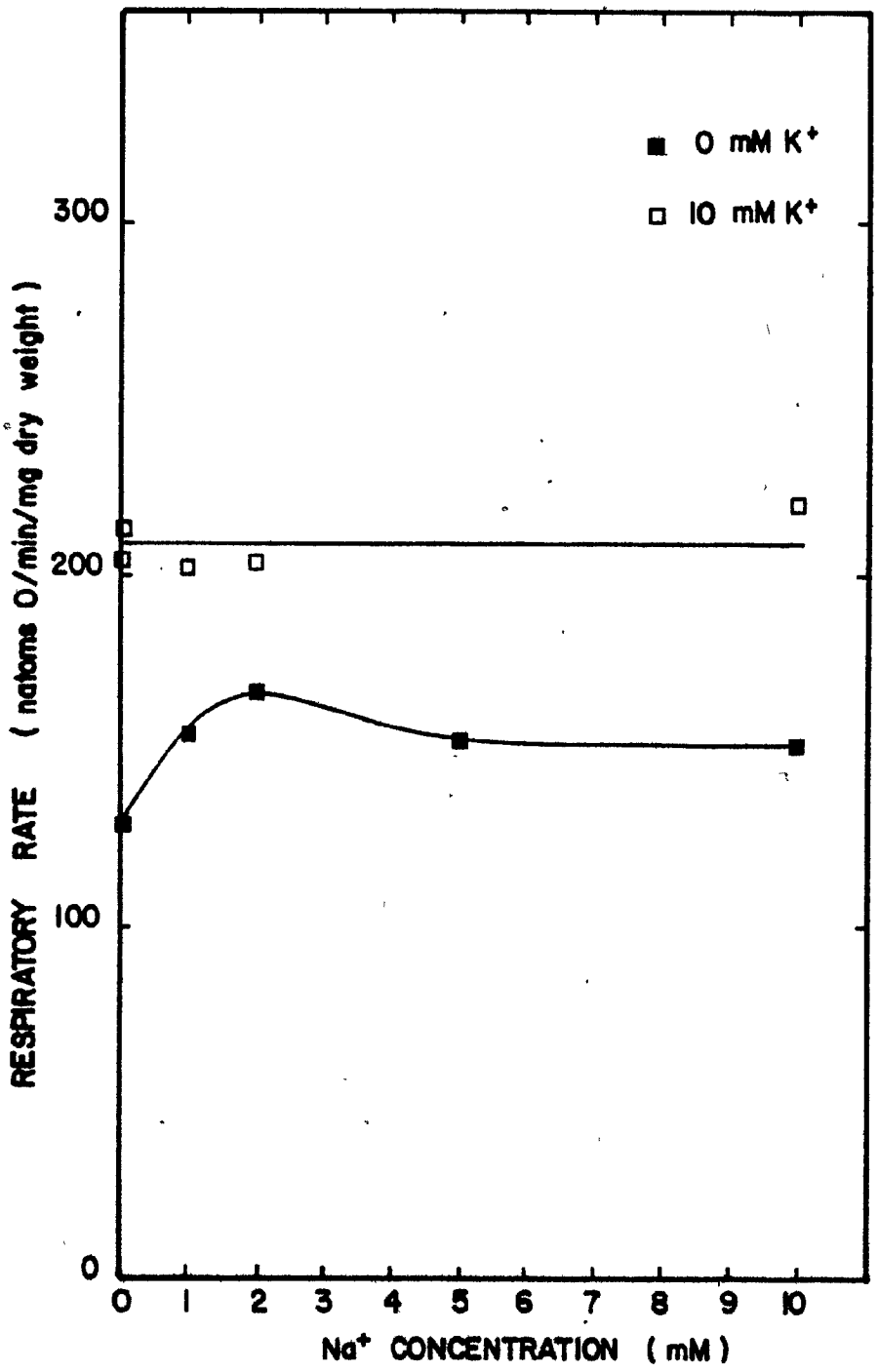

0 mM K+
10 mM K+
300
200
100
0
RESPIRATORY RATE (natoms O/min/mg dry weight)
0 1 2 3 4 5 6 7 8 9 10
Na+ CONCENTRATION (mM)

Figure 3. Effect of K^+ on the oxidation of NADH by whole cells, in the presence and absence of added (10 mM) NaCl.

In the determination of K^+-activation of NADH oxidation in the absence of NaCl (■), cells were washed and suspended in a complete salt solution without K^+ and Na^+, the composition of which is described in Figure 2. In the determination of the K^+ effect on NADH oxidation in the presence of NaCl (□), cells were washed and suspended in a salt solution consisting of 50 mM Tris/HCl, 10 mM NaCl, 300 mM choline chloride, 50 mM $MgSO_4$, 0.006% v/v phosphoric acid, pH 7.2. In each case K^+ was added to the reaction medium as KCl-salt solution (50 mM Tris/HCl, 30 mM KCl, 280 mM choline chloride, 50 mM $MgSO_4$, 1 mM KH_2PO_4, pH 7.2) in amounts sufficient to give the desired final concentration of K^+ shown. NADH was added to a final concentration of 2.5 mM. The respiratory rates were all corrected for endogenous oxidation at every level of K^+ tested.

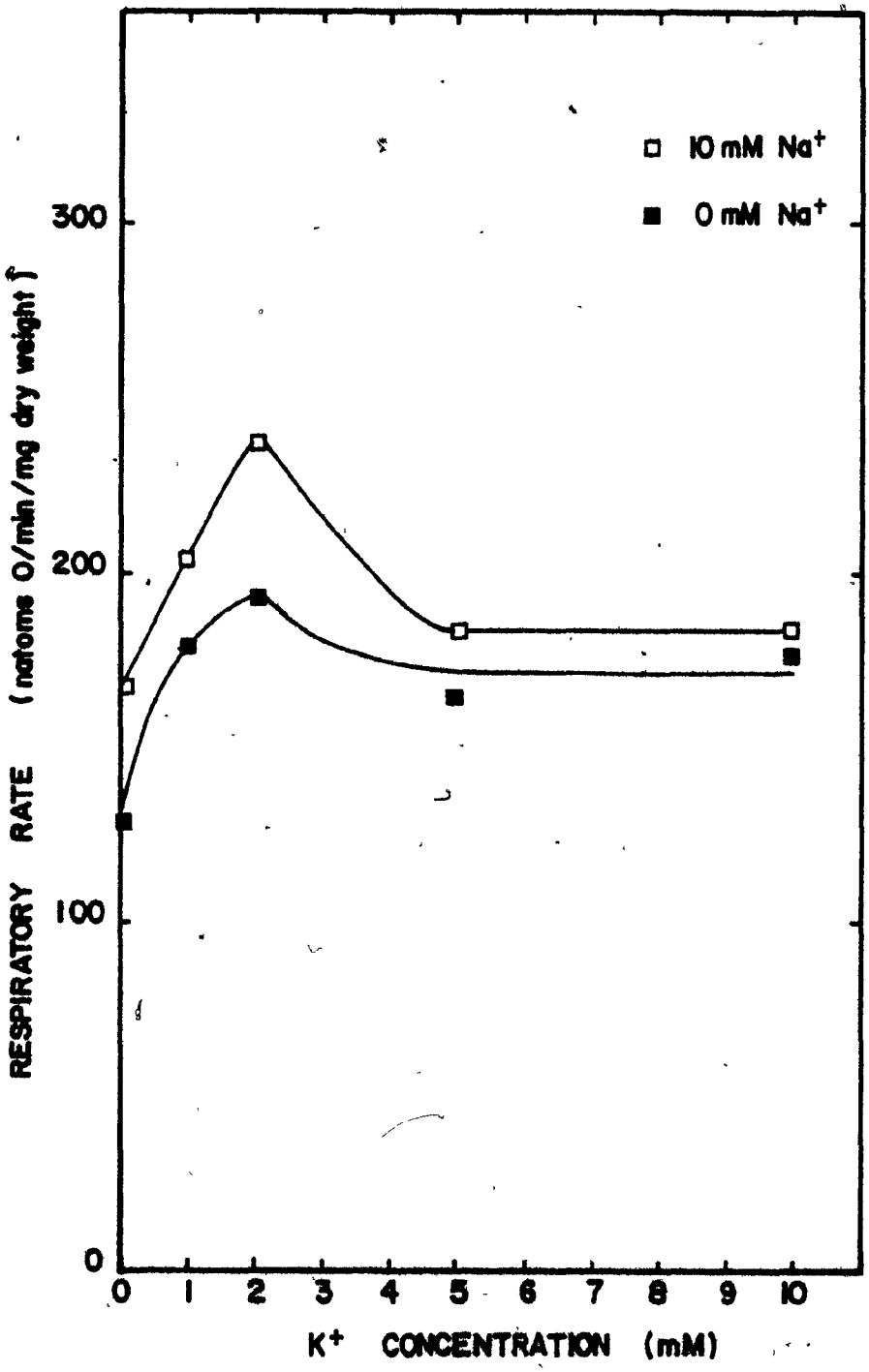
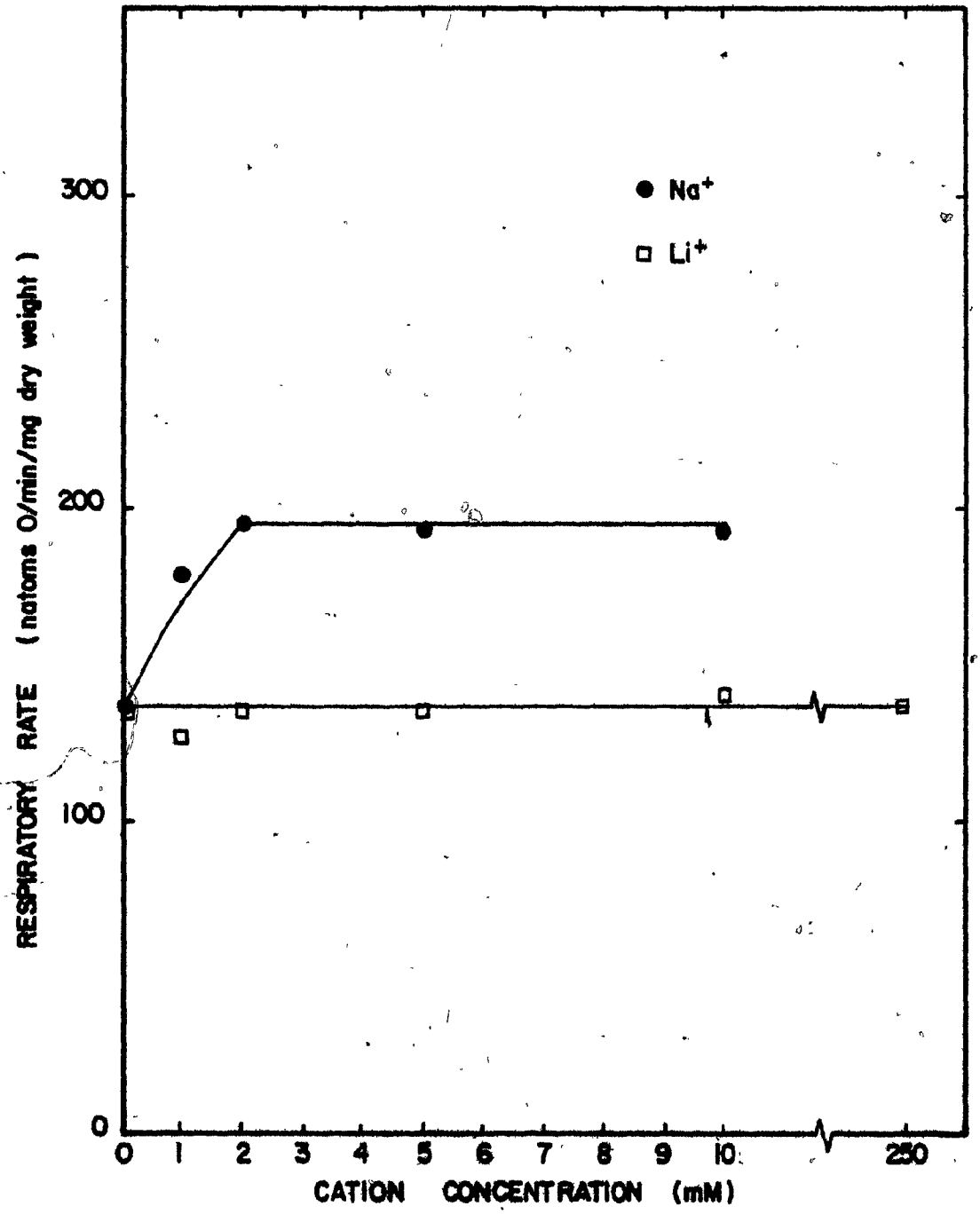

10 mM Na+
0 mM Na+
RESPIRATORY RATE (natoms O/min/mg dry weight)
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
K+ CONCENTRATION (mM)

Figure 4. Effect of Li^+ on the oxidation of NADH by intact cells in the absence of potassium.

Experimental conditions were as described in Figure 2. Monovalent cations, Na^+ (●) and Li^+ (□) were added to the reaction medium as NaCl salt solution without K^+, and LiCl-salt solution without K^+ (50 mM Tris/HCl, 300 mM LiCl, 10 mM choline chloride, 50 mM $MgSO_4$, 0.006% v/v phosphoric acid, pH 7.2), respectively. The respiratory rates were corrected for the endogenous oxidation rates at each concentration of monovalent cation tested.


300
200
100
0
● Na+
□ Li+
RESPIRATORY RATE (natoms O/min/mg dry weight)
0 1 2 3 4 5 6 7 8 9 10 250
CATION CONCENTRATION (mM)

of Na^+ on toluene-treated cells is shown in Figure 5. NADH oxidation by toluenized cells was found to require 100 mM Na^+ for maximal activity. While the NADH dehydrogenase was stimulated by Na^+, considerable activity was observed, even in the absence of the cation. However, since the treatment with toluene is exposing both faces of the membrane to the substrate, this activity probably corresponded to the oxidation of NADH by the Na^+-independent NADH oxidase on the outer surface of the cytoplasmic membrane. Figure 6 reveals that no concentration of K^+ could reduce the requirement for Na^+ for maximum activity of the NADH oxidase on the inner surface of the cytoplasmic membrane. Li^+, in contrast, could activate to about 50% the rate at which Na^+ stimulated NADH oxidase activity inside the cell (Figure 7).

In a recent paper, De Smet et al. (1978) presented evidence to demonstrate that intact cells of E. coli treated with toluene in the presence of Mg^{+2} remained relatively impermeable to pyridine nucleotides. This, however, does not appear to be the case with A. haloplanktis. When these organisms, resuspended in complete salt solution containing Mg^{+2}, were disrupted by passage through a French pressure cell and cell free extracts prepared, NADH oxidation by these extracts was qualitatively similar to that obtained by toluenized cells with respect to Na^+ (Figure 8). Quantitatively, however, the relative rate of NADH oxidation by the cell free extracts was lower than by toluenized cells. This implied that toluenization is a gentler treatment. Furthermore, the observation that treating whole

Figure 5. Effect of Na^+ concentration on the rate of NADH oxidation by intact and toluene-treated cells of A. haloplanktis.

□ - intact cells; ■ - toluene-treated cells.
Conditions were as described in Figure 1. The substrate, NADH, was added to a final concentration of 2.5 mM in the reaction medium. With both intact cells as well as toluenized cells, the respiratory rates were corrected for endogenous oxidation at each level of Na^+ tested.

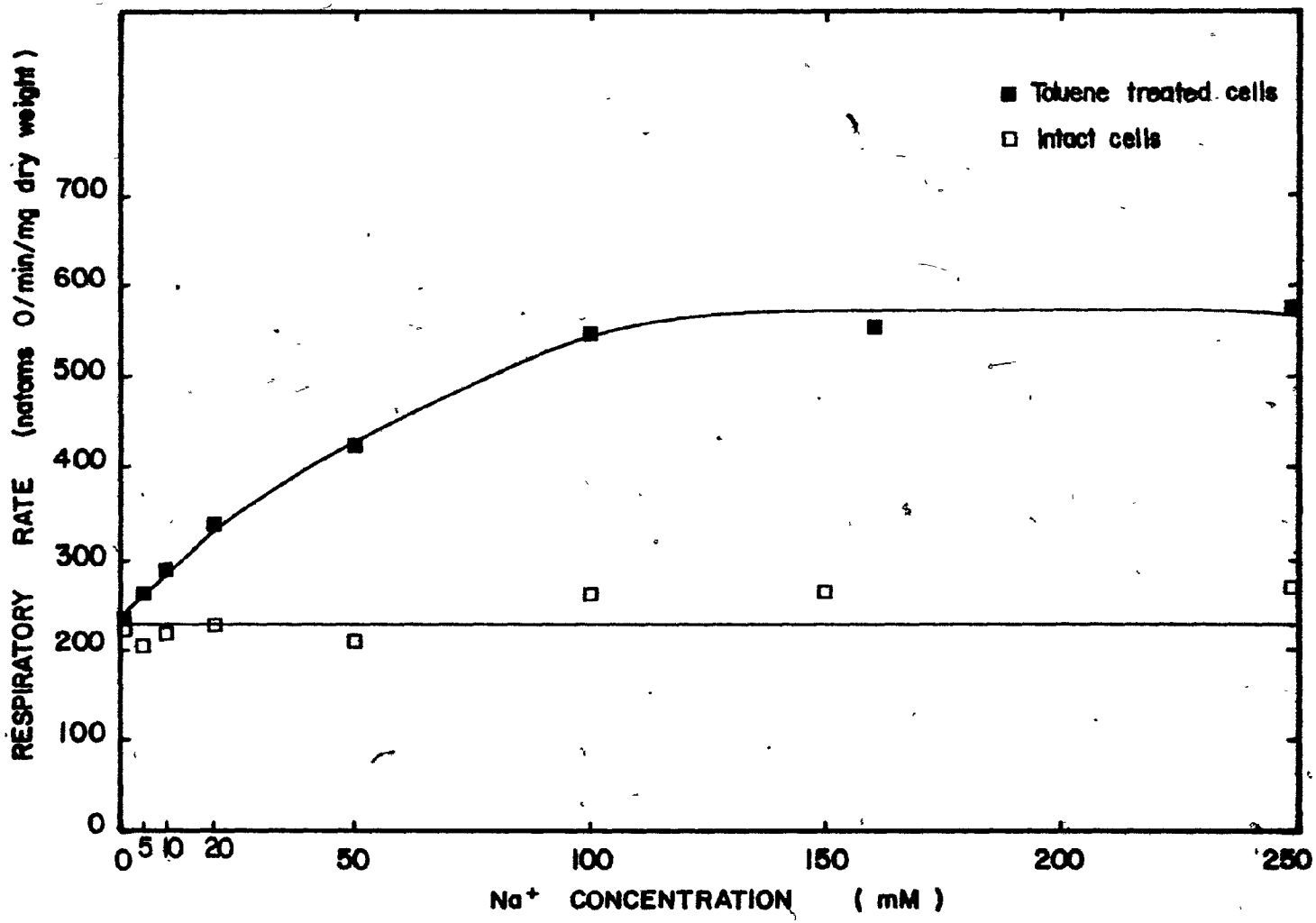

■ Toluene treated cells
□ intact cells
RESPIRATORY RATE (natoms O/min/mg dry weight)
0
100
200
300
400
500
600
700
0
5
10
20
50
100
150
200
250
Na+ CONCENTRATION (mM)

Figure 6. Effect of K^+ on the quantitative requirement for Na^+ to stimulate NADH oxidation by toluene-treated cells.

Cells were washed, toluenized and suspended in different salt solutions, the K^+ concentrations of which were either 0 mM K^+ (●), 10 mM K^+ (■) or 400 mM K^+ (o), maintaining the concentrations of all other salts constant. Na^+ was added to the reaction mixture, in all cases, as NaCl-salt solution. The substrate, NADH, was added to a final concentration of 2.5 mM. The respiratory rates were all corrected for endogenous respiration.

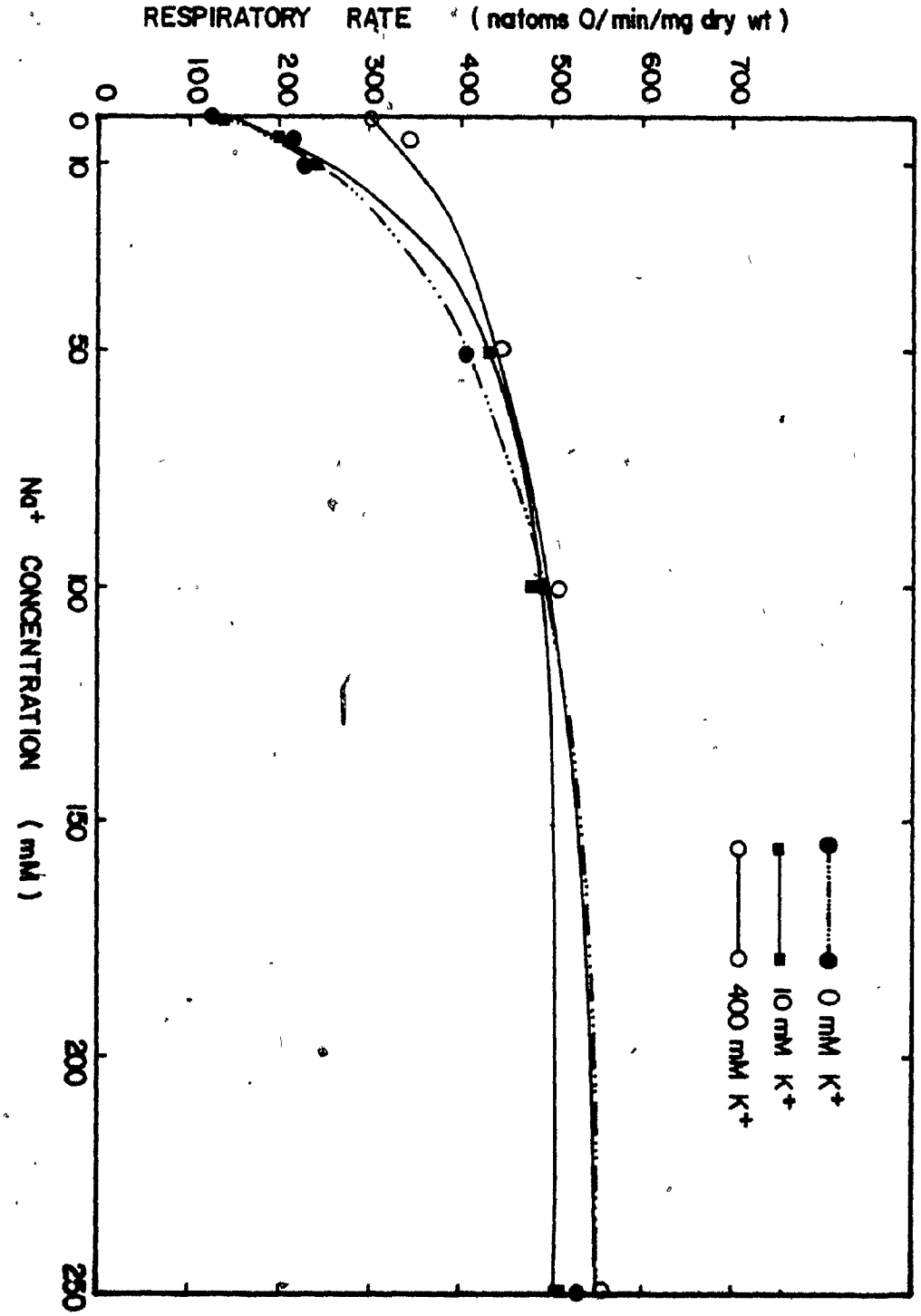

RESPIRATORY RATE (natoms O/min/mg dry wt)
0
100
200
300
400
500
600
700
Na+ CONCENTRATION (mM)
0
10
50
100
150
200
250
0 mM K+
10 mM K+
400 mM K+

Figure 7. Na^+ (■) and Li^+ (□) activation of NADH oxidation in toluene-treated cells.

Both curves represent respiratory rates in the presence of 2.5 mM NADH corrected for endogenous respiration at each level of cation tested. Na^+ and Li^+ were each added as NaCl- and LiCl-salt solutions. Other conditions were as described in Figure 4.

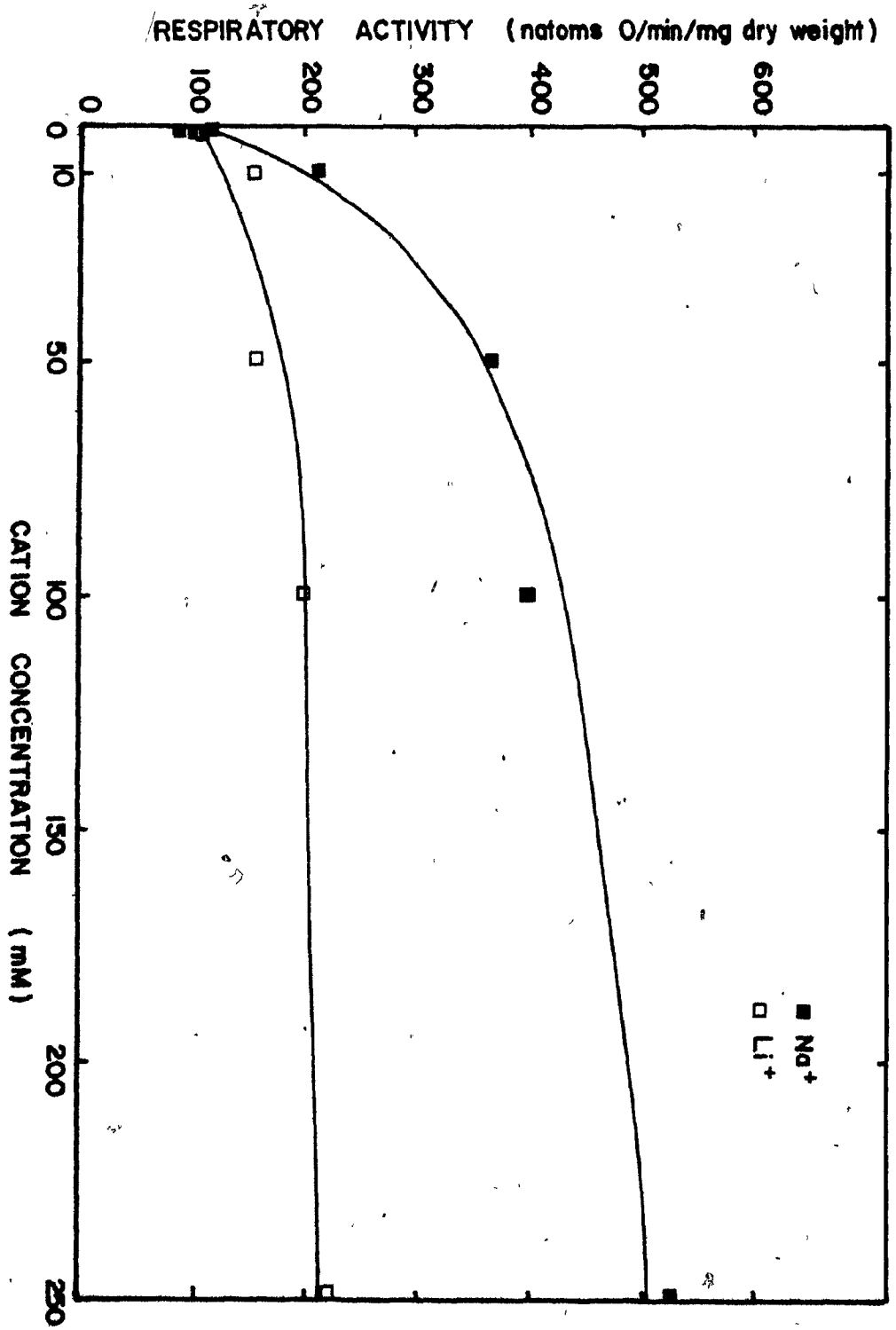
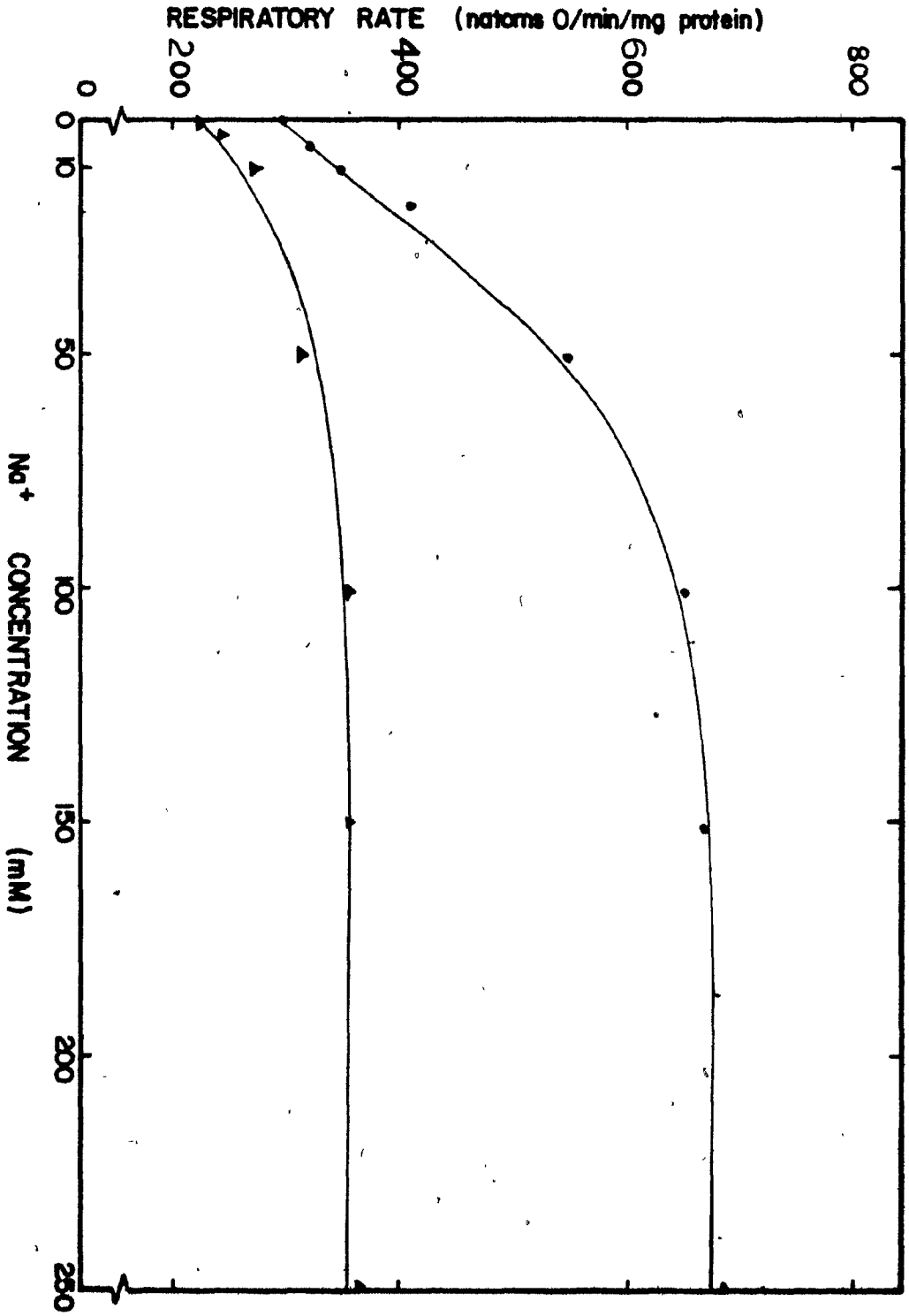

RESPIRATORY ACTIVITY (natoms O/min/mg dry weight)
0
100
200
300
400
500
600
CATION CONCENTRATION (mM)
0
10
50
100
150
200
250
Li+
Na+

Figure 8. Respiratory response of toluene-treated cells (●) and cell-free extracts (▲) of A. haloplanktis to the oxidation of NADH in the presence of increasing NaCl concentrations.

The substrate, NADH, was added to the reaction mixture to a final concentration of 2.5 mM. All other conditions were as described in Figure 1. The respiratory rates, adjusted for endogenous oxidation, are expressed as natoms O/min/mg protein.


RESPIRATORY RATE (natoms O/min/mg protein)
0
200
400
600
800
Na+ CONCENTRATION (mM)
0
10
50
100
150
200
250

cells with toluene resulted in an increase in NADH oxidase activity provided adequate evidence to suggest that the cytoplasmic membrane of this organism is permeable to NADH.

C. Oxidation of ethanol

a. Oxidation of ethanol by intact cells

Previous work conducted in this laboratory has shown that ethanol is oxidized actively by whole cells of A. haloplanktis (Thompson and MacLeod, 1974). Figure 9 demonstrates the effect of Na^+ on the oxidation of ethanol by intact cells. It was found that ethanol oxidase activity was stimulated in the presence of 2 mM Na^+. Since the activation of ethanol oxidation by Na^+ was observed in the presence of K^+, it was of interest to investigate whether K^+ had a response on Na^+ effect. Figure 10 shows that in the absence of added K^+ in the reaction medium, maximum activity of ethanol oxidase was obtained in the presence of 5 mM Na^+. Hence, it appears that K^+ can spare partially the Na^+ requirement for ethanol oxidation by intact cells; however, it cannot reduce the requirement below 2 mM Na^+. That K^+ could stimulate ethanol oxidation, both in the presence as well as in the absence of Na^+, suggested that there is a specific requirement for K^+ besides the requirement for 2 mM Na^+ for the maximum respiration of ethanol (Figure 11). This requirement was shown to be for 1 mM K^+. Li^+ was shown to spare the requirement for Na^+ by 50% (Figure 12). The data shown in Figure 12 are the

Figure 9. Effect of Na^+ on the oxidation of ethanol by whole cells.

Ethanol was added to a final concentration of 10 mM, all other conditions were as described in Figure 1. The respiratory rate recorded was adjusted for endogenous respiration at each concentration of Na^+ tested.

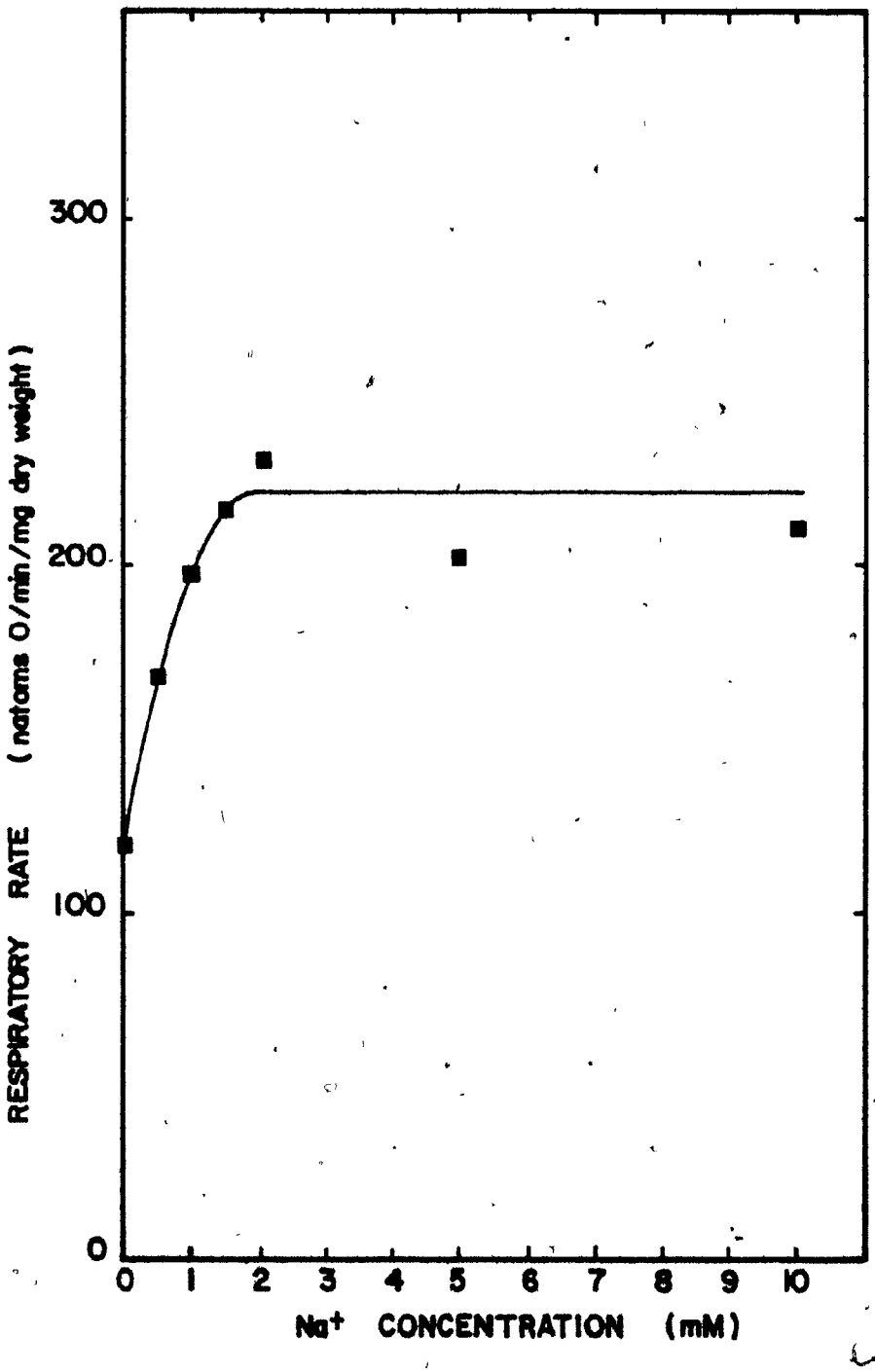

RESPIRATORY RATE (natoms O/min/mg dry weight)
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
Na+ CONCENTRATION (mM)

Figure 10. Effect of Na^+ on the oxidation of ethanol by intact cells in the presence (■) and absence (□) of potassium.

Experimental conditions were as described in Figure 2. The substrate ethanol was added to the reaction mixture to a final concentration of 10 mM.

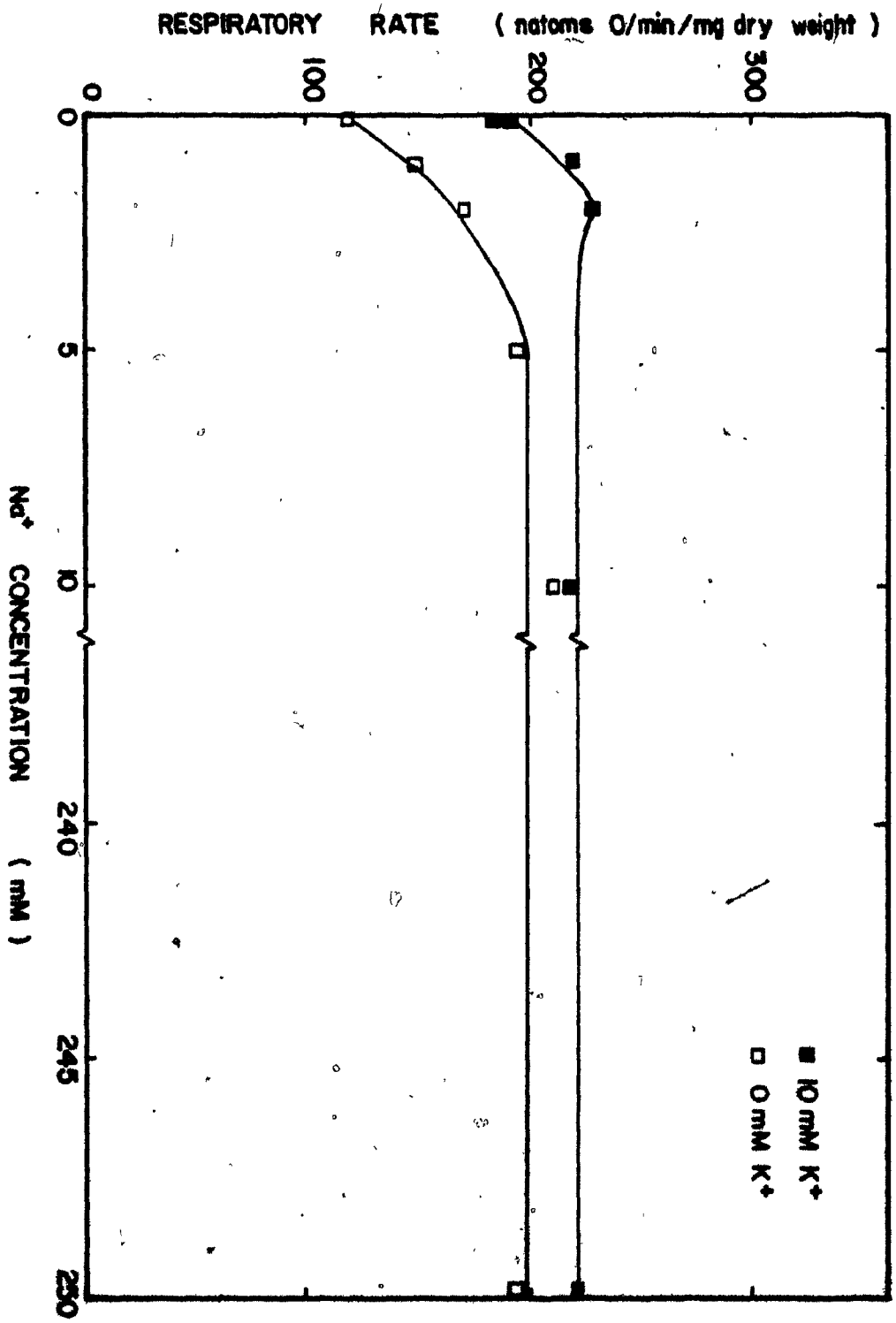

RESPIRATORY RATE (natoms O/min/mg dry weight)
0
100
200
300
0
5
10
240
245
250
Na+ CONCENTRATION (mM)
10 mM K+
0 mM K+

Figure 11. Effect of K^+ on the oxidation of ethanol by whole cells, in the presence (■) and absence (□) of 10 mM NaCl.

Experimental conditions were as described in Figure 3, except for the determination of the effect of 250 mM K^+ on ethanol oxidase activity. Both in the presence and absence of Na^+, the K^+ was added to the reaction mixture as KCl salt solution consisting of 50 mM Tris/HCl, 310 mM KCl, 50 mM $MgSO_4$, 1 mM KH_2PO_4, pH 7.2. The substrate, ethanol, was added to a final concentration of 10 mM ethanol. The ethanol oxidase activity at each concentration of K^+ tested, was adjusted for endogenous respiration.

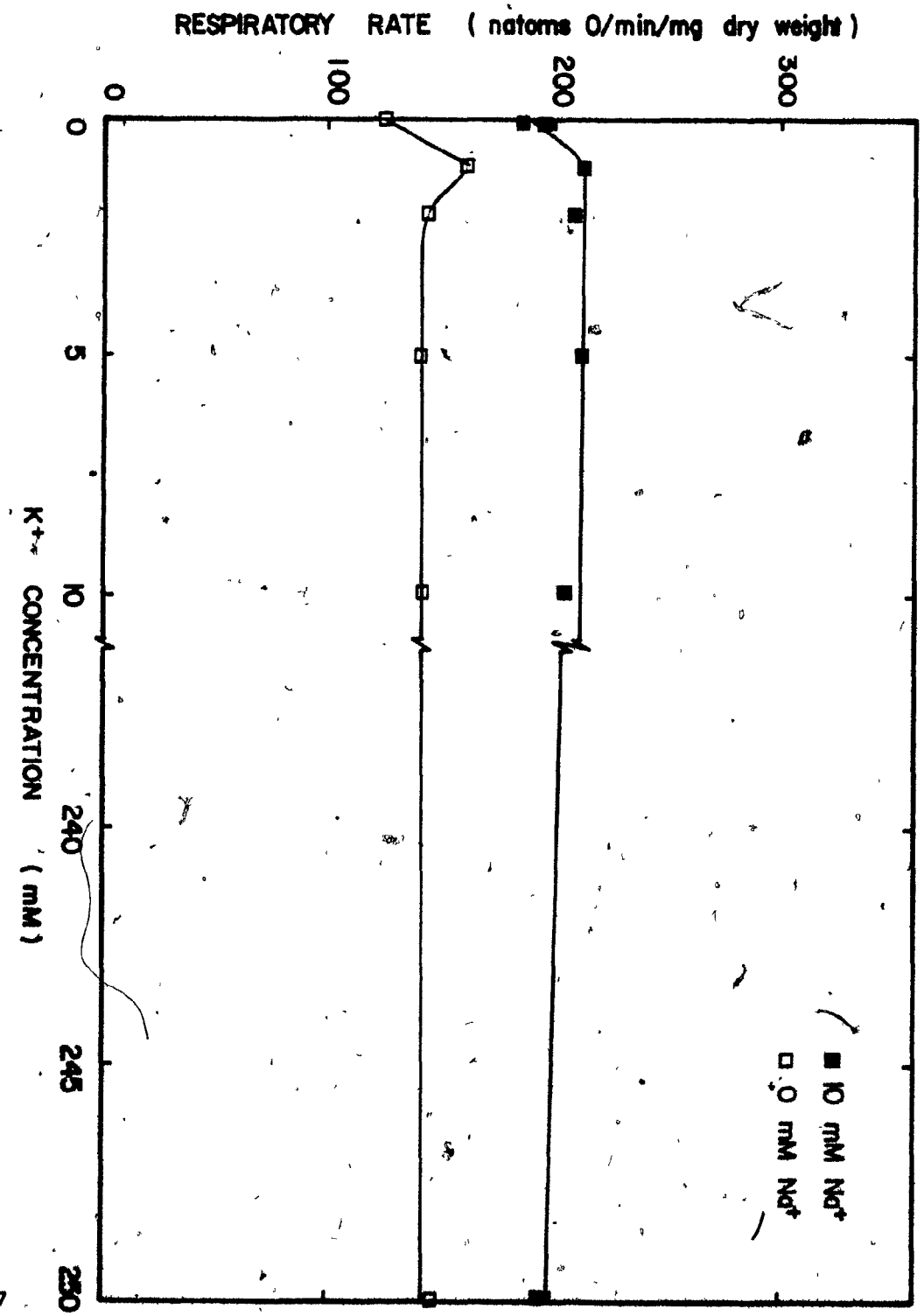
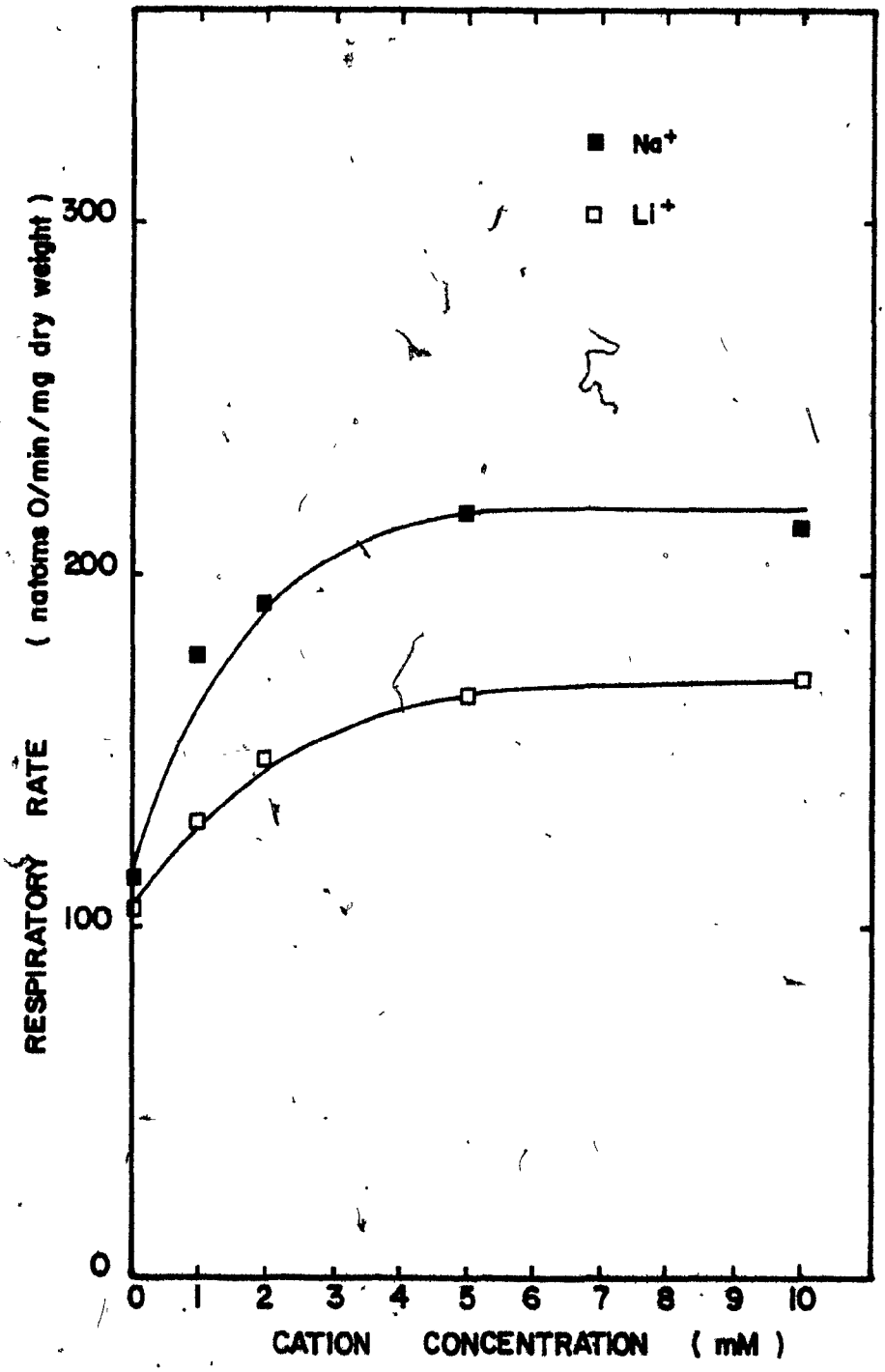

RESPIRATORY RATE (natoms O/min/mg dry weight)
0
100
200
300
0
5
10
240
245
250
K+ CONCENTRATION (mM)
■ 10 mM Na+
□ 0 mM Na+

Figure 12. Effect of Li^+ (□) and Na^+ (■) on the oxidation of ethanol by intact cells, in the absence of added potassium.

The composition of the reaction media was as described in Figure 4. The respiratory rates were all adjusted for endogenous rates at each concentration of monovalent cation tested.


■ Na+
□ Li+
RESPIRATORY RATE (natoms O/min/mg dry weight)
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
CATION CONCENTRATION (mM)

effect of Na^+ and Li^+ on ethanol oxidation by intact cells in the absence of K^+ - this was done to avoid the interfering sparing effect of K^+ on the Na^+ requirement.

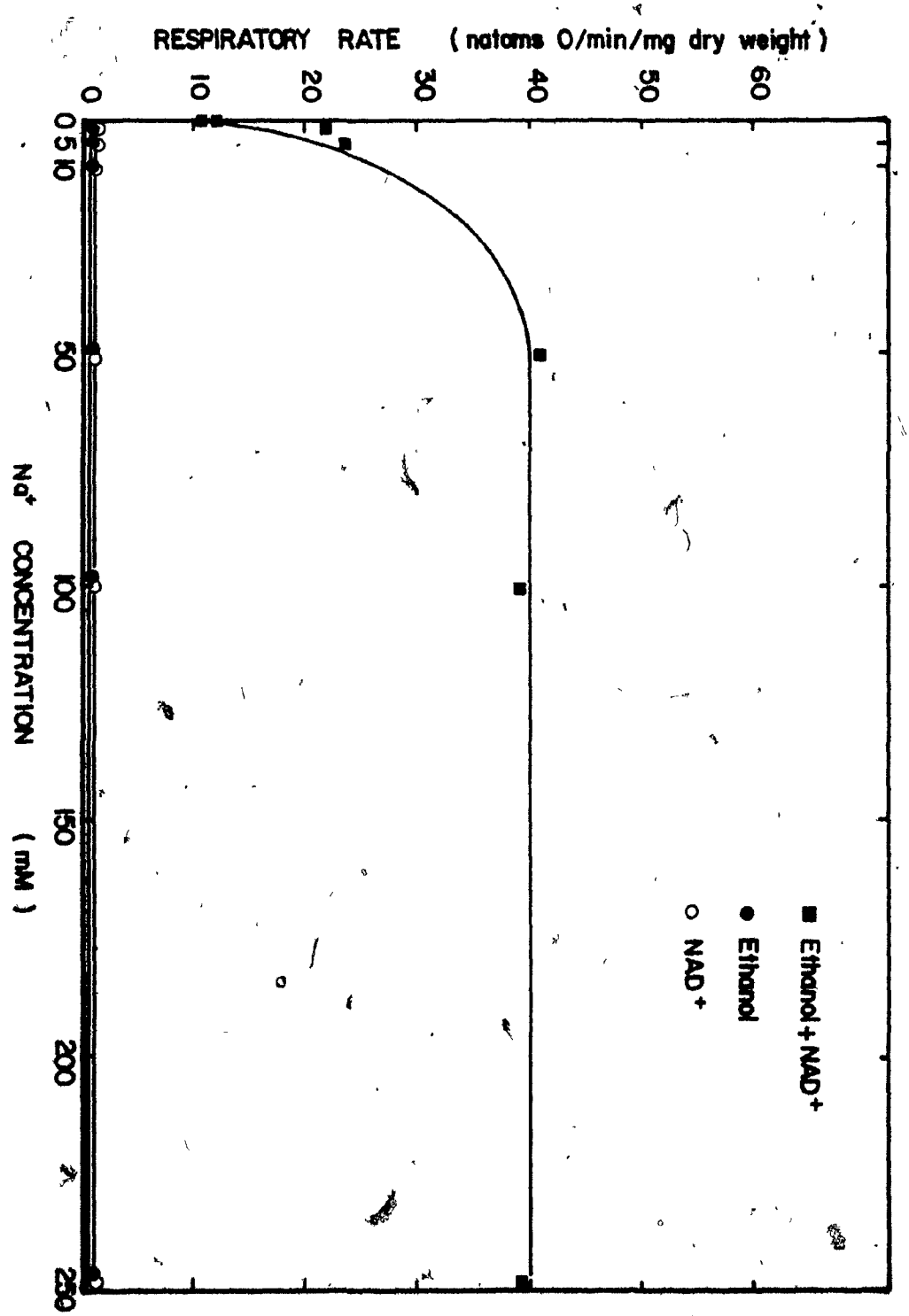
Recent studies done on the specific coupling of ethanol to transport have led to the conclusion that there are possibly two alcohol dehydrogenases in this organism. One of these enzymes has been shown to be an NAD^+-linked alcohol dehydrogenase present in the cytoplasm (Sprott and MacLeod, 1974). Since the Na^+ requirement for the inner NADH oxidase activity (100 mM Na^+) far exceeded the quantitative Na^+ requirement for ethanol oxidation (2 mM), it seemed likely that ethanol oxidation by whole cells is catalyzed by an enzyme other than a cytoplasmic NAD^+-linked ethanol dehydrogenase - perhaps another alcohol dehydrogenase located on the outer surface of the cytoplasmic membrane. This suggestion is consistent with the previous findings of Sprott and MacLeod (1974) that ethanol could energize the uptake of metabolites into membrane vesicles which lack the cytoplasmic alcohol dehydrogenase.

b. Oxidation of ethanol by toluenized cells

Following the results obtained thus far, it was of interest to investigate the possible effect of Na^+ on the intracellular NAD^+-linked alcohol dehydrogenase present in this organism. To explore this possibility, intact cells were treated with 1% toluene and tested for the response of ethanol oxidation to Na^+. Figure 13 revealed

Figure 13. Effect of Na^+ on NAD^+, ethanol and NAD^+ + ethanol oxidation in toluene-treated cells.

Experimental conditions were as described in Figure 1. The substrates were added to final concentrations of 2.5 mM NAD^+ (o); 10 mM ethanol (●); 2.5 mM NAD^+ plus 10 mM ethanol (■). The respiratory rates were all corrected for endogenous respiration at each concentration of Na^+ examined.


RESPIRATORY RATE (natoms O/min/mg dry weight)
0
10
20
30
40
50
60
Na+ CONCENTRATION (mM)
0
50
100
150
200
250
■ Ethanol + NAD+
● Ethanol
○ NAD+

that there was no oxidation of ethanol by toluenized cells, nor was there any oxidative response by the cells to NAD^+. However, upon addition of both ethanol and NAD^+ to the reaction vessel containing an aerobic suspension of toluenized cells, there was a significant stimulation of O_2 uptake. This stimulation was Na^+-dependent, with a requirement for 50 mM Na^+ for maximum activation. It was observed that even in the presence of 10 mM K^+, the Na^+ requirement for maximum ethanol oxidase activity in toluenized cells was not reduced (Figure 14). In contrast, Li^+, at low concentrations, could partially spare this Na^+ requirement; however, at high concentrations, it could activate ethanol oxidation to a comparable rate at which Na^+ stimulated ethanol oxidase activity inside the cell (Figure 15). Thus, these results show that there are two alcohol dehydrogenases present in this organism. One of these is a cytoplasmic NAD^+-linked enzyme with a Na^+ requirement for 50 mM, and the other a membrane alcohol dehydrogenase with a requirement for 2 mM Na^+.

An interesting point to note is the relative rates of ethanol oxidation at 50 mM Na^+ ($\sim$40 natoms O/min/mg dry wt) and that of NADH oxidation at the same Na^+ concentration ($\sim$300 natoms O/min/mg dry wt), both in toluenized cells. As can be seen (Figures 5 and 13), the ethanol + NAD^+ respiratory rate was significantly lower than that of NADH oxidation. The possible explanation for this is that the respiration of ethanol plus NAD^+ is limited by the rate at which the intracellular alcohol dehydrogenase can function.

Figure 14. Relative capacity of potassium to spare the Na^+ requirement for oxidation of NAD^+ plus ethanol in toluene-treated cells.

The conditions were as described in Figure 6, except that the K^+ concentrations at which the effect of Na^+ on NAD^+ plus ethanol oxidation was examined were 0 mM K^+ (□) and 10 mM K^+ (■). The concentrations of substrates used were 2.5 mM NAD^+ and 10 mM ethanol. The NAD^+ plus ethanol oxidation rates were all adjusted for endogenous respiration at each concentration of Na^+ tested.

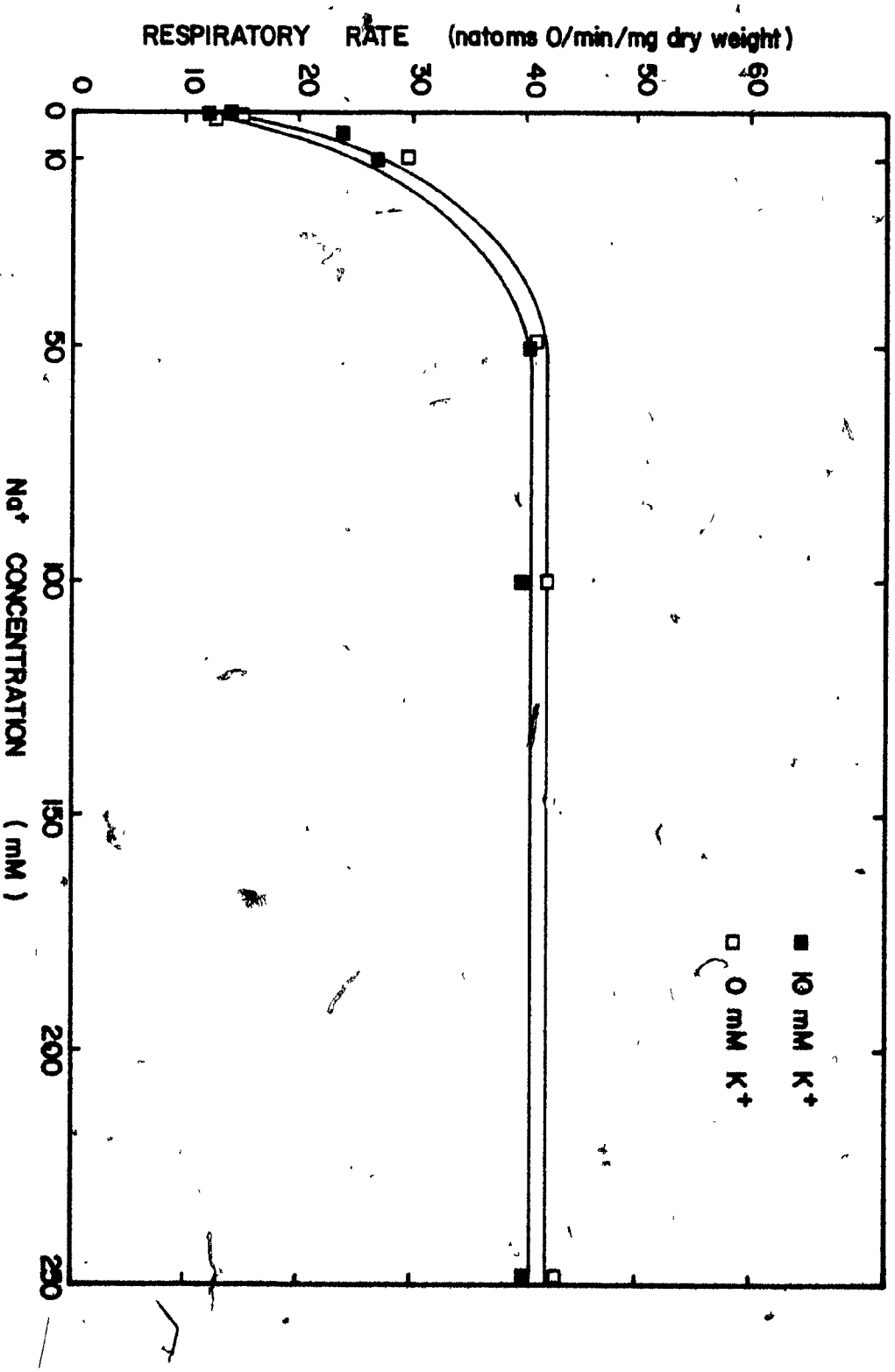
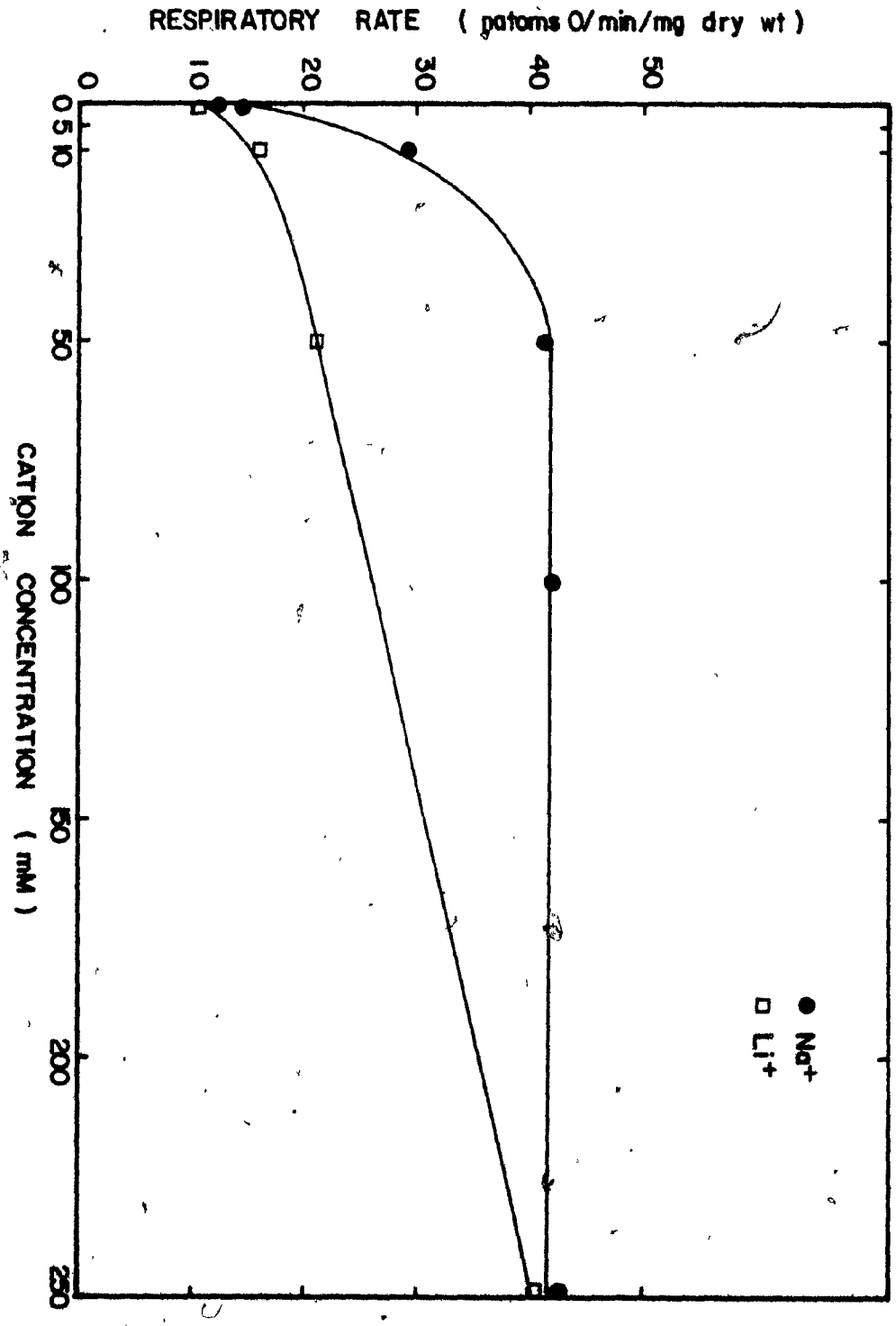

RESPIRATORY RATE (natoms O/min/mg dry weight)
0
10
20
30
40
50
60
Na+ CONCENTRATION (mM)
0
50
100
150
200
250
10 mM K+
0 mM K+

Figure 15. Effect of Li^+ on the oxidation of NAD^+ plus ethanol in toluene-treated cells.

Na^+ (●) and Li^+ (□) were added to the reaction mixture as NaCl- and LiCl-salt solutions, respectively, as described in Figure 7. NAD^+ was present at 2.5 mM and ethanol at 10 mM. The respiratory rates recorded were all corrected for endogenous respiration.


RESPIRATORY RATE (patoms O/min/mg dry wt)
0
10
20
30
40
50
CATION CONCENTRATION (mM)
0.5
10
50
100
150
200
250
Li+
Na+

D. Oxidation of succinic acid

a. Oxidation of succinate by intact cells

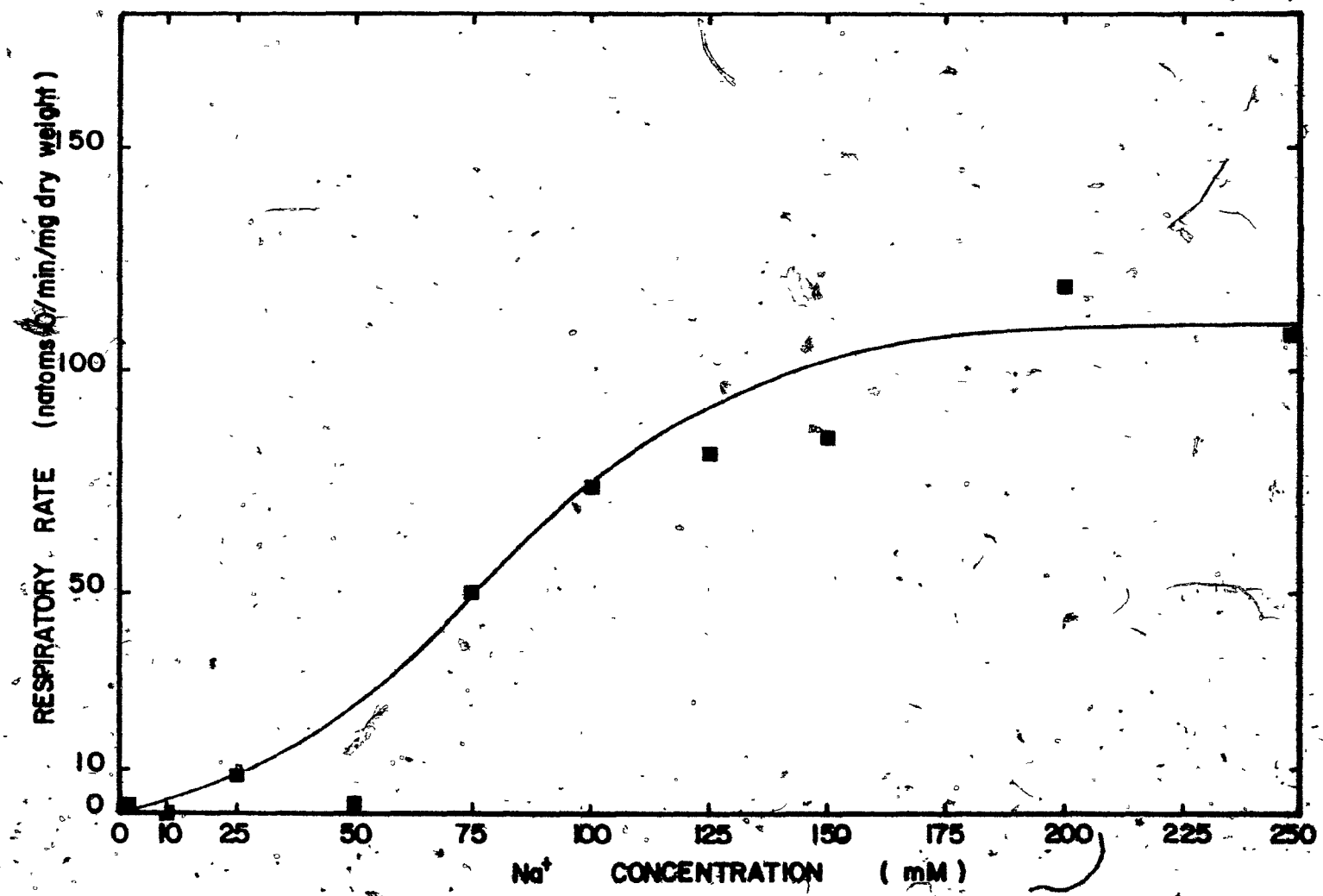
Earlier studies conducted on marine bacterium, A. haloplanktis had shown that Na^+ was required for maximum succinate oxidation (MacLeod et al., 1958). A more detailed investigation, however, had shown that the intracellular enzymes of the tricarboxylic acid cycle did not require Na^+ (MacLeod, 1965). These findings were thus extended by investigating the effect of Na^+ on succinate oxidase activity in whole cells and cell-free extracts. Figure 16 shows that the maximum succinate oxidation rate, by intact cells, was achieved in the presence of 150 mM Na^+. The activation of succinate respiration exhibited sigmoidal kinetics with respect to Na^+ concentration, indicating that there is some cooperative interaction between Na^+ and succinic acid. A point of interest was the finding that no respiratory activity was present with succinate as substrate in the absence of Na^+.

b. Oxidation of succinate by cell-free extracts

Since the Na^+ requirement for succinate oxidation was so much in excess of that required for maximum ethanol and NADH oxidation by whole cells, it seemed necessary to examine whether this requirement for Na^+ did not, in fact, reflect the involvement of Na^+ in the transport of the substrate. In order to test this possibility, whole cells were disrupted to produce cell-free extracts (CFE).

Figure 16. Quantitative requirement for Na^+ for the oxidation of succinate by whole cells.

Conditions were as described in Figure 1. The substrate was added to a final concentration of 10 mM succinate in the reaction medium. The respiratory rate recorded was corrected for endogenous respiration at each level of Na^+ tested.


RESPIRATORY RATE (natoms O/min/mg dry weight)
150
100
50
10
0
0
10
25
50
75
100
125
150
175
200
225
250
Na+ CONCENTRATION (mM)

The effect of Na^+ on the oxidation of succinate by CFE was subsequently measured. Since the cell-free extracts consist of membrane fragments, the question of transporting the substrate into the cell is thus overcome. The results obtained indicated that succinic oxidase activity was Na^+-independent (Figure 17).

The respiratory rate of succinate in the CFE was considerably lower than even the endogenous rate in whole cells. This could be because either the succinate dehydrogenase is soluble, or it is loosely bound to the membrane such that upon passage through the French pressure cell or during the washing procedure, some component of the succinate oxidase system is released from the membrane, thereby becoming diluted in concentration.

Hence, it is evident from the data presented that there is a requirement for 150 mM Na^+ for the uptake of succinate by whole cells. However, there appears to be no requirement for this cation in the oxidation of succinate. These findings are in accordance with results obtained by Droniuk et al. (unpublished data, 1979) showing that the uptake of ^{14}C-succinate is Na^+-dependent with a specific requirement for 150 mM Na^+ for maximum uptake.

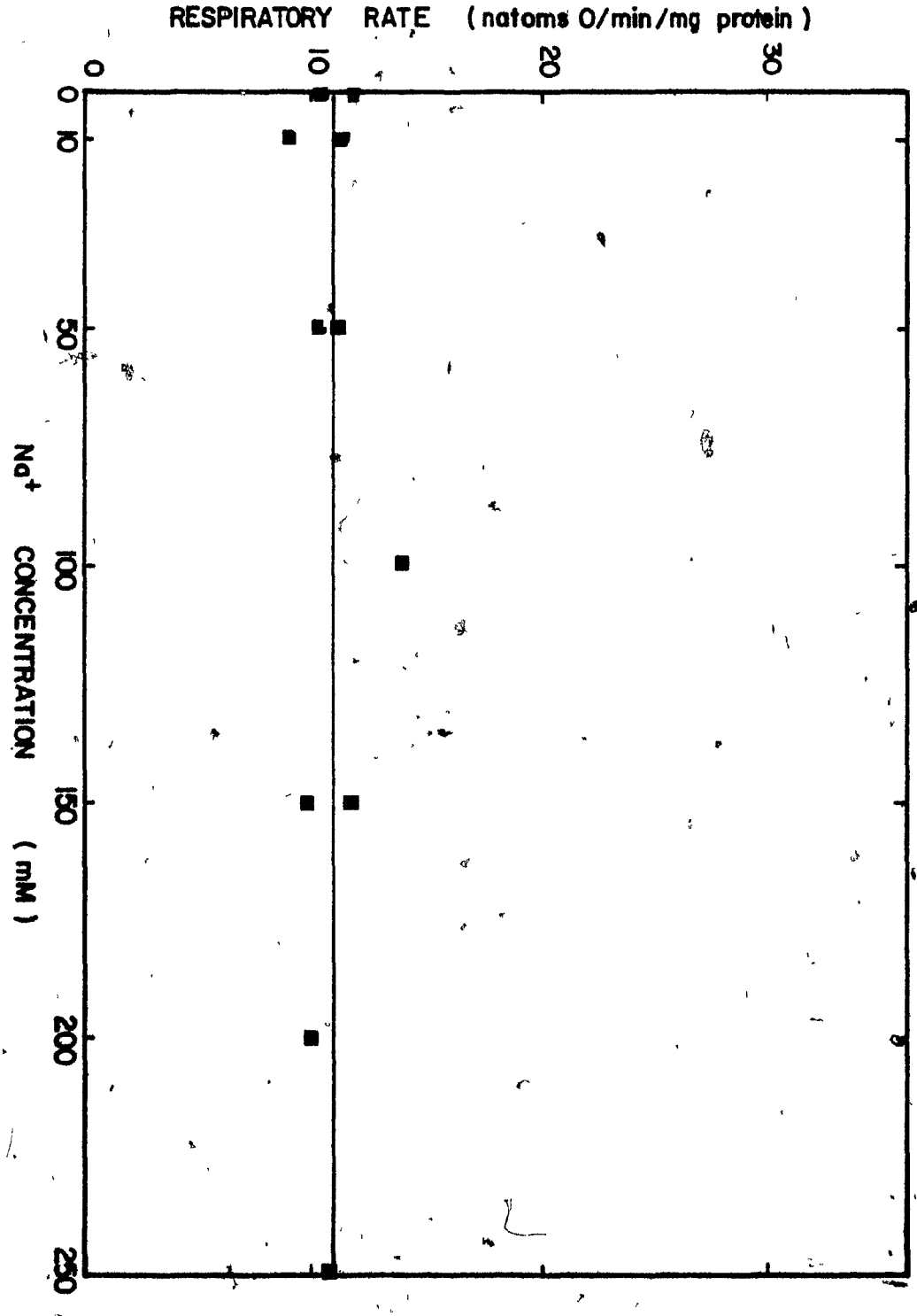
E. Oxidation of ascorbate/TMPD

a. Oxidation of ascorbate/TMPD by whole cells

Studies with A. haloplanktis have revealed that the artificial electron donor, ascorbate/TMPD, can be oxidized by the organism and

Figure 17. Effect of Na^+ on the oxidation of succinate by cell-free extracts of A. haloplanktis.

The composition of the suspending medium was as in Figure 1. The substrate was added to a final concentration of 10 mM succinate. Respiratory rates recorded have been corrected for the endogenous respiration at each level of Na^+ tested.


RESPIRATORY RATE (natoms O/min/mg protein)
0
10
20
30
Na+ CONCENTRATION (mM)
0
50
100
150
200
250

appears to introduce electrons into the respiratory chain at the level of cytochrome c (Sprott and MacLeod, 1974). Before attempting to examine the effect of Na^+ on the cytochrome c oxidase activity in whole cells, it seemed necessary to determine the optimum concentrations of ascorbate/TMPD for maximum oxidation rates. Figure 18 demonstrates that at 250 mM Na^+, the optimum concentration of ascorbate/TMPD for maximum oxidase activity was 10 mM ascorbate/ 0.5 mM TMPD. Once the optimum concentration had been established, the ascorbate/TMPD oxidase activity by whole cells was determined as a function of the Na^+ concentrations. The salt-dependent ascorbate/ TMPD oxidase activity is shown in Figure 19. As is evident from the results, maximum oxidase activity is obtained with 5 mM Na^+. In an attempt to examine whether ascorbate/TMPD oxidase is specifically activated by Na^+, other cations as K^+ and Li^+ were examined for their ability to stimulate ascorbate/TMPD oxidation. Figure 20 shows that the oxidation of ascorbate/TMPD is activated equally by Na^+ and Li^+; that Li^+ can replace Na^+ entirely. K^+, however, had no effect in reducing the requirement for Na^+ for maximum ascorbate/ TMPD oxidation (Figure 21). Following the observation that the presence of K^+ in the reaction medium increased the relative rate of ascorbate/TMPD oxidation, it was decided to investigate whether ascorbate/TMPD oxidase had a requirement for K^+. Figure 21 demonstrates that in the absence of Na^+, ascorbate/TMPD oxidation had a requirement for 2 mM K^+. Thus, for the maximum oxidation of the artificial substrate ascorbate/TMPD by whole cells, there is a

Figure 18. Determination of the concentrations of Ascorbate (■) and TMPD (●) required for the maximum respiratory activity of whole cells, in the presence of 250 mM Na^+.

In Graph A, the concentration of Ascorbate was maintained at 10 mM, and the amount of TMPD varied. In Graph B, the concentration of TMPD was maintained at 0.5 mM and the concentrations of Ascorbate varied. All other conditions are as described in Materials and Methods. The respiratory rates at each level of Ascorbate/TMPD tested were adjusted for endogenous and autoxidation rates.

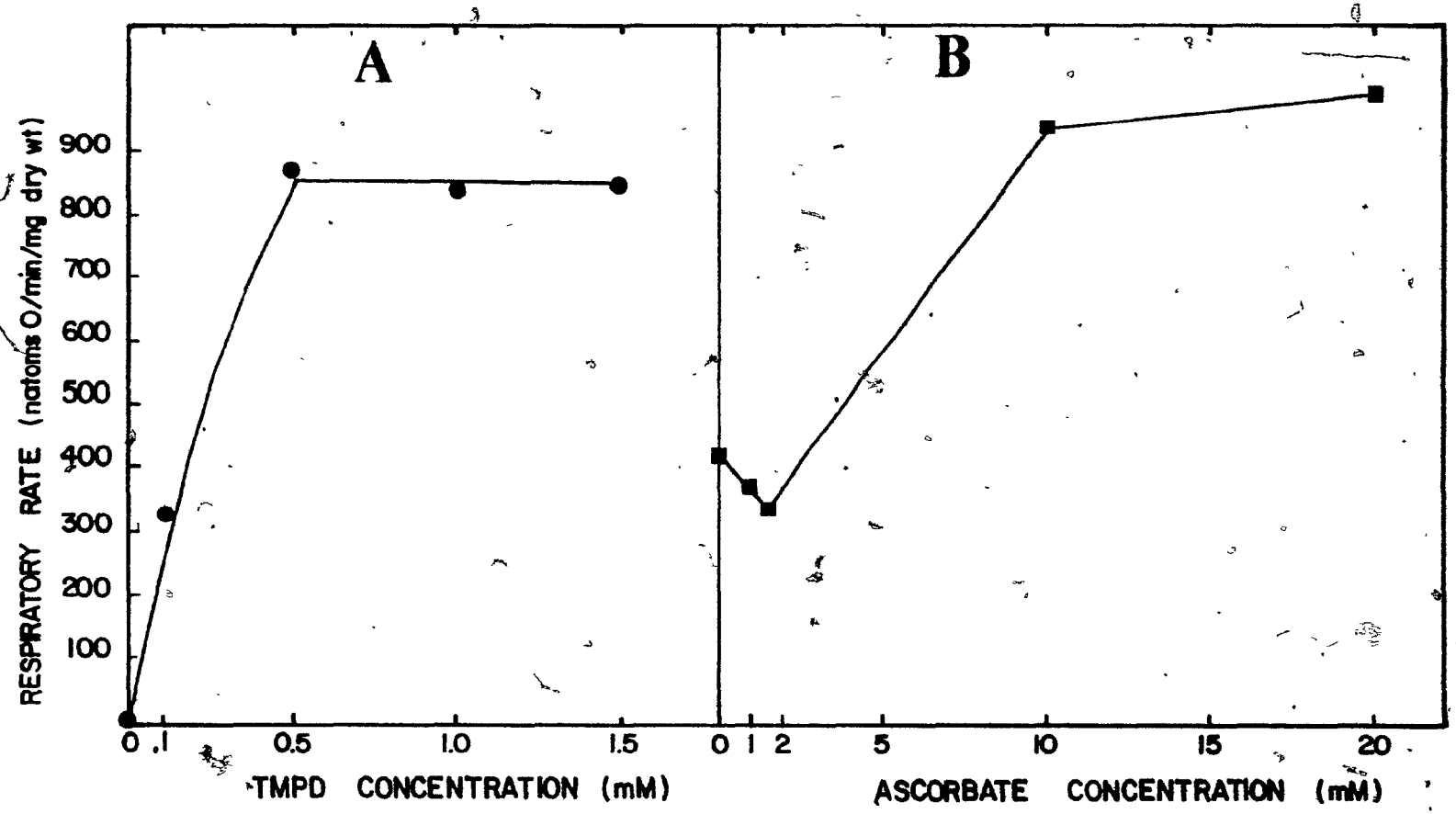

A
B
RESPIRATORY RATE (natoms O/min/mg dry wt)
900
800
700
600
500
400
300
200
100
0 .1 0.5 1.0 1.5
TMPD CONCENTRATION (mM)
0 1 2 5 10 15 20
ASCORBATE CONCENTRATION (mM)

Figure 19. Effect of Na^+ on the Ascorbate/TMPD oxidase activity in intact cells.

Cells were suspended in a reaction medium containing complete salt solution and different volumes of NaCl-salt solution, such that the concentration of Na^+ present in the reaction mixture was that which was required under the conditions of assay. At each concentration of Na^+, the ASC/TMPD autoxidation rate was determined in the absence of cells. The respiratory rates recorded were corrected for both endogenous oxidation and autoxidation rate at each concentration of Na^+ tested. The concentration of substrate used was 10 mM Ascorbate/0.5 mM TMPD.

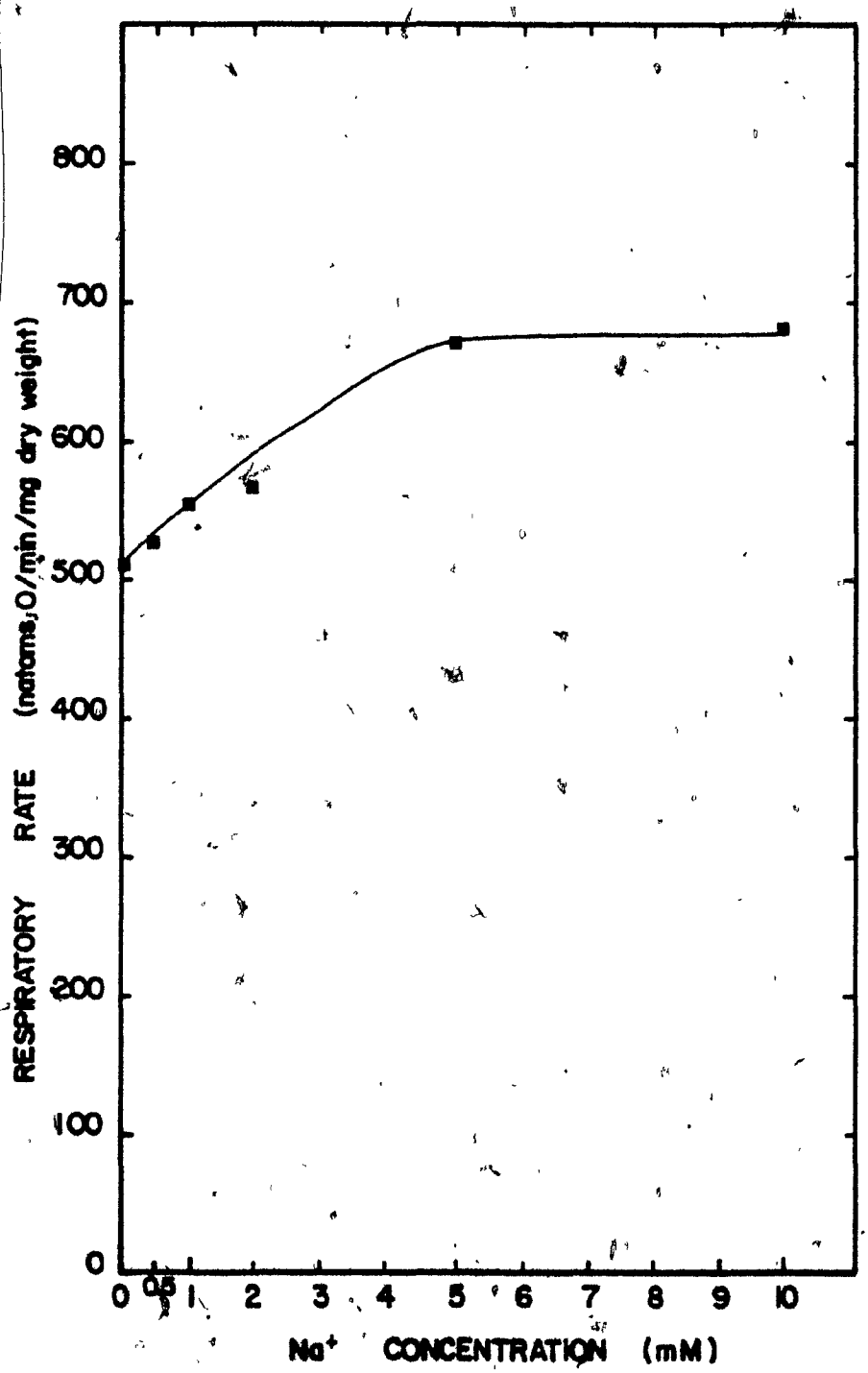

RESPIRATORY RATE (natoms,O/min/mg dry weight)
800
700
600
500
400
300
200
100
0
0 0.5 1 2 3 4 5 6 7 8 9 10
Na+ CONCENTRATION (mM)

Figure 20. Ascorbate/TMPD oxidase activity in whole cells in the presence of increasing concentrations of NaCl (■) and LiCl (□).

Conditions were as described in Figure 19, except that Li^+ was added as a LiCl salt-solution. In both curves, the respiratory rates were adjusted for endogenous respiration and Ascorbate/TMPD autoxidation rate at each concentration of cation tested.

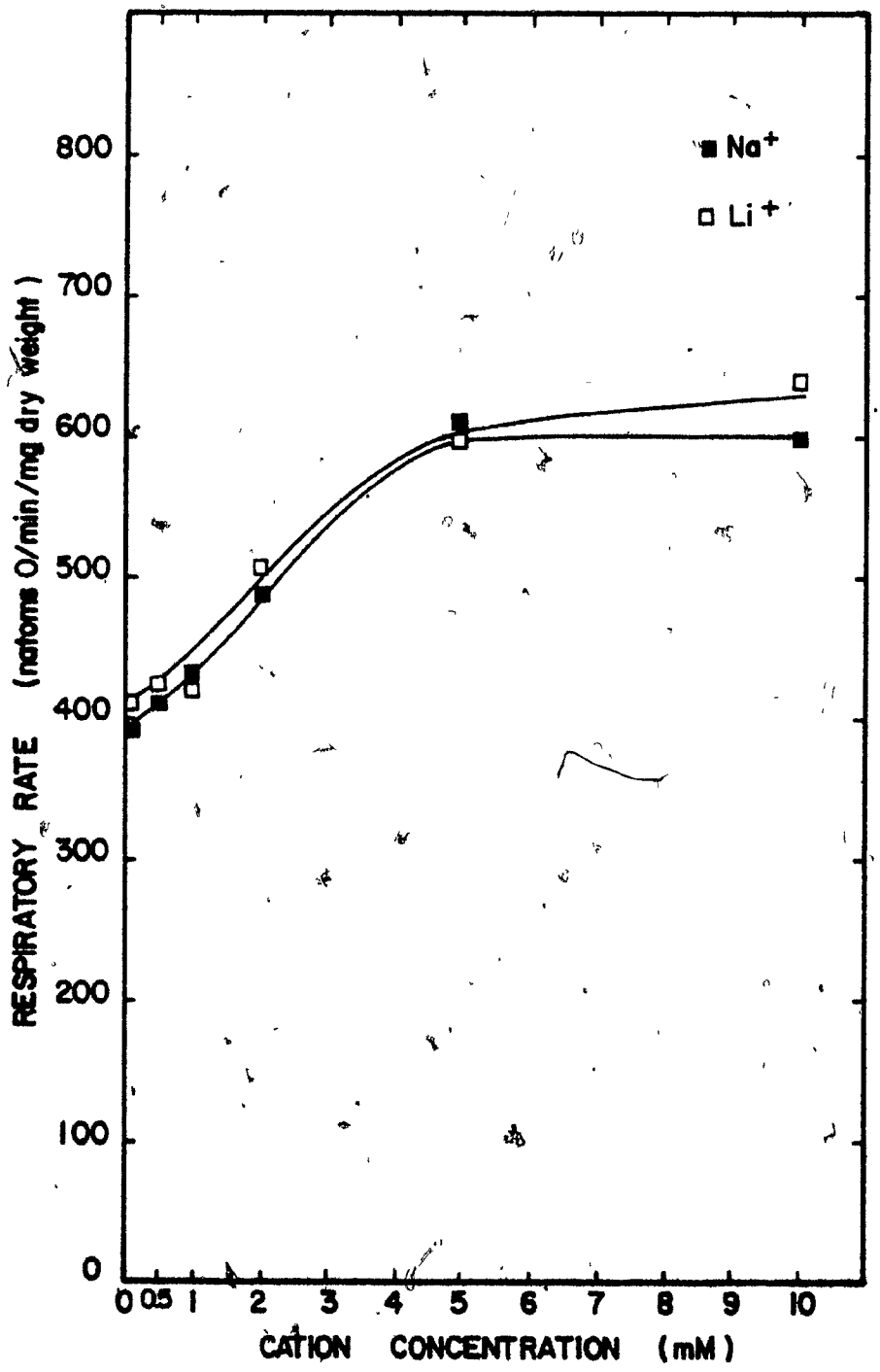
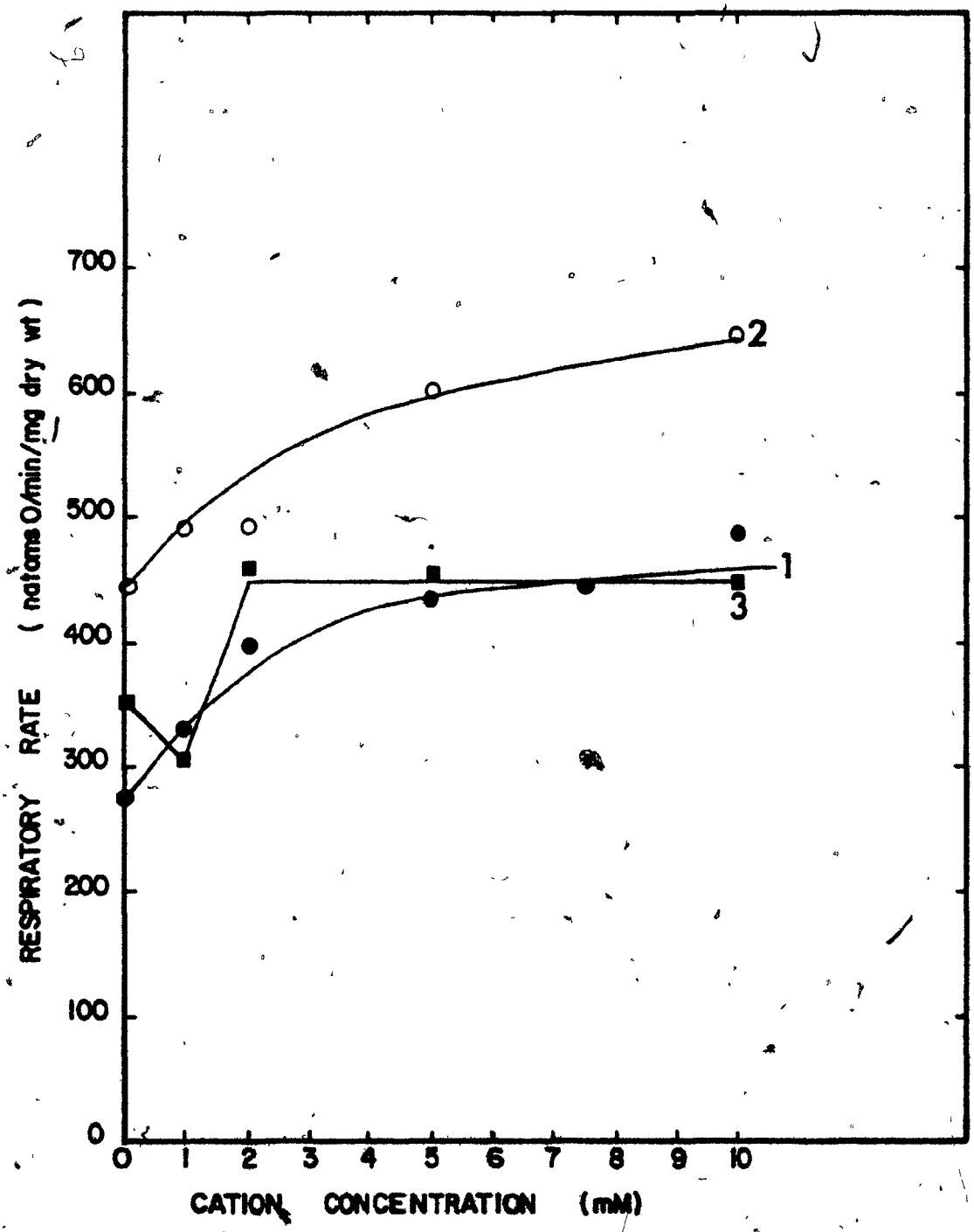

■ Na+
□ Li+
RESPIRATORY RATE (natoms O/min/mg dry weight)
800
700
600
500
400
300
200
100
0
0 0.5 1 2 3 4 5 6 7 8 9 10
CATION CONCENTRATION (mM)

Figure 21. Capacity of Na^+ and K^+ to stimulate the oxidation of Ascorbate/TMPD by intact cells of A. haloplanktis.

Curve 1 - response to Na^+ in the absence of added K^+.
Curve 2 - response to Na^+ in the presence of 10 mM K^+.
Curve 3 - response to K^+ in the absence of Na^+.
The conditions for the determination of the Na^+ effect on the Ascorbate/TMPD oxidase activity in the absence and presence of K^+ were as described in Figure 2. The conditions for the determination of the K^+ effect on the Ascorbate/TMPD oxidase activity in the absence of Na^+ were as described in Figure 3. In all three assays, the concentrations of substrates in the reaction medium were 10 mM Ascorbate/0.5 mM TMPD. The respiratory rates recorded at each level of cation examined, were adjusted for endogenous and autoxidation rates.


RESPIRATORY RATE (natoms O/min/mg dry wt)
700
600
500
400
300
200
100
0
0 1 2 3 4 5 6 7 8 9 10
CATION CONCENTRATION (mM)
1
2
3

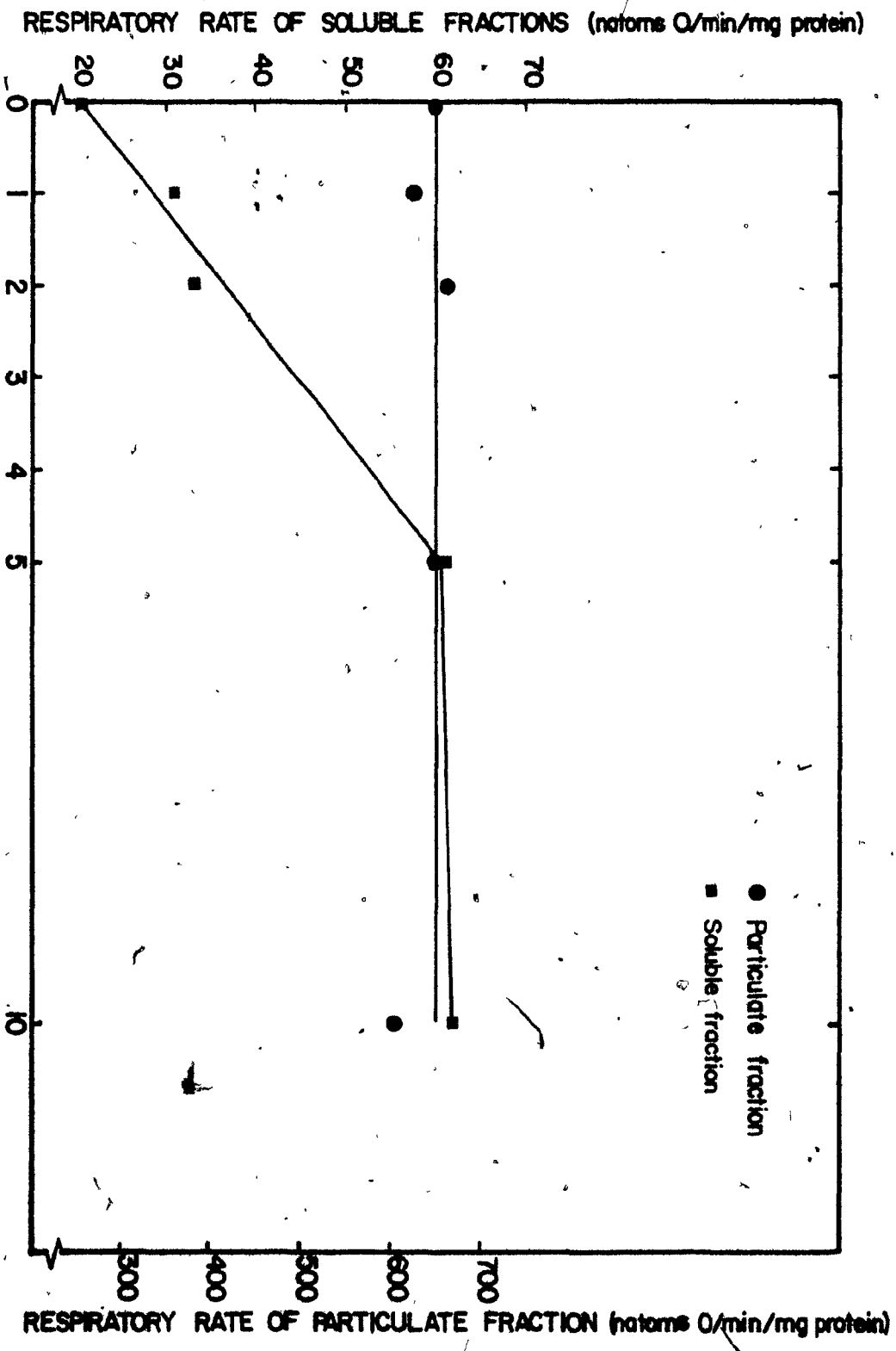
requirement for 5 mM Na^+ and 2 mM K^+; K^+ appeared to have no function in sparing Na^+.

b. Oxidation of ascorbate/TMPD by cell-free extract

Evidence provided by Calcott and MacLeod (1974, unpublished data) suggests that there are two terminal oxidases in A. haloplanktis: an o-type cytochrome and a c-type oxidase. In addition, there appears to be a soluble periplasmic cytochrome c_{549} which acts as a terminal oxidase only for the oxidation of artificial substrate ascorbate/TMPD (Knowles and MacLeod, 1974). In view of these data, it became of interest to investigate which of the two ascorbate/TMPD oxidases, the membrane-bound oxidase or the soluble cytochrome c, was Na^+-dependent. Figure 22 demonstrates the respiratory activities obtained of the oxidation of ascorbate/TMPD by the particulate and soluble fractions of the cell-free extracts. As is evident from the data, there is considerable ascorbate/TMPD oxidase activity in the membrane fraction. However, this activity was not stimulated further by Na^+. The high speed supernatant fraction was also shown to have ascorbate/TMPD oxidase activity. This activity, in contrast, was shown to be Na^+-dependent. Several experiments conducted under identical conditions of assay revealed that the Na^+ requirement for maximum activity of ascorbate/TMPD oxidase was either 2 mM or 5 mM. The absolute Na^+ requirement for ascorbate/TMPD oxidation by the soluble fraction was difficult to ascertain. However, since

Figure 22. Effect of Na^+ on the oxidation of Ascorbate/TMPD by the particulate (●) and soluble fractions (■) of A. haloplanktis.

Composition of the suspending medium was as described in Figure 1. All other conditions were as described in Materials and Methods. The concentration of the substrates in the reaction medium was 10 mM Ascorbate/0.5 mM TMPD. The respiratory rates, recorded as natoms 0/min/mg protein, were all corrected for the endogenous and autoxidation rates at each level of Na^+ tested.


RESPIRATORY RATE OF SOLUBLE FRACTIONS (natoms O/min/mg protein)
20
30
40
50
60
70
0
1
2
3
4
5
10
Na+ CONCENTRATION (mM)
● Particulate fraction
■ Soluble fraction
300
400
500
600
700
RESPIRATORY RATE OF PARTICULATE FRACTION (natoms O/min/mg protein)

cytochrome levels and their functional activities are highly susceptible to change with minimum alteration in aeration and O_2 tension (Haddock and Jones, 1977; Jones, 1978; M. Jones 1975; Weston, Knowles and Collins, 1974), it is possible that some such factor is functioning in the case of ascorbate/TMPD oxidation by the soluble fraction of this bacterium. However, this remains only as a conjecture.

2. Na^+ requirement for the uptake of ^{14}C-AIB into whole cells

Previous studies have shown that the primary function of Na^+ in Alteromonas haloplanktis is to permit transport of metabolites into the cell. Among the substrates examined was ^{14}C-α-aminoisobutyric acid (^{14}C-AIB), a non-metabolizable analogue of L-alanine, which was found to require 200 mM Na^+ in order to be transported into the cell (Drapeau and MacLeod, 1963, 1966). Further work done by Sprott et al. (1972) demonstrated that the Na^+-dependent transport process is an active one energized by the oxidation of electron donors. Data have already been presented in the earlier section of this thesis that oxidation of various electron donors appear also to have a requirement for Na^+. In view of these findings, it was of interest to test the Na^+ requirement for ^{14}C-AIB uptake into whole cells stimulated by the respiration of several substrates.

A. Uptake of ^{14}C-AIB as energized by oxidative substrates

A comparison of the Na^+ requirement for the transport of ^{14}C-AIB using various energy sources is shown in Figure 23. As is evident from the data, the initial rate of uptake of AIB required about 250 mM Na^+, irrespective of the electron donor oxidized to energize the process.

The concentrations of the substrates used were those which gave maximum oxidative activity with the exception of the artificial electron donor ascorbate/TMPD, which appeared maximally to energize transport at concentrations of 10 mM ascorbate/1 mM TMPD (Figure 24).

Ethanol stimulated ^{14}C-AIB uptake at a rate comparable to that obtained with ascorbate/TMPD, whereas NADH energized transport to a slightly greater extent, particularly at lower Na^+ concentrations. The possible reason for this is that NADH is oxidized at a faster rate, thereby generating a greater proton gradient and hence the higher uptake rate. In the case of ethanol, ascorbate/TMPD and NADH, these exogenous substrates are oxidized primarily by the enzymes located on the outer surface of the membrane and need not be transported into the cell. Succinate, in contrast, is oxidized by an enzyme located on the cytoplasmic side of the membrane and thus has to be transported into the cell prior to its oxidation. Succinate transport has been demonstrated to require 150 mM Na^+. In view of these results, initial rate of AIB uptake would be limited

Figure 23. Effect of Na^+ concentration on the initial rates of AIB uptake by A. haloplanktis in the presence of various energy sources.

Cells were suspended at a cell density of 100 µg dry weight per ml in a reaction medium containing complete salt solution and different volumes of NaCl-salt solution such that the final concentration of Na^+ in the reaction mixture was that required under the conditions of the assay, maintaining the concentrations of all other salts constant. All other conditions were as described in Materials and Methods. The final concentration of the various oxidizable substrates used to stimulate the uptake of ^{14}C-AIB were:

●—·—·—● : NADH, 2.5 mM

△—··—··—△ : Ethanol, 10 mM

■———■ : Ascorbate, 10 mM/TMPD, 1.0 mM

□———□ : Succinate, 10 mM

○———○ : No added energy source - endogenous substrates

Radioactivity of the cells was measured after 3 minutes of incubation in the presence of 200 µM ^{14}C-AIB (0.5 µCi/µmole specific activity).

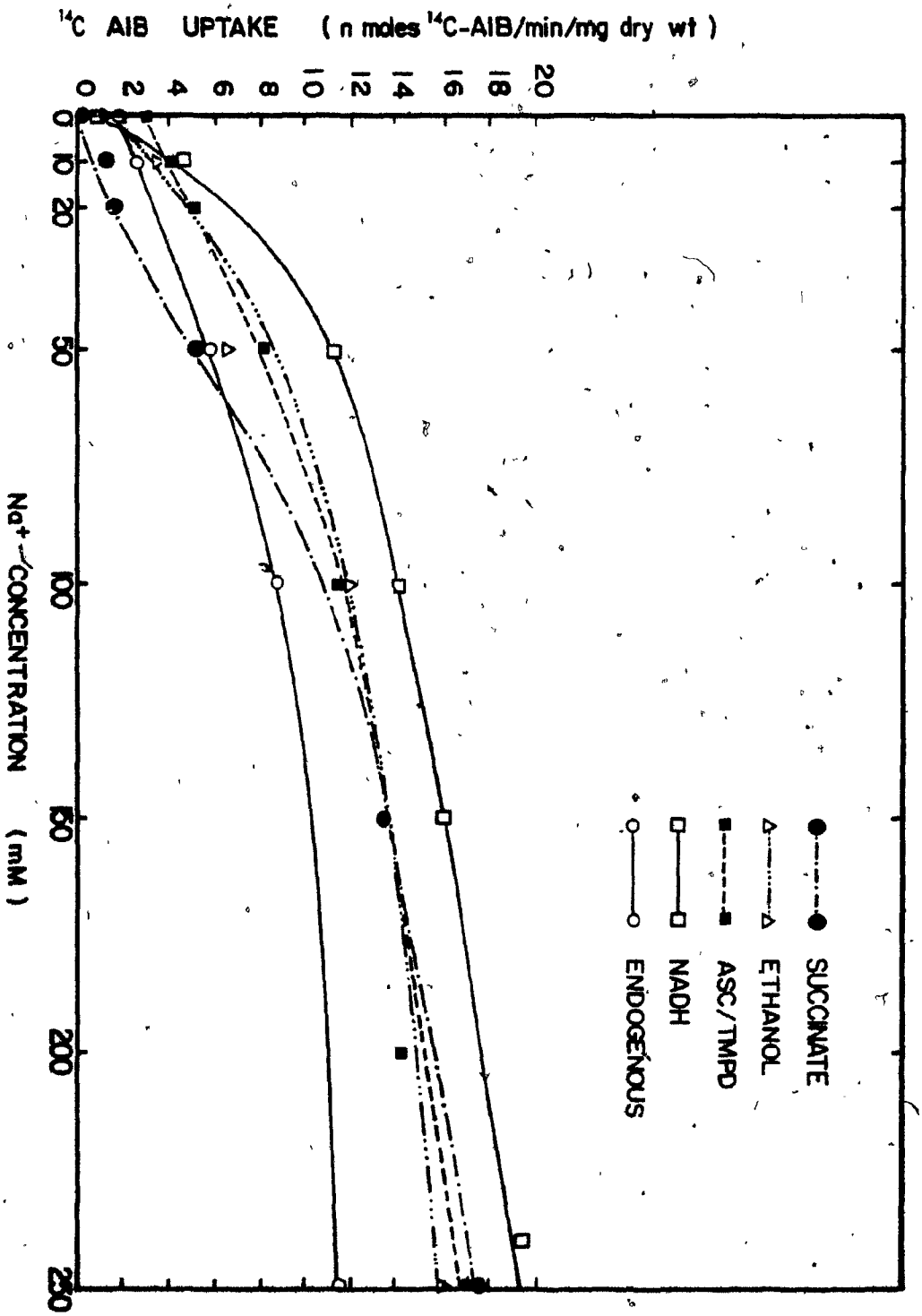
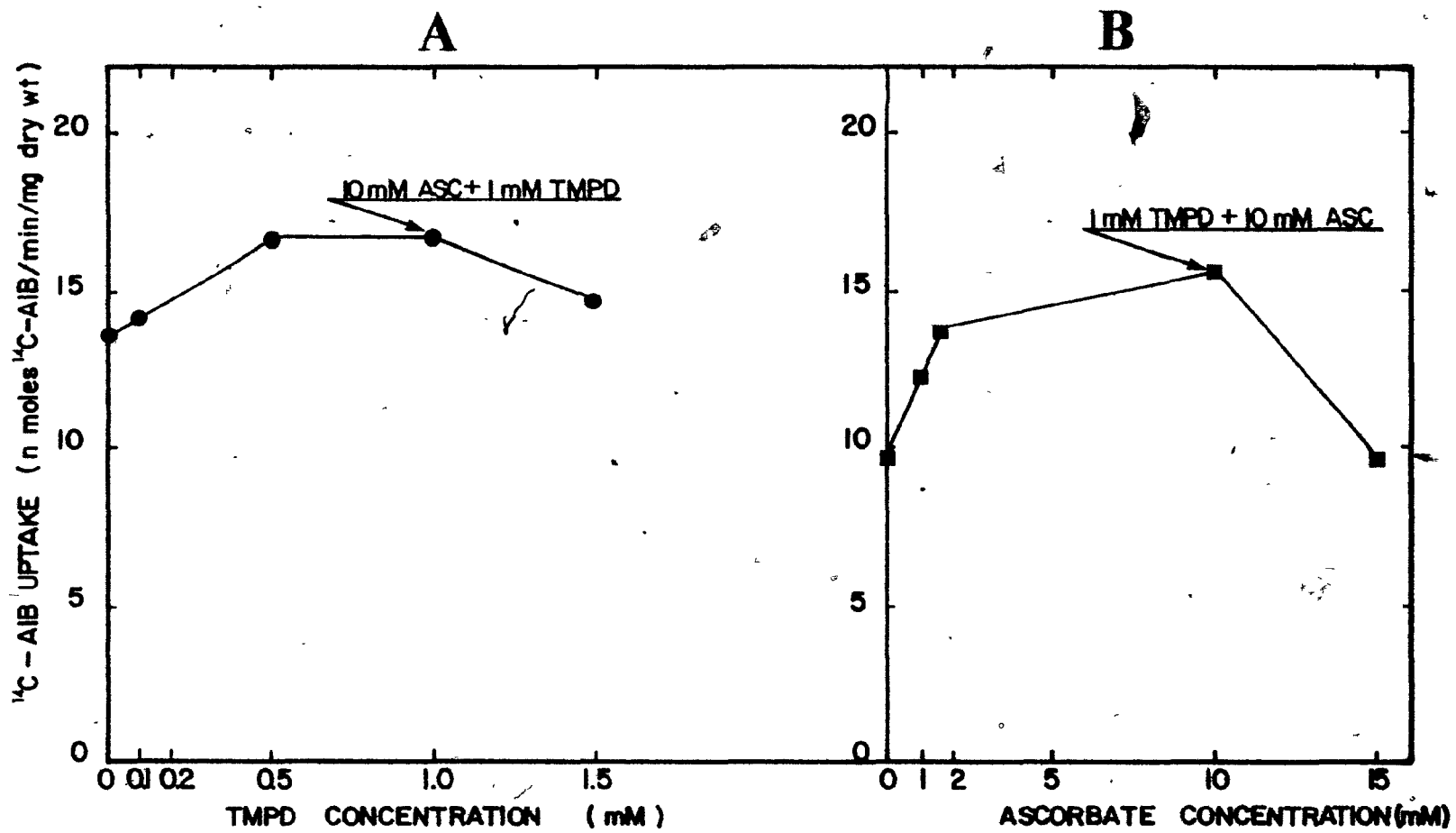

^{14}C AIB UPTAKE (n moles ^{14}C-AIB/min/mg dry wt)
0 2 4 6 8 10 12 14 16 18 20
0 10 20 50 100 150 200 250
Na+ CONCENTRATION (mM)
SUCCINATE
ETHANOL
ASC/TMPD
NADH
ENDOGENOUS

Figure 24. Determination of the concentrations of Ascorbate (■) and TMPD (●) required to stimulate maximum uptake of ^{14}C-AIB into whole cells of A. haloplanktis, in the presence of 250 mM NaCl.

In Graph A, the concentration of Ascorbate was maintained at 10 mM and the amounts of TMPD were varied. In Graph B, the concentration of TMPD was kept constant at 1.0 mM and the concentrations of Ascorbate were varied. The radioactivity of the cells was determined after 3 minutes incubation in the presence of 200 µm ^{14}C-AIB (0.5 µCi specific activity).


A
B
^{14}C – AIB UPTAKE (n moles ^{14}C-AIB/min/mg dry wt)
0
5
10
15
20
10 mM ASC + 1 mM TMPD
1 mM TMPD + 10 mM ASC
0 0.1 0.2 0.5 1.0 1.5
TMPD CONCENTRATION (mM)
0 1 2 5 10 15
ASCORBATE CONCENTRATION (mM)

by the Na^+ requirement for succinate transport. It is evident from Figure 23 that at low Na^+ concentrations, the initial rate of AIB uptake is low and it is only at concentrations greater than 50 mM Na^+ that any significant increase in uptake rate is apparent, reaching a rate comparable to that obtained with other oxidizable substrates at high Na^+ concentrations.

It was observed that a considerable amount of AIB is taken up even in the absence of added electron donors; this is probably due to the endogenous activity of the cells. Even though endogenous oxidation has been shown to be much lower than oxidation of various substrates, it appears to be at an adequate rate to create a protonmotive force (pmf) sufficient to drive ^{14}C-AIB uptake.

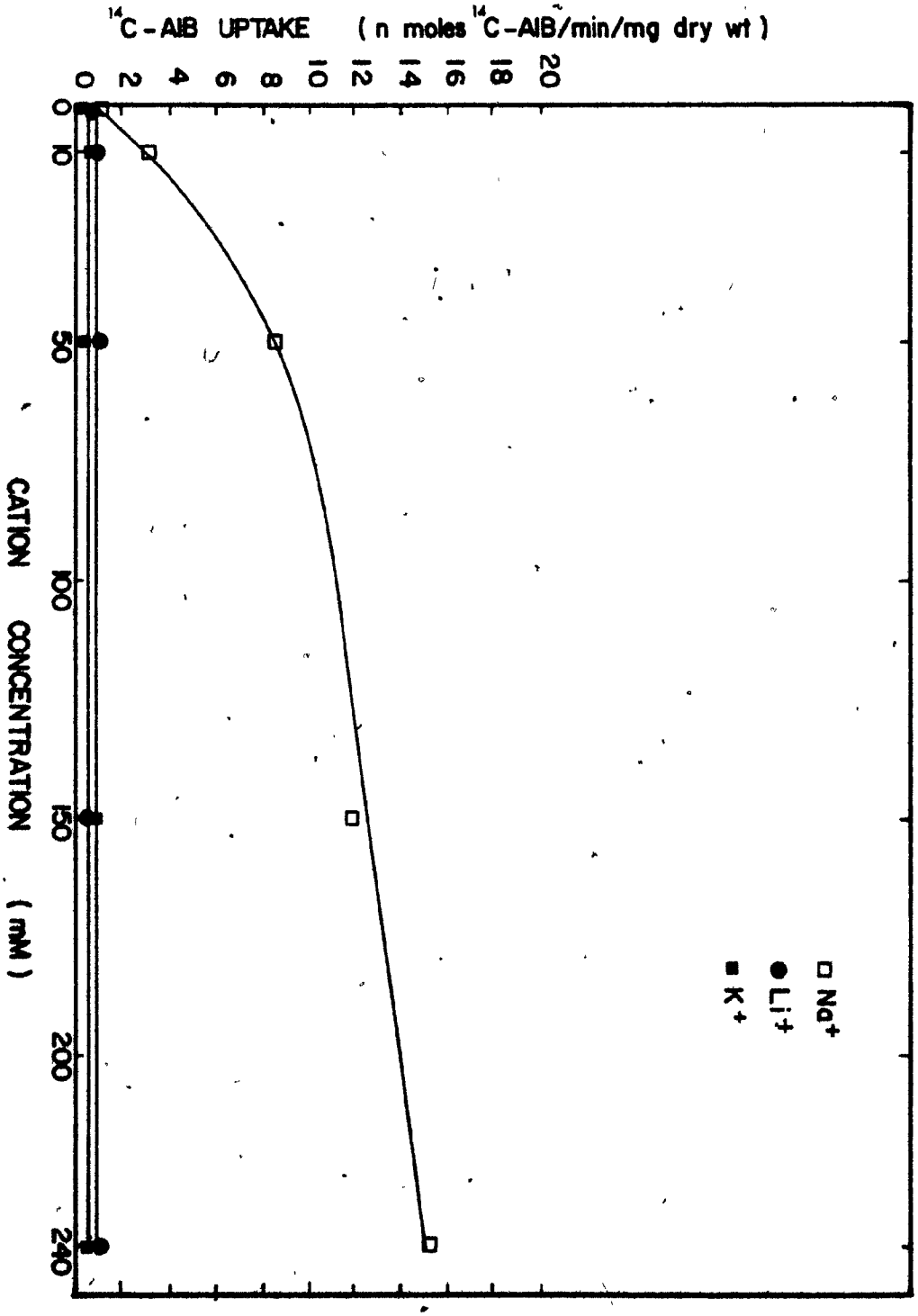
B. Specificity of Na^+-dependent AIB uptake

Previous studies conducted in this laboratory have shown that there is a requirement for K^+, in addition to Na^+ for the maximum uptake of AIB into whole cells (Thompson and MacLeod, 1971). K^+ was shown to act at the intracellular level to bring about the accumulation of AIB against a concentration gradient. However, this function of K^+ was demonstrated to be separate from the role of Na^+ in transport, since, even in the presence of 200 mM K^+, the requirement for Na^+ was not decreased (Drapeau and MacLeod, 1966; Wong, 1968).

This highly specific requirement for Na^+ for AIB transport was confirmed, since neither K^+ nor Li^+ showed any significant

Figure 25. Effect of various monovalent cations on the uptake of ^{14}C-AIB into intact cells of A. haloplanktis.

Na^+ (□) was added to the reaction medium as NaCl-salt solution; Li^+ (●) and K^+ (■) was added as LiCl- and KCl-salt solution; all other conditions were as described in Figure 23. The oxidative substrate used to stimulate uptake of ^{14}C-AIB was 10 mM ethanol. Radioactivity of the cells was measured after 3 minutes of incubation.


^{14}C-AIB UPTAKE (n moles ^{14}C-AIB/min/mg dry wt)
0 2 4 6 8 10 12 14 16 18 20
0 10 50 100 150 200 240
CATION CONCENTRATION (mM)
□ Na+
● Li+
■ K+

capacity to spare the requirement for Na^+. There was no uptake of ^{14}C-AIB, even in the presence of 250 mM LiCl or KCl (Figure 25).

It is thus concluded, based on the observations that Na^+ requirement for AIB uptake was several times greater than the Na^+ requirement for respiration, that Na^+ has a role in transport which is separate from its role in the oxidative metabolism of this marine bacterium. However, Na^+ acts as a cofactor, both for the active transport of metabolites (AIB) as well as for the maximum respiratory activity of A. haloplanktis.

DISCUSSION

The Na^+-dependent activation of the respiratory system of A. haloplanktis was examined by determining the effect of Na^+ on the oxidation of several exogenously added substrates. The oxidizable substrates examined, by virtue of their redox potentials, could donate electrons directly to various electron carriers along the respiratory chain. Results obtained in the present study demonstrated that NADH was actively oxidized by whole cell suspensions of A. haloplanktis; Na^+ had no stimulatory effect. However, when the cells were toluenized, exposing the inner surface of the cytoplasmic membrane to the substrate, there was a significant increase in NADH oxidase activity, and the maximum rate of oxidation was obtained in the presence of 100 mM Na^+ (Figure 5). These observations suggest that there are two respiratory-linked NADH dehydrogenases in this organism; one is located on the outer surface of the cytoplasmic membrane and the other on the inner surface. Based on the results obtained, the characteristic which distinguishes these two enzymes is their response to Na^+. Na^+-dependent activation of NADH oxidase has also been observed in another marine bacterium, Vibrio alginolyticus. Unemoto et al. (1977) reported that the lysed cells of this organism exhibited maximum oxidation of exogenously added NADH in the presence of 0.3 M NaCl.

Although NADH dehydrogenase in most procaryotes is located only on the inner surface of the membrane, there have been some reports on the presence of this enzyme on the outer surface of the membrane. Lombardi et al. (1974) have observed NADH oxidation by intact cells of E. coli. Further studies on the fractionation of the electron transport chain of E. coli revealed that there are two NADH dehydrogenases, differing in their ability to reduce various dye acceptors (Hendler and Burgess, 1974). Preliminary experiments conducted with ferricyanide in A. haloplanktis suggested that there are two NADH dehydrogenases in this bacterium (Calcott et al., unpublished data).

Besides the observation that the two NADH dehydrogenases in this marine organism differ in their response to Na^+, the possibility that they may also differ in the nature of their prosthetic groups still needs to be examined. Studies on the membrane-bound NADH dehydrogenase of Photobacterium phosphoreum have shown that this enzyme contains FAD as prosthetic group. This NADH dehydrogenase was found to be activated by monovalent cations. These characteristics were in contrast to the nature of a NADH-FMN reductase, a similar enzyme obtained from the soluble fraction of the same organism. The activity of the latter enzyme was almost independent of Na^+ or K^+ concentrations in the reaction medium (Imagawa et al., 1978; Watanabe et al., 1977).

Earlier work conducted with whole cells of A. haloplanktis (Sprott et al., 1975) suggested that under normal physiological conditions where the intracellular K^+ concentration is high, Na^+ is expelled from within the cell. More recently, Niven and MacLeod (1978) established that this organism possesses a Na^+/H^+ antiporter; the transmembrane proton gradient effected by respiration could then be used to drive the antiporter-mediated extrusion of Na^+. Although the K^+ concentration in actively respiring cells is high, the intracellular Na^+ concentration would be maintained at a low level. As the NADH dehydrogenase on the inner surface of the cytoplasmic membrane requires 100 mM Na^+ for maximum activity, it would seem reasonable to suggest that this enzyme may be involved in the regulation of respiratory activity. Under normal physiological conditions where the intracellular Na^+ concentration is low, the enzyme would be functioning at a suboptimal rate. However, under certain abnormal conditions where the physiology of the organism is altered, resulting in an increase in the intracellular Na^+ concentration, the Na^+-dependent NADH dehydrogenase would be activated. The resulting increase in the respiration-driven proton translocation would stimulate Na^+ extrusion via the Na^+/H^+ antiporter and the intracellular Na^+ concentration would be reduced again.

In recent studies on the specific coupling of ethanol to transport, it was suggested that A. haloplanktis contains two ethanol dehydrogenases (Sprott and MacLeod, 1974; Thompson and MacLeod, 1974). An NAD^+-linked alcohol dehydrogenase was shown to be present in the cytoplasm, but such an enzyme was not detected in membrane vesicles. Based on the observation that ethanol could still stimulate transport in membrane vesicles, it was suggested that there is a second ethanol dehydrogenase present in the membrane of this organism. Results obtained in this present study support this conclusion. It was observed that 2 mM Na^+ was required for the maximum oxidation of ethanol by whole cells, whereas in toluenized cells, 50 mM Na^+ was required for maximum oxidation of the substrate. That toluenized cells could not oxidize ethanol unless NAD^+ was present in the reaction medium, confirmed that the intracellular alcohol dehydrogenase was NAD^+-linked. As the Na^+ requirement for the cytoplasmic ethanol oxidase activity (50 mM Na^+) far exceeded the quantitative Na^+ requirement for maximum ethanol oxidation by whole cells (2 mM Na^+), it seemed likely that ethanol respiration by whole cells is catalyzed by an enzyme other than the cytoplasmic NAD^+-linked alcohol dehydrogenase - most probably through an enzyme located on the outside surface of the membrane. With the data available, it is not possible to ascertain whether the membrane-bound alcohol dehydrogenase is either NAD^+-linked via the NADH dehydrogenase located on the outer surface of the membrane, or alternatively, whether it is a flavin-linked enzyme. The

observation that the ethanol oxidase activity in toluenized cells (~ 40 natoms O/min/mg dry wt) was much less than the respiratory rate on ethanol in intact cells (~ 200 natoms O/min/mg dry wt) suggested that treatment with toluene destroys the membrane-bound alcohol dehydrogenase. Hence, the respiratory rate on ethanol measured in toluene-treated cells would reflect only the oxidation of ethanol by the soluble NAD^+-linked ethanol dehydrogenase.

The NAD^+ dependency of the cytoplasmic ethanol oxidase activity supports the conclusion that ethanol oxidation generates endogenous NADH in whole cells. Since the cytoplasmic membrane is impermeable to NADH, the NADH generated intracellularly would probably be oxidized by the NADH dehydrogenase located on the inner surface of the membrane. Since the maximum rate of NADH oxidation (~400 natoms O/min/mg dry wt) is tenfold greater than that of ethanol (~ 40 natoms O/min/mg dry wt) in toluenized cells, under conditions of optimum Na^+ concentrations, the respiratory rate with ethanol is apparently limited by the activity of the alcohol dehydrogenase. Furthermore, the Na^+ requirement for maximum ethanol oxidase activity (50 mM) is considerably greater than the Na^+ concentrations required for the activation of the internal NADH oxidase to produce a corresponding respiratory rate. This suggested that 50 mM Na^+, in fact, reflects the Na^+ requirement for maximum intracellular ethanol dehydrogenase activity.

Early studies on cell-free extracts of A. haloplanktis showed that none of the enzymes of the tricarboxylic acid cycle required Na^+ specifically for activity (MacLeod et al., 1958; MacLeod and Hori, 1960). Results reported in the present study confirm these observations for succinate dehydrogenase as the succinate oxidase activity of this organism was shown not to be stimulated by Na^+. However, the oxidation of the non-physiological substrates, ascorbate/TMPD, by whole cells was shown to be stimulated when 5 mM Na^+ was included in the assay system (Figure 19). A more detailed investigation revealed that the Na^+-dependent activation of ascorbate/TMPD oxidase in whole cells corresponds to the Na^+ requirement for the maximum ascorbate/TMPD oxidation by the soluble fraction obtained from cell-free extracts. A recent study on the cytochrome composition of A. haloplanktis demonstrated the presence of a soluble CO-binding cytochrome c_{549} in the periplasm of this organism (Knowles et al., 1974). The observation that there was active respiration of NADH, succinate and ascorbate/TMPD by the particulate fraction of A. haloplanktis, led to the suggestion that the soluble cytochrome c is associated with a side branch of the main respiratory chain. In a more detailed study, Calcott and MacLeod (unpublished data) demonstrated that the periplasmic CO-binding cytochrome c, present in the soluble fraction obtained from cell-free extracts, was able to accept electrons from ascorbate/TMPD and these electrons could be used in the subsequent reduction of oxygen. However, the soluble cytochrome c was unable to accept

electrons from NADH, succinate or a variety of other potential electron donors. Furthermore, it was observed that the respiratory activity associated with this c-type cytochrome was sensitive to several electron-transport inhibitors to which other terminal oxidases are usually resistant. These observations confirmed the earlier suggestion that the soluble cytochrome c_{549} is not located on the main respiratory chain of this organism, but is part of a branch pathway. This periplasmic CO-binding c-type cytochrome, although unusual, is not unique as similar cytochromes have been found in other bacteria including the marine organism, Beneckea natriegens (Weston and Knowles, 1973). However, the Na^+-dependent activation of ascorbate/TMPD oxidase in the soluble fraction of cell-free extracts has only been examined and reported for A. haloplanktis.

Results obtained in this study on the effect of Na^+ on endogenous respiration of whole cells revealed that maximum oxidation of endogenous substrates was achieved in the presence of 50 mM Na^+. Ethanol and NADH oxidases located on the inner surface of the cytoplasmic membrane have been shown to require 50 mM and 100 mM Na^+ respectively for maximum activity; however, since even in the presence of 50 mM Na^+ in the reaction medium, the intracellular concentration of Na^+ in whole cells is maintained at a low level, the reason for the Na^+-dependent activation of endogenous respiration cannot be explained.

When the effect of other monovalent cations on the respiratory system of A. haloplanktis was examined, it was observed that Li^+ could stimulate oxidation of most substrates tested, albeit at a slower rate than that activated by Na^+. Results on the ascorbate/TMPD oxidase revealed that Li^+ and Na^+ could stimulate ascorbate/TMPD oxidation to the same extent. When the effect of K^+ on respiratory activity was examined, it was found that K^+ was relatively ineffective in replacing the Na^+ requirement for maximum oxidation of NADH and ethanol in toluene-treated cells (Figures 6 and 14) and for maximum ascorbate/TMPD oxidase activity in whole cells (Figure 21). However, in the absence of K^+, the concentration of Na^+ required for maximum ethanol oxidase activity by whole cells was increased, suggesting that K^+ could partially satisfy the Na^+ requirement for ethanol oxidation (Figure 10). In the presence of 10 mM K^+, Na^+ activation of NADH oxidase in intact cells was eliminated, suggesting that the effect of Na^+ on the external NADH oxidase is not entirely specific (Figure 2). Besides a Na^+ requirement, oxidation of NADH, ethanol and ascorbate/TMPD by whole cells was shown also to require K^+ for maximum activity. This K^+ requirement was shown to be specific since even in the presence of 10 mM Na^+, the concentration of K^+ needed to activate the maximum oxidation of substrates was not reduced.

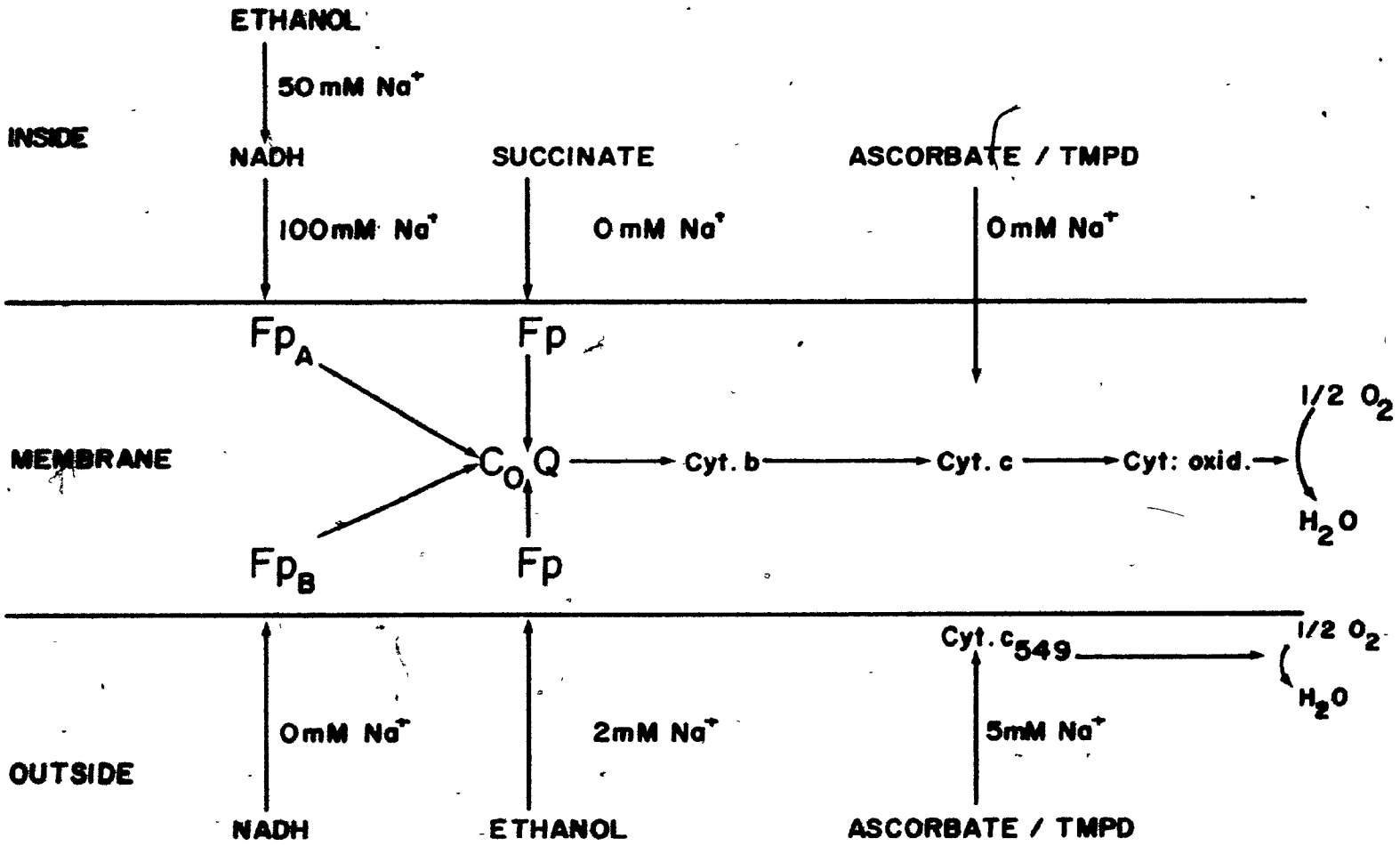
An earlier study by Sprott et al. (1975) demonstrated that the oxidation of ethanol and ascorbate/TMPD by membrane vesicles of A. haloplanktis was not stimulated by Na^+. In these experiments

substrate oxidation in the absence of Na^+ was determined in a reaction medium containing 200 mM LiCl. Results presented in this study have shown that the membrane-bound ascorbate/TMPD oxidase activity is not stimulated by Na^+, thereby confirming the observations made in membrane vesicles. In contrast, Na^+ has been shown to be required for maximum ethanol oxidase activity; Li^+ could partially replace Na^+. However, since the concentration of Na^+ required for maximum ethanol oxidation is so low (2 mM Na^+), perhaps being even less in the presence of 200 mM LiCl, it is possible that such small concentrations of Na^+ were present in contaminating amounts in a reaction medium containing no added Na^+. This would seem more likely under conditions where particular attention was not given to the possibility that such small concentrations of Na^+ would be sufficient to stimulate maximum oxidation of substrates. Thus, under the conditions of the experiment where the reaction medium contained no added NaCl, but 200 mM LiCl, it seems reasonable to suggest that the membrane vesicles were oxidizing ethanol at maximum rate, such that in the presence of higher concentrations of Na^+, no further stimulation was apparent.

A tentative scheme, showing the possible sites of Na^+-dependent activation in the respiratory chain of A. haloplanktis is presented in Figure 26. The scheme summarizes the data on Na^+ activation as presented in this study with respect to the tentative sites at which substrates could introduce electrons into the chain. However, since the composition of the respiratory chain of

Figure 26. Tentative scheme of the respiratory chain of A. haloplanktis showing the sites of Na^+-dependent activation.

Cyt, cytochrome; CoQ, coenzyme Q; Fp, flavoprotein.


ETHANOL
50 mM Na+
INSIDE
NADH
SUCCINATE
ASCORBATE / TMPD
100mM Na+
0 mM Na+
0 mM Na+
FpA
Fp
MEMBRANE
CoQ
Cyt. b
Cyt. c
Cyt: oxid.
1/2 O2
H2O
FpB
Fp
Cyt. c549
1/2 O2
H2O
0mM Na+
2mM Na+
5mM Na+
OUTSIDE
NADH
ETHANOL
ASCORBATE / TMPD

A. haloplanktis has still not been fully elucidated, the nature and sequence of the redox carriers remain only speculative at this stage.

NADH oxidase associated with the NADH dehydrogenase located on the outer surface of the cytoplasmic membrane, succinate oxidase, and the membrane-bound ascorbate/TMPD oxidase, all showed no specific Na^+ requirement for maximum activity. However, 100 mM Na^+ was required specifically for NADH oxidation via the NADH dehydrogenase located on the inside surface of the membrane. Ethanol oxidation was catalyzed by two enzymes, a membrane bound alcohol dehydrogenase which was shown to require 2 mM Na^+ for maximum activity and an NAD^+-linked cytoplasmic ethanol dehydrogenase which exhibited maximum activity in the presence of 50 mM Na^+. Ascorbate/TMPD oxidation by the soluble fraction of cell-free extracts showed maximum stimulation in the presence of 5 mM Na^+. Since of all the redox carriers subject to Na^+ activation, only the inner NADH oxidase is part of the main respiratory chain, it can be suggested that the primary site of Na^+-dependent activation in the respiratory chain of A. haloplanktis is at the reaction step of NADH-quinone oxidoreductase located on the inner surface of the membrane. Recent studies by Unemoto and Hayashi (1979) demonstrated that the site of Na^+-dependent activation in the respiratory chain of Vibrio alginolyticus is at the level of NADH: quinone oxidoreductase and more specifically at the quinone reduction portion of this step. They observed that the rate of menaquinone reduction by NADH was activated specifically by Na^+.

They further showed that the Ka value for Na^+ for menaquinone reductase was the same as that of NADH oxidase. Hence, based on these results, they concluded that the site of Na^+ activation in the electron transport chain of this organism was at the step of NADH: quinone oxidoreductase. Results obtained in the present study suggest that the primary site of Na^+ activation in the respiratory chain of A. haloplanktis is also at the level of NADH: quinone oxidoreductase located on the inside surface of the membrane. This conclusion was based on the observations that the oxidation of succinate, which introduces electrons at the level of CoQ, is Na^+-independent, whereas the NADH oxidation system located on the inner face of the cytoplasmic membrane requires 100 mM Na^+ for maximum activity. However, the present data do not allow us to conclude whether the effect of Na^+ is on the NADH dehydrogenase or the quinone reductase portion of the NADH-quinone oxidoreductase.

It is noteworthy that since all the substrates examined could be oxidized even in the absence of added Na^+ or K^+, reaching a maximum rate only when Na^+ is present in the reaction medium, the respiratory activity of A. haloplanktis would appear to be a Na^+-activated process. However, since Na^+ could be present as a contaminant in a reaction medium containing no added Na^+ at a concentration sufficient to activate the oxidation of the substrates, it is difficult to ascertain whether respiration of A. haloplanktis is, in fact, a Na^+-dependent or a Na^+-activated process. Likewise, substrate oxidations shown to be Na^+-independent do not exclude the

possibility that Na^+ is present at sufficient concentrations to satisfy the requirement of the enzyme systems for Na^+.

When the Na^+ requirement for maximum transport of metabolites was examined, results reported here provided strong support for the earlier conclusion that transport of α-AIB into intact cells is a Na^+-dependent process (Drapeau et al., 1966; Drapeau and MacLeod, 1963). It was observed that 250 mM Na^+ was specifically required for the maximum uptake of α-AIB, irrespective of the oxidizable substrate used to stimulate the process. Other cations such as K^+ and Li^+ were ineffective in activating transport of the non-metabolizable substrate (Figure 25), confirming the Na^+ specificity of the transport system(s).

According to the chemiosmotic hypothesis, the transmembrane proton gradient effected by respiration is used to extrude Na^+ from the bacterial cells by means of a Na^+/H^+ antiporter, resulting in the formation of an inwardly directed transmembrane Na^+ gradient. The chemical and electrical components of this gradient could then be used to drive the intracellular accumulation of metabolites (Mitchell, 1963, 1973). In a recent study, Niven and MacLeod (1978) provided evidence for the presence of an Na^+/H^+ antiporter in A. haloplanktis. More recently, it has been shown that both the chemical and electrical components of the trans-membrane Na^+ gradient are responsible for driving the transport of AIB into the cells, by a Na^+/AIB symport mechanism (Niven and MacLeod, 1979,

unpublished data). Results from the present study suggest that this symporter has a specific requirement for 250 mM Na^+_A for maximum activity.

In conclusion, the quantitative Na^+ requirement for transport of metabolites is separate from that for maximum respiratory activity in the marine bacterium, A. haloplanktis. Evidence for this is provided by the observation that 250 mM Na^+ was specifically required for the maximum rate of α-aminoisobutyric acid uptake, whereas depending on the substrate, the Na^+ requirement for maximum rate of respiration could be as little as zero (succinate oxidase) or as much as 100 mM Na^+ for an NAD^+-linked substrate being oxidized on the surface of the cytoplasmic membrane. The primary site of Na^+-dependent activation in the respiratory chain was shown to be at the level of NADH:quinone oxidoreductase located on the inside surface of the cytoplasmic membrane.

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