Short Title:

ENERGY COUPLING AND KINETICS OF $Na^+$-DEPENDENT TRANSPORT

Sprott

# MECHANISM OF ENERGY COUPLING AND KINETICS OF $Na^+$-DEPENDENT TRANSPORT IN CELLS AND IN ISOLATED MEMBRANE VESICLES OF A MARINE PSEUDOMONAD

by

G. Dennis Sprott

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for
the degree of Doctor of Philosophy

Department of Microbiology
Macdonald College of McGill University
Montreal, Quebec

August 1973

# ENERGY COUPLING AND KINETICS OF $Na^+$-DEPENDENT TRANSPORT

Sprott

A B S T R A C T

Ph.D. G.Dennis Sprott Microbiology

MECHANISM OF ENERGY COUPLING AND KINETICS OF $Na^+$-DEPENDENT TRANSPORT IN CELLS AND IN ISOLATED MEMBRANE VESICLES OF A MARINE PSEUDOMONAD

Transport in marine pseudomonad ATCC 19855 was studied using isolated membrane vesicles. When $Na^+$ was treated as a substrate in the transport process, kinetic analysis revealed a sequential bi bi mechanism. Short chain primary alcohols, NADH and ascorbate-TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) energized L-alanine transport. With ethanol, initial rates of oxygen uptake were approximately 4% of that obtained with NADH or ascorbate-TMPD, yet the extent of transport was similar for each energy source. Succinate oxidation was not coupled to transport. Unlike results using NADH and ascorbate-TMPD, excellent correlation was obtained between inhibition of ethanol oxidation and inhibition of ethanol driven transport. Transport inhibition by 2,4-dinitrophenol reflected a specific inhibition of ethanol oxidation. Approximately 25% of cytochromes b and c were reduced by ethanol. It was concluded that only that part of the respiratory activity coupled to ethanol oxidation is also coupled to transport in vesicles.

# RÉSUMÉ

Ph.D. G. Dennis Sprott Microbiologie

LE MÉCANISME DU COUPLAGE DE L'ÉNERGIE ET LA CINÉTIQUE DU TRANSPORT LIÉ AU $Na^+$, CHEZ DES CELLULES ET DES VÉSICULES MEMBRANEUSES D'UNE PSEUDOMONADE MARINE

Le phénomène de transport a été étudié chez la pseudomonade marine ATCC 19855, à l'aide de vésicules membraneuses. Lorsque l'ion $Na^+$ fut considéré comme substrat du processus de transport, l'analyse cinétique révéla un mécanisme séquentiel binaire-binaire. Les alcools primaires à courte chaîne, le NADH et l'ascorbate-TMPD (N,N,N',N'-tetraméthyl-p-phénylènediamine) activèrent le transport de la L-alanine. Avec l'éthanol, les taux initiaux d'incorporation d'oxygène furent approximativement de 4% des taux obtenus avec le NADH ou l'ascorbate-TMPD, bien que le niveau de transport fût similaire pour chaque source d'énergie. L'oxydation du succinate n'était pas couplée au transport. À l'opposé des résultats obtenus avec le NADH et l'ascorbate-TMPD, l'inhibition de l'oxydation de l'éthanol et celle du transport activé par l'éthanol montrèrent une excellente corrélation. L'inhibition du transport par le 2,4-dinitrophénol a put être directement reliée à une inhibition spécifique de l'oxydation de l'éthanol. L'éthanol réduisait environ 25% des cytochromes b et c. Ceci permit de conclure que seulement une partie de l'activité respiratoire couplée à l'oxidation de l'éthanol était aussi couplée au processus de transport dans les vésicules.

# TABLE of CONTENTS

# A C K N O W L E D G E M E N T S

It is with sincerity that the author thanks his research director, Dr. R.A. MacLeod, Professor, Department of Microbiology, Macdonald College, for rewarding discussions, for his enthusiasm, and for advice given during the course of this investigation.

Dr. John Thompson and Dr. Jordan Ingram, Department of Microbiology, Macdonald College, are gratefully acknowledged for helpful discussions concerning $Na^+$-dependent transport.

The help of Dr. E.L. Martin, University of Nebraska, Nebraska, U.S.A., in designing and performing some of the introductory experiments on vesicle transport is acknowledged. Special thanks are extended to Mr. Joseph Drozdowski with whom fruitful discussions were held and whose valuable technical assistance contributed to the kinetic and cotransport studies.

Thanks are extended to Dr. A.R. Wasserman, Biochemistry Department, McGill University, for kindly providing advice and assistance in performing the cytochrome analysis.

Mr. W.J. Crosby of the Ste-Anne-de-Bellevue Veterans Hospital is acknowledged for kindly developing X-ray films on several occasions.

Sincere appreciation is extended to Mrs. Edna Rowell for promptly and skilfully typing this manuscript.

This research was supported by a grant from the National Research Council of Canada. The author sincerely expresses gratitude to the National Research Council of Canada for a Postgraduate Scholarship and to McGill University for a McConnell Memorial Fellowship.

# G L O S S A R Y.

| | | |
|---|---|---|
| 1. | AIB | α-aminoisobutyric acid |
| 2. | PEA | phenethyl alcohol |
| 3. | TMPD | N,N,N',N'-tetramethyl-p-phenylenediamine di HCl. |
| 4. | PMS | phenazine methosulfate |
| 5. | HOQNO | 2-heptyl-4-hydroxyquinoline-N-oxide |
| 6. | DNP | 2,4-dinitrophenol |
| 7. | PCMB | p-chloromercuribenzoate |
| 8. | NEM | N-ethylmaleimide |
| 9. | IAA | iodoacetic acid |
| 10. | DMSO | dimethyl sulfoxide |

# LIST of TABLES

# L I S T  of  F I G U R E S

## C L A I M  of  C O N T R I B U T I O N S  to  K N O W L E D G E

1. Unlike cells a low rate of L-alanine metabolism was exhibited by vesicles, thus allowing transport studies without the use of non-metabolized analogues.

2. The complete absence of endogenous metabolism in the vesicle system was conducive to a study of energy sources for transport. Short chain primary alcohols, NADH, and ascorbate-TMPD were found to be coupled to electron flow and to transport.

3. Studies using various energy sources and transport inhibitors strongly indicated that only part of that respiratory activity coupled to NADH and ascorbate-TMPD oxidation was coupled to transport. Ethanol was efficiently coupled to transport. The extent of ethanol oxidation, but not that of NADH, succinate or ascorbate-TMPD, was directly related to transport. Oxygen was required for transport energized by ethanol.

4. The first case of a $Na^+$-specific transport system in vesicles of a bacterium is described. No $Na^+$-requirement for oxygen uptake induced by ethanol or ascorbate-TMPD was found.

5. A $K^+$-requirement for transport in vesicles could not be demonstrated. Sufficient $K^+$ remained bound to the vesicles to account for the level of amino acid transported. Under these

conditions a true $Na^+$ effect on transport kinetics was determined. The transport process followed a sequential bi bi mechanism, where $Na^+$ and amino acid are the two substrates.

6. Bisubstrate transport kinetics were shown for $Na^+$ and AIB in cells, but only if steps were taken to hold the intracellular $K^+$ concentration constant. In this case, the kinetic parameters for transport in cells and vesicles with the exception of $V_{max}$ were nearly identical.

7. $^{22}Na$ appeared to penetrate only to the cytoplasmic membrane in Complete-Salts washed cells. When the ion gradients were disrupted by washing the cells in 50 mM $MgSO_4$ and the cells suspended in Complete Salts, the internal $Na^+$ was rapidly extruded from the cell interior. The accumulation of AIB within the cells was not accompanied by detectable levels of intracellular $Na^+$.

8. Cell wall fractions isolated from this organism bound L-alanine and L-leucine as determined by equilibrium dialysis techniques. The same fractions when added back to cells increased the initial rate of uptake of either L-alanine or L-leucine. No activity was observed for AIB.

# INTRODUCTION

Three major findings, two of which were discovered in this laboratory, led to the initiation of the present study. These findings were that membrane vesicles of *Escherichia coli* could actively transport amino acids, that clean cytoplasmic membrane preparations could be prepared from the marine pseudomonad (ATCC 19855), and that $Na^+$ was required for the active transport of amino acids into cells of the marine pseudomonad.

Convincing evidence has been presented for the active transport of amino acids (Kaback and Milner, 1970) and β-galactosides (Barnes and Kaback, 1970) into isolated membrane vesicles isolated from *E. coli*. Uptake specifically required the conversion of D-lactate to pyruvate, and could be prevented by inhibitors of electron flow or by anaerobiosis. The membrane preparations used, however, were not pure as shown by electron micrographs in which two double track structures were present (Kaback and Deuel, 1969).

When cells of a marine pseudomonad (ATCC 19855) were washed in 0.5 M NaCl and suspended in 0.5 M sucrose, non-dialyzable material was released from the outer cell wall layer (Buckmire, Ph.D. Thesis, 1967). Later, Forsberg (Ph.D. Thesis, 1969) analyzed the material released and observed the cell forms remaining after these treatments by electron microscopy. From this study it was concluded that

several discrete wall layers were present and a model of the cell wall structure was formulated (Forsberg *et al.*, 1970a,b). Further, lysozyme treatment of the cell forms (mureinoplasts) remaining after 0.5 M NaCl and 0.5 M sucrose treatments resulted in the formation of true protoplasts (Costerton *et al.*, 1967). This latter finding was used to advantage by Martin and MacLeod (1971). These authors disrupted the protoplasts by French pressure cell treatment and isolated a clean membrane fraction by differential centrifugation. This membrane fraction gave a single band in a sucrose density gradient and contained about 63% protein and 31% lipid. Both chemical analysis and electron microscopy revealed the absence of contaminating cell wall or cytoplasmic constituents. Furthermore, nearly all of the membrane fraction was vesiculated when examined as thin-sections.

Previous studies using cells of the marine pseudomonad have shown a $Na^+$ requirement for the transport of amino acids (Drapeau and MacLeod, 1963a; Wong *et al.*, 1969). Two functions for $Na^+$ in AIB uptake were observed, one for the prevention of leakage of material from the cells, and the other for the uptake of AIB into the cells (Wong *et al.*, 1969). The cells possessed sufficient internal reserves to energize transport. This fact accounted for the absence of studies concerning transport energetics.

It was hoped at the outset of this project that the membrane

fraction isolated by Martin and MacLeod (1971) would actively transport amino acids as did *E. coli* vesicles. Furthermore, it was hoped that the high level of endogenous energy for transport present in these cells would be lost, allowing a study of the energetics of transport. Finally, such a purified transport system could be used to further elucidate the role of $Na^+$ and $K^+$ in the transport process.

# LITERATURE REVIEW

## Transport into isolated membrane vesicles

In dilute saline solution or water, erythrocytes swell with the loss of most of their cytoplasmic constituents. The cell may be restored by the addition of appropriate levels of salt to form ghosts (Stein, 1967a). Such ghosts exhibit permeability characteristics similar to the original cells. Considerable progress made recently in the field of bacterial transport is largely attributable to the adoption of the isolated membrane technique to bacteria (Kaback, 1970). Membrane vesicles of *E. coli* actively transported proline in the presence of glucose, with transport activity subject to inhibition by anaerobiosis or compounds known to inhibit either electron flow or oxidative phosphorylation (Kaback and Stadtman, 1966). Furthermore, the activity observed was not attributable to undisrupted cell forms, since sonication of the membrane preparation did not prevent proline uptake (Kaback and Deuel, 1969).

An extensive survey has been made to identify the compounds which can be utilized by vesicles of *E. coli* to energize transport. D-lactate stimulated proline uptake most dramatically being converted stoichiometrically to pyruvate (Kaback and Milner, 1970). Of all the metabolites and cofactors tested only succinate, L-lactate, D,L-α-hydroxybutyrate, and NADH could partially replace D-lactate. The

transport of several other amino acids showed the same trend. Similar studies proved the transport of β-galactosides was coupled to a membrane bound D-lactate dehydrogenase (Barnes and Kaback, 1970) through an electron transport chain. High concentrations of arsenate or oligomycin did not inhibit transport suggesting that oxidative phosphorylation was not involved. Like the transport systems for amino acids and β-galactosides, D-lactate oxidation was coupled to an inducible galactose transport system in vesicles of *E. coli* (Kerwar *et al.*, 1972).

Konings and Freese (1971) pointed out the necessity for constant aeration of vesicles of *B. subtilis* during transport studies, and implied that some of the work done by Kaback and coworkers should be reconsidered in view of these findings. In vesicles of *B. subtilis* 90% oxygen consumption was recorded 4.3 seconds after NADH addition. Under aeration conditions NADH was found to be a very effective energy source for L-serine transport. Further, these authors showed that the artificial electron donor system ascorbate-PMS could fully energize transport in the presence of oxygen.

In a more detailed study, Konings and Freese (1972) showed amino acids to be actively transported by membrane vesicles of *B. subtilis* using the physiological electron donors NADH, NADPH, L-α-glycerol phosphate, L-lactate, and succinate. In contrast to *E. coli* vesicles, D-lactate had little stimulating effect on transport in

vesicles of *B. subtilis*. The efficiency of the electron donors in energizing transport was determined by comparing rates of oxidation and transport. No relation was found between the rate of oxidation of a compound and its ability to energize transport, suggesting to the authors an ability of certain oxidizable substrates to donate electrons more specifically to the transport coupling sites.

A significant contribution was made by Hirata *et al.* (1971) using electron transport particles from *Mycobacterium phlei*. Uptake of proline against a gradient occurred with succinate, NADH or an artificial electron donor ascorbate-TMPD. Since the latter two substrates were most effective for transport but least efficient for oxidative phosphorylation, and since arsenate and the absence of coupling factors or phosphate did not prevent transport, it was concluded that transport was independent of oxidative phosphorylation. Glucose, D-lactate, fumarate and ATP had no effect on transport.

The conversion of L-α-glycerol-phosphate to dihydroxyacetone phosphate was coupled to the active transport of 16 amino acids in vesicles of *Staphylococcus aureus* (Short *et al.*, 1972a). Other electron donors, with the exception of ascorbate-PMS, did not replace α-glycerol-phosphate. These authors also found no relation between rates of oxidation of electron donors and their ability to stimulate

amino acid transport. Since the Michaelis constant for α-glycerol-phosphate:dichloroindophenol reductase, for α-glycerol-phosphate oxidation, and for α-glycerol-phosphate stimulated transport were all the same, it was concluded by Short *et al.* (1972b) that the rate limiting step for transport was α-glycerol-phosphate oxidation.

Membranes prepared from *E. coli*, *S. typhimurium*, *Pseudomonas putida*, *Proteus mirabilis*, *B. megaterium* and *B. subtilis* concentrated proline in the presence of ascorbate-PMS (Konings *et al.*, 1971). Further, ascorbate-PMS was found to reduce the respiratory chain in *E. coli* vesicles before cytochrome $b_1$, but after 80% of the flavoprotein.

A membrane fraction was isolated from *Azotobacter vinelandii* which contained highly active L-malate oxidase not linked to pyridine nucleotide (Jones and Redfearn, 1966). A supernatant fraction did contain $NAD^+$-linked malate dehydrogenase. In this respect, a similarity is observed to the *E. coli* system where membrane bound D-lactate dehydrogenase is flavin linked, while the cytoplasmic enzyme is a pyridine nucleotide dependent enzyme (Kaback, 1972). It was not surprising then, that membrane vesicles from *A. vinelandii* actively accumulated glucose by the conversion of L-malate to oxalacetate via L-malate dehydrogenase, or that flavin adenine dinucleotide was required for maximum transport stimulation (Barnes, 1972).

Membrane vesicles of *E. coli*, *B. subtilis* and a *Pseudomonas* sp.

transported D-lactate, L-lactate and succinate in the presence of an electron donor (Matin and Konings, 1973). Four electron donors were tested, L-glutamate, D-lactate, L-lactate and succinate. In the case of *B. subtilis* membranes, only NADH was oxidized significantly and only NADH energized the uptake of the three substrates. However, in vesicles of *E. coli* and the *Pseudomonas* sp. NADH, L-lactate and D-lactate energized the transport of succinate, while NADH and succinate energized the transport of D-lactate and L-lactate. At concentrations of D-lactate, L-lactate and succinate in the micromolar range, little transport occurred unless an additional energy source was added. It is possible, the authors conclude, that at high concentrations these compounds may provide the energy for their own transport. This point was technically difficult to test.

Vesicles prepared from *P. aeruginosa* were found to actively transport gluconate, but only if during the isolation the use of Tris and ethylenediaminetetraacetic acid was avoided. Thus, a new procedure for membrane isolation was developed by Stinnett *et al.* (1973) involving osmotic lysis of cells treated with LiCl and lysozyme. In these preparations active gluconate transport occurred via a flavin adenine dinucleotide-linked L-malate dehydrogenase and via a D-glucose dehydrogenase. The authors claim credit for the first demonstration of a physiological role for glucose dehydrogenase.

## Mechanism of energy coupling to active transport

### a. *General*

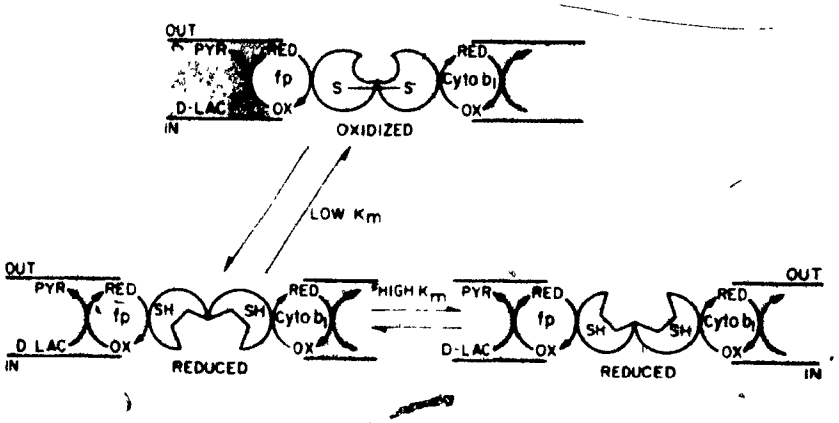
Since the cytoplasmic membrane is permeable allowing simple diffusion (Wilbrandt, 1954), Cohen and Monod (1957) have suggested that at the steady state there is a balance between active uptake and leakage of the substrate. The coupling of energy to a facilitated diffusion system may thus occur in two possible ways. The energy source may either reduce the rate of efflux or it may increase the rate of influx of the solute. Early experiments by Winkler and Wilson (1966) showed that metabolic inhibitors reduced the $K_t$ of exit of β-galactosides from cells of *E. coli*, whereas the $K_t$ of entrance remained constant. Manno and Schachter (1970) developed a rapid procedure to separate thick cell suspensions from the medium by centrifuging through silicone. In contrast to Winkler and Wilson, these authors reported energy uncoupled cells not only to have a decreased maximal velocity, but to show an increased $K_m$ for influx. Kaback and Barnes (1971) found lactose accumulation in vesicles of *E. coli* to represent an equilibrium state between influx and efflux at a variety of temperatures. Temperature-induced efflux and KCN-induced efflux showed an apparent affinity constant which was about sixty times higher than the affinity constant for influx.

That electron flow is the ultimate source of energy for transport has been well established. How the flow of electrons through

the cytochrome system is coupled to transport is still controversial. Three possible mechanisms are through cation gradients, through high energy phosphate bond intermediates, or by a novel new mechanism suggested by Kaback and Barnes (1971) concerning a redox carrier protein.

b. *The redox carrier concept*

Kaback and Barnes (1971) presented the following scheme for D-lactate dehydrogenase coupled transport systems in *E. coli:*




The basic premise in explaining how electron flow could be coupled to transport is that a redox carrier protein is coupled between a flavoprotein and cytochrome $b_1$. The carrier is shown to exist in either a high affinity (oxidized) state or a low affinity (reduced) state. Electrons from D-lactate were hypothesized to reduce a

critical disulfide in the carrier, resulting in a marked decrease in the affinity of the carrier with release of the ligand on the inside of the membrane. The cycle could be completed by reoxidation of the disulfide by cytochrome $b_1$ and ultimate formation of $H_2O$. When the substrate concentration inside the membrane became high enough to saturate the reduced form of the carrier the rate of influx and efflux would be equal. To explain carrier mediated efflux in energy poisoned membranes it was necessary to include in the model a step to show that the reduced form of the carrier could "vibrate".

Experimental evidence for the model is based primarily on the lack in correlation between the rates of oxidation of various electron donors and transport, and on the effect of inhibitors on influx and efflux. D-lactate, NADH and succinic dehydrogenases were shown to couple through different flavoproteins to cytochrome $b_1$ (Barnes and Kaback, 1971). Since D-lactate was the primary electron donor for transport, the carrier was positioned in the branch from the main chain between D-lactic dehydrogenase and cytochrome $b_1$. Further positioning of the carrier and evidence on its redox nature was obtained using inhibitors. It was found that only inhibitors acting after the D-lactate flavoprotein caused efflux. In other words, reduction of the respiratory components between D-lactate dehydrogenase and cytochrome $b_1$ caused lactose efflux. Amytal, an inhibitor at the flavin level, and oxamate, an inhibitor of D-lactic dehydro-

genase, caused little or no efflux. Consequently, the carrier was positioned between flavoprotein and cytochrome $b_1$. Inhibition of transport by PCMB was reversed by dithiothreitol. Furthermore, PCMB inhibited temperature induced efflux of lactose and lactose exchange. These findings were interpreted as evidence for the sulfhydryl dependent nature of the carrier protein.

It has been shown in vesicles of *E. coli* that far more D-lactate is oxidized than glucose-6-phosphate transported (Dietz, 1972). In fact, Kaback and Barnes (1971) reported that the $V_{max}$ for the D-lactic dehydrogenase can account for the sum of the $V_{max}$ values of all the transport systems found in the membranes. More recently the model presented was found to require slight revision, since Hong and Kaback (1972) isolated mutants able to oxidize D-lactate but defective in transport. The authors suggested that the carrier proteins may be components of shunts from the main portion of the respiratory chain.

c. *High energy phosphate compounds*

In cells of *E. coli* treated with cold Tris-HCl buffer to deplete them of intracellular nucleotides and $K^+$, a stimulation of the lactose transport system was obtained by adding purine nucleoside triphosphates, especially ATP (Scarborough *et al.*, 1968). Klein and Boyer (1972) concluded that cells of *E. coli* actively transport certain amino acids, carbohydrates, and cations using either oxidative

energy or phosphate bond energy. Their conclusions were based on studies where ATP levels in cells were dramatically lowered by incubation with arsenate and low phosphate. Under this condition aerobic proline transport was uninhibited, as was the case with vesicles, while anaerobic transport was sharply depressed. This finding seems to answer the apparent inconsistency of the Kaback and Barnes (1971) model, since that model does not explain anaerobic transport. One may argue that a physiological electron acceptor is functional in place of oxygen in facultative anaerobes growing in the absence of oxygen. However, a transport mechanism in strict anaerobes based on the model for *E. coli* (Kaback and Barnes, 1971) is difficult to envisage.

Berger (1973) arrived at similar conclusions to Klein and Boyer (1972). Proline uptake into cells of *E. coli* could be driven by oxidative energy derived from D-lactate or by substrate level processes driven by glucose. The oxidative pathway was sensitive to cyanide but not to arsenate and did not require the Ca, Mg-dependent ATPase. The substrate level process was sensitive to arsenate but not to cyanide, and required a functional ATPase. In contrast to proline, the transport of glutamine was driven directly by phosphate-bond energy formed by either oxidative phosphorylation or glycolysis.

Further attempts were made to relate the membrane vesicle behaviour to that of cells of *E. coli*. Intact cells of mutants defective in D-lactate dehydrogenase and electron transport were found

to transport proline and alanine normally (Simoni and Shallenberger, 1972). Mutants defective in Ca, Mg-stimulated ATPase were also defective in proline and alanine transport. These results clearly showed transport in *E. coli* cells could occur in the absence of D-lactate oxidation or electron flow. The authors suggest that in vesicles the Ca, Mg-ATPase may in some mysterious way "couple electron transport energy directly to active transport without going through ATP".

β-galactoside accumulation in mutants of *E. coli* defective in the Ca, Mg-ATPase was studied by Schairer and Haddock (1972). The ATPase was found necessary for transport in the presence of cyanide.

Convincing evidence against the involvement of high energy phosphate compounds in proline transport into electron transport particles of *M. phlei* has been presented (Hirata *et al.*, 1971). When depleted of coupling factor the particles could not carry out oxidative phosphorylation but could take up oxygen in the presence of NADH or ascorbate-TMPD. Under these conditions proline transport was stimulated about 4-fold.

d. *Proton gradient theory*

This theory has arisen largely from the finding that uncouplers of oxidative phosphorylation block many transport processes. The inhibition of galactoside transport by 2,4-dinitrophenol was explained by Mitchell (1963) in his chemiosmotic hypothesis as an

effect of the uncoupler to allow protons to diffuse across the lipid membrane. He thus formulated the idea of proton-sugar symport. As ATP is hydrolyzed by the Mg-dependent ATPase protons are ejected giving rise to a proton gradient across the membrane. It is this potential which Mitchell (1963 and 1970) suggested could maintain the concentration gradient of sugar. The energy required for transport would be obtained from the passage of a proton in the same direction as the lactose molecule down its electrochemical potential gradients. In the language of Mitchell, "the lactose porter is a proton symporter".

The effect of uncouplers on transport is not an effect on alteration of ATP levels in the cell (Harold, 1972a). Active transport of many solutes is abolished by uncouplers under anaerobic conditions, yet the carrier remains functional as a facilitated diffusion system (Pavlasova and Harold, 1969). Further, membrane vesicles giving active transport without ATP involvement are subject to inhibition by uncouplers (Barnes and Kaback, 1970).

West (1970) evaluated the theory of Mitchell in *E. coli* in relation to lactose transport as follows. In metabolizing cells if protons flow into the cell during transport, then an equal flow of protons out of the cell should occur because of the flow of electrons down the respiratory chain and the ATPase. When lactose was added to the cells protons were taken up by parental cells but not by a mutant lacking the permease. The flow of protons was roughly

proportional to that of lactose. Energy uncoupled transport mutants were isolated which showed a lower ratio of proton to β-galactoside uptake than the parent (West and Wilson, 1973).

Harold and coworkers have studied transport in *Streptococcus faecalis*. The organism lacks cytochromes and the capacity for oxidative phosphorylation, obtaining energy from glycolysis and arginine degradation (Harold, 1972b). The authors propose that during fermentation of glucose proton extrusion occurs, the proton movement being coupled to active transport (Harold and Baarda, 1968; Harold *et al.*, 1970). Uncouplers collapsed the proton gradient and abolished active transport.

Studies by Eddy and Nowacki (1971) using ATP-depleted yeast cells have shown proton uptake and $K^+$ extrusion to accompany amino acid accumulation. In the anaerobe *Streptococcus lactis*, which lacked oxidative phosphorylation and required the addition of a metabolizable substrate before active transport could occur, the active transport of thiomethyl-β-galactoside was energized by net ionic movements (Kashket and Wilson, 1972). This was accomplished by adding valinomycin in the absence of metabolizable substrates. The data supported the view that $K^+$ induced efflux was associated with the entry of protons and the sugar into the cells via the sugar transport carrier.

Membrane vesicles of *E. coli* generated a proton gradient in the

presence of D-lactate (Reeves, 1971). This observation does not account for active transport in the vesicles, since lactose or amino acids had no effect on the degree of acidification and since vesicles treated with phosphorylase leaked solute but still gave pH effects (Kaback, 1972).

## $Na^+$-dependent transport

### a. *Mammalian cells*

A common feature of transport systems in most mammalian cells is the requirement for both $Na^+$ and $K^+$ (Schultz and Curran, 1970). Evidence suggests that the gradients of $Na^+$ and $K^+$ are established by the operation of a Na, K-ATPase often called the $Na^+$ pump (Skou, 1965). Crane (1965) and Crane *et al.* (1965) proposed a model for intestinal transport in which the $Na^+$ gradient was an essential element. The mobile carrier was depicted as having two separate binding sites, one for $Na^+$ and one for the ligand. Binding of $Na^+$ to the carrier resulted in a high affinity form, while the release of $Na^+$ at the inside surface of the membrane, where the $Na^+$ concentration was relatively low, resulted in ligand release. Evidence was presented for $K^+$ competition with $Na^+$ for the $Na^+$ binding site. The Crane hypothesis, however, has fallen short in several instances where abolition of the $Na^+$ and $K^+$ gradients reduced but did not eliminate transport (Kimmich, 1970; Schultz and Curran, 1970). However, the affinities for $Na^+$ and $K^+$ need not be the same on each

side of the membrane (Johnstone, 1972). Kimmich (1970) suggested that the Na,K-ATPase generated a high energy intermediate coupled to active transport.

Cotransport of $Na^+$ and ligand has been shown to occur in many mammalian transport systems (Crane, 1965; Schultz and Curran, 1970; Thomas and Christensen, 1971), and has been taken as evidence for the formation of a ternary complex (Schultz and Zalusky, 1965; Schafer and Jacquez, 1967).

b. *Bacterial cells*

The requirement for $Na^+$ and $K^+$ for transport in animal cells has been extended to marine bacteria and recently to several terrestrial bacteria. Tomlinson and MacLeod (1957) found both $Na^+$ and $K^+$ to be essential for oxidation of added metabolizable compounds in a marine pseudomonad. Both $Na^+$ and $K^+$ were required for indole production from tryptophan by a marine vibrio (Pratt and Happold, 1960). Drapeau and MacLeod (1965) and Drapeau *et al.* (1966) presented the first definitive experiments showing $Na^+$ and $K^+$ to be required for transport of solutes into the marine pseudomonad and into *Photobacterium fischeri*.

The transport of certain solutes into several terrestrial bacteria has also been found to require $Na^+$. Glutamate uptake into cells of *E. coli* was stimulated by $Na^+$ (Frank and Hopkins, 1969), although $Na^+$ did not specifically enhance succinate transport into

vesicles (Rayman *et al.*, 1972). Glutamate transport into cells and isolated cytoplasmic membrane vesicles of *B. licheniformis* was also markedly stimulated by $Na^+$ (MacLeod *et al.*, 1973). For anaerobic growth on citrate *Aerobacter aerogenes* required $Na^+$ (O'Brien and Stern, 1969). The citrate transport system of *Aerobacter* requires $Na^+$ as does oxalacetate decarboxylase, the proposed carrier protein (Sachan and Stern, 1971). In a study of the $Na^+$-dependent melibiose permease system of *Salmonella typhimurium*, Stock and Roseman (1971) showed $Na^+$ to cotransport with the solute. The possibility of $Na^+$ gradients being involved in transport was therefore suggested. Thompson and MacLeod (1973a) have shown this not to be the case in a marine pseudomonad, since abolition of both the $Na^+$ and $K^+$ gradients did not affect transport. Cotransport was not tested by the latter authors. In summary, the $Na^+$-requirement for transport in terrestrial bacteria, where present, seems less specific for $Na^+$ and is satisfied by a lower level of $Na^+$ than in the marine bacteria examined.

Several bacteria isolated from the rumen have an obligate requirement for $Na^+$ for growth (Bryant *et al.*, 1959; Hudson and Caldwell, 1972).

In the marine pseudomonad, kinetics of AIB uptake into the cells revealed $Na^+$ to change the $K_m$ (Wong *et al.*, 1969). This was interpreted as evidence for an effect of $Na^+$ in altering the affinity of an AIB carrier protein. Similar findings were reported by

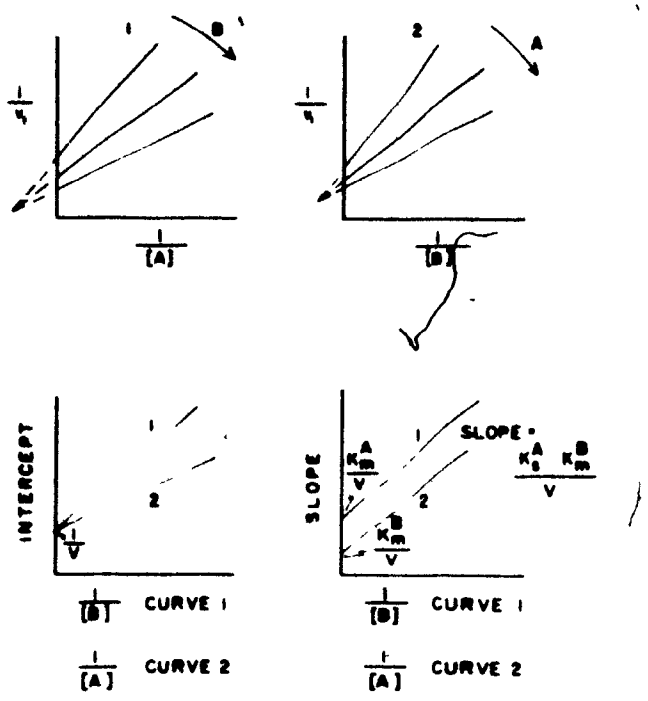
Halpern *et al.* (1973a) for glutamate transport by *E. coli*. A $K^+$-requirement for transport was also demonstrated by the latter authors.

c. *Application of bisubstrate kinetics to $Na^+$-dependent transport*

Graphic determination of the four parameters required to specify a bisubstrate reaction has been described (Florini and Vestling, 1957; Dalziel, 1957). The initial rate equation relating these parameters is shown below:

$$\frac{V}{v_1} = 1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K_s^A K_m^B}{AB}$$

The two substrates are represented by A and B, $K_m^A$ and $K_m^B$ are the limiting Michaelis constants for A or B, and $K_s^A$ represents the dissociation constant for A. The limiting maximum velocity is represented by V and the initial velocity as $v_1$. With one substrate concentration constant, the other is varied giving one primary Lineweaver-Burk plot (Lineweaver and Burk, 1934) for each substrate (graphs 1 and 2 in following illustration). Two secondary plots are then made from each primary plot as described in Mahler and Cordes (1971). As shown in the following illustration, a hypothetical case for a bisubstrate mechanism involving a ternary complex, curves 1 and 2 in the intercept plot must intersect on the ordinate and have equal slopes in the slope plot.




The nomenclature used for reactions of two or more substrates is that of Cleland (1963). A reaction with two substrates and two products is a Bi Bi reaction. When both substrates must add to the enzyme before any products are released the mechanism is called "sequential". Such reactions are designated "ordered" if the substrates must add in a definite way and the products leave similarly and are designated "random" if the substrates do not react in any definite order.

Recently Thomas and Christensen (1971) applied $Na^+$-dependent transport kinetics of serine uptake in pigeon erythrocyte to a two substrate mechanism. Both primary Lineweaver-Burk plots were linear and the sequential reaction was apparently random. Previous experiments (Vidaver, 1964a,b) had shown similar effects in pigeon erythrocyte where two $Na^+$ atoms cotransported with each glycine molecule.

d. *Isolation of transport components*

Fox and Kennedy (1965) first demonstrated the solubilization of a membrane bound transport component. Using the finding that N-ethyl maleimide (NEM) irreversibly inhibited the β-galactoside permease but only in the absence of β-galactosides, the authors developed a dual-label technique to identify the NEM binding component. Solubilization with Triton X-100 released this component which they called the M protein. The M protein was considered the permease because it reacted with NEM, was induced by inducers of the Lac operon and bound β-galactosides. Also, the M protein was absent in a permease-less mutant of *E. coli* (Fox *et al.*, 1966).

Membrane vesicles of *E. coli* when partially solubilized using Brij 36-T, released protein components which bound proline (Gordon *et al.*, 1972). The solubilized crude extract was passed through a Sephadex G-100 column. Three 280 nm absorbing peaks emerged. All three fractions bound proline but peak III had the highest

specific activity. Little if any phospholipid was associated with peak III. Furthermore, only proline itself inhibited [$^{14}$C] proline binding. Many of the characteristics of the vesicle transport system were observed for binding. Proline binding was inhibited by PCMB and the inhibition was reversed by dithiothreitol. Electron transfer inhibitors and DNP did not inhibit.

A second type of proteins believed to be part of the transport system have been extensively studied. These are the proteins released from bacterial cells by osmotic shock. Unlike the M protein or proline-binding protein described, the shock proteins are water soluble and are located external to the cytoplasmic membrane (Heppel, 1967 and 1969). The osmotic shock procedure was described for *E. coli* and related gram-negative organisms (Neu and Heppel, 1964; Nossal and Heppel, 1966) and is as follows. Cells are suspended in 0.5 M sucrose containing ethylenediaminetetraacetate, centrifuged, and rapidly dispersed in cold dilute $MgCl_2$. Several hydrolytic enzymes and binding proteins were released from the cells, while the shocked cells remained viable growing in fresh medium after a lag phase.

The sulfate-binding protein from *Salmonella typhimurium* (Langridge *et al.*, 1970), and the leucine-binding protein from *E. coli* (Penrose *et al.*, 1968) have been crystallized. A second leucine-binding protein from *E. coli*, which did not bind either isoleucine

or valine was crystallized by Furlong and Weiner (1970). Recently the shock-released proteins were discussed in a review (Kaback, 1970).

Arguments in favour of these binding-proteins being involved in active transport are as follows:

(a) osmotic shock largely inhibited transport in the shocked cells with a simultaneous release of binding protein;

(b) transport-negative mutants lack the binding protein;

(c) the affinity constants for binding and transport are similar;

(d) the binding is reversible;

(e) Boos and Gordon (1971) have shown the galactose-binding protein of *E. coli* to exist in two conformational states. Furthermore, over the cell growth cycle the degree of galactose binding protein synthesized closely paralleled the transport capacity of the cell for galactose (Shen and Boos, 1973).

This evidence has not established the exact location of the binding proteins in the cell or their exact function in the transport sequence of events, nevertheless, the evidence is very much in favour of their participation in active transport.

# M A T E R I A L S and M E T H O D S

## Organism

The organism used and designated B-16 (ATCC 19855, NCMB 19) has been classified as a *Pseudomonas* sp. type IV by the Torry Research Group, Aberdeen, Scotland. Recently, Baumann *et al.* (1972) classified this organism as an *Alteromonas marinopraesens* strain 214. A number of variants arise during growth on laboratory media (Gow, Ph.D. Thesis, 1973). In this study the rough variant-3 was used (Gow *et al.*, 1973).

## Growth Medium

Cells were grown in a medium consisting of nutrient broth, 0.8%; yeast extract, 0.5%; $MgCl_2$, 0.026 M; KCl, 0.01 M; and $Fe(NH_4)_2(SO_4)_2$, 0.1 mM. The culture was maintained by monthly transfer to slants of medium containing 1.5% agar.

## Cell Growth

Cells were grown by the procedure of DeVoe *et al.* (1970) resulting in a high yield of late-log cells. Cells stored at 4 C on an agar slant were inoculated into 10 ml of broth medium in a 50 ml Erlenmeyer flask. After growth for 8 hours, the flask contents were used to inoculate a 250-ml volume of broth medium contained in a 2-l Erlenmeyer flask. After a 5 hour growth interval, 40-ml aliquots of this latter culture were transferred to fresh

250-ml samples of medium and these were incubated for 5 hours. At each step broth cultures were incubated at 25 C on a rotary shaker.

Complete Salts

Complete salts-Tris (CS-T) refers to a salts buffer solution comprised of $MgSO_4$, 50 mM; KCl, 10 mM; Trizma base, 50 mM adjusted to pH 8.3 with HCl; and NaCl at indicated concentrations.

Membrane isolation

Following the final 5 hour incubation the cells were harvested at 16,000 x g at 4 C and washed three times by resuspension in, and centrifugation from, volumes of 0.5 M NaCl equal to half the volume of the growth medium. The cells were then resuspended into half volume of 0.5 M sucrose and incubated 30 min on a rotary shaker. These cells were then centrifuged, resuspended into the same volume of 0.5 M sucrose and centrifuged immediately. The resulting cell forms have been called mureinoplasts, since they still retain the peptidoglycan layer (DeVoe *et al.*, 1970). Mureinoplasts were then converted to protoplasts by resuspension into CS-T containing 150 µg lysozyme per ml. Protoplast formation was routinely checked by phase microscopy, however, throughout over 100 membrane preparations protoplasting was judged well over 90% efficient within 5 min or less. Protoplasts were collected by centrifuging at 10,000 x g at

4 C, and resuspended into CS-T using a glass tissue homogenizer. This material was then passed through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 15,000 to 16,000 lb/in$^2$ directly to a precooled CS-T solution containing lysozyme (150 µg/ml), deoxyribonuclease (50 µg/ml), and ribonuclease (50 µg/ml). Material sedimentable within 10 min at 4,080 x g was discarded. The supernatant was centrifuged at 75,000 x g at 4 C for 20 min and the membranes which sedimented were washed twice with CS-T. The product was primarily vesicles (Martin and MacLeod, 1971), which were pigmented a deep pink. These vesicles when resuspended into CS-T (a glass homogenizer had to be used) to an optical density of 0.450 using a Spectronic 20 (1 cm light path, 660 nm source) gave a dry weight of 20 mg membranes per ml.

During vesicle isolations CS-T contained either NaCl (75 mM) or LiCl (200 mM) in place of the NaCl. Care was taken during the isolation to keep the material cold whenever possible.

Viable cell counts in vesicle preparations

The number of viable cells was determined by spreading dilutions of the membrane preparations on a solid medium containing the same components as that used to grow the organism.

Transport assay using Vesicles

Membranes adjusted to 20 mg/ml were added in 50-µl amounts to a series of serological tubes stored in ice, each tube correspond-

ing to one transport assay. Where potential inhibitors were tested, solutions of the compounds in CS-T were added in a volume of 50 µl. In tubes without inhibitor, 50 µl of CS-T was added. Immediately thereafter the tubes were placed in a New Brunswick water bath at 25 C operating at 250 rpm for 15 min. Finally, 50 µl either of the energy source in CS-T or CS-T alone was added, followed immediately by 20 µl of a solution of labeled amino acid in CS-T. Each complete assay mixture, thus, contained 1.0 mg membranes in a final volume of 170 µl. Energy sources and inhibitors were added at molarities recorded in the legends based on the final volume of the assay. In most experiments NADH, ethanol and ascorbic acid (neutralized with Tris) were added at 25 mM; TMPD at 150 µM and PMS at 100 µM. Following the addition of label, the tube was incubated for specified times and the reaction mixture diluted with 0.4 ml CS-T. The suspension was filtered quickly and washed with 4 ml of CS-T. Wash fluids in kinetic experiments contained the level of NaCl present in the uptake medium.

In the first section of "Results" L-[$^{14}$C]alanine was 1.8 µM and either 156 or 173 µCi/µmole. In other experiments the details are given in the legends.

Transport assay using cells

Cells were harvested and washed twice in CS-T (NaCl 200 mM). Separate cell pellets were resuspended to 200 µg dry weight cells

per ml. Resuspension fluids consisted of CS-T varying the NaCl concentration for each pellet as desired and including chloramphenicol dissolved at 100 µg/ml. Transport was assayed at 25 C using 0.5 ml of cell suspension per serological tube. The sequence of additions was as described for vesicle transport. Following uptake of the labeled substrate the entire tube contents were drawn into a Pasteur pipette (as in the vesicle assay) and filtered using 0.45 µ Millipore filters. Filtered cells were washed with 5 ml CS-T (NaCl 200 mM).

Radioactive counting

The filters in vesicle and cell assays were dried slowly with an infra red lamp and counted in a Nuclear Chicago Isocap/300 liquid scintillation spectrometer using 5 ml scintillation fluid (5 g 2,5-diphenyloxazole/1 toluene). Samples were counted with an efficiency of 87% for carbon-14 and 65% for tritium.

Recording transport data

Results from duplicate assays were averaged. Using the calculated specific activity (Wang and Willis, 1965), transport was reported as either pmoles or nmoles of amino acid per mg dry weight of vesicles or cells. Since uptake of AIB or L-alanine into cells and vesicles was linear for at least the first minute at the concentrations used, initial rates in kinetic studies were based on 1.0 min uptakes.

Metabolism of L-alanine by cells and vesicles

Cells were resuspended into salts-chloramphenicol solution as previously described. L-alanine ($1 \times 10^{-5}$ M, 14.2 μCi/μmole) or AIB ($1 \times 10^{-4}$ M, 0.78 μCi/μmole) were added to the cells. After vortexing, a time of 1.0 min was allowed for the cells to take up the amino acid. At this point the cells were filtered and washed. A total of 6 filters were immersed into 35 ml of hot water contained in a 50 ml Erlenmeyer flask and the flask immersed into boiling water for 20 min. The fluid containing cell debris was spun at 39,000 x g for 30 min. Extraction efficiency was calculated by counting samples of the supernatant fluid and of the pellet resuspended in water. Extracts were flash evaporated at 50 C to approximately 2 ml, and desalted by passing through a column of Ion Retardation Resin AG 11 A8 (BioRad) with bed dimensions of 0.9 cm x 20.5 cm and equilibrated with water (flow rate 9 ml/min). The entire extract was applied to the column and 5.0 ml fractions collected. Each fraction was tested for $Cl^-$ with $AgNO_3$ and 0.1 ml counted for radioactivity. Most of the radioactivity which could be recovered emerged in the first few fractions and $Cl^-$ was not found in these fractions. Fractions containing radioactivity were pooled and concentrated to about 0.5 ml by flash evaporation. Aliquots of these final concentrates were counted for quantitation of radioactivity spotted on thin layer plates.

Glass plates (20 x 20 cm) were spread with Cellulose powder

(MN 300) 300 μ in thickness. Extracts and standards (about 2,000 DPM), were spotted and the plates developed by two dimensional chromatography (Jones and Heathcote, 1966). The first solvent was propan-2-ol-formic acid-water (40:2:10, v/v) and the second was t-butanol-methyl ethyl ketone-ammonia-water (50:30:10:10, v/v). Each developed plate was spotted with marker radioactive material and placed in intimate contact with X-ray film (RP/SX-Omat, rapid processing medical X-ray film, RPS-2, Kodak). After two weeks exposure time in a dark room, the film was developed using the facilities at the Ste.-Anne-de-Bellevue Veterans Hospital.

Each developed X-ray film was placed over the original thin layer plate and aligned with the marker spots. Exposed areas on the X-ray film corresponding to radioactive areas on the adsorbent were marked and removed to counting vials. Samples were counted using aquasol (a product of New England Nuclear Corp.).

The metabolism of L-alanine by vesicles was determined in a similar manner to that described for cells. Uptake assays were performed using 1.0 mg membranes per assay, 1.8 μM L-alanine (156 μCi/μmole), and 2 mM ethanol as energy source. Hot water extraction followed a 10 min uptake interval.

Cytochrome analysis

A Phoenix dual wavelength split beam scanning spectrophotometer was used to obtain difference spectra as described by Chance (1953)

and by Chance and Williams (1955). Potential electron donors and inhibitors were added as described in figure legends, giving a final volume of 3.1 ml.

Oxygen uptake

Oxygen consumption measurements were performed using a Clark electrode (Table 12) or a Beckman electrode, connected to a Heath recorder. Reaction mixtures were prepared as for cytochrome analysis in 3.1 ml, added to an enclosed chamber made from plexiglass.

ATP assay

The various energy sources were added at the concentrations used in transport assays to tubes containing 3.8 mg membranes in a final volume of 0.6 ml. After 1.0 min, ATP was extracted with boiling Tris buffer (50 mM, pH 8.3) and assayed by the luciferin luciferase reaction (Holm-Hansen and Booth, 1966).

Estimation of intravesicular volume

The double label technique of Hunter and Brierley (1969) was used with some modification. Approximately 3.5 µCi of [$^{14}$C]inulin and 10 µCi of $^{3}H_2O$ were added to 75 mg membranes in a volume of 7.5 ml CS-T (NaCl 75 mM). After incubation for 3 min with occasional mixing the membranes were spun at 36,000 x g ($R_{max}$) for 10 min to obtain a tightly packed pellet. The wet weight of the

pellet was determined and assumed to be its volume. The membranes in the pellet were resuspended and diluted to 2.0 ml with 1.0 M $HClO_4$, membrane debris was removed by centrifugation and the radioactivity of the supernatant was determined. Correction was made for overlap of $^{14}C$ into the $^{3}H$ channel. Intravesicular space was taken as the difference in space penetrated by $^{3}H_2O$ and by [$^{14}C$] inulin.

Quantitation of $Na^+$ and $K^+$

Determination of residual $Na^+$ and $K^+$ in membrane and cell preparations was performed as follows. Samples were digested with $HNO_3$ and $HClO_4$ (Sanui and Pace, 1959) using Vycor digestion flasks as described by Rayman (Ph.D. Thesis, 1970). Pellet digests were resuspended into distilled water.

Supernatant fluids and pellet digests were analyzed for $Na^+$ and $K^+$ using a SP 90 Unicam flame emission spectrophotometer. Correction was made for the effect of $Na^+$ on the $K^+$ assay. Reference standards were purchased from Fisher Scientific Co. Before use in the assays, glassware was soaked overnight in a solution of sulfuric and nitric acids (2:1, v/v), and thoroughly rinsed with glass distilled water.

Attempt to demonstrate $Na^+$ and AIB cotransport

a. *Filter assay*

Cells were washed once in a solution comprised of NaCl, 300 mM;

$MgSO_4$, 50 mM; and Tris buffer, 50 mM, pH 7.2. A thick cell suspension was made using the above salts solution, and incubated with shaking at 25 C. Uptake assays were performed using 50 ml Erlenmeyer flasks each containing 5 ml of a solution of the following salts: LiCl, 200 mM; $MgSO_4$, 50 mM; NaCl, 47 mM; and Tris, 50 mM, pH 7.2.

For demonstration of [$^{14}$C]AIB uptake, the 5 ml salts were supplemented with AIB (150 µM, 1.0 µCi/µmole). At zero time cells were quickly added to give 1.0 mg dry weight per ml ($Na^+$ carry over was 3 mM), and 0.5 ml samples were filtered at intervals. Rapid filtration of 0.5 ml was obtained using Millipore prefilters without any 0.45 µ filter. [Cells were reproducibly retained by these filters to 80% of control values, as determined using labeled cells and filtering on prefilters with or without an underlying 0.45 µ filter.] Immediately following filtration a 5 ml wash was added containing KCl, 200 mM; $MgSO_4$, 50 mM; and Tris, 50 mM, pH 7.2.

For $^{22}$Na uptakes two flasks were used, each containing 5 ml of salts and one containing 150 µM [$^{14}$C]AIB. Each flask contained 5 µCi $^{22}$Na. The assay was begun by adding 1.0 mg cells per ml. Filtration and washes were as for [$^{14}$C]AIB uptakes.

$^{22}$Na was counted with an efficiency of 5% in vials containing 10 ml water (Parker and Elrick, 1970).

b. *Thick cell suspension assay*

Cells were harvested and washed once in either 0.05 M $MgSO_4$ or in CS-T (NaCl 200 mM, pH 7.5) in volumes equal to those of the growth medium. Thick cell suspensions were prepared by resuspending the pellets into CS-T (NaCl 200 mM, pH 7.5) to a dry weight of 10 to 25 mg per ml.

Assays were performed in 7.5 ml CS-T using 50 ml Erlenmeyer flasks. Separate flasks contained 10 µCi $^{3}H_2O$, 4 µCi [$^{14}$C]sucrose plus 9 mM [$^{12}$C]sucrose, approximately 3.5 µCi [$^{14}$C]inulin, or 5 µCi $^{22}Na$. Where required [$^{12}$C]AIB was 150 µM and [$^{14}$C]AIB was present at 1.0 µCi. Approximately 60 mg dry weight cells, added as 4.0 ml of the thick cell suspension, were added to start the assay. Incubation was continued at 25 C with shaking. The contents of each Erlenmeyer flask were then transferred after specified times to 10 ml centrifuge tubes and centrifuged for 10 min at 36,000 x g. As described for the estimation of intravesicular volume, the supernatant fluids were removed, diluted 10 times with 1 M $HClO_4$, cell debris was removed at 36,000 x g, and 0.2 ml samples were counted directly.

Equilibrium dialysis

Binding activity of cell wall fractions was tested using two equilibrium dialysis techniques. In the first case, citrate buffer (Gomori, 1955) containing 25 mM NaCl (7.0 ml) was placed in the

tube followed by a small stirring magnet. The extract (1.0 ml) was placed in boiled dialysis tubing (Fisher), and this in turn was placed in the test tube. After 1.0 hour equilibration at 4 C, the amino acid was added as the undiluted isotope to the fluid outside the dialysis sac. Incubation was continued for 20 hours at 4 C. Outside fluid and the dialysis sac contents (0.2 ml volumes) were counted using aquasol. Quenching was corrected for by the Channels ratio method (Wang and Willis, 1965), and the ratio of DPM in an equal volume of inside fluid to outside fluid calculated.

In the second method, an equilibrium dialysis unit with 40 chambers was constructed using plexiglass (Weiner and Heppel, personal communication). The chambers were separated by boiled dialysis tubing (Fisher). Extract was added to one side of the chamber (0.75 ml) and an equal volume of citrate buffer, pH 5.5, added to the other side. Labeled amino acid was added to the extract side. The unit was placed at 4 C with rotation for approximately 20 hours. Mixing was facilitated by adding a glass bead to each side of the chamber. From each side of the chamber 0.1 ml volumes were withdrawn and counted in aquasol.

Sepharose chromatography

A Pharmacia column (K 25/45, 2.5 x 45 cm) was packed with Sepharose 4B (Pharmacia, fractionation range 300,000 to 3,000,000

MW). One part of the Sepharose was diluted with two parts of water and poured into the column using a long glass tube as the reservoir (2.5 cm diameter). The packed column had a bed volume of 200 ml, and flow rate of 10 ml water per hour with a 15 cm pressure head.

Blue dextran or the outer-double-track component of the cell wall was added to the top of the column and eluted with distilled water. Fractions of 3.4 ml were collected using an Isco PUP fraction collector.

Radioactive materials

L-[U-$^{14}$C]alanine was purchased from Amersham Searle (156 µCi/µmole) or New England Nuclear (173 µCi/µmole). Tritiated-$H_2O$ (1.0 mCi/g), $^{3}$H-AIB (235 µCi/µmole), [carboxyl-$^{14}$C]inulin (2 mCi/g), [carboxyl-$^{14}$C]AIB (10.4 µCi/µmole), $^{22}$Na, and $^{42}$K were purchased from New England Nuclear Corporation.

Alcohol dehydrogenase assay

The method of Kersters and DeLey (1966) was used. Reduction of $NAD^+$ in the presence of ethanol was measured at 340 nm. Calculations were based on an extinction of 6.2 using a 1.0 mM solution read at 340 nm and using a 1 cm light path. A unit of enzyme is defined as that enzyme concentration required to reduce 1 µmole $NAD^+$ per min. The detection limit was 1-2 units per 100 ml using 0.1 ml of extract in an assay volume of 1.0 ml.

# RESULTS

## SECTION I.

## MECHANISM OF TRANSPORT

### Filtration Assay

Since Kaback and co-workers had demonstrated that isolated membranes of *E. coli* vesiculated and were able to concentrate amino acids and sugars, the possibility was examined that isolated membranes of marine pseudomonad B-16 would behave in a similar manner. Attempts to filter membranes of the marine pseudomonad on 0.45 μ Millipore filters resulted in severe problems with filter clogging. For this reason Whatman #3 prefilters were used to overlay Millipore filters of various pore diameters. It was found that a 0.8 μ Millipore filter overlaid with a Whatman prefilter gave rapid filtration of up to 1.0 mg dry weight of membranes. To test for the retention of the vesicles on this filter pad combination, the experiment shown in Table 1 was performed. The cells were labeled by adding [$^{14}$C] oleic acid to the growth medium (Nelson and MacLeod, unpublished) and the membrane fraction isolated. Either 1.0 mg membranes were counted directly or 1.0 mg samples filtered and the filter pads counted. The filters used retained 90% of the labeled membranes and in 6 filtrations proved to be very reproducible. Later it was found

TABLE 1.

*Retention of [$^{14}$C]oleic acid labeled vesicles using a filtration assay.*

Six 0.1 ml volumes each containing 1 mg (dry wt) of [$^{14}$C]oleic acid labeled membranes were filtered through separate filters. The radioactivity retained by the filters was compared with that obtained when six 0.1 ml volumes of membrane were added directly to filters and counted.

| Sample Treatment | Vesicles cpm/mg | Retention % |
|---|---|---|
| Not filtered | 23,641 ± 591 | - |
| Filtered | 21,599 ± 240 | 91 |

that Millipore prefilters were more absorbent than the Whatman paper discs and consequently these were used for most of the experiments.

General characteristics of the Vesicle Transport System

a. *Uptake of L-alanine by membrane preparations*

Using the filtration assay for transport it was found that L-alanine was taken up by membrane preparations incubated in a salts solution containing NaCl (Table 2). Uptake of the amino acid continued as the incubation time lengthened.

b. *Lability of the vesicle transport system*

When isolated membranes were exposed to L-[$^{14}$C]alanine, they either bound or transported the amino acid. The activity was reduced considerably if the membranes were stored, especially if stored above 3.5 C (Table 3). Although not shown, it was also found that L-alanine uptake was considerably higher if the membranes were washed only twice during the isolation rather than five times as was the case in Table 3. The lability of the isolated membrane system was of importance in planning the experiments to follow. Assays to be compared were closely spaced in time to each other making it unnecessary to correct for losses in activity.

c. *Optimum temperature for L-alanine uptake*

The effect of temperature on the uptake of AIB into cells of

TABLE 2.

*Uptake of L-[$^{14}$C]alanine by membrane vesicles.*

Uptake was measured by the filtration assay using 1.8 μM L-[$^{14}$C] alanine in the presence of 2 mM ethanol. The amino acid was added to the membranes at zero time and the assay mixtures filtered at various times thereafter.

| Uptake time min | Uptake of Radioactivity cpm/mg membranes |
|---|---|
| 0.5 | 1,744 |
| 1.0 | 2,767 |
| 1.5 | 3,639 |
| 2.0 | 4,212 |
| 4.0 | 6,002 |
| 6.0 | 7,683 |
| 10.0 | 8,566 |

TABLE 3.

*Loss in L-alanine uptake capacity of vesicles over time comparing two storage temperatures.*

Uptake of L-[$^{14}$C]alanine (1.8 µM; 2 mM ethanol) was measured beginning after five washes in CS-T, corresponding to zero storage time and taken as 100% uptake capacity.

| Time of storage hours | Temperature of storage 25 C | 3.5 C |
|---|---|---|
| | % | |
| 0 | 100 | 100 |
| 12 | 37 | 69 |
| 24 | 32 | 56 |

the marine pseudomonad has been presented (Wong, Ph.D. thesis, 1968). Uptake was best at 25 C with less activity at 35 C and still less at 4 C. No activity was observed at 45 C. The uptake of L-alanine by the membrane preparation (Fig. 1) was similar to that reported for cells, although the membrane activity was depressed more strikingly at temperatures above 25 C. After exposure to 35 C for 10 minutes the membranes flocculated and showed little remaining activity for L-alanine uptake.

d. *Optimum pH for L-alanine uptake*

The optimum pH for uptake of L-alanine by both intact cells and membrane vesicles was pH 8.3-8.5 (Fig. 2). Intact cells were less subject to transport depression at pH values toward the acid side of pH 8.3 than were vesicles. For example, if the rate of transport at the optimum pH is taken as 100%, at pH 6.0 intact cells still retained 58% of their uptake activity while vesicles retained only 25%.

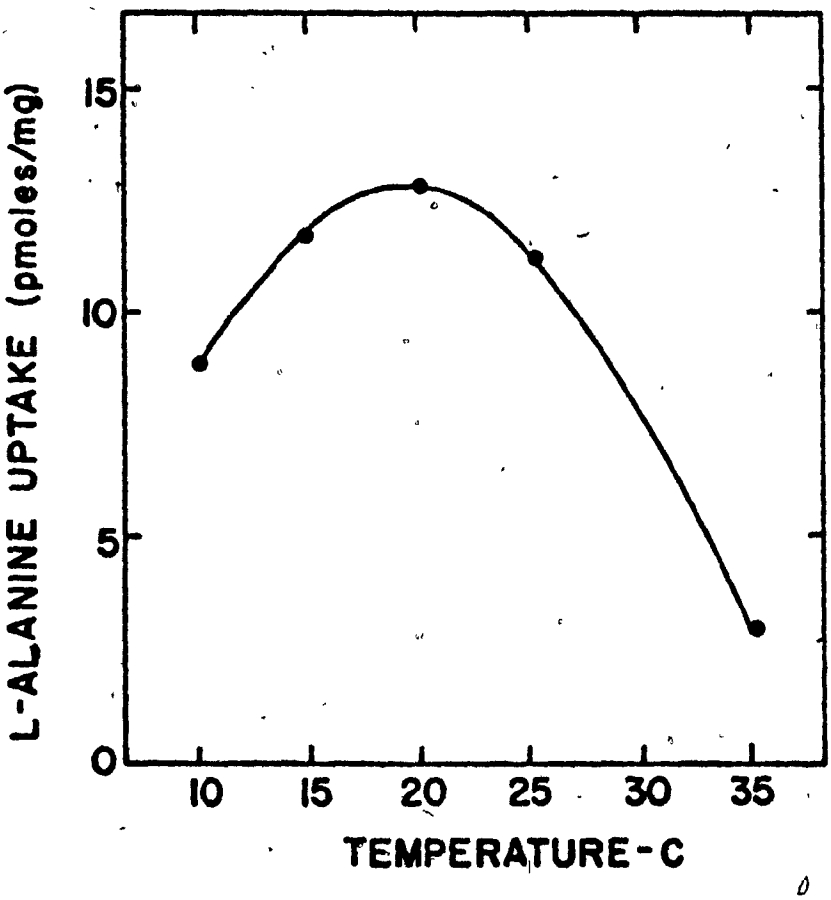
e. *Metabolism of L-alanine*

Amino acid transport studies require that a distinction be made between transport across the membrane and subsequent incorporation of the label. For this reason the nonmetabolized amino acid, AIB, has been used in previous studies (Wong *et al.*, 1969; Thompson and MacLeod, 1971). The necessity for this procedure was evaluated in this study by comparing the metabolism of AIB and L-alanine by the

FIGURE 1.

Fig. 1. Effect of temperature on the uptake of L-alanine into vesicles.

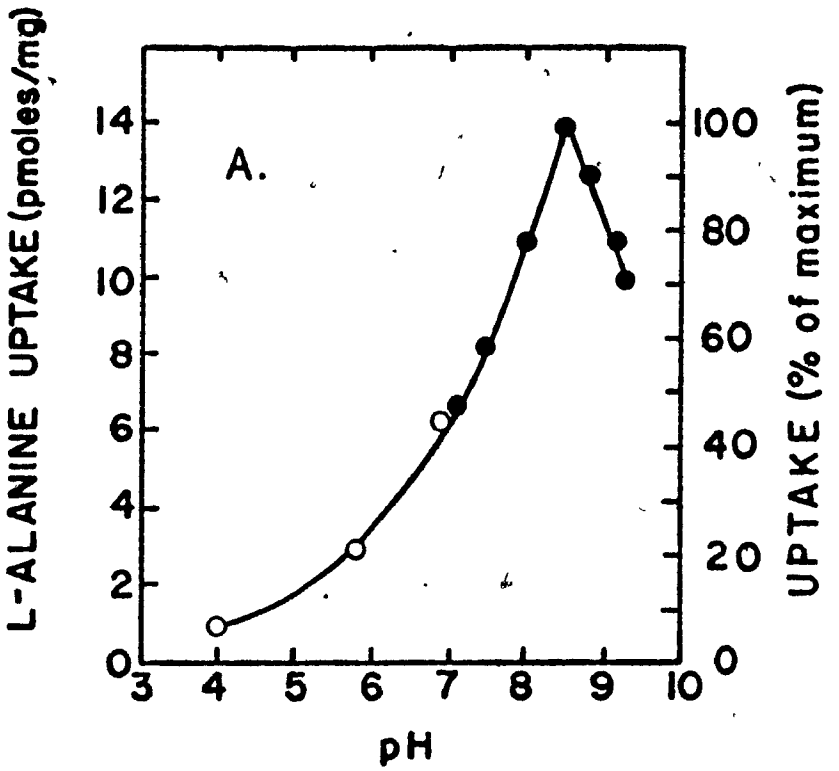
Following equilibration of vesicles at the specified temperature for 15 min, L-alanine (1.8 μM, 156 μCi/μmole) and ethanol (2 mM) were added. Uptake was terminated after 10 min at the temperature tested.

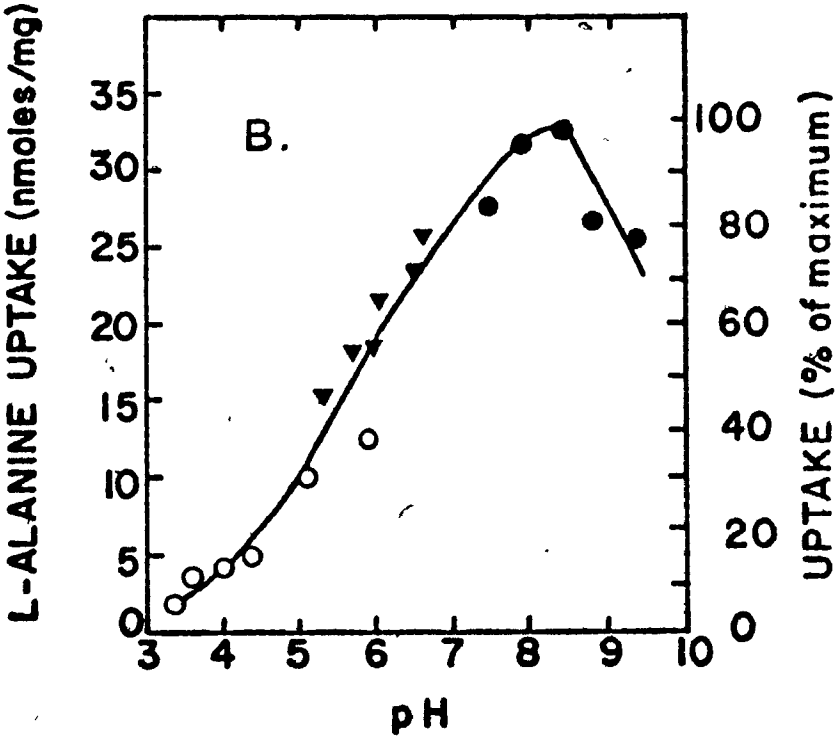

L-ALANINE UPTAKE (pmoles/mg)
15
10
5
0
10
15
20
25
30
35
TEMPERATURE-C

FIGURE, 2.

Fig. 2. Effect of pH on L-alanine uptake by (A) vesicles and (B) intact cells.

Buffers were added just before the addition of ethanol (10 mM) and L-alanine. pH values were measured in reaction mixtures prepared as were those used to measure transport, but with omission of the radioactive compound. The pH remained constant for a period which exceeded the 1.0 min alloted for uptake. In (A) L-alanine was 1.8 μM and in (B) 0.5 mM, 1.55 μCi/μmole. Citrate-phosphate buffer (Gomori, 1955) using the $K^+$ salts, O O ; maleate buffer (Gomori, 1955) using $K^+$ salts, ▼ ▼ ; Tris, 50 mM, adjusted with HCl, ● ● .


A.
L-ALANINE UPTAKE (pmoles/mg)
UPTAKE (% of maximum)
pH
0
2
4
6
8
10
12
14
20
40
60
80
100
3
4
5
6
7
8
9
10


B.
L-ALANINE UPTAKE (nmoles/mg)
UPTAKE (% of maximum)
pH
0
5
10
15
20
25
30
35
20
40
60
80
100
3
4
5
6
7
8
9
10

cells. Hot water extraction of cells filtered after 1.0 min exposures to either of the amino acids, yielded extracts containing about 95% of the label. Column chromatography of the extracts gave recoveries of 72% for the AIB extract and 25% for the alanine extract. Radioactivity not recovered remained firmly bound to the resin. The desalted extracts were subjected to thin layer chromatography and the results recorded in Table 4. The AIB extract gave only one spot, with $R_f$ values which compared well with the standard. In contrast, the alanine extract gave three spots on autoradiography. If spot one is taken as alanine, then only 27% of the radioactivity spotted on the thin layer plate was not altered over the 1.0 min exposure time to the cells.

Using similar techniques but with omission of the desalting step, metabolism of L-alanine by the membrane vesicles was determined (Table 5). Extraction of radioactivity from filter pads containing the membranes was 88% efficient. On two duplicate thin layer plates five spots were found, number one spot having $R_f$ values similar to L-alanine and accounting for 92 or 98% of the total DPM spotted. Heated membranes on the other hand, yielded an extract with only one spot, showing that the appearance of spots two to five were metabolism products and not formed as decomposition products during the extraction. This finding allows the study of L-alanine transport into membrane vesicles without the use of nonmetabolized transport analogues.

TABLE 4.

*Tabulation of $R_f$ values for AIB and L-alanine extracted from cells of a marine pseudomonad.*

| Amino acid | Sample | Spot number | $R_f$ value Solvent 1 | $R_f$ value Solvent 2 | % radioactivity remaining as AIB or alanine |
|---|---|---|---|---|---|
| AIB: | | | | | |
| | Standard | 1 | .77 | .22 | --- |
| | Filter extract | 1 | .71 | .20 | 100 |
| L-alanine: | | | | | |
| | Standard | 1 | .58 | .12 | --- |
| | Filter extract | 1 | .48 | .15 | 27 |
| | | 2 | .39 | .10 | |
| | | 3 | .30 | .09 | |

TABLE 5.

*Tabulation of $R_f$ values for L-alanine extracted from membranes.* Between 4,000 and 6,000 DPM were spotted per TLC plate. From 91 to 98 per cent of spotted radioactivity was accounted for on removal of radioactive adsorbent.

| Sample | Spot number | $R_f$ Value Solvent 1 | $R_f$ Value Solvent 2 | % radioactivity remaining as alanine (Spot 1) |
|---|---|---|---|---|
| Filter extract, plate 1 | 1 | 0.59 | 0.10 | |
| | 2 | 0.20 | 0.00 | |
| | 3 | 0.42 | 0.00 | 92 |
| | 4 | 0.59 | 0.00 | |
| | 5 | 0.00 | 0.00 | |
| Filter extract, plate 2 | 1 | 0.60 | 0.15 | |
| | 2 | 0.20 | 0.00 | |
| | 3 | 0.38 | 0.00 | 98 |
| | 4 | 0.52 | 0.00 | |
| | 5 | 0.00 | 0.00 | |
| Filter extract, heated membranes | 1 | 0.61 | 0.15 | 100 |
| Standard, L-alanine | 1 | 0.60 | 0.13 | 100 |

If L-[$^{14}$C]alanine is transported by the vesicles and not incorporated into protein or otherwise changed then the accumulated amino acid should exchange with high levels of externally added L-[$^{12}$C] alanine (Stein, 1967b). The results of an experiment to test this are shown in Fig. 3 where radioactivity was lost from the membranes in the presence but not in the absence of unlabeled amino acid.

f. *Whole cell contamination of the membrane fraction*

It was important to determine whether bacteria present as contaminants in the membrane preparations could account for the uptake activity recorded. The number of viable cells was determined by spreading dilutions of the membrane preparations on a solid medium containing the same components as that used to grow the organisms. Plate counts established that the number of viable cells present in a typical incubation mixture containing the usual concentration of membranes did not exceed 2 x $10^3$ cells. Of these, 1 x $10^2$ failed to grow on the plating medium containing no added $Na^+$ and hence could be cells of the marine pseudomonad. Separate experiments established that a concentration of at least 1 x $10^6$ cells of the marine pseudomonad were required per assay to demonstrate transport activity.

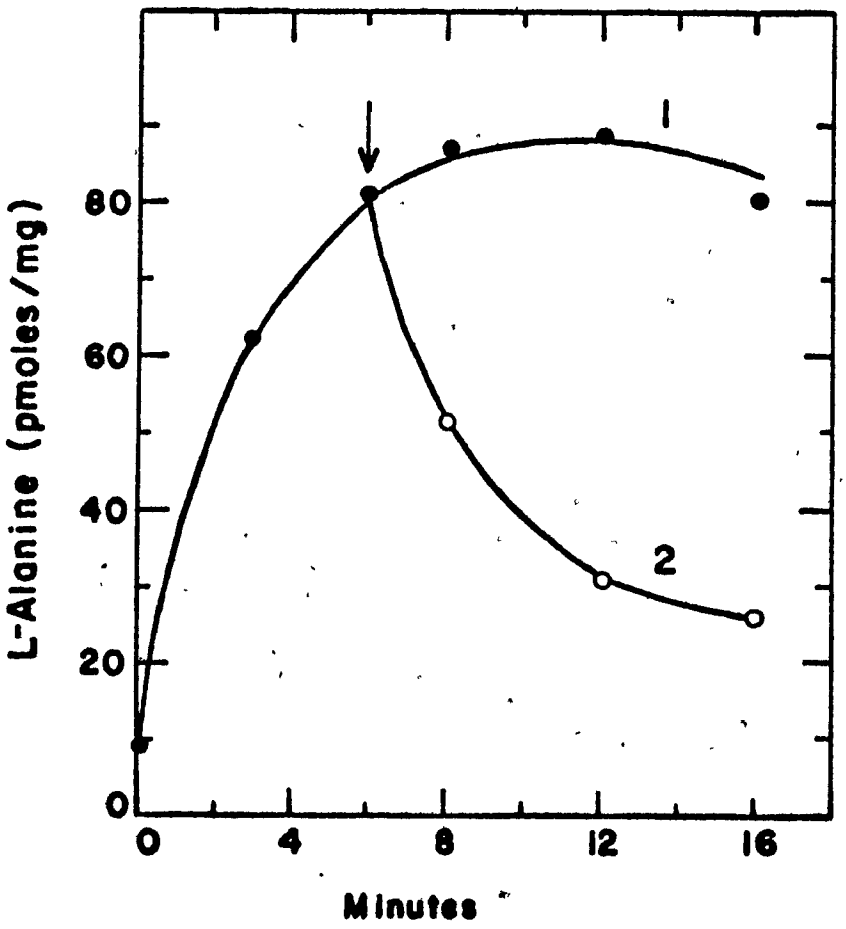
g. *Distinction between transport or binding of L-alanine by membranes*

It has been shown using intact cells of this organism that

FIGURE 3.

Fig. 3. Counterflow experiment showing displacement of L-[$^{14}$C] alanine by L-[$^{12}$C]alanine.

L-[$^{14}$C]alanine at 1.6 µM was taken up by membranes in the presence of 2 mM ethanol. The arrow indicates addition of 10 µl CS-T (Curve 1) or 10 µl L-[$^{12}$C]alanine (2.6 mM) in CS-T (Curve 2).


L-Alanine (pmoles/mg)
0
20
40
60
80
0
4
8
12
16
Minutes
1
2

cells preloaded with AIB exposed to 0.25% phenethyl alcohol (PEA) lose both their intracellular $K^+$ and AIB (Thompson and DeVoe, 1972). The effects of the alcohol were completely reversible and were correlated to structural changes within the cell envelope. These findings were applied in the present study to distinguish active transport of L-alanine from the mere binding of L-alanine to the membranes. Membrane vesicles were allowed to accumulate L-[$^{14}$C]alanine to the maximum level. PEA was then added to the reaction mixture and the level of radioactivity associated with the membranes measured with time (Fig. 4). In the presence of the alcohol, but not in its absence, radioactivity was quickly lost from the membrane vesicles. When the membranes exposed to PEA were dialyzed against CS-T in the absence of the alcohol they were found to be capable of taking up L-alanine again to levels prevailing before PEA treatment (Table 6). This latter finding shows that radioactivity was not released by a dissolution of the membrane.

h. *Amino acid competition*

Radioactivity was reversibly released from the vesicles, indicating active transport. This finding did not say anything about the specificity of the accumulation, however. It was necessary, therefore, to establish that the amino acid uptake into vesicles exhibited similar amino acid competition patterns to that found for intact cells of the marine pseudomonad. The results in Table 7 show

FIGURE 4.

Fig. 4. Phenethyl alcohol (PEA) induced loss of L-alanine from vesicles.

Vesicles were allowed to accumulate L-[$^{14}$C]alanine (1.8 µM, 156 µCi/µmole) for 10 min in the presence of 2 mM ethanol. At zero time either CS-T solution (Curve 1) or PEA in CS-T (Curve 2) was added. The final PEA concentration was 0.25%. On the basis of an internal space of 0.4 µl/mg membranes (Table 21) the internal/external concentration of L-alanine changed from 10.9 at zero time to 2.9 after 36 min exposure to PEA.

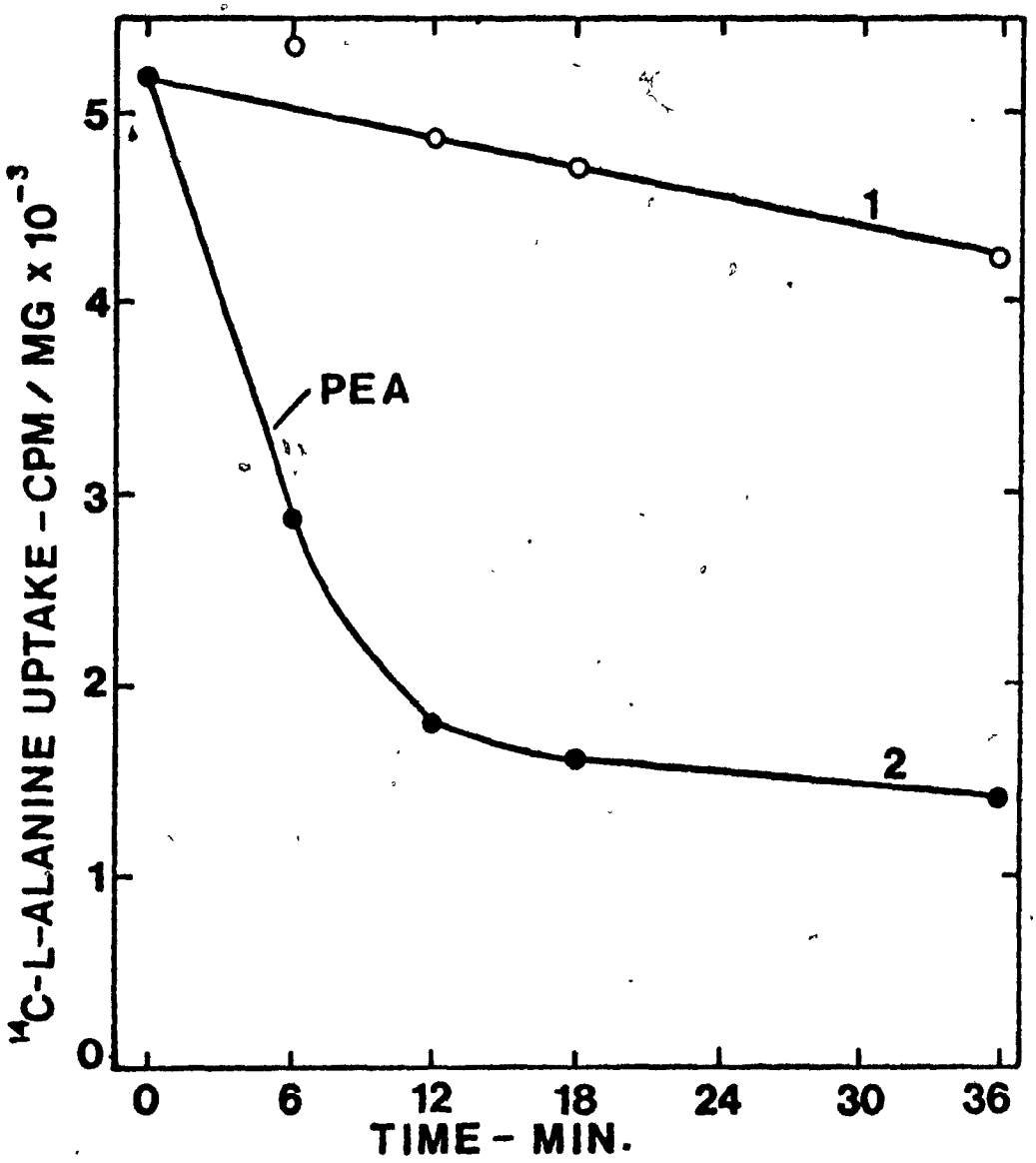

$^{14}$C-L-ALANINE UPTAKE - CPM / MG x $10^{-3}$
0
1
2
3
4
5
0
6
12
18
24
30
36
TIME - MIN.
PEA
1
2

TABLE 6.

*Reversibility of the effect of 0.25% phenethyl alcohol on L-alanine uptake in vesicles.*

Vesicles (10 mg) were exposed to PEA for 18 min. These vesicles, and non-exposed vesicles, were each dialyzed against separate 1-litre volumes of CS-T over 2 hours with one change of the dialysis fluids. Uptakes were performed as in Fig. 4.

| Exposure to PEA | Uptake time min | |
|---|---|---|
| | 2 | 6 |
| | pmoles/mg | |
| - | 9.9 | 14.9 |
| + | 9.3 | 15.0 |

T A B L E 7.

*Effect of non-radioactive amino acids on the uptake of L-[$^{14}$C] alanine by membranes.*

[$^{12}$C]amino acids were added at 180 μM 15 min prior to the addition of 1.8 μM L-[$^{14}$C]alanine. Uptake was continued for 10 min in the presence of ethanol added initially at 2 mM.

| [$^{12}$C]amino acid | % inhibition of uptake |
|---|---|
| L-alanine | 90 |
| L-serine | 86 |
| Glycine | 60 |
| AIB | 59 |
| D-alanine | 58 |
| D-serine | 56 |
| L-threonine | 53 |
| L-valine | 26 |
| L-leucine | 22 |
| L-proline | 5 |
| DL-phenylalanine | 4 |
| Glycinamide | 0 |

that a number of amino acids structurally related to L-alanine did, in fact, inhibit L-[$^{14}$C]alanine transport into the membrane vesicles. Similar results for intact cells using [$^{14}$C]AIB have been reported (Drapeau *et al.*, 1966). Amino acid systems and their overlapping nature in these cells have been much more thoroughly studied by Fein (Fein and MacLeod, unpublished).

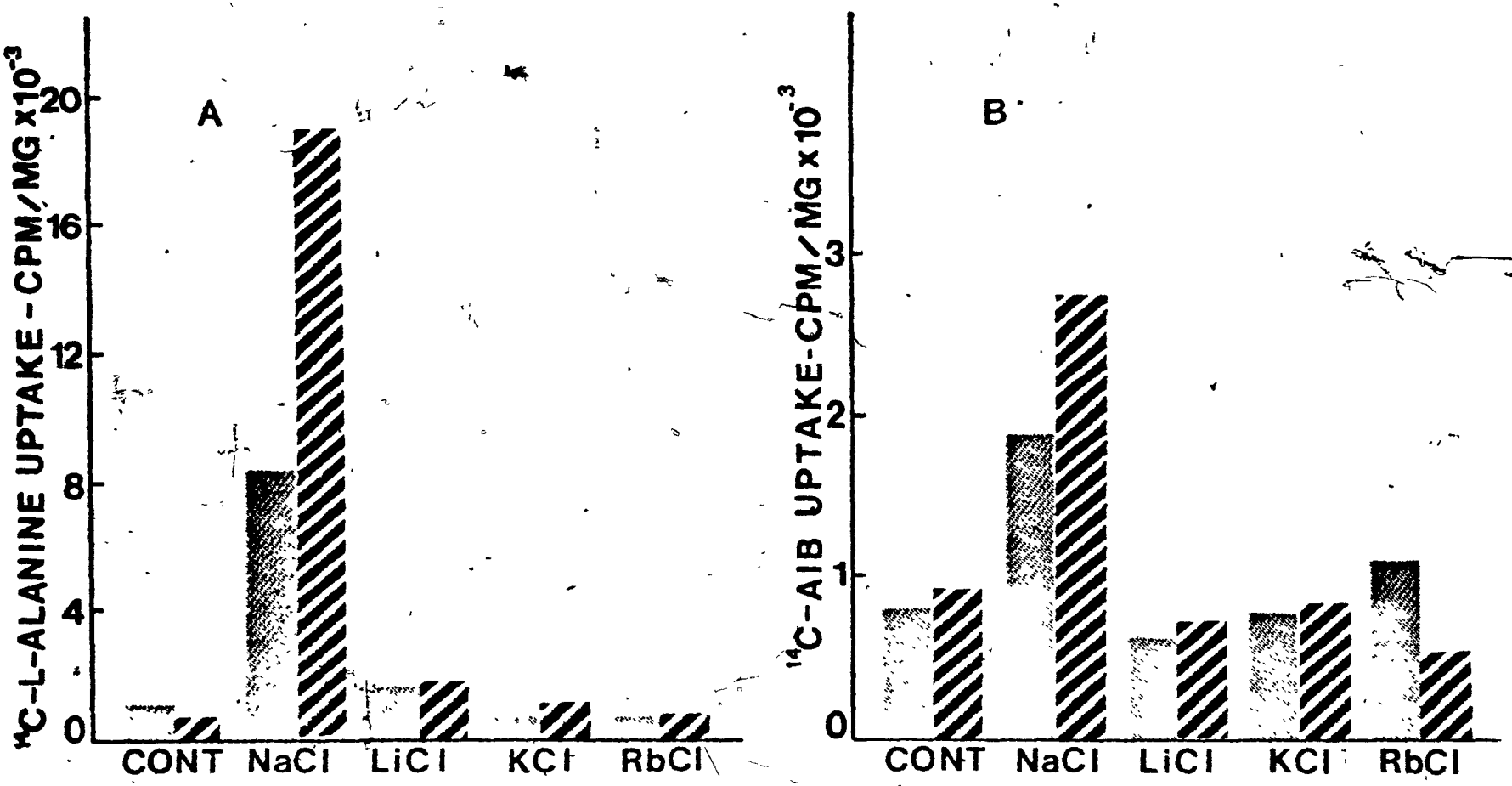
i. *Requirement for $Na^+$ in amino acid transport into isolated membranes*

Because of the data already shown it was felt that the uptake of L-alanine by the membranes did represent the transport system operative in intact cells. Cells of this organism will neither grow in the absence of NaCl (Gow, Ph.D. thesis, 1973) nor will they transport AIB (Drapeau and MacLeod, 1963a). This finding allowed a further test as to the physiological importance of the isolated membrane system, since $Na^+$ should also be required for the transport activity observed with isolated membranes. A highly specific requirement for $Na^+$ for the uptake of L-alanine or AIB into isolated membranes could, in fact, be demonstrated in the presence of either of two energy sources (Fig. 5). As compared to the control (CONT), only in the presence of NaCl did uptake of the amino acids occur. Neither $Li^+$, $K^+$, $Rb^+$ nor $Cl^-$ had any ability to replace this function of $Na^+$. The level of residual $Na^+$ in the assay medium containing membranes was determined by flame photometry after digestion to be

FIGURE 5.

Fig. 5. Requirement for $Na^+$ for the uptake of L-alanine and AIB by vesicles.

Vesicles were suspended in Mg-K-Tris solution, and diluted with this same solution (Control) or with this solution containing sufficient NaCl, LiCl, KCl or RbCl to give 200 mM. L-[$^{14}$C]alanine, 1.8 μM, 156 μCi/μmole (A); [$^{14}$C]AIB, 55 μM, 8.6 μCi/μmole (B). Shaded bars, ethanol at 2 mM; hatched bars, ascorbate-TMPD. Electron donors and amino acids were added in Mg-K-Tris solution. Following 5 min uptake intervals, vesicles were filtered and washed with CS-T solution containing LiCl (200 mM) in place of NaCl.


A
$^{14}$C-L-ALANINE UPTAKE-CPM/MG x10$^{-3}$
0
4
8
12
16
20
CONT
NaCl
LiCl
KCl
RbCl
B
$^{14}$C-AIB UPTAKE-CPM/MG x10$^{-3}$
0
1
2
3
CONT
NaCl
LiCl
KCl
RbCl

1.1 mM. In the experiment described NaCl was added at 200 mM, since this was the concentration found optimum for AIB uptake into intact cells (Wong *et al.*, 1969). It was considered necessary to quantitate the $Na^+$ required by the isolated system. This experiment was performed in two ways, by a tube dilution technique (Fig. 6, Curve 1) and by dialysis (Curve 2). The dialysis procedure was time consuming allowing time for activity losses, and gave more erratic results than by the tube dilution technique. In contrast to the intact cells the $Na^+$ optimum was found to be about 75 mM using either technique. That this lower level of $Na^+$ was not an effect of the energy source (ethanol) used to activate transport is shown in Fig. 7. Transport energized by either ethanol or ascorbate-TMPD showed quantitatively similar $Na^+$ concentrations for optimum activity. This is particularly interesting since reduced TMPD was found in mitochondria to couple at the level of cytochrome c (Jacobs, 1960).

Electron Donors for Transport

Recent studies with intact cells of the marine pseudomonad have shown that ethanol and various straight chain alcohols stimulate the concentrative uptake of AIB (Thompson and MacLeod, 1973b). A comparison between various potential energy sources for transport is shown in Fig. 8. Ethanol stimulated L-alanine transport into membrane vesicles to an extent comparable to that obtained with NADH or ascorbate-TMPD. It was observed previously that a considerable

FIGURÆ 6.

Fig. 6. Quantitative requirement for $Na^+$ for L-alanine uptake into vesicles.

Vesicles prepared in the absence of added $Na^+$ were re-exposed to this ion in two ways. In the tube dilution assay (Curve 1) vesicles were diluted 1:1 in test tubes with CS-T containing sufficient NaCl to give the desired concentration. The dialysis method (Curve 2) allowed a slower addition of NaCl. Vesicles in dialysis sacs (10 mg per sac) were dialyzed 1 hour in CS-T containing the final desired NaCl level. Each sac was dialyzed at 4 C against 1-liter volumes changed every 20 min. Uptakes were for 1.0 min using L-alanine (1.8 μM) and ethanol (2 mM).

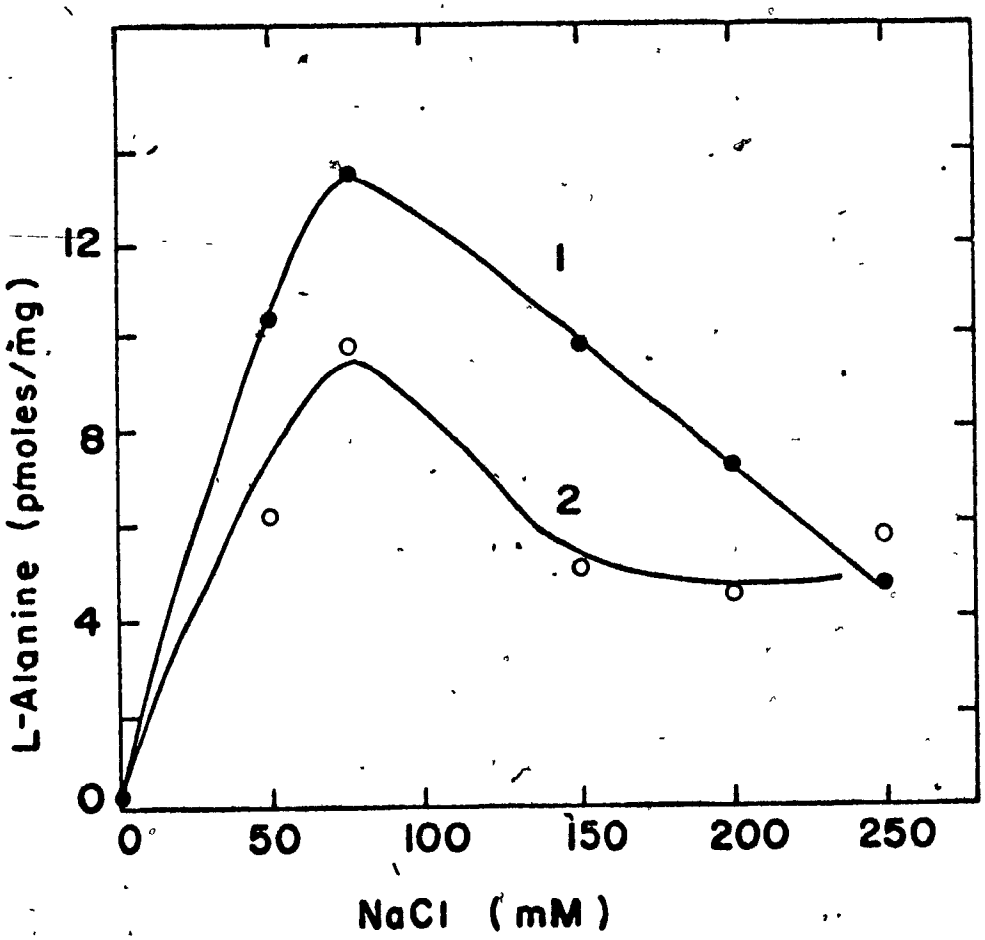

L-Alanine (pmoles/mg)
12
8
4
0
0
50
100
150
200
250
NaCl (mM)
1
2

FIGURE 7.

Fig. 7. Quantitative response to $Na^+$ of L-alanine uptake into vesicles.

Using the tube dilution method $Na^+$ was added to vesicles at the concentrations shown. Uptakes were for 1:0 min using L-alanine (1.8 μM) and either ascorbate-TMPD (Curve 1) or 2 mM ethanol (Curve 2).

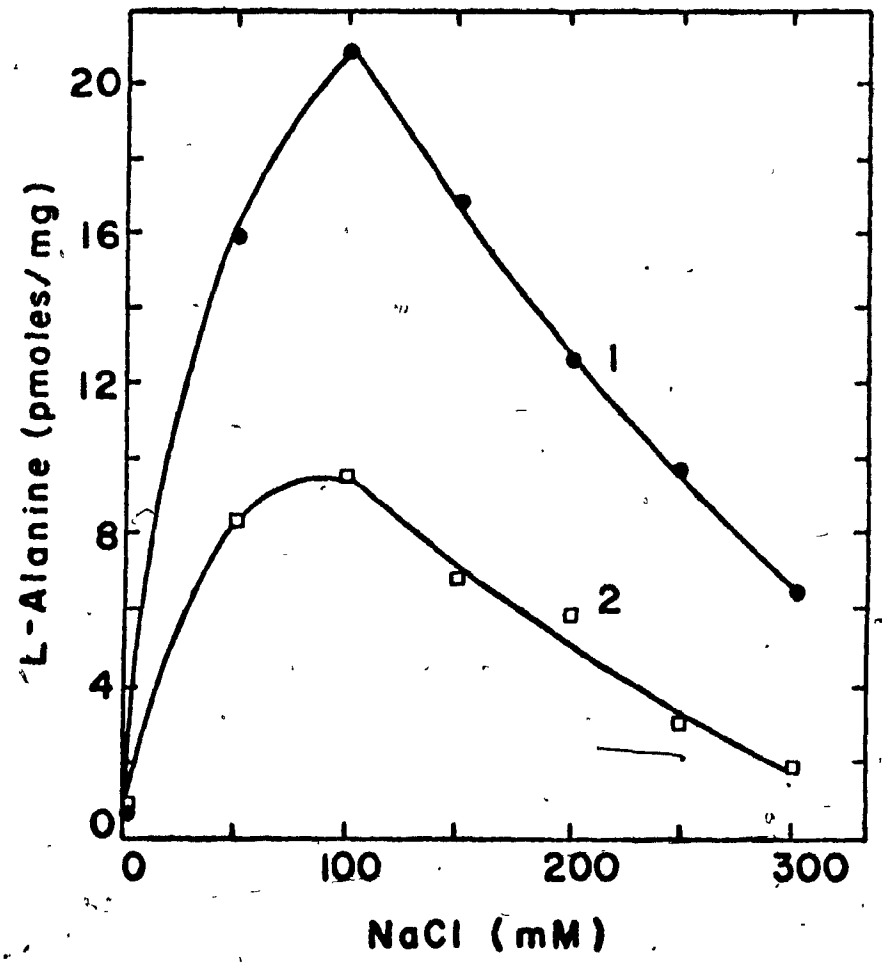
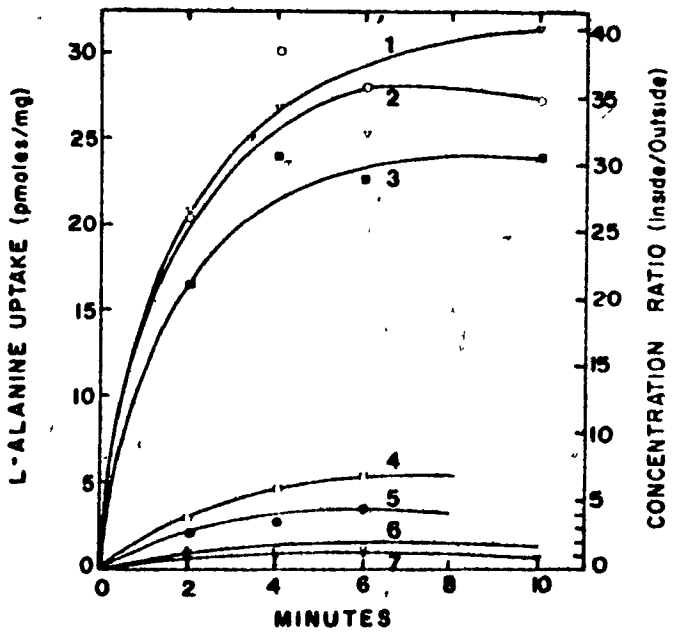

L-Alanine (pmoles/mg)
20
16
12
8
4
0
0
100
200
300
NaCl (mM)
1
2

FIGURE 8.

Fig. 8. Comparison of the ability of selected compounds to stimulate the accumulation of L-alanine into vesicles.

Curve 1, ascorbate-TMPD; Curve 2, ethanol at 25 mM; Curve 3, NADH; Curve 4, ascorbate-PMS; Curve 5, ascorbate; Curve 6, no added energy source; Curve 7, no added energy source, plus KCN at 1.0 mM.

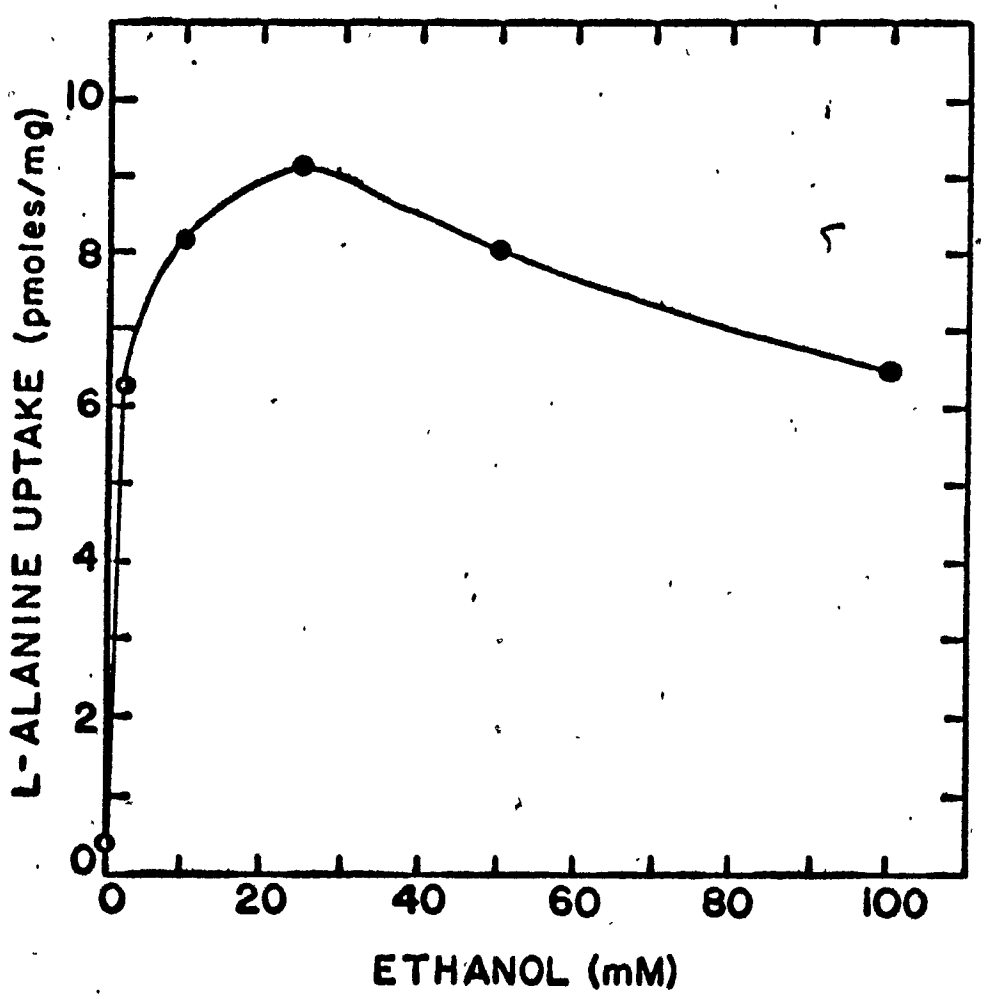

L-ALANINE UPTAKE (pmoles/mg)
CONCENTRATION RATIO (Inside/Outside)
MINUTES
0
2
4
6
8
10
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7

amount of L-alanine was taken up by vesicles in the absence of added electron donors. The radioactive preparation used had been supplied as a solution containing ethanol, thus resulting in the addition of 1-2 mM ethanol to the incubation medium. As can be seen in Fig. 9, this amount of ethanol is capable of stimulating transport appreciably and could account for the high level of activity obtained previously. The results in Fig. 8 were obtained with a radioactive L-alanine preparation from which ethanol had been removed by evaporation under vacuum. Uptake in the absence of an added electron donor was very low (Curve 6) and this endogenous activity was reduced only slightly by the addition of KCN. Also shown is the low activity induced by ascorbate-PMS or by ascorbate alone.

Intravesicular space was determined to be 0.7 µl per mg of protein. This value is low compared to those reported for other bacterial membrane preparations (Hirato *et al.*, 1971; Kaback and Barnes, 1971; Konings and Freese, 1972) and may be attributed either to a lower percentage of the membranes existing as vesicles, or to partial collapse of these vesicles as a result of centrifugation in the course of determining the intravesicular space. The latter possibility seems unlikely and our intravesicular space correct, since L-alanine can be calculated on the basis of the above space to equilibrate across the membrane in the presence of KCN (Fig. 8, Curve 7). The capacity of the vesicles to concentrate L-alanine in the presence of energy sources, expressed as a ratio of internal

Fig. 9. Effect of ethanol concentration on the rate of uptake of L-alanine by vesicles.

Ethanol additions were just before the addition of L-[$^{14}$C]alanine (1.8 μM), and uptakes were measured after 1.0 min.


L-ALANINE UPTAKE (pmoles/mg)
0
2
4
6
8
10
0
20
40
60
80
100
ETHANOL (mM)

to external concentration, is also shown. It is evident that added electron donors produced concentrations of the amino acid in the vesicles which were 30 to 40 times that present in the suspending medium.

Data had been obtained comparing various compounds for their ability to energize transport, prior to the discovery that ethanol could serve in this capacity. This data is shown in Table 8 and was obtained with 1-2 mM ethanol in all the assay systems. Consequently, in the absence of any test compound considerable activity was observed. Of the compounds tested only NADH and ascorbate-TMPD stimulated transport over the endogenous. The reason for this additional stimulation over that obtained with 1-2 mM ethanol is evident from the data in Fig. 9 showing transport to be only partially activated by 1-2 mM ethanol. No stimulation was noted for D-lactate and D,L-α-glycerophosphate, compounds known to be effective, respectively, in vesicles of *E. coli* (Kaback and Milner, 1970) and *Staphylococcus aureus* (Short *et al.*, 1972).

## Specificity of Alcohol energized Transport

In order to gain some insight into the possible mechanism of action of alcohols in energizing transport the alcohol specificity was tested. Alcohols tested for their capacity to promote transport (Table 9) showed a chain length of at least 2 carbon atoms to be

TABLE 8.

*Effect of potential electron donors and ATP on the uptake of L-alanine and AIB by membrane vesicles.*

Compounds were tested at 20 mM except for NADH at 25 mM, TMPD at 150 µM, and PMS at 100 µM. Uptake period, 5 min. Uptakes were expressed as the ratio of the uptake in the presence, to uptake in the absence, of an added test compound. L-alanine, 1.8 µM; AIB 55 µM.

| Compound tested | L-alanine | | AIB | |
|---|---|---|---|---|
| | Uptake | Ratio | Uptake | Ratio |
| | cpm/mg | | cpm/mg | |
| None | 5,558 | 1.00 | 1,732 | 1.00 |
| NADH | 9,554 | 1.72 | 5,496 | 3.17 |
| D(-)-Lactate | 3,983 | 0.72 | 1,051 | 0.61 |
| D,L-α-Glycerophosphate | 3,981 | 0.72 | 1,218 | 0.70 |
| Ascorbate-TMPD | 15,100 | 2.72 | 5,667 | 3.27 |
| Ascorbate-PMS | 2,593 | 0.47 | 2,321 | 1.34 |
| Ascorbate | 4,836 | 0.87 | 1,279 | 0.74 |
| TMPD | 1,360 | 0.25 | not tested | |
| PMS | 1,480 | 0.25 | 819 | 0.47 |
| Succinate | 5,956 | 1.07 | 1,301 | 0.75 |
| ATP | 4,241 | 0.76 | 932 | 0.54 |

TABLE 9.

*Comparison of ability of various alcohols to energize L-alanine transport into membrane vesicles of the marine pseudomonad.*

Alcohols tested at 25 mM were added to the membranes just before L-[$^{14}$C]alanine.

| Alcohol | Transport pmoles/mg/min |
|---|---|
| none | 0.70 |
| methanol | 0.73 |
| ethanol | 2.60 |
| 1-propanol | 3.04 |
| 1-butanol | 2.90 |
| 1-pentanol | 2.21 |
| 2-propanol | 1.43 |
| 2-methyl-1-propanol | 0.86 |
| 2-methyl-2-butanol | 0.65 |
| 1,4-butanediol | 1.04 |

essential for activity. Transport with 1-propanol and 1-butanol was even slightly better than with ethanol. A further increase in chain length beyond four carbon atoms, or the presence of methyl or hydroxyl groups on the carbon atoms other than carbon-1, reduced L-alanine transport stimulating activity. A close parallel exists between the transport promoting capacity of the alcohols observed here and the specificity of the alcohol dehydrogenase isolated by Kersters and DeLey (1966) from *Gluconobacter*.

An attempt to energize L-alanine transport by NADH generated endogenously by $NAD^+$, ethanol and alcohol dehydrogenase (Sigma) is shown in Table 10. This technique was effective in energizing proline transport into *Mycobacterium phlei* electron transport particles (Hirato *et al.*, 1971). The addition of $NAD^+$ and enzyme in the presence of ethanol actually reduced the uptake of L-alanine over that obtained with ethanol alone. As can be seen, this reduction was due to the presence of the added enzyme. However, it was also possible that the presence of the membranes interfered with the alcohol dehydrogenase reaction. To test this possibility two assays were set up as in Table 10 containing ethanol, $NAD^+$ and enzyme, either with or without membranes. The reaction was started by adding the enzyme. After 1.0 min at 25 C the volumes of each assay were diluted to 2.0 ml and centrifuged 10 min at 36,000 g. The two supernatant fluids both gave an absorbance of 0.60 at 340 nm using the Unicam SP 800. Thus the presence of membranes did not interfere

T A B L E 10.

*Effect of components of the alcohol dehydrogenase reaction on L-alanine transport into membrane vesicles.*

NADH, $NAD^+$ and ethanol were added at 25 mM. Enzyme refers to alcohol dehydrogenase (E.C. 1.1.1.1.) added to give 6 µg protein (1.06 International Units) to the incubation medium.

| Addition | Transport pmoles/mg/min |
|---|---|
| Experiment 1: | |
| none | 0.92 |
| $NAD^+$ | 1.04 |
| enzyme | 1.08 |
| enzyme + $NAD^+$ | 0.83 |
| ethanol | 4.16 |
| ethanol + $NAD^+$ | 4.00 |
| ethanol + enzyme | 2.34 |
| ethanol + $NAD^+$ + enzyme | 1.43 |
| NADH | 3.90 |
| Experiment 2: | |
| ethanol | 4.65 |
| ethanol + enzyme | 2.13 |
| NADH | 3.83 |
| NADH + enzyme | 2.05 |
| ascorbate-TMPD | 2.38 |
| ascorbate-TMPD + enzyme | 1.71 |
| ethanol + NADH | 5.31 |
| ethanol + NADH + enzyme | 2.09 |
| ethanol + $NAD^+$ + enzyme | 1.37 |

with the production of NADH by the added alcohol dehydrogenase. Since the enzyme inhibited transport in the presence of ethanol, NADH, ascorbate-TMPD or with combinations of ethanol and NADH, Table 10, it would appear that the enzyme is nonspecifically blocking alanine uptake.

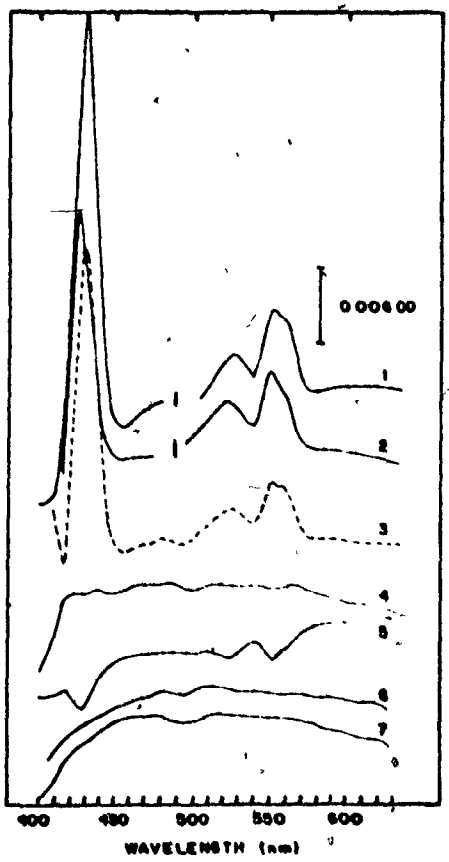
Cytochrome Analysis and Alcohol dehydrogenase Assays

The finding that electron donors such as NADH and ascorbate-TMPD could energize transport of alanine into membrane vesicles (Fig. 8) convincingly linked electron flow to active transport. For this reason, and because of a complete lack of information regarding the electron transfer components in the marine pseudomonad, a study of the cytochromes was initiated.

Membrane components reduced by either NADH or by dithionite and scanned under steady state conditions yielded the results shown in Fig. 10. The alpha peaks associated with cytochromes b and c (Chance and Williams, 1955; Appleby, 1969) appeared at 560 and 552 nm, respectively. An absorption maximum for the beta peak was observed at 524 nm and for the Soret peak at 428 nm. The Soret peak and the beta peak represent a mixture of those peaks from cytochrome b and cytochrome c. Although not shown, the addition of ferricyanide added to ensure complete oxidation of membranes in the reference cuvette, had no effect on the dithionite reduced spectrum.

Fig. 10. Analysis of respiratory components present in vesicles of the marine pseudomonad.

The oxidized-oxidized baseline was obtained by scanning freshly aerated membranes (5.2 mg) present in sample and reference cuvettes. Sodium dithionite (1.0 mg/ml), ascorbate-TMPD, NADH and ethanol were tested by adding each to a suspension of vesicles and recording the scan after peak heights became constant. The ethanol scan was recorded 10 min after the addition of ethanol. The reduced-reduced baseline was obtained by adding dithionite to both reference and sample cuvettes. CO was then bubbled for 3 min through the contents of the sample cuvette and the dithionite-reduced plus CO minus dithionite-reduced scan recorded. Curve 1, dithionite; Curve 2, ascorbate-TMPD (2.7 mM - 16 μM); Curve 3, NADH (2.7 mM); Curve 4, ethanol (2.7 mM); Curve 5, dithionite plus CO minus dithionite; Curve 6, reduced-reduced; Curve 7, oxidized-oxidized.


0.006 00
1
2
3
4
5
6
7
400
450
500
550
600
WAVELENGTH (nm)

With ascorbate-TMPD as the electron donor, cytochrome c was reduced to a slightly greater degree than with NADH. This suggests that the added NADH does not reach all of the available NADH dehydrogenase coupling sites. Also, with ascorbate-TMPD the 560 nm peak decreased considerably compared to the peak at 552 nm, indicating comparatively less cytochrome b reduction than had been obtained with NADH or dithionite. This observation was verified by a shifting of the Soret peak from 428 to 425 nm. It can be concluded that reduced TMPD couples primarily to cytochrome c; however, cytochrome b was partially reduced. Either some electrons are donated from TMPD directly to cytochrome b, or a back flow of electrons occurs from cytochrome c. A back flow of electrons from ascorbate-TMPD has been observed (Klingenberg, 1968). Also, the trough at about 455 nm, tentatively identified as flavoprotein, was of interest. This component was not reduced with ascorbate-TMPD but did show up in dithionite and NADH reduced membranes.

Membranes of this organism do not contain detectable levels of cytochrome a using our experimental conditions. Peaks which could be attributed to this type of oxidase (Castor and Chance, 1959) were absent on reduction of the respiratory components by dithionite, ascorbate-TMPD, or NADH. The dithionite-reduced plus CO minus dithionite-reduced spectrum shown (Curve 5) is, however, characteristic of a cytochrome o, having a peak at 415 nm and a trough at 426 nm

(Castor and Chance, 1959). The 415 nm peak may be low because of the dropping baseline in this region (Curves 6 and 7).

Considering the previous observation that alcohols energized transport and showed similar specificities to *Gluconobacter* alcohol dehydrogenase (Table 9), it was surprising to find that no detectable cytochrome reduction was found in membranes using ethanol as the electron donor (Fig. 10, Curve 4). When whole cells were analyzed for cytochrome reduction (Fig. 11) it was observed that NADH and ethanol gave similar results, with peaks appearing at 550 and 560 nm. These cells were expected to contain considerable alcohol dehydrogenase since this organism can grow using ethanol as sole carbon source (Baumann *et al.*, 1972). Unlike the cells, no production of NADH was noted when ethanol and $NAD^+$ were added to the membranes. Assays were performed using membranes poisoned with 1.0 mM KCN or suspended in the absence of any inhibitor. As shown in Table 11, an NAD-linked alcohol dehydrogenase was not released from the cells until they were broken open. Some release during protoplasting was likely the result of lysis of a low proportion of the protoplasts. Assays performed using membranes poisoned with 1.0 mM KCN, to prevent the reoxidation of reduced NAD, were negative.

These results indicate a production of NADH on the addition of ethanol to cells. The NADH formed by cytoplasmic alcohol dehydrogenase would be expected to cause the reduction of cytochromes b and

Fig. 11. Analysis of respiratory components in cells reduced by ethanol and by dithionite.

Each cuvette contained 1.5 mg dry weight of cells. Cells in reference cuvettes were oxidized with ferricyanide. Scans were recorded when peak heights remained constant. Curve 1, dithionite; Curve 2, ethanol (2.5 mM); Curve 3, no electron donor.

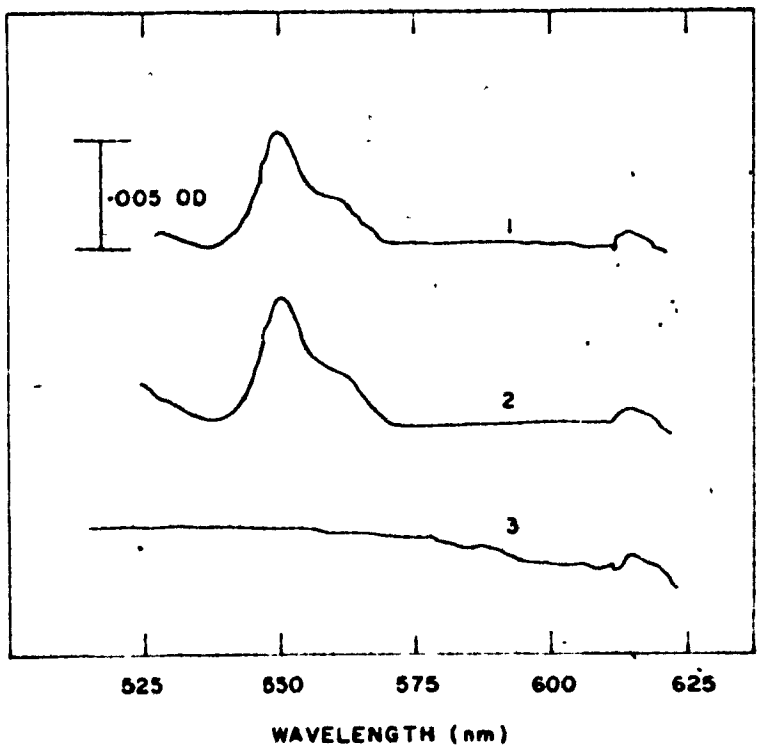

.005 OD
1
2
3
525
550
575
600
625
WAVELENGTH (nm)

TABLE 11.

*Release of NAD-linked alcohol dehydrogenase from cells of the marine pseudomonad during membrane isolation.*

Total units represent the total enzyme released from 375 mg cells.

| Assay material | Total units |
|---|---|
| Sonicated cell extract | 43.7 |
| Sonicated cell extract + 1 mM KCN | 34.9 |
| NaCl supernatant fluids | none detected |
| Sucrose supernatant fluids | none detected |
| Protoplast supernatant fluid | 12.4 |
| Membrane wash fluid | 0.4 |
| Cytoplasmic contents | 42.1 |
| Membranes (5.3 mg) + 1 mM KCN | none detected |

c. In membranes not containing the cytoplasmic enzyme no cytochrome reduction would be expected. Previous data has, however, indicated a membrane bound alcohol dehydrogenase (Table 11). Two alcohol dehydrogenases have been observed in acetic acid bacteria (Nakayama and DeLey, 1965). One is membrane bound and is an alcohol-cytochrome 553 reductase, while the other is an NADH-linked soluble enzyme in the cytoplasm.

Sites of HOQNO and KCN inhibition in the respiratory chain

Because of the unexpected lack of correlation between the ability of ethanol to energize L-alanine transport on the one hand and to reduce the cytochrome components on the other, it was decided to test the ability of various energy sources for transport to induce cyanide and HOQNO sensitive oxygen uptake (Table 12). In the absence of inhibitors it is evident that NADH gave more oxygen uptake than ascorbate-TMPD, while the oxygen consumed was low when ethanol was the electron source. Oxygen uptake in the presence of alcohol in particulate preparations containing the entire respiratory chain has been taken as a measure of alcohol dehydrogenase activity (Kersters and DeLey, 1966). Since considerable oxygen uptake was found with NADH it is possible to conclude that alcohol dehydrogenase activity in these membrane preparations is quite low.

Oxygen uptake data show HOQNO to inhibit NADH induced uptake

TABLE 12.

*Rate of oxygen consumption by membranes of the marine pseudomonad in the presence of various energy sources and inhibitors.*

Oxygen was taken up at a linear rate for at least 1.0 min and calculations were based on the initial rates. Data obtained with ascorbate-TMPD was corrected for oxygen taken up in the absence of membranes.

| Energy source | Inhibitor | Oxygen consumed ng atoms O/min/mg |
|---|---|---|
| None | None | 0 |
| NADH | None | 56 |
| NADH | HOQNO | 21 |
| NADH | KCN | 4 |
| Ascorbate-TMPD | None | 40 |
| Ascorbate-TMPD | HOQNO | 37 |
| Ascorbate-TMPD | KCN | 0 |
| Ethanol | None | 2 |
| Ethanol | HOQNO | < 1 |
| Ethanol | KCN | < 1 |
| Methanol | None | 0 |

by 62.5% and ascorbate-TMPD induced uptake by only 7.5% (Table 12). Since reduced TMPD couples primarily to cytochrome c (Fig. 10), it may be deducted that HOQNO acts on the NADH dehydrogenase side of this cytochrome. That the site of HOQNO inhibition probably lies between flavoprotein and cytochrome b is shown in Table 13 where the percentage reduction of the respiratory components by NADH was compared in the presence and absence of HOQNO and KCN. The percentage reduction of flavoprotein by NADH was not affected by HOQNO while the percentage reduction of cytochromes b and c were about equal and less than in the anaerobic control. In *E. coli* electron flow is inhibited by HOQNO at the level of the quinone (Cox *et al.*, 1970). Thus in the marine pseudomonad a quinone component may be involved in electron flow between a flavoprotein and cytochrome b.

Similar considerations lead to the conclusion that KCN acts at or near the terminal oxidase. KCN strongly inhibited both NADH and ascorbate-TMPD stimulated oxygen uptake (Table 12). Also, the percentage reduction of respiratory components by NADH (Table 13) was similar whether recorded in the presence of KCN or in an anaerobic control.

## Effect of inhibitors on uptake of L-alanine

Because of the low alcohol oxidase activity in the membranes, the involvement of electron flow in ethanol energized amino acid transport became of considerable interest. The possibility that

TABLE 13.

*Effect of inhibitors on the percentage reduction of respiratory components under anaerobic steady state conditions using NADH as the electron donor.*

Steady state values were determined using a split beam spectrophotometer. Reaction mixtures containing no inhibitor were scanned after anaerobic steady state was achieved. Steady state conditions were rapidly obtained in the presence of either inhibitor. Values for per cent reduction were determined from peak heights or flavoprotein trough, as compared to dithionite (1.0 mg/ml) reduced samples containing no inhibitors.

| Component | Inhibitor added | | |
|---|---|---|---|
| | None | HOQNO | KCN |
| | % reduction | | |
| Soret | 65 | 18 | 59 |
| Cytochrome b | 71 | 36 | 67 |
| Cytochrome c | 64 | 32 | 60 |
| Flavoprotein | 60 | 55 | 50 |

electron flow was perhaps not involved in alcohol driven transport was tested using various inhibitors.

The effect of replacing air with $N_2$ is shown in Fig. 12. Serological tubes containing membranes were sealed with serum caps and flushed with $N_2$ for several minutes. Either ascorbate-TMPD or ethanol, plus L-[$^{14}$C]alanine, were added through the serum cap and transport measured with time. As compared to controls tested for transport with normal aeration (Curves 1 and 2), the $N_2$ flushed samples gave lowered activity for ascorbate-TMPD or ethanol.

The effect of concentration of various inhibitors is shown in Table 14. Although an accurate $K_i$ cannot be determined from this data, roughly 50% inhibition occurred for DNP at 3 mM, for KCN at 0.5 mM, and for $NaN_3$ at 11 mM. Ouabain, an inhibitor of the ($Na^+$ + $K^+$)- activated ATP'ase (Schultz and Curran, 1970), was found to show no inhibition. This is consistent with the previous finding that cells of this organism lack an ATP'ase specifically activated by the combination of $Na^+$ and $K^+$ (Drapeau and MacLeod, 1963b).

The effect of a wider range of inhibitors on transport is shown in Table 15. Inhibitions were similar both quantitatively and qualitatively when NADH or ethanol were the electron donors. Marked differences were apparent when ascorbate-TMPD energized transport. Transport was inhibited by HOQNO with either NADH or with ethanol but not with ascorbate-TMPD as electron donor. Both KCN and DNP

Fig. 12. Effect of a $N_2$ atmosphere on L-alanine uptake by vesicles. Membrane suspensions contained in serological tubes were either left open to the atmosphere or sealed with serum caps and flushed with $N_2$ for 5 min. In the case of sealed tubes subsequent additions were made by injection through the cap. Curve 1, air, ascorbate-TMPD; Curve 2, air, ethanol at 25 mM; Curve 3, 4, $N_2$, with ascorbate-TMPD or ethanol. L-alanine, 1.8 μM, 156 μCi/μmole.

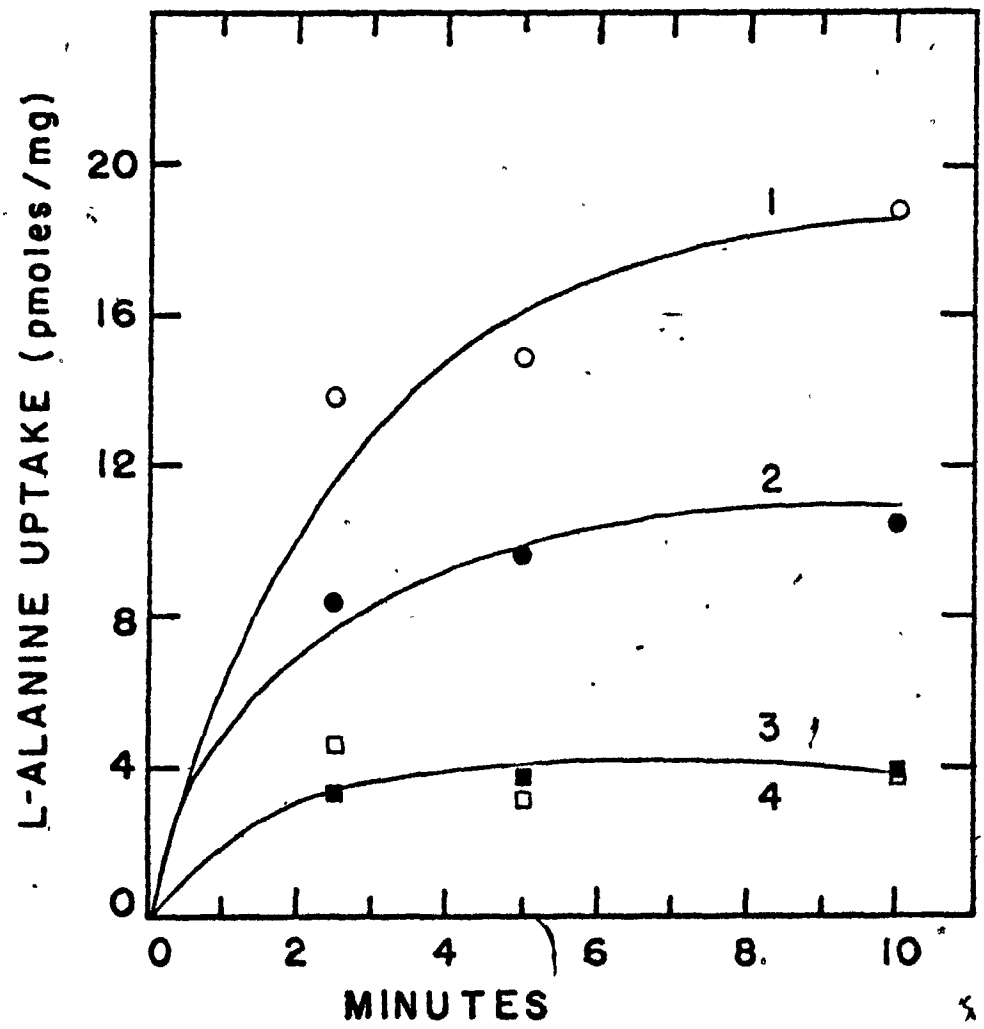

L-ALANINE UPTAKE (pmoles/mg)
20
16
12
8
4
0
0
2
4
6
8.
10
MINUTES
1
2
3
4

TABLE 14.

*Effect of metabolic inhibitors on the uptake of L-[$^{14}C$]alanine by membranes.*

Inhibitors were added 15 min before the addition of energy source (ethanol, 1-2 mM) and L-[$^{14}C$]alanine (1.8 μM, 156 μCi/μmole). Uptake in this experiment was concluded after 10 min. Reaction mixtures contained CS-T, $Na^+$ 200 mM.

| Inhibitor | Concentration mM | % Inhibition |
|---|---|---|
| DNP | 10 | 82 |
| | 1 | 40 |
| | 0.1 | 36 |
| KCN | 10 | 87 |
| | 1 | 68 |
| | 0.1 | 24 |
| $NaN_3$ | 10 | 46 |
| | 1 | 14 |
| | 0.1 | 2 |
| PCMB | 1 | 79 |
| | 0.1 | 39 |
| IAA | 10 | 42 |
| | 1 | 6 |
| Ouabain | 1 | (-11) |

TABLE 15.

*Effect of metabolic inhibitors on the NADH, ethanol, and ascorbate-TMPD energized transport of L-alanine into membrane vesicles of the marine pseudomonad.*

The negative values recorded (in brackets) in the column under ascorbate-TMPD indicate stimulation of transport. DMSO (dimethyl sulfoxide) was added where indicated at 2.9%.

| Inhibitor | Concentration mM | Electron donor added | | |
|---|---|---|---|---|
| | | NADH | Ethanol | Ascorbate-TMPD |
| | | Inhibition of transport (%) | | |
| HOQNO | 0.02 | 65 | 59 | (-11) |
| KCN | 1.0 | 93 | 90 | 96 |
| PCMB | 1.0 | 96 | 92 | 51 |
| IAA | 10.0 | 32 | 22 | (-14) |
| NEM | 10.0 | 88 | 87 | (-26) |
| DNP | 10.0 | 88 | 87 | 90 |
| $NaN_3$ | 10.0 | 30 | 41 | 93 |
| DMSO | | 66 | 71 | (-22) |
| DMSO + valinomycin | 0.1 | 76 | 78 | (-4) |
| DMSO + antimycin A | 0.1 | 71 | 78 | (-23) |
| DMSO + rotenone | 0.1 | 68 | 74 | (-25) |

inhibited transport essentially completely with all three energy sources. The sulfhydryl inhibitors PCMB and NEM inhibited transport essentially completely and iodoacetate partially, when NADH or ethanol served as electron donors. With ascorbate-TMPD, PCMB inhibited transport only 50% and the other two compounds not at all. Several of the compounds tested stimulated L-alanine transport in the presence of ascorbate-TMPD.

Dimethyl sulfoxide (DMSO) was used to help dissolve valinomycin, antimycin A, and rotenone. DMSO itself proved to be inhibitory when NADH or ethanol were the electron donors but not when ascorbate-TMPD was used. The combinations with DMSO of valinomycin, antimycin A, and rotenone inhibited only slightly more than the DMSO alone when NADH or ethanol drove transport, but were not inhibitory when ascorbate-TMPD was the energy source. In a separate experiment ethanol was used in place of DMSO as solvent for valinomycin, antimycin A and rotenone, again, no inhibition was observed with these compounds.

As noted in Table 12, oxygen uptake with NADH was inhibited by 62.5% by HOQNO. This value compares well to the 65% inhibition of NADH stimulated transport by HOQNO (Table 15). Furthermore, HOQNO did not inhibit transport with ascorbate-TMPD and inhibited ascorbate-TMPD induced oxygen uptake by only 7.5%.

It has been shown that 360 nm light destroys oxidative phosphory-

lation in *Mycobacterium phlei* particles and that vitamin $K_2$ can restore the activity (Brodie and Ballantine 1960; Kashket and Brodie, 1963). Furthermore, membrane vesicles of *B. licheniformis* lost transport activity on exposure to 360 nm light and the activity was recovered by adding vitamin $K_2$ of appropriate chain length (MacLeod *et al.*, 1973). Transport activity in vesicles of the marine pseudomonad as a function of exposure time to 366 nm light is shown in Fig. 13. Because of the sensitivity of the membrane transport system to heat (Fig. 1 ) the irradiation was performed on membranes stored on ice. If inactivation occurred by destruction of a quinone component then ascorbate-TMPD should overcome the inhibition of transport. This experiment is shown in Table 16. The per cent inhibition by 366 nm light on transport driven by NADH or ethanol was the same, a result to be expected if a quinone had been destroyed or damaged. Irradiated membranes were partially restored for transport by ascorbate-TMPD. As compared to ethanol and NADH, passing electrons directly to cytochrome c overcame about 14% of the inhibition by 366 nm light. In membranes of *Aerobacter aerogenes* light of 354 nm was found to destroy ubiquinone primarily, but also in some cases to alter protein structures (Knook and Planta, 1971). Furthermore, in *B. licheniformis* FAD and FMN had to be added back in addition to quinone to restore full transport activity (MacLeod *et al.*, 1973).

These results do implicate electron flow as the driving force for transport although the mechanism of coupling of electron flow to

Fig. 13. Loss of L-alanine transport activity in vesicles on exposure to 366 nm light.

Vesicles (4 ml, 10 mg/ml) in a beaker of 3 cm internal diameter were exposed at a distance of 5 cm to a 366 nm source. The source was 110v, 50-60 cy., 9 watts, made by Ultra-Violet Products, Inc. Uptakes were measured using 2 mM ethanol as energy source.

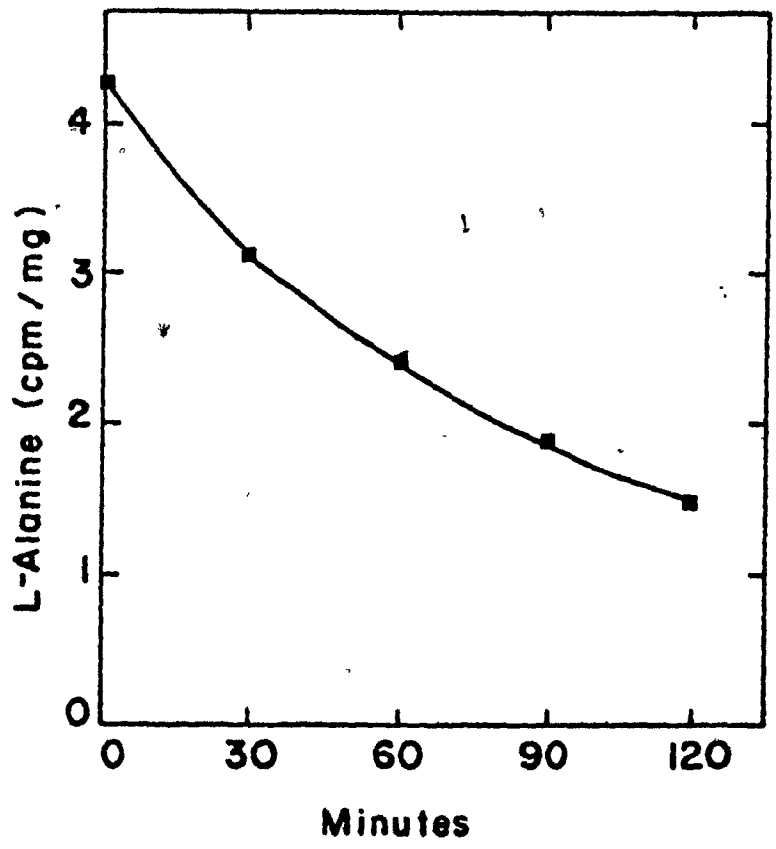

L-Alanine (cpm / mg)
0
1
2
3
4
0
30
60
90
120
Minutes

TABLE 16.

*Effect of the electron donor on recovery of L-alanine transport into irradiated vesicles.*

Vesicles irradiated with 366 nm light in Fig. 13 for 120 min were tested for transport and compared to non-irradiated membranes. Ethanol 2 mM; uptake time 5 min.

| Electron Donor | Irradiated | L-alanine uptake cpm/mg | Inhibition % |
|---|---|---|---|
| Ethanol | - | 2,893 | |
| Ethanol | + | 862 | 70 |
| NADH | - | 3,192 | |
| NADH | + | 953 | 70 |
| Ascorbate-TMPD | - | 5,184 | |
| Ascorbate-TMPD | + | 2,302 | 56 |

transport is unclear.

Mechanism of energy coupling

Several possible mechanisms exist for coupling of the potential energy of electron flow through the respiratory chain to transport. One such possibility is through oxidative phosphorylation. Scarborough *et al.* (1968) have implicated ATP in the transport of β-galactose into cells of *E. coli*. In membranes of the marine pseudomonad neither ATP (Table 8) nor ADP plus inorganic phosphate (Table 18) had any stimulating effect. More direct evidence for the lack of ATP effects in the membrane vesicle transport system is shown in Table 17. The electron donors able to stimulate L-alanine transport failed to stimulate ATP formation. DNP, an uncoupler of oxidative phosphorylation (Harold, 1972a) and an inhibitor of L-alanine transport (Table 15), had little or no effect on the amount of ATP found present in the membranes. It should be emphasized that the conditions of the assay were the same as used in transport assays where no ADP or inorganic phosphate were added. Under these conditions electron flow appeared uncoupled from oxidative phosphorylation.

A further point of interest was the finding that ethanol in combination with ascorbate-TMPD did not give additive effects on transport.

TABLE 17.

*ATP content of membrane preparations and L-alanine uptake into membrane vesicles in the presence and absence of electron donors and DNP.*

Dashes (-) indicate assays not performed.

| Additions | | L-alanine uptake | ATP |
|---|---|---|---|
| Electron donor | Inhibitor | pmoles/mg/min | pmoles/mg/min |
| None | None | 0.88 | 38 |
| None | DNP | - | 35 |
| Ethanol | None | 9.87 | 31 |
| Ethanol | DNP | - | 26 |
| Ascorbate-TMPD | None | 11.14 | 24 |
| Ascorbate-TMPD | DNP | - | 23 |
| NADH | None | 9.73 | 19 |
| Ascorbate-TMPD + Ethanol | None | 14.22 | 29 |

A second possible mechanism of energy coupling was described by Kaback and Barnes (1971). This involved the positioning of a redox carrier protein within the electron transport chain. Part of the evidence for the hypothesis that the reduced form of the carrier mediated efflux was based on inhibitor studies in which only those inhibitors acting on the terminal oxidase side of the carrier induced efflux. In the marine pseudomonad the inhibitors HOQNO, IAA and NEM do not give inhibition when electrons are donated directly to the cytochromes by ascorbate-TMPD (Table 15). Thus, an opportunity for testing the theory devised for *E. coli* was apparently afforded.

Membrane vesicles preloaded with L-[$^{14}$C]alanine were exposed to various inhibitors and the level of radioactivity remaining in the vesicles determined at intervals (Fig. 14). It can be seen that those inhibitors which inhibited uptake of L-alanine in Table 15 also caused the release of the amino acid. With ascorbate-TMPD as electron donor, KCN, which prevented uptake of the amino acid, also caused its rapid release. PCMB, however, which produced 50% inhibition of uptake caused no more release than compounds which did not prevent uptake. Certain of the inhibitors caused an initial stimulation of uptake of L-alanine whether or not they subsequently gave release of the compound. In a separate experiment (Fig. 15), DNP was found to induce rapid efflux in both ethanol and ascorbate-TMPD systems.

Fig. 14. Effect of metabolic inhibitors on retention of preloaded L-alanine in vesicles.

Vesicles were allowed to take up L-[$^{14}$C]alanine (1.8 μM) for 6 min at which time a steady state concentration in the vesicles had been achieved. Either 50 μl of CS-T or 50 μl of CS-T containing the appropriate inhibitor was then added. Reaction mixtures were then filtered at the indicated time intervals after the addition of the inhibitor. Data is reported as percentage of L-alanine present in the vesicles in the absence of the inhibitor at each time interval. Ethanol, 25 mM. Inhibitor concentrations as in Table 15. Curve 1, no inhibitor; Curve 2, NEM; Curve 3, IAA; Curve 4, PCMB; Curve 5, HOQNO; Curve 6, KCN.

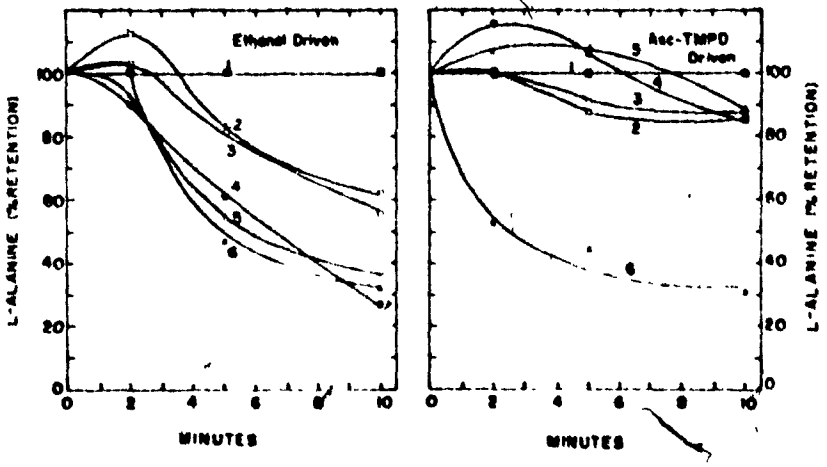
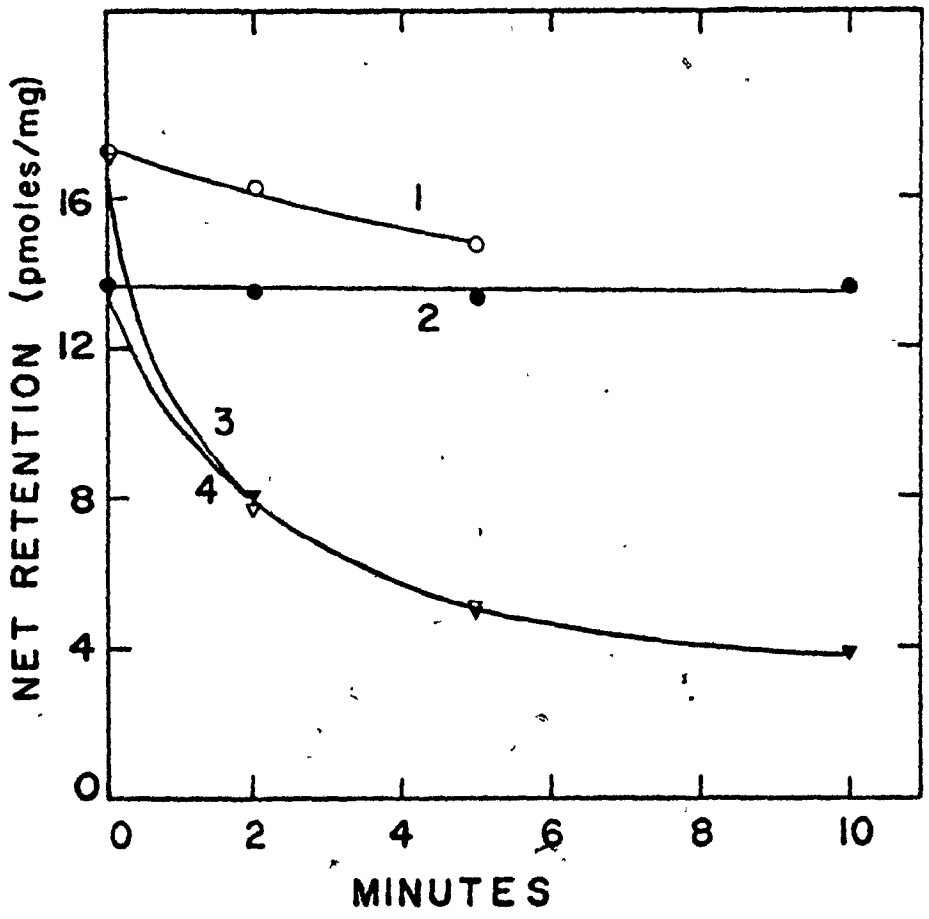

Ethanol Driven
Asc-TMPD Driven
L-ALANINE (%RETENTION)
MINUTES
0
20
40
60
80
100
2
4
6
8
10
1
2
3
4
5
6

Fig. 15. Effect of 2,4-dinitrophenol (DNP) on retention of pre-loaded L-alanine in vesicles.

Conditions were as in Fig. 14. Curve 1, ascorbate-TMPD, no inhibitor; Curve 2, ethanol, no inhibitor; Curve 3, ascorbate-TMPD plus DNP; Curve 4, ethanol plus DNP.


NET RETENTION (pmoles/mg)
16
12
8
4
0
0
2
4
6
8
10
MINUTES
1
2
3
4

These results show an absolute requirement for electron flow from ascorbate-TMPD coupling to the terminal oxidase in order to prevent efflux. In ethanol driven systems efflux occurred irrespective of the point of inhibition.

Lack of correlation between oxygen uptake and transport

Previous experiments have shown ethanol to induce a low level of oxygen uptake compared to NADH or ascorbate-TMPD, yet to give the same rate and extent of transport. A lack in correlation between rates of oxidation of various substrates and their ability to activate transport was reported for *E. coli* vesicles (Barnes and Kaback, 1971), although such dramatic effects as reported here were not observed. A more detailed study is shown in Table 18. Ethanol induced about 4% as much oxygen uptake as did NADH or ascorbate-TMPD, yet promoted similar rates of transport. Of particular interest was succinate. The oxidation of this compound was not appreciably coupled to transport.

The site of action of transport inhibitors was investigated by spectrophotometric analysis of the membrane cytochromes. In this experiment, the effect of various inhibitors on the reduction of cytochromes b and c by ethanol is shown (Table 19). Compared to a dithionite reduced control, at steady state (anaerobic or in the presence of cyanide) only about 25% of the cytochromes were reduced.

TABLE 18.

*Oxygen uptake and L-alanine transport induced by various compounds and inability of ADP and $K_2HPO_4$ to energize transport in membrane vesicles.*

Correction was made for oxygen consumption occurring with reduced-TMPD in the absence of membranes. No $O_2$ uptake occurred with the other compounds in the absence of membranes. Transport was measured after 1.0 min using L-alanine (0.3 mM, 5.3 μCi/μmole), and the transport values recorded were corrected for background observed in the absence of any added energy source. Energy sources 2.7 mM, TMPD 16 μM; $K_2HPO_4$, 12 mM; ADP, 2 mM.

| Compound | Oxygen consumed ng atoms O/min/mg | Transport pmoles/min/mg |
|---|---|---|
| None | 0 | 0 |
| NADH | 103 | 75 |
| Ascorbate-TMPD | 74 | 92 |
| Ethanol | 4 | 77 |
| Succinate | 46 | 4 |
| ADP + $K_2HPO_4$ | not tested | 0 |

TABLE 19.

*Effect of metabolic inhibitors and anaerobiosis on the steady state reduction of vesicle respiratory components by ethanol.*

Vesicles (12 mg dry weight per cuvette) were mixed with the inhibitor (concentrations as in Table 15) and ethanol (10 mM). Scans were recorded at steady state 60 min later using ferricyanide oxidized vesicles as reference. Per cent reduction by ethanol was determined by comparison to dithionite reduced vesicles, based on peak heights. A trough at about 477 nm, suggested to be flavoprotein, was observed in the control (no inhibitor) and in the cyanide treated vesicles.

| Inhibitor | Reduction | |
|---|---|---|
| | Cytochrome b (560 peak) | Cytochrome c (552 peak) |
| | % | |
| None (anaerobic) | 26 | 26 |
| KCN | 26 | 34 |
| HOQNO | 7 | 13 |
| PCMB | 2 | 3 |
| NEM | 8 | 6 |
| IAA | 13 | 11 |

The inhibitors HOQNO, PCMB, NEM and IAA act before cytochromes b and c since under anaerobic conditions the inhibitors partially prevented the reduction of both cytochromes. IAA, an inhibitor to the extent of only 22% with regard to ethanol energized transport (Table 15), was also less effective in blocking cytochrome b and c reduction than the other inhibitors used. A second experiment, shown in Table 20, was performed using the same membrane preparation used in Table 19. Inhibition of oxygen uptake by several transport inhibitors was compared. Some of the inhibitors had considerably different effects dependent on the energy source. In fact, only when ethanol was used was there a correlation between inhibition of oxygen uptake and inhibition of transport (Table 15). This finding was indicated quite remarkably for every inhibitor tested.

Gradients of $Na^+$ and $K^+$ in membrane vesicles

Cells of this organism actively accumulate $K^+$ with the active expulsion of $Na^+$ from the cells (Thompson and MacLeod, 1973a). Further, the internal concentration of $K^+$ governs the extent of AIB accumulation (Thompson and MacLeod, 1971). It was of interest then to test for the presence of cation gradients in membranes using the energy sources found most effective in energizing transport (Table 21). By assuming that the $Na^+$ and $K^+$ released by digestion of the membrane pellets was not bound significantly to the membranes, and

TABLE 20.

*Effect of inhibitors on initial rates of oxygen uptake in vesicles exposed to either NADH, ethanol, or ascorbate-TMPD.*

For NADH and ascorbate-TMPD assays 6 mg vesicles per assay were used, and 30 mg used in ethanol assays. NADH and ascorbate, 2.7 mM; TMPD, 16 µM; ethanol, 10 mM. Inhibitors at concentrations used in Table 15 were added 1.0 min before the energy sources. Negative values represent per cent stimulation compared to the controls lacking inhibitors.

| Inhibitor | Inhibition | | |
|---|---|---|---|
| | NADH | Ethanol | Ascorbate-TMPD |
| | | % | |
| None | 0 | 0 | 0 |
| HOQNO | 97 | 56 | 10 |
| PCMB | 99 | 87 | (-11) |
| IAA | (-10) | 38 | (-11) |
| NEM | (-3) | 78 | (-14) |
| DNP | 18 | 82 | 23 |

TABLE 21.

*$Na^+$ and $K^+$ gradients in membranes in the presence or absence of energy sources known to energize amino acid transport.*

Ascorbate, NADH and ethanol were used at 25 mM and TMPD at 150 µM. Note: the procedure from the time of addition of energy sources took 17 min, a period too short for depletion of the energy sources.

| Energy Source | Internal space µl/mg | $Na^+$ - mM | | | $K^+$ - mM | | |
|---|---|---|---|---|---|---|---|
| | | Inside | Outside | Inside / Outside | Inside | Outside | Inside / Outside |
| None | | 180 | 74 | 2.4 | 14 | 12 | 1.2 |
| Ascorbate-TMPD | | 97 | 76 | 1.3 | 42 | 10 | 4.2 |
| NADH | | 256 | 122 | 2.1 | 37 | 10 | 3.7 |
| Ethanol | 0.36 | 180 | 74 | 2.4 | 30 | 10 | 3.0 |
| Ethanol | 0.43 | 185 | 74 | 2.5 | 46 | 27 | 1.7 |

by knowing the intravesicular space to be about 0.4 µl/mg membranes, an internal to external cation ratio was calculated. The relatively high level of external $Na^+$ used for the NADH treatment was a consequence of adding the Na-salt of NADH. In spite of this and regardless of whether an energy source was included, the Inside/Outside ratio of $Na^+$ was either equal to or greater than 1.0. With ethanol as energy source, increasing external $K^+$ from 10 to 27 mM had no effect on the $Na^+$ gradient. As compared to $Na^+$, the $K^+$ bound by membranes is less (5 to 6 µg compared to 0.15 µg ion/mg membranes), and the Inside/Outside $K^+$ ratio in the absence of energy source was consequently closer to unity. A small gradient did form in the presence of energy sources, but largely disappeared as external $K^+$ was increased to 27 mM. The small $K^+$ gradient observed seems not to be involved in amino acid transport, since increasing the external $K^+$ level dissipated the $K^+$ gradient (Table 21) but had no effect on transport (Table 22).

The transport of $^{42}K$ into $K^+$-depleted cells of this marine pseudomonad is stimulated by the same electron donors which promote the transport of L-alanine into membrane vesicles (Thompson and MacLeod, 1973b). Because membranes were found to have little ability to concentrate $K^+$ using the previous technique, it was decided to check this observation using $^{42}K$. The uptake of $^{42}K$ by $K^+$-depleted cells and membrane vesicles was compared (Fig. 16) using ethanol as

TABLE 22.

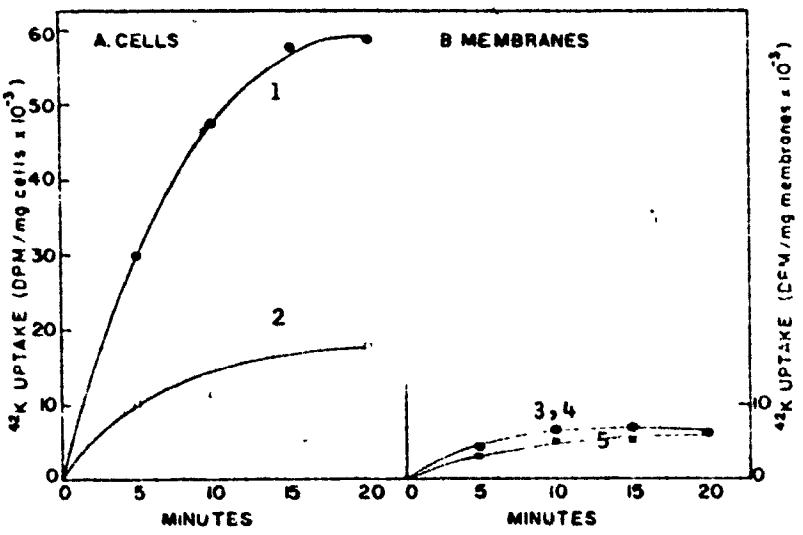
*Effect of external $K^+$ concentrations on L-alanine transport into membrane vesicles of a marine pseudomonad.*

NaCl was maintained at 20 mM. L-alanine was used at 1.8 µM and 173 µCi/µmole.

| $K^+$ added mM | Transport pmoles/mg/min |
|---|---|
| 4 | 29 |
| 28 | 31 |
| 55 | 32 |
| 115 | 31 |
| 230 | 28 |

Fig. 16. Uptake of $^{42}K$ by (A) $K^+$-depleted intact cells and by (B) $K^+$-depleted vesicles in the presence and absence of NaCN.

Ethanol (25 mM) and NaCN (10 mM) were added to cell and vesicle suspensions just prior to the addition of sufficient $^{42}K$ solution to give 1 mM $K^+$ (specific activity 8.99 μCi/μmole for A, and 3.38 μCi/μmole for B). Curve 1, ethanol; Curve 2, ethanol plus NaCN; Curve 3, ethanol; Curve 4, ethanol plus NaCN; Curve 5, no ethanol.


A. CELLS
B MEMBRANES
1
2
3, 4
5
$^{42}$K UPTAKE (DPM/mg cells x $10^{-3}$)
$^{42}$K UPTAKE (CPM/mg membranes x $10^{-3}$)
MINUTES
MINUTES
0
10
20
30
40
50
60
0
5
10
15
20

the electron donor. Under conditions permitting the cyanide sensitive uptake of $^{42}K$ by the intact cells, no uptake of $^{42}K$ was detected in the vesicles. Valinomycin induced uptake of $^{42}K$ has been reported for *E. coli* vesicles (Bhattacharyya *et al.*, 1971). In a separate experiment similar to that shown in Fig. 16 using membrane vesicles, the addition of 0.1 mM valinomycin added as a solution in ethanol, failed to stimulate the uptake of $^{42}K$.

## $K^+$-requirement for L-alanine transport

Cells of this organism depleted of intracellular $K^+$ by washing with 50 mM $MgSO_4$ show a requirement for $K^+$ for AIB uptake (Thompson and MacLeod, 1971). This also applies to L-alanine uptake into cells as shown in Fig. 17. To determine whether membrane vesicles required $K^+$ for transport, the vesicles were prepared in the usual manner except that the solutions used contained no added K-salts. These membranes contained 0.15 µg $K^+$ per mg of membranes, and transported L-alanine normally in a medium containing no added $K^+$ (Fig. 18,A). In fact, 10 mM external KCl slightly depressed uptake. Attempts to displace the $K^+$ remaining in the preparations by 10 mM RbCl is shown in Fig. 18, B and C. Washing with RbCl resulted in a loss in transport activity, which was not restored by adding 10 mM KCl, and not further reduced by including 10 mM RbCl in the medium during the transport assays. When analyzed, the $K^+$ remaining in RbCl washed preparations was the same as that in membranes washed in the absence of both KCl and RbCl.

Fig. 17. Requirement for $K^+$ in the uptake of L-alanine and AIB into $K^+$-depleted cells.

Cells were depleted of $K^+$ by washing once in 50 mM $MgSO_4$, washing again in CS-T (NaCl 200 mM and no KCl), and resuspending in the latter solution but containing chloramphenicol. The cells were then diluted into CS-T with or without 10 mM KCl. L-alanine, 0.01 mM, 14.2 μCi/μmole; AIB, 0.01 mM, 0.44 μCi/μmole.

Dotted bars, no KCl;

Striped bars, KCl.

Following the addition of the labeled amino acid, uptake of L-alanine (left graph) and AIB (right graph) was measured.

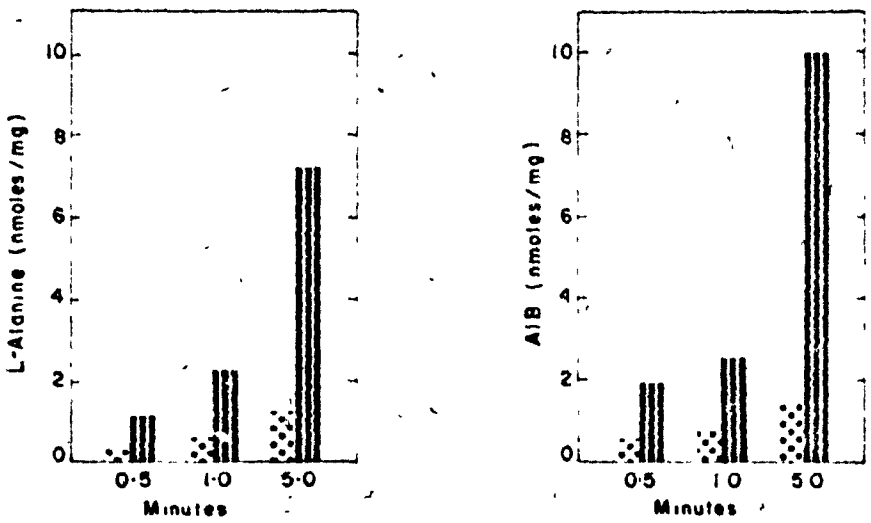
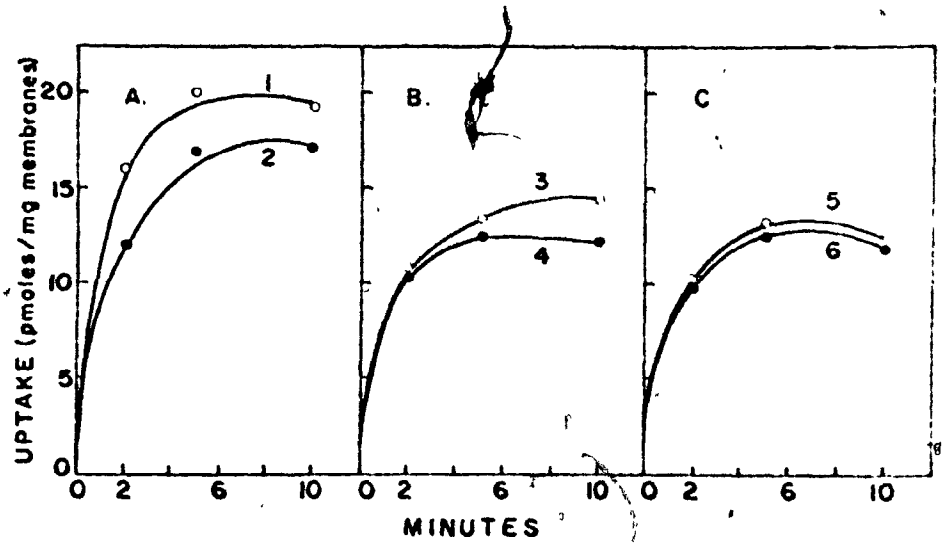

L-Alanine (nmoles/mg)
10
8
6
4
2
0
0·5
1·0
5·0
Minutes
AIB (nmoles/mg)
10
8
6
4
2
0
0·5
1·0
5·0
Minutes

Fig. 18. Effect of KCl and RbCl on the transport of L-alanine into vesicles.

Vesicles were washed twice in $K^+$ free CS-T (A), or in $K^+$ free CS-T containing 10 mM RbCl (B,C). Energy source, ascorbate-TMPD; L-alanine, 1.8 μM, 174 μCi/μmole. Transport assays contained the following additions: Curves 1 and 3, no KCl; Curves 2 and 4, KCl at 10 mM; Curve 5, RbCl at 10 mM; Curve 6, KCl and RbCl each at 10 mM.

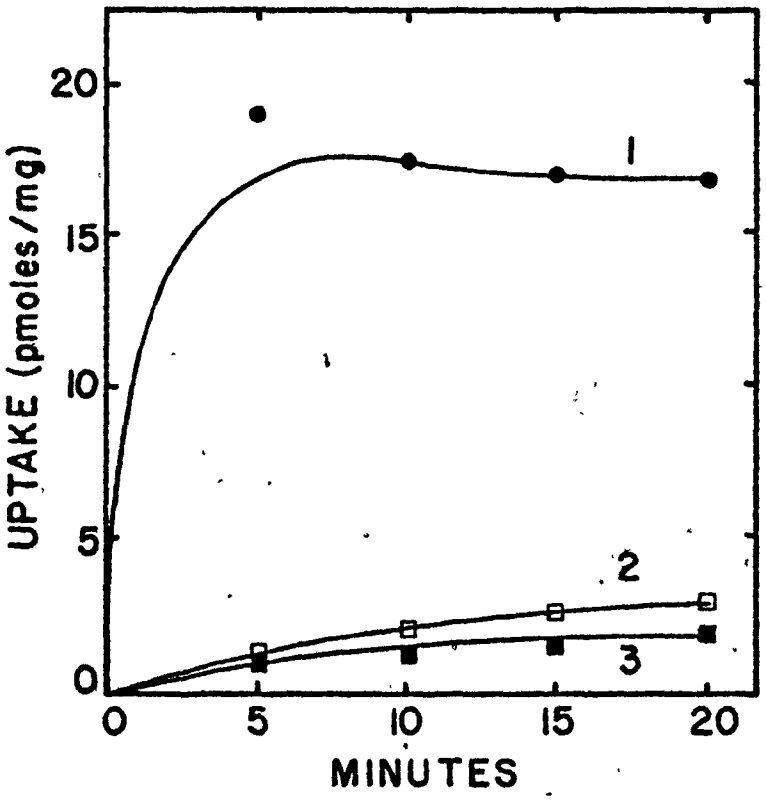

A.
B.
C
1
2
3
4
5
6
UPTAKE (pmoles/mg membranes)
0
5
10
15
20
0 2 6 10
MINUTES

Effect of $K_3$ $Fe(CN)_6$

In intact cells of the marine pseudomonad containing their normal complement of intracellular $K^+$, the inhibition of AIB transport by cyanide can be largely overcome by adding $K_3Fe(CN)_6$. This electron acceptor when added as the Na-salt did not overcome cyanide inhibited $^{42}K$ uptake (Thompson and MacLeod, 1973b). Since in membrane vesicles no effect of externally added $K^+$ was detected, it should be possible to restore cyanide inhibited L-alanine uptake by ferricyanide. This was tried in Fig. 19 but no appreciable recovery in alanine transport was obtained. Similar results were found in a separate experiment, where $K_3Fe$ $(CN)_6$ at concentrations from 0.01 mM to 1.0 mM was added to the vesicle suspension immediately after the KCN addition.

Fig. 19. Effect of ferricyanide on the cyanide inhibited transport of L-alanine in vesicles.

KCN was added 15 min prior to the addition of $K_3Fe(CN)_6$ and L-[$^{14}$C]alanine (1.8 μM). Curve 1, no KCN or $K_3Fe(CN)_6$; Curve 2, KCN and $K_3Fe(CN)_6$ each at 6 mM; Curve 3, KCN at 6 mM.


UPTAKE (pmoles/mg)
20
15
10
5
0
1
2
3
0
5
10
15
20
MINUTES

# SECTION II.

## KINETICS OF $Na^+$-DEPENDENT TRANSPORT

### Preparation of $Na^+$-free membranes

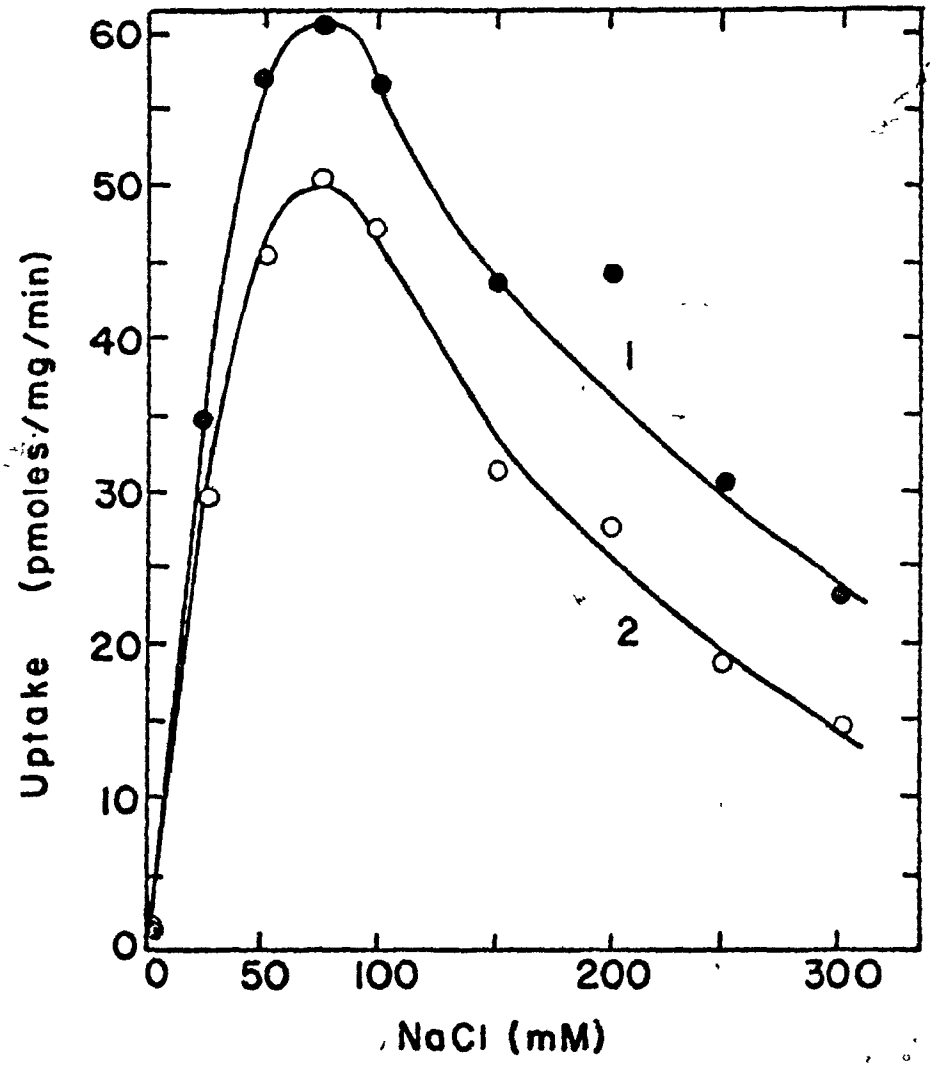
In the absence of any response to external $K^+$ in the vesicle transport system, it was possible to obtain a true $Na^+$ effect uninfluenced by $K^+$. The effect of NaCl on L-alanine transport is shown in Fig. 20. Part of the membrane suspension was washed in the presence of 0.2 M NaCl (Curve 1) and the remainder was washed with a salts solution where 0.2 M LiCl had replaced the NaCl (Curve 2). Both types of membranes were centrifuged and resuspended in CS-T containing neither NaCl nor LiCl. The membranes were then diluted with CS-T containing various concentrations of NaCl (tube dilution assay, Fig. 6). When tested for L-alanine transport it can be seen that similar responses to $Na^+$ were obtained with either membrane preparation. It is also evident that membranes prepared in the absence of NaCl, even when LiCl was used as a substitute, did not regain full transport capacity. The procedure shown for Curve 2 was used to prepare $Na^+$-free membranes in the following transport experiments. Using this method possible variations in the amount of $Na^+$ carried over from the final membrane pellet was avoided.

### Effect of wash fluid on retention

In transport studies using cells of this organism the salt com-

Fig. 20. Response of L-alanine transport to $Na^+$ concentration comparing vesicles prepared in the presence or absence of NaCl.

Ascorbate-TMPD served as the energy source and L-[$^{14}$C] alanine was used at 2.3 μM (173 μCi/μmole). Curve 1, vesicles prepared in CS-T containing 200 mM NaCl; Curve 2, vesicles prepared in CS-T with 200 mM LiCl replacing the NaCl.


60
50
40
30
20
10
0
Uptake (pmoles/mg/min)
0
50
100
200
300
NaCl (mM)
1
2

position of the fluid used to wash the filtered cells is very important. In the absence of NaCl in the wash fluid, for example, AIB was dramatically lost from the cells (Drapeau and MacLeod, 1965). No such dramatic effect was observed with vesicles (Table 23). In this experiment vesicles were preloaded with L-alanine, filtered, and then washed with CS-T containing from 0 to 500 mM NaCl. Although some effect was seen at 0 NaCl, with a possible optimum of 150 mM, the level of NaCl in the wash fluid had little effect on retention of alanine.

Transport kinetics using vesicles exposed to high $Na^+$ concentrations

At $Na^+$ concentrations above 75 mM, transport was dramatically reduced (Fig. 21). The results in Fig. 21 show this effect of high $Na^+$ levels not to be through an effect of $Na^+$ on the apparent $K_m$ for transport. The $K_m$ remained at 0.08 mM even at 500 mM NaCl, while $V_{max}$ varied dramatically.

Transport kinetics in vesicles using L-alanine

Because of the $Na^+$-dependent nature of this transport system it seemed quite reasonable that $Na^+$ could be considered as a substrate for the reaction rather than merely as an ion activator. Cotransport of $Na^+$ and solute has been shown to occur in mammalian cells (Schultz and Curran, 1970) probably by the formation of a

TABLE 23.

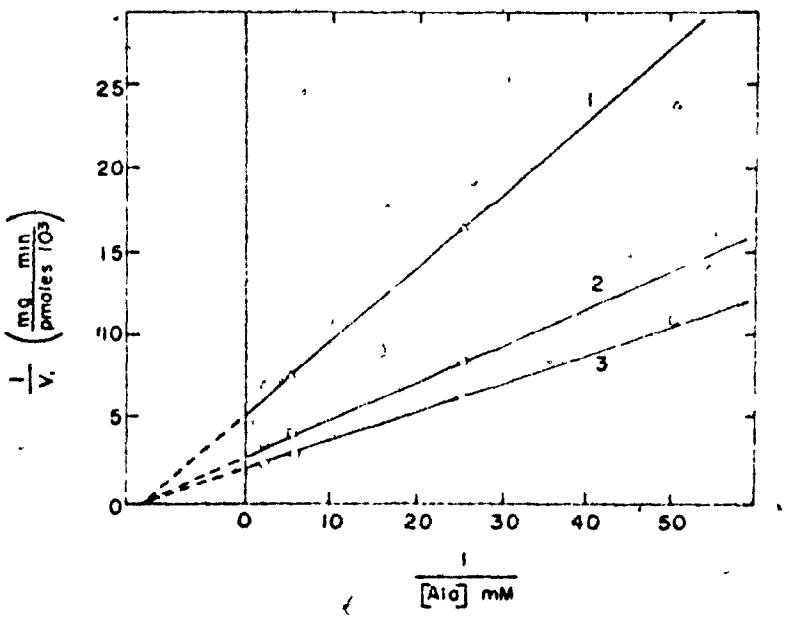
*Effect of various NaCl concentrations on the retention of L-alanine in membrane vesicles of the marine pseudomonad.*

Membranes were preloaded with L-alanine (0.3 mM, 5.3 µCi/µmole) for 6 min using ascorbate-TMPD as energy source and NaCl at 75 mM. The membranes were then filtered and washed with 5 ml CS-T (25 C) containing the indicated NaCl concentrations.

| NaCl in wash fluid mM | L-alanine retained pmoles/mg |
|---|---|
| 0 | 83 |
| 10 | 105 |
| 25 | 105 |
| 50 | 111 |
| 75 | 110 |
| 100 | 115 |
| 150 | 138 |
| 200 | 124 |
| 250 | 119 |
| 300 | 133 |
| 500 | 103 |

Fig. 21. Effect of high $Na^+$ concentrations on L-alanine transport kinetics in vesicles.

The energy source was ascorbate-TMPD. L-alanine, 5.3 μCi/μmole. Curve 1, NaCl 500 mM; Curve 2, NaCl 200 mM; Curve 3, NaCl 75 mM.


25
20
15
10
5
0
0
10
20
30
40
50
1
2
3
$\frac{1}{V_i}\left(\frac{mg}{pmoles}\frac{min}{10^3}\right)$
$\frac{1}{[Alo]}$ mM

ternary complex (Schafer and Jacquez, 1967). If $Na^+$-dependent transport in the marine pseudomonad involves the formation of a ternary complex then transport should exhibit bisubstrate kinetics. Of crucial importance in these studies is the fact that no appreciable $K^+$ gradient or $K^+$ requirement in vesicles for transport has been observed (Table 21, Fig. 18). The amount of internal $K^+$ in these cells controls the rate of transport (Thompson and MacLeod, 1971), and the amount of internal $K^+$ is in turn a function of the external $Na^+$ concentration (Gow and Thompson, unpublished). These considerations appear to account for the fact that bisubstrate kinetics are not observed with cells when using the procedure of Wong *et al.* (1969) as will be shown later. The difficulties originally observed with cells should not exist in the vesicle system. This in fact, was found to be the case. Reciprocals of the rates of alanine transport are plotted against the reciprocals of alanine (Fig. 22) or $Na^+$ (Fig. 23) concentrations. In both cases linear plots were obtained giving a point of intersection on or above the abscissa. If $Na^+$ is taken as substrate "A" and alanine as "B" then $K_m^A$ calculated from Fig. 23 is 18 mM. The intercepts taken from the ordinate of Fig. 23 when plotted against the reciprocal of alanine concentration also gave a straight line from which the limiting maximum velocity was calculated (Fig. 24). The slope of Curve 1 in Fig. 24 is equal to $\frac{K_m^B}{V}$ and this provided a means of calculating $K_m^B$. This method of determining $K_m^B$ seemed more accurate than employing the

Fig. 22. Effect of $Na^+$ concentration on kinetics of L-alanine transport into vesicles of the marine pseudomonad. Energy source and other conditions were as for Fig. 21. At low $Na^+$ levels the activity observed in the absence of $Na^+$ plus KCN (1.0 mM) became significant, and was subtracted before plotting the results shown. Curve 1, NaCl 50 mM ($K_m$ 0.1 mM); Curve 2, NaCl 25 mM ($K_m$ 0.10 mM); Curve 3, NaCl 12 mM ($K_m$ 0.14 mM); Curve 4, NaCl 4.5 mM ($K_m$ 0.25 mM).

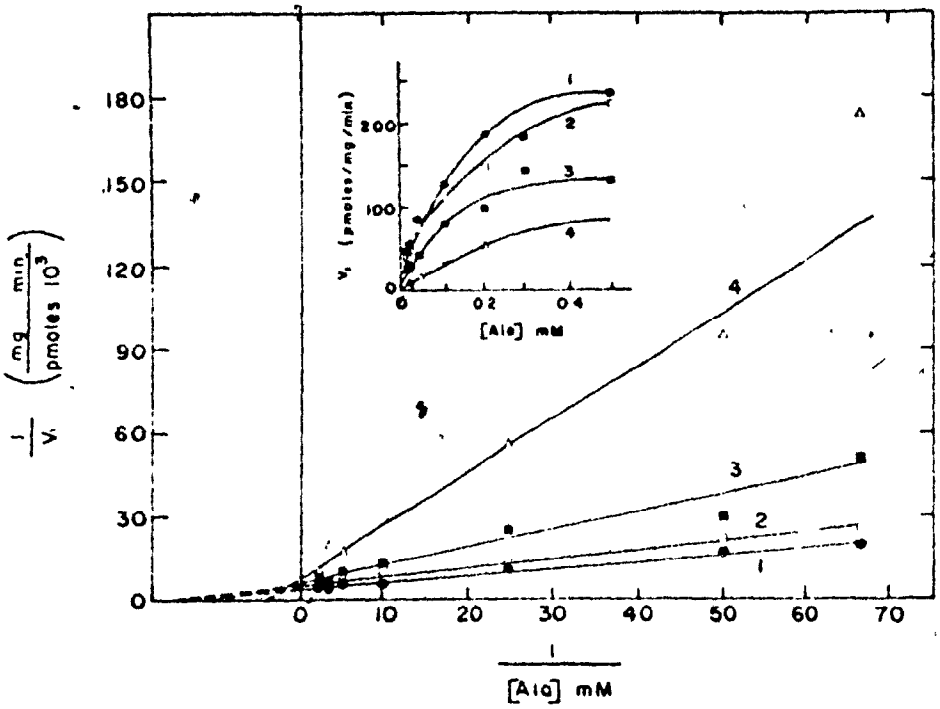

180
150
120
90
60
30
0
0
10
20
30
40
50
60
70
4
3
2
1
200
100
0
0.2
0.4
[A1a] mM
4
3
2
1

Fig. 23. Variation of the reciprocal of L-alanine transport velocity with the reciprocal of NaCl concentration for several constant L-alanine concentrations (mM):

Curve 1, 0.015; Curve 2, 0.02; Curve 3, 0.04; Curve 4, 0.1; Curve 5, 0.2; Curve 6, 0.3; Curve 7, 0.5. The point of intersection on the abscissa is $\frac{1}{-K_m^A}$

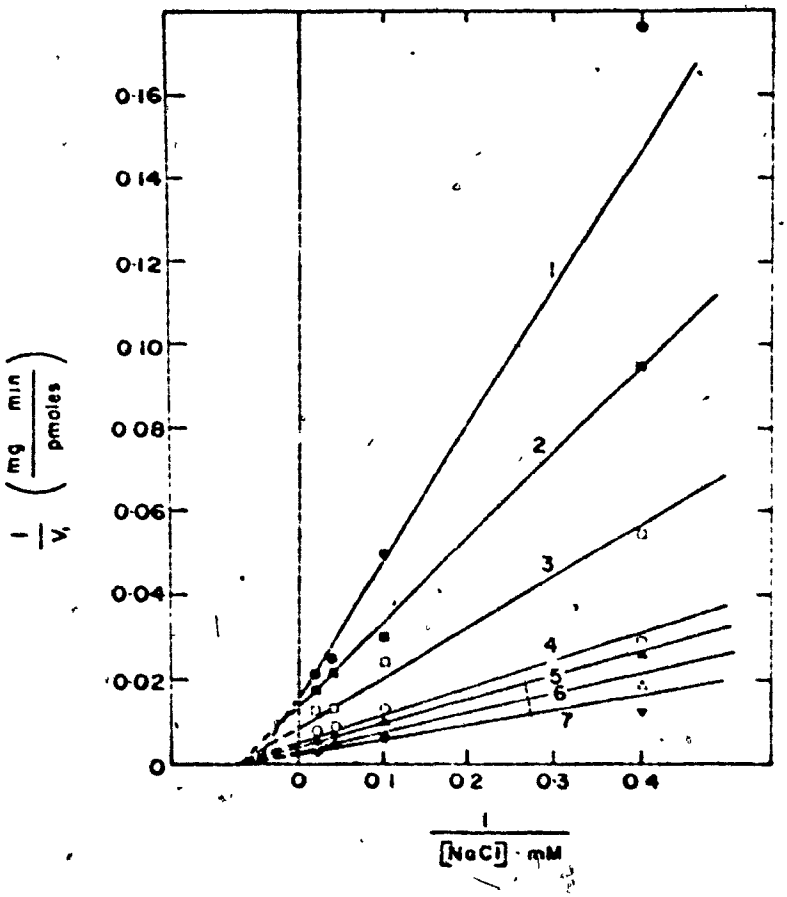
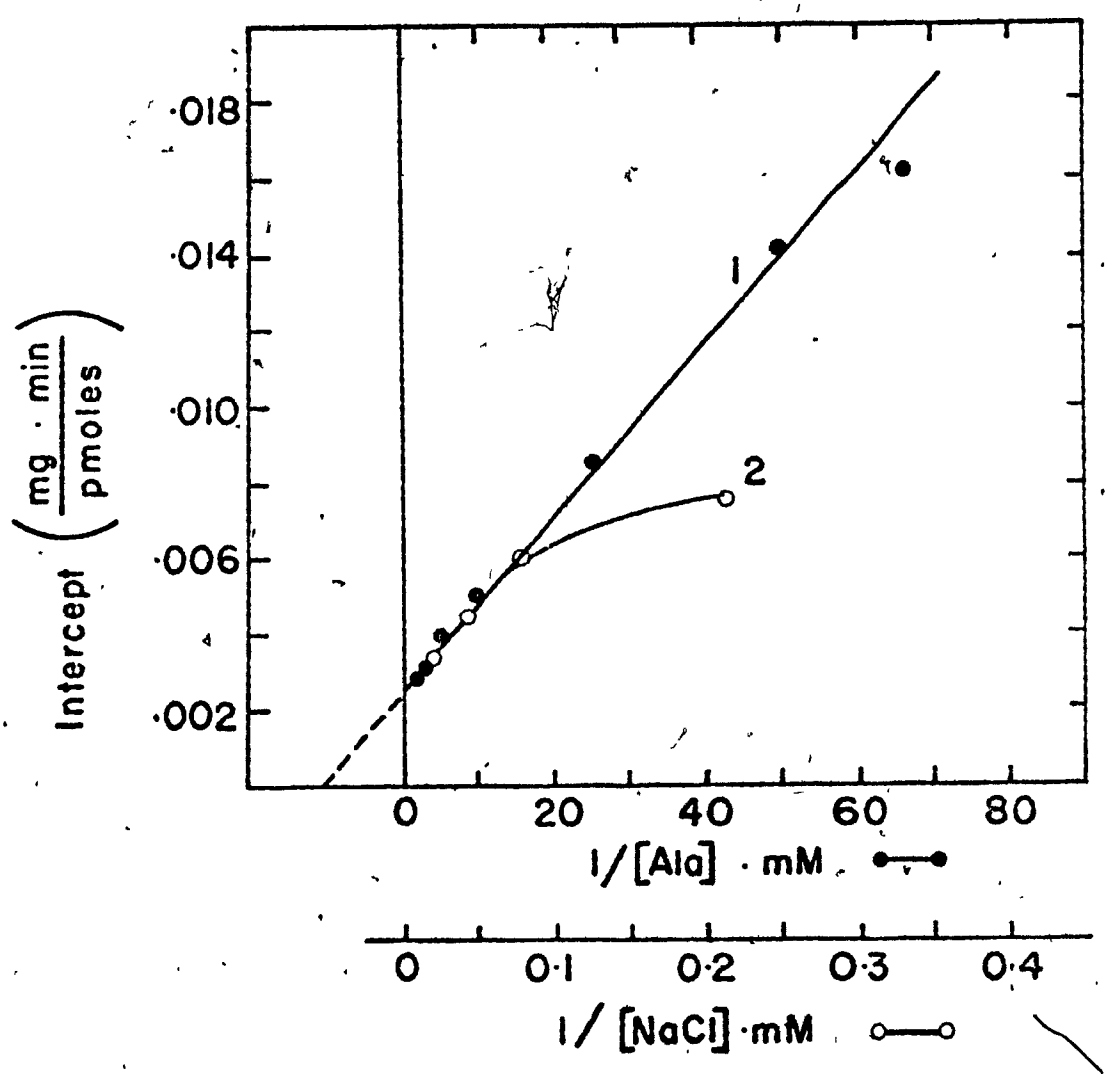

0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
0
0·1
0·2
0·3
0·4
1
2
3
4
5
6
7
$-\frac{1}{V_1}\left(\frac{mg\ min}{pmoles}\right)$
$\frac{1}{[NaCl]\cdot mM}$

Fig. 24. Secondary plots of the intercepts of the Lineweaver-Burk plots shown in Fig. 23 against the reciprocals of L-alanine concentration (Curve 1), or the intercepts from Fig. 22 against the reciprocals of NaCl concentration (Curve 2). The point of intersection on the ordinate is 1/V.


·018
·014
·010
·006
·002
Intercept (mg · min / pmoles)
1
2
0
20
40
60
80
1/[Ala] · mM
0
0·1
0·2
0·3
0·4
1/[NaCl] · mM

double reciprocal plot in Fig. 22, since in that plot only an approximate point of intersection was obtained. The plot in Fig. 24 of intercepts taken from Fig. 22 against the reciprocal of $Na^+$ concentration, deviated from the linear plot expected. In Fig. 25 the slopes of the lines from the two primary Lineweaver-Burk plots were plotted against the reciprocals of the opposite substrates. Straight lines with similar slopes were obtained. By knowing $K_m^A$, $K_m^B$ and V, a value for $K_s^A$ could be calculated. Experimentally determined kinetic constants are shown in Table 26 (page 138).

Using these kinetic constants the theoretical initial transport rates were calculated from the relationship for a bisubstrate reaction (Dalziel, 1957; Florini and Vestling, 1957), and these results are shown in Fig. 26. For $Na^+$ concentrations between 50 and 12 mM the theoretical and observed data (compare Fig. 22 and 26) correlate well. At 4.5 mM $Na^+$, however, with the higher concentrations of alanine used, the observed velocity was higher than theoretical. Attempts to attribute this finding to allosterism were negative. As shown in Fig. 27 increasing alanine to 2 mM did not overcome the $Na^+$ requirement for transport. The previous transport data shown (Fig. 22 ) had been corrected for alanine bound to the membranes in the absence of $Na^+$ plus 1.0 mM KCN. As can be seen in Fig. 27, considerable alanine was associated with membranes in the absence of $Na^+$ and this association was not

Fig. 25. Secondary plots of the slopes of the Lineweaver-Burk plots shown in Fig. 22 against the reciprocals of NaCl concentration (Curve 1), or the slopes from Fig. 23 against the reciprocals of L-alanine concentration (Curve 2). The point of intersection on the ordinate is $\frac{K_m^B}{V}$ (Curve 1) and $\frac{K_m^A}{V}$ (Curve 2). Slopes were 0.0040 (Curve 1) and 0.0042 (Curve 2).

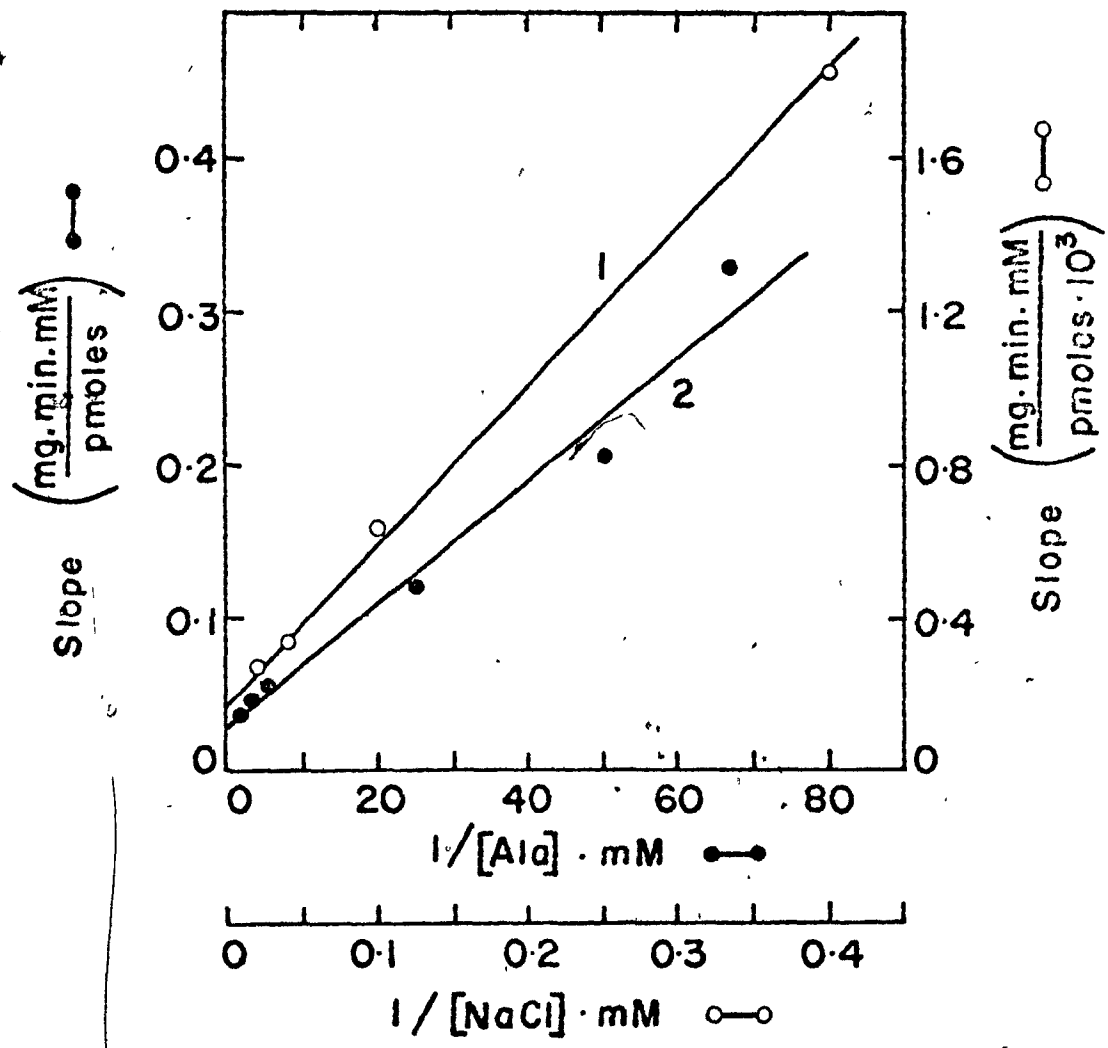

Slope (mg.min.mM / pmoles)
0·4
0·3
0·2
0·1
0
1·6
1·2
0·8
0·4
0
Slope (mg.min.mM / pmoles·10³)
1
2
0
20
40
60
80
1/[Ala]·mM
0
0·1
0·2
0·3
0·4
1/[NaCl]·mM

Fig. 26. Theoretical plots of L-alanine transport into membrane vesicles using the data calculated for a bisubstrate mechanism. The kinetic parameters used were calculated from Figs. 22 to 25 and are recorded in Table 26.

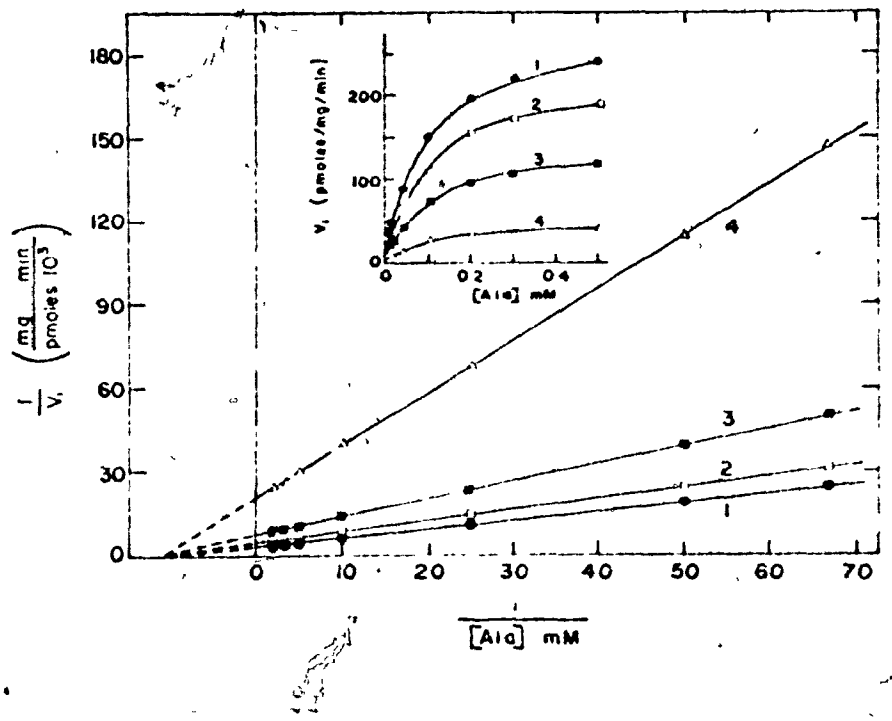
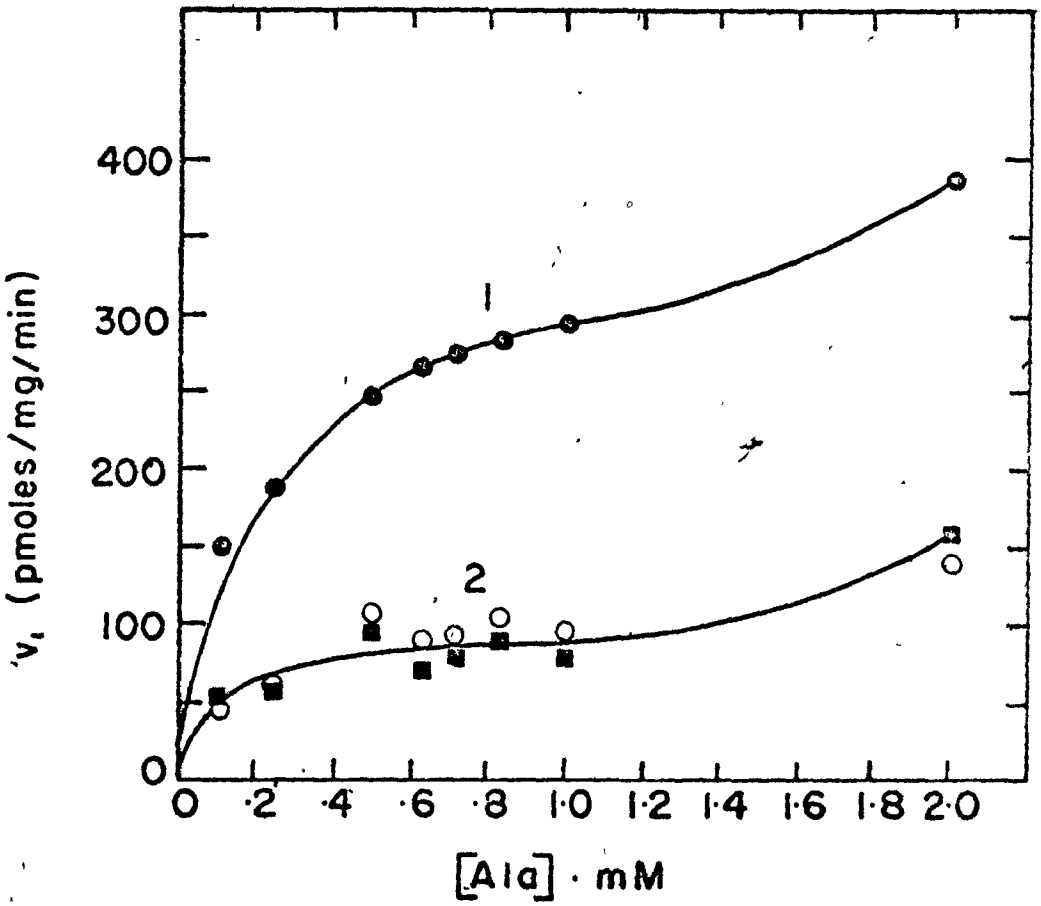

180
150
120
90
60
30
0
0
10
20
30
40
50
60
70
[Ala] mM
200
100
0
0 2 0 4
[Ala] mM
1
2
3
4

Fig. 27. Requirement for energy and $Na^+$ for the transport of L-alanine into membrane vesicles as a function of L-alanine concentration.

Specific activity, 0.586 μCi/μmole. Curve 1, NaCl 75 mM plus ascorbate-TMPD; Curve 2, no added NaCl with either ascorbate-TMPD (squares) or with no energy source (open circles).


400
300
200
100
0
$v_i$ (pmoles/mg/min)
1
2
0
·2
·4
·6
·8
1·0
1·2
1·4
1·6
1·8
2·0
[Ala] · mM

energy dependent. At amino acid concentrations above 1.0 mM an energy independent increase in uptake has been observed and is shown here.

Transport kinetics in vesicles using AIB

Because of the extensive work done in this laboratory with intact cells using the nonmetabolized compound AIB (Table 4), transport kinetics of AIB in membranes was compared to those obtained for L-alanine. As shown in Fig. 28 through 32, the same effects as observed for alanine were found to apply to AIB transport kinetics.

Requirement for $Na^+$ for electron flow

Studies with certain halophilic bacteria have shown $Na^+$ to stabilize and to activate some of the electron transport chain components (Lanyi, 1969). Since active transport of amino acids into vesicles of this organism is dependent on electron flow, it became important to see if $Na^+$ was involved in this process. Oxygen uptake induced by either ascorbate-TMPD or by ethanol (Table 24) was not dependent on NaCl. It should be emphasized that even 60 mg membranes per assay gave no trace of endogenous oxygen uptake.

Optimum NaCl for L-alanine uptake in cells

The relation between NaCl concentration and uptake of L-alanine in cells of the marine pseudomonad is shown in Fig. 33. An optimum of 200 mM $Na^+$ was found, and this supports the finding of Wong *et al.*

Fig. 28. Effect of $Na^+$ concentration on kinetics of AIB transport into membrane vesicles.

The energy source was ascorbate-TMPD, and specific activity of $^3H$-AIB was 39.2 μCi/μmole. Curve 1, NaCl 50 mM ($K_m$ 0.02 mM); Curve 2, NaCl 25 mM ($K_m$ 0.03 mM); Curve 3, NaCl 10 mM ($K_m$ 0.05 mM); Curve 4, NaCl 5 mM ($K_m$ 0.12 mM).

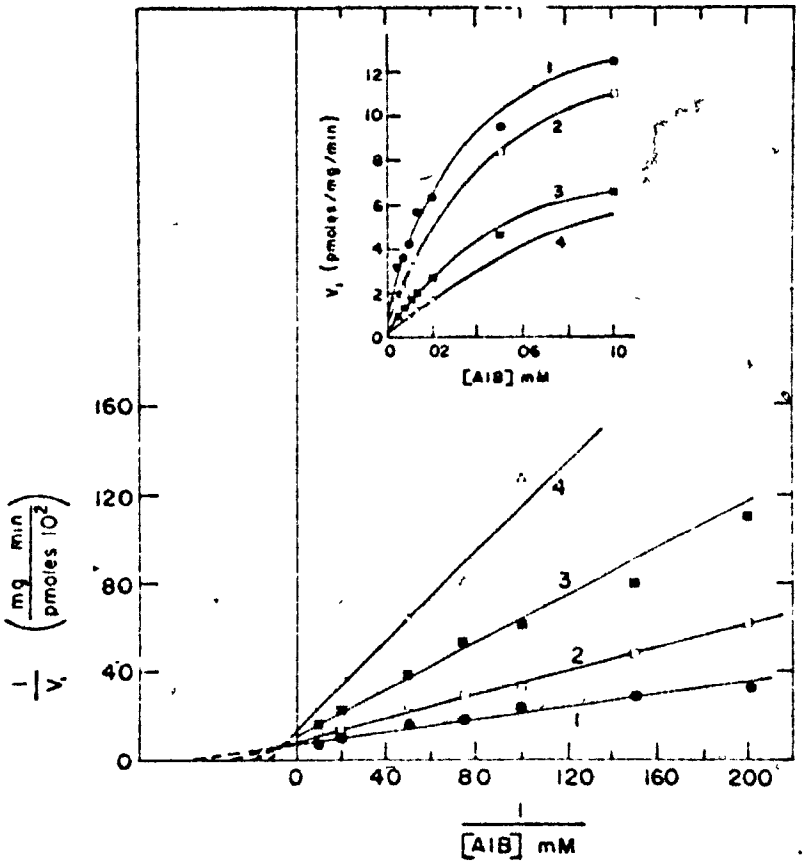

$-\frac{1}{v}\left(\frac{mg}{pmoles}\frac{min}{10^2}\right)$
160
120
80
40
0
0
40
80
120
160
200
$\frac{1}{[AIB]}$ mM
4
3
2
1
$V_i$ (pmoles/mg/min)
12
10
8
6
4
2
0
0
02
06
10
[AIB] mM
1
2
3
4

Fig. 29. Variation of the reciprocal of AIB transport velocity with the reciprocal of NaCl concentration for several constant AIB concentrations (mM):

Curve 1, 0.005; Curve 2, 0.0066; Curve 3, 0.01; Curve 4, 0.0133; Curve 5, 0.02; Curve 6, 0.05; Curve 7, 0.10.

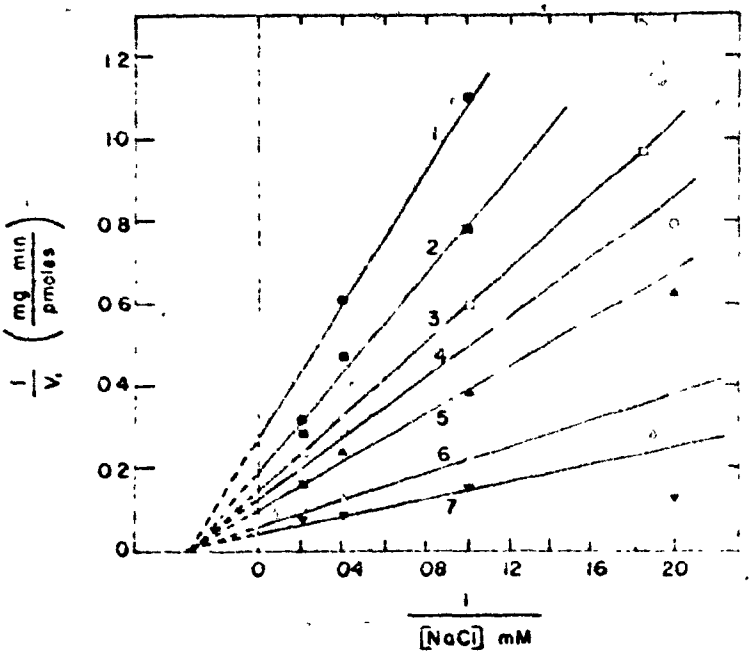

1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.4
0.8
1.2
1.6
2.0
1
2
3
4
5
6
7
$\frac{1}{V_i}\left(\frac{mg\ min}{p moles}\right)$
$\frac{1}{[NaCl]\ mM}$

Fig. 30. Secondary plots of the intercepts of the Lineweaver-Burk plots shown in Fig. 29 against the reciprocals of AIB concentration (Curve 1), or the intercepts from Fig. 28 against the reciprocals of NaCl concentration (Curve 2).

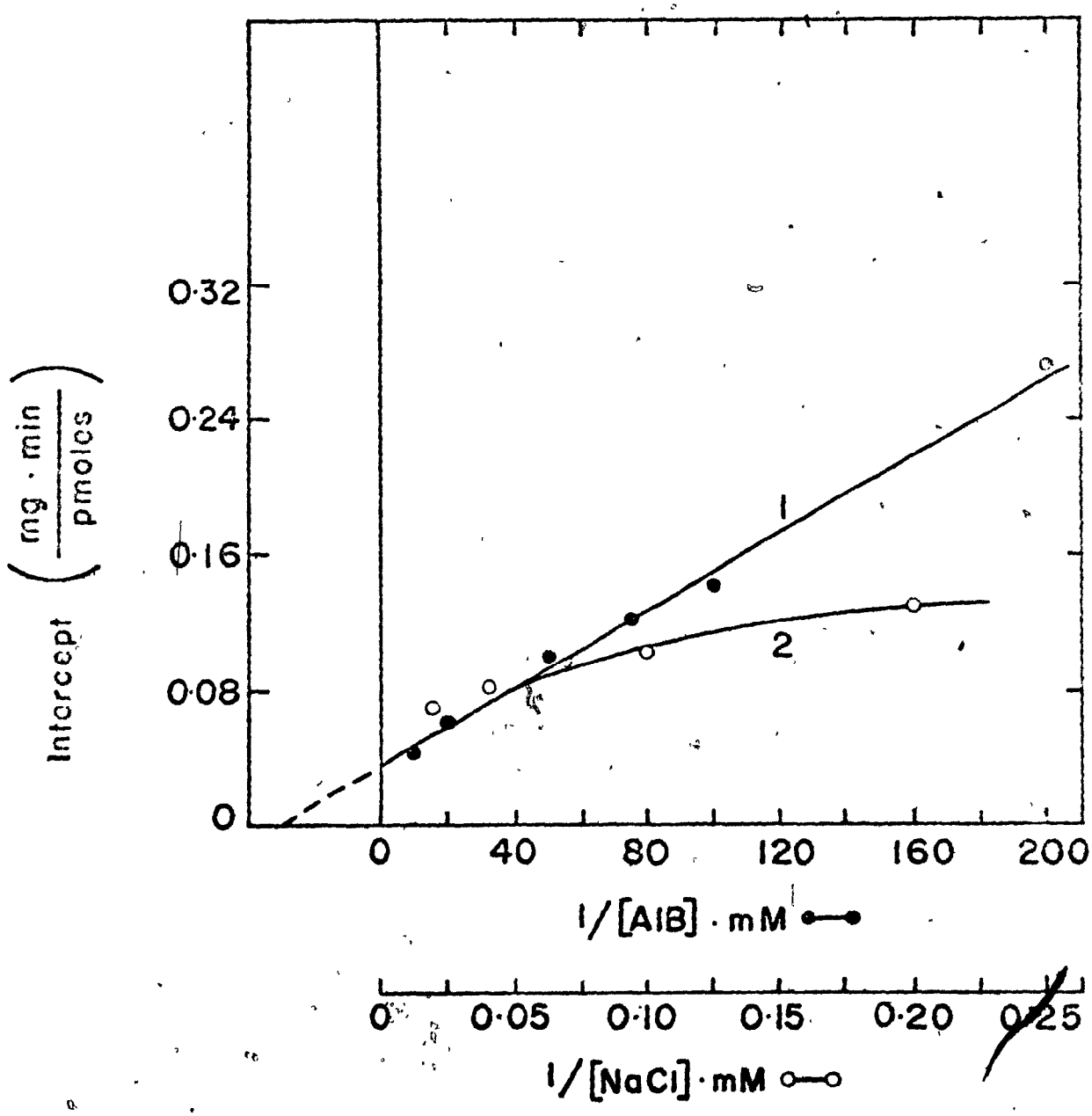

Intercept (mg · min / pmoles)
0·32
0·24
0·16
0·08
0
1
2
0
40
80
120
160
200
1/[AIB] · mM
0
0·05
0·10
0·15
0·20
0·25
1/[NaCl] · mM

Fig. 31. Secondary plots of the slopes of the Lineweaver-Burk plots shown in Fig. 28 against the reciprocals of NaCl concentration (Curve 1), or the slopes from Fig. 29 against the reciprocals of AIB concentration (Curve 2). Slopes were 0.0485 (Curve 1) and 0.0380 (Curve 2).

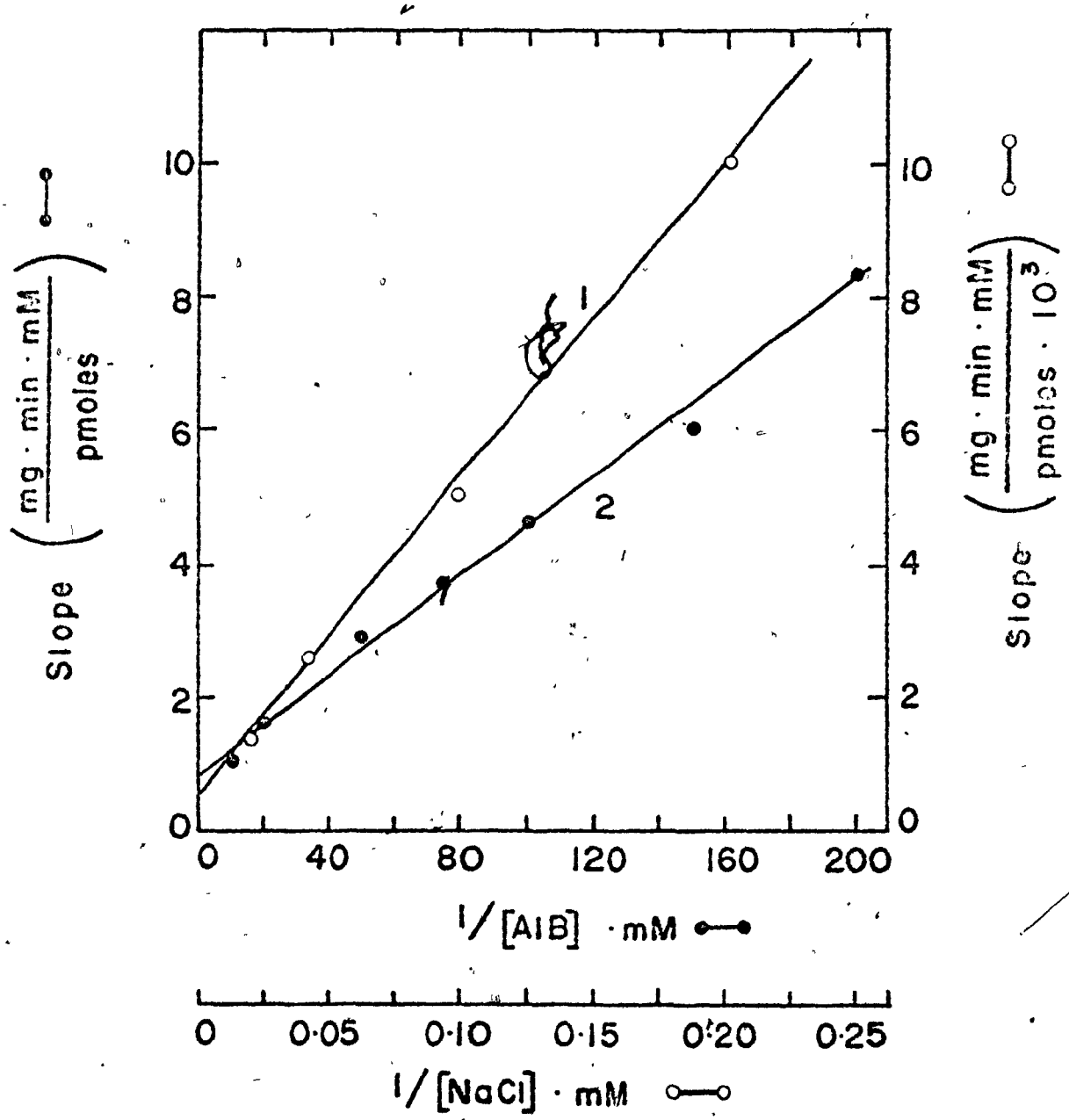

Slope ( mg · min · mM / pmoles )
Slope ( mg · min · mM / pmoles · 10³ )
0
2
4
6
8
10
1
2
0
40
80
120
160
200
1/[AIB] · mM
0
0·05
0·10
0·15
0·20
0·25
1/[NaCl] · mM

Fig. 32. Theoretical plots of AIB transport into membrane vesicles using the data calculated for a bisubstrate mechanism. The kinetic parameters used were calculated from Figs. 28 to 31 and are recorded in Table 26.

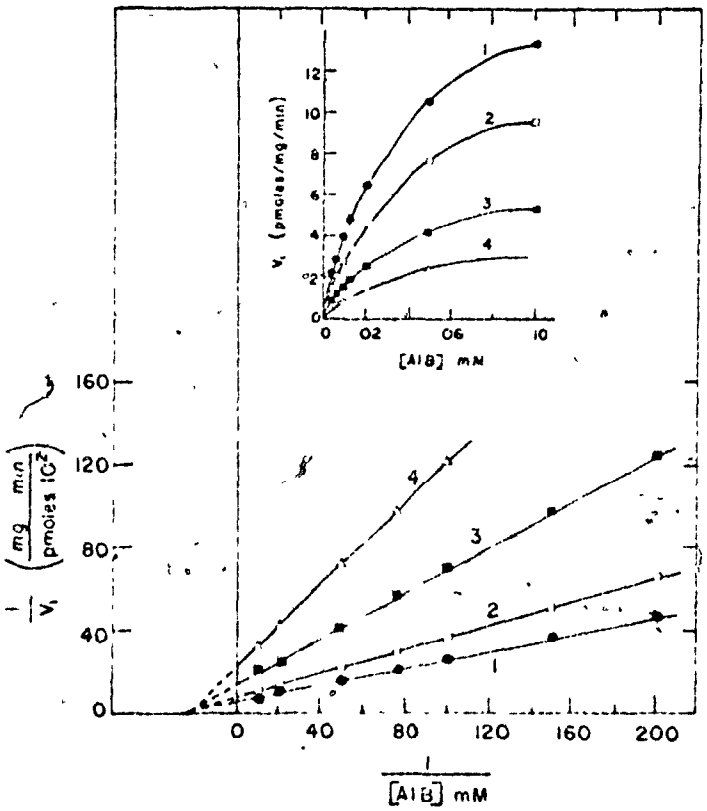

160
120
80
40
0
0
40
80
120
160
200
1
[AIB] mM
$-\frac{1}{V_i}\left(\frac{mg\ min}{pmoles\ IC^2}\right)$
4
3
2
1
12
10
8
6
4
2
0
0
02
06
10
[AIB] mM
$V_i$ (pmoles/mg/min)
1
2
3
4

TABLE 24.

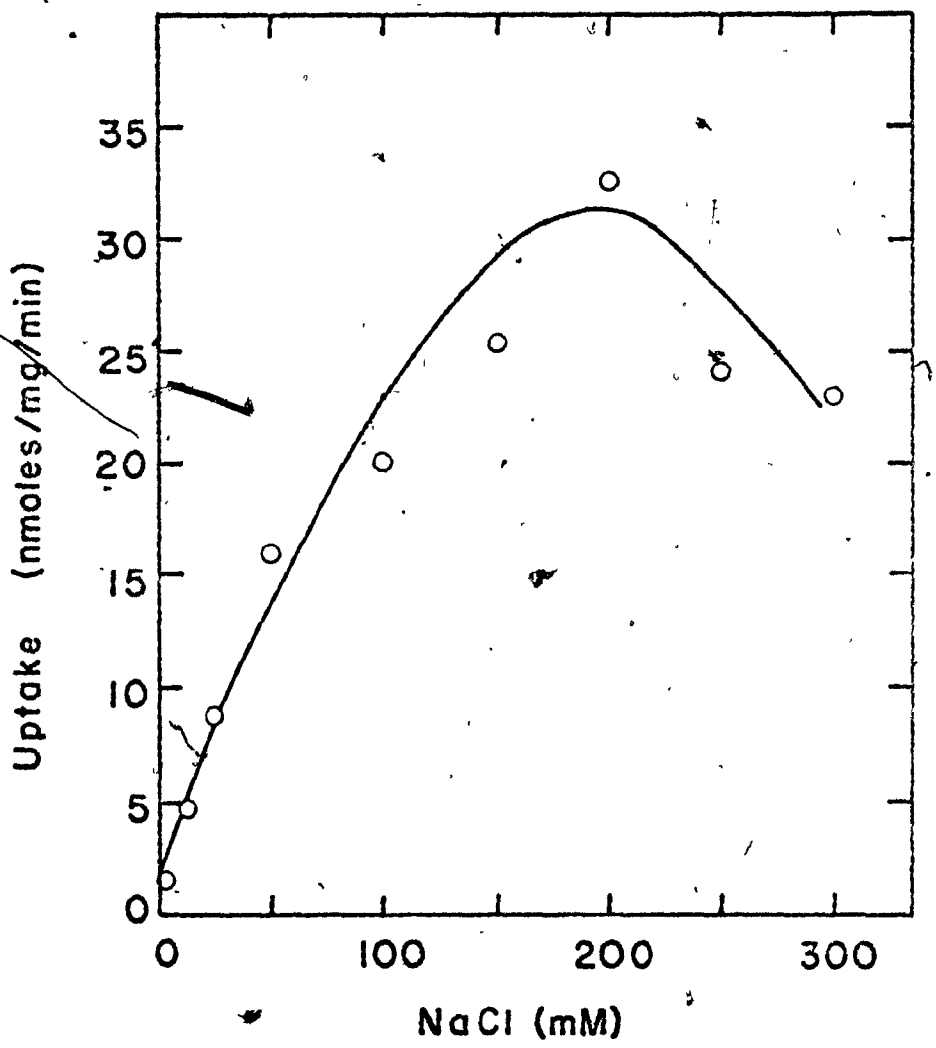
*Effect of NaCl on the ethanol and ascorbate-TMPD induced oxygen uptake in membranes.*

In assays involving ascorbate (2.7 mM) and TMPD (16 μM), membranes were used at 6 mg per assay. Results were corrected for oxygen taken up in the absence of membranes. Membranes were used at 14 mg per assay when using ethanol (2.7 mM). No oxygen was consumed with ethanol in the absence of membranes, or in the presence of 14 mg membranes without added energy sources.

| Energy Source | NaCl mM | Oxygen Uptake ng atoms O/min/mg |
|---|---|---|
| Ascorbate-TMPD | none | 55 |
| Ascorbate-TMPD | 75 | 65 |
| Ethanol | none | 1.4 |
| Ethanol | 75 | 1.4 |

Fig. 33. Response to $Na^+$ concentration of L-alanine uptake into cells of the marine pseudomonad.

After the third wash of cells in CS-T (NaCl 200 mM) individual cell pellets were resuspended in CS-T containing the indicated NaCl concentrations. The $Na^+$ carried over from the pellets to the final cell suspensions was 0.78 mM. L-alanine uptake was assayed after 1.0 min using 25 mM ethanol and 0.5 mM L-alanine, 4.8 μCi/μmole.


Uptake (nmoles/mg/min)
35
30
25
20
15
10
5
0
0
100
200
300
NaCl (mM)

(1969) for AIB. In the absence of added NaCl, essentially no activity was observed. Analysis of these cells showed the $Na^+$ carried over from the cell pellets during resuspension was 0.78 mM.

Transport kinetics in cells using L-alanine

The kinetics of alanine uptake into cells is plotted using the method of Lineweaver and Burk (1934) in Figs. 34 and 35. At the optimum $Na^+$ level of 200 mM, the apparent $K_m$ was 0.03 mM. No change in $K_m$ was noted until NaCl was lowered below 10 mM.

It is known for this organism that plasmolysis of the cells is accompanied by the loss of intracellular $K^+$ (Thompson *et al.*, 1970). Following the kinetic analysis at each $Na^+$ level shown in Fig. 35, some of the remaining cells were tested for plasmolysis using phase microscopy. These results are shown in Table 25. Plasmolysis bays became evident when $Na^+$ was reduced to 5 mM. The cells exposed to low NaCl concentrations did not lyse to any extent, and this is shown also in Table 25 where readdition of $Na^+$ was found to restore transport to a level comparable with that obtained in the cells continually exposed to 200 mM NaCl.

Transport kinetics in cells using AIB

The assay used to measure transport in cells was considerably different than that used by Wong *et al.* (1969). Rather than using

Fig. 34. Effect of $Na^+$ on kinetics of L-alanine uptake into cells of the marine pseudomonad.

Ethanol (25 mM) was added just prior to L-alanine (4.8 μCi/μmole). Curve 1, NaCl 200 mM ($K_m$ 0.03 mM); Curve 2, NaCl 50 mM, LiCl 150 mM; Curve 3, NaCl 10 mM, LiCl 190 mM; Curve 4, NaCl 5mM, LiCl 195 mM.

Note: external KCl was 10 mM in this experiment.

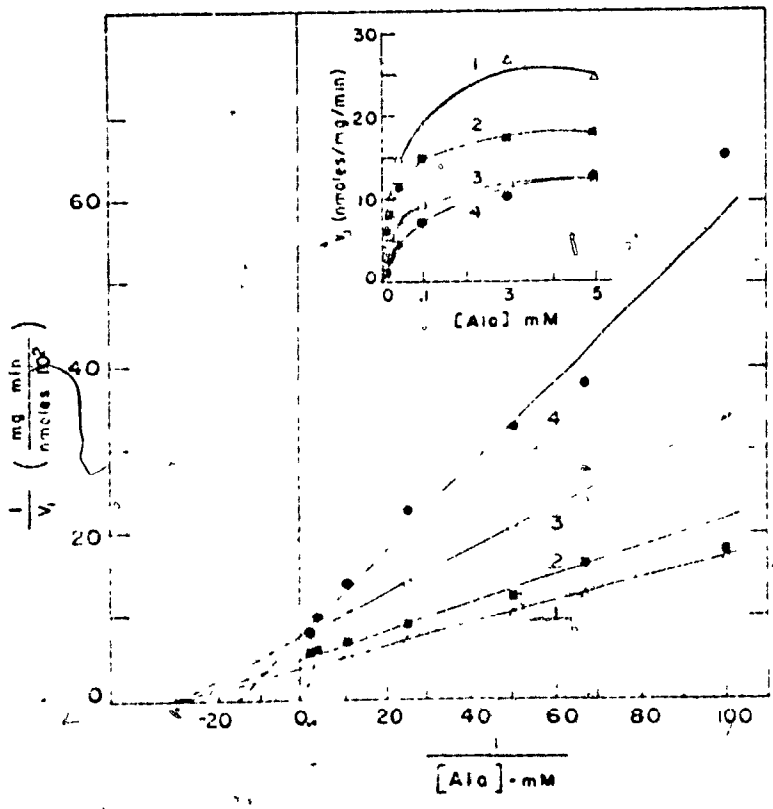

30
20
10
0
$v_i$ (nmoles/mg/min)
1
2
3
4
0
.1
3
5
[Ala] mM
60
40
20
0
$\frac{1}{v_i}$ ($\frac{mg\ min}{nmoles}$)
-20
0
20
40
60
80
100
$\frac{1}{[Ala]}$ - mM

Fig. 35. Effect of low $Na^+_i$ concentrations on the kinetics of L-alanine uptake into cells.

Experimental conditions were as described for Fig. 34. Curve 1, NaCl 10 mM, LiCl 190 mM ($K_m$ 0.03 mM); Curve 2, NaCl 5 mM, LiCl 195 mM ($K_m$ 0.17 mM); Curve 3, NaCl 2.5 mM, LiCl 198 mM ($K_m$ 0.25 mM); Curve 4, NaCl 1.0 mM, LiCl 199 mM ($K_m$ 0.50 mM).

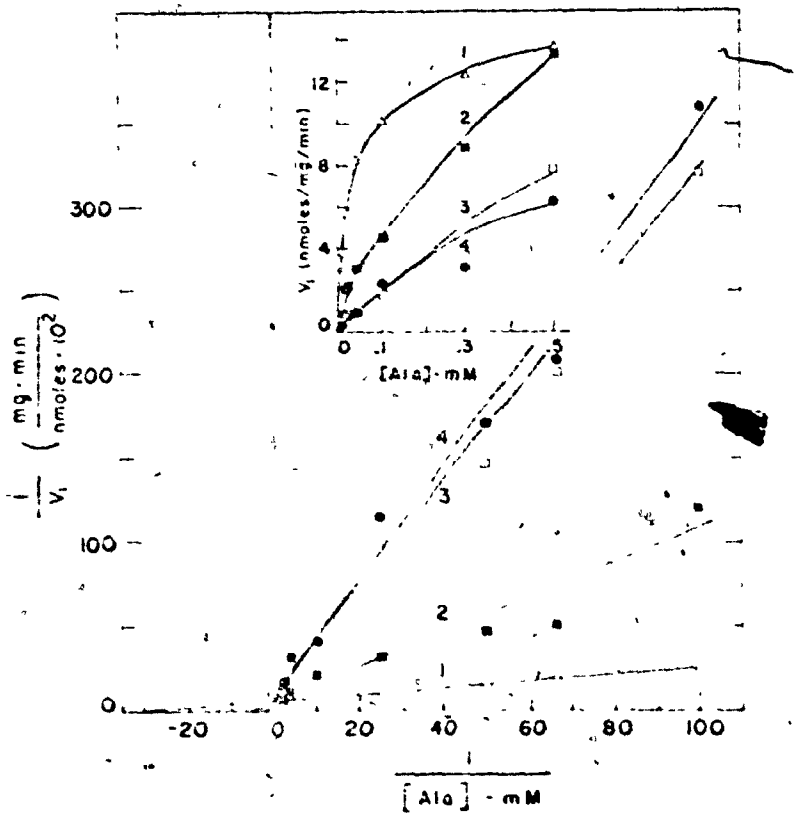

$\frac{1}{V_i}\left(\frac{mg \cdot min}{nmoles \cdot 10^2}\right)$
300
200
100
0
-20
0
20
40
60
80
100
$\frac{1}{[Ala]}$ - mM
$V_i$ (nmoles/mg/min)
12
8
4
0
.1
.3
.5
[Ala] - mM
1
2
3
4

TABLE 25.

*Cell morphology on exposure of cells of the marine pseudomonad to low NaCl concentrations and subsequent capacity to transport alanine.*

The cells in separate packed cell preparations were resuspended into CS-T containing the indicated NaCl concentrations. *Immediately after obtaining the data for each kinetic curve shown in Fig. 35, the cells were examined by phase contrast microscopy for the presence of plasmolysis bays. Plasmolysis bays in "partially plasmolyzed" cells were less numerous and less evident than in "plasmolyzed cells". L-alanine uptake was measured for 1 min, 15 min after adjusting the cell suspension to contain 200 mM $Na^+$.

| NaCl (mM) | Cell Morphology* | L-alanine uptake nmoles/mg/min |
|---|---|---|
| 200 | normal | 25 |
| 10 | normal. | 24 |
| 5 | partially plasmolyzed | 29 |
| 2.5 | plasmolyzed | 30 |
| 1.0 | plasmolyzed | 28 |

10 ml of uptake medium only 0.5 ml was used. This was an advantage not only in the saving of labeled substrates, but also in minimizing time and effort required in performing large numbers of assays as were needed for kinetic analysis. Using the modified technique (Fig. 36) similar results to those of Wong *et al.* (1969) were observed. The apparent $K_m$ was found to shift from 0.03 mM to 0.5 mM as $Na^+$ was reduced from 50 to 10 mM. No notable change in $V_{max}$ occurred until the $K_m$ shift was complete.

In the experiments described in Figs. 34 to 36 and in those of Wong *et al.* (1969), external KCl was added at 10 mM. Using the data reported by Wong (Ph.D. thesis, 1968) the second primary Lineweaver-Burk plot is shown in Fig. 37 for AIB in cells. Linear plots were not obtained either for this data or for that reported in Fig. 36. The obtained values deviate from the expected linear curves by showing a lower velocity than expected as $Na^+$ concentrations are decreased. Since plasmolysis of the cells occurs if $Na^+$ levels are decreased below 10 mM, it seems reasonable that the observed lack of linearity could be through a variation in internal $K^+$ concentrations dependent on the level of external NaCl. Very recent experiments by Gow and Thompson have confirmed this. Finally, Thompson and MacLeod (1971) have shown the amount of AIB taken up by cells is proportional to the internal $K^+$ concentration. In order to prevent the loss of $K^+$ from the cells during the kinetic study, external KCl was added to the uptake medium at the concentration found inside the cells by

Fig. 36. Effect of $Na^+$ on kinetics of AIB transport into cells of the marine pseudomonad.

No externally added energy source was used. Specific activity 0.78 μCi/μmole. Assays performed at pH 8.3. Curve 1, NaCl 200 mM ($K_m$ 0.03 mM); Curve 2, NaCl 50 mM, LiCl 150 mM ($K_m$ 0.03 mM); Curve 3, NaCl 10 mM, LiCl 190 mM ($K_m$ 0.5 mM).

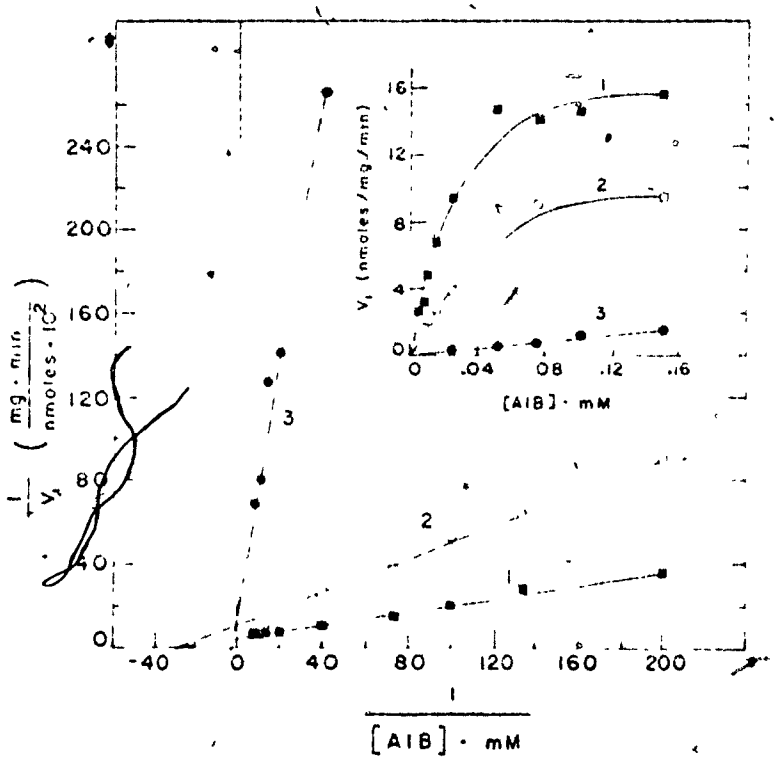
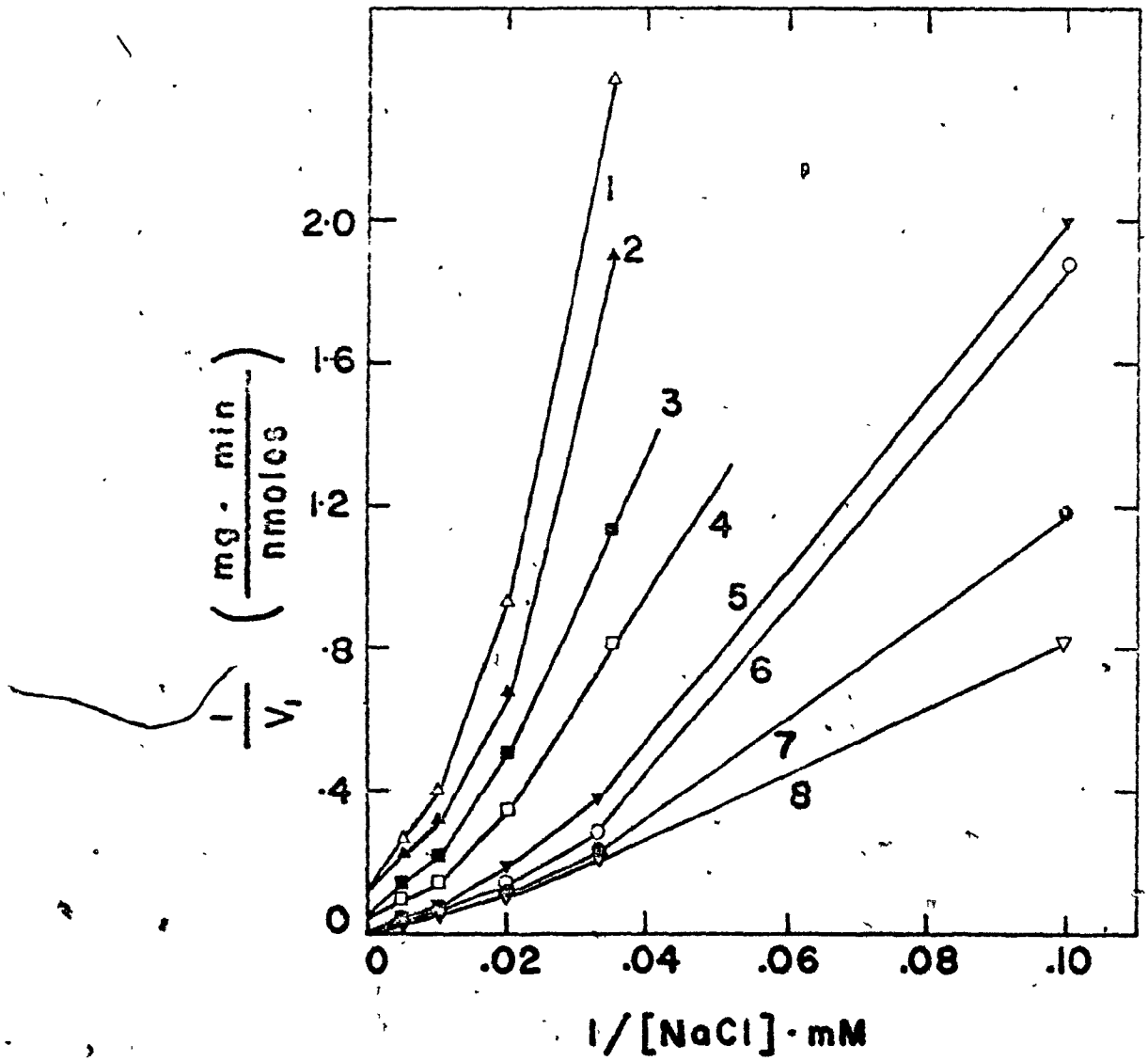

240
200
160
120
80
40
0
-40
0
40
80
120
160
200
1
[AIB] · mM
3
2
1
$\frac{1}{V_1}$ ( $\frac{mg \cdot min}{nmoles \cdot 10^2}$ )
16
12
8
4
0
$V_1$ (nmoles/mg/min)
0
.04
.08
.12
.16
[AIB] · mM
1
2
3

Fig. 37. Double reciprocal plot of AIB transport velocity and NaCl concentration for cells using the data of Wong *et al.* (1969).

AIB concentrations were (μM): Curve 1, 7.5; Curve 2, 10; Curve 3, 15; Curve 4, 25; Curve 5, 50; Curve 6, 75; Curve 7, 100; Curve 8, 150. Note: external KCl was 10 mM and LiCl was used to maintain constant molarity.


2·0
1·6
1·2
·8
·4
0
0
.02
.04
.06
.08
.10
1/[NaCl]·mM
1
2
3
4
5
6
7
8
$-\frac{1}{V_1}\left(\frac{mg \cdot min}{nmoles}\right)$

Thompson and MacLeod (1973a). These results are shown in Fig. 38 and in Fig. 39 where linear plots were in fact, obtained. The secondary plots in Fig. 40 and 41 confirmed the results obtained using membrane vesicles. A comparison in the kinetic parameters for transport into cells and vesicles is shown in Table 26. As an addition to this experiment it was shown that external KCl as high as 0.45 M did not affect the rate of transport (Table 27). It should be noted that KCl concentrations above 0.45 M decreased the rate of AIB uptake into cells.

Cotransport of AIB and $Na^+$ in cells

The simplest interpretation of ion effects in this transport system would be that $Na^+$ and AIB cotransport. Internal $K^+$ could then displace the $Na^+$ from the ternary complex, and in turn AIB would be released. Attempts to show $^{22}Na$ and AIB cotransport were negative as shown in Table 28. Using a similar method and *S. typhimurium*, Stock and Roseman (1971) had shown an initial flux of $^{22}Na$ entry in the presence but not in the absence of thiomethyl β-D-galactopyranoside. Calculations based on the filtration data shown in Table 28 assuming an internal space of 1.0 µl/mg cells seemed to indicate that the $^{22}Na$ was not penetrating the cytoplasm. In order to investigate this further the thick cell suspension technique was used (Buckmire and MacLeod, 1970). In the first experiment shown (Fig. 42) the penetration of $^{22}Na$ was compared for CS-T washed or

Fig. 38. Effect of $Na^+$ concentration on kinetics of AIB transport into cells using 0.5 M KCl in the uptake medium.

Cells washed x 3 in CS-T (NaCl 200 mM) were suspended into CS-T containing various NaCl concentrations. The molarity was kept constant using LiCl. Uptake of [$^{14}$C]AIB (0.78 μCi/μmole) was followed over 1.0 min intervals using 0.5 M KCl, Tris, pH 7.5, and $MgSO_4$ as usual at 50 mM. In all cases filtered cells were washed with CS-T (KCl 10 mM and NaCl 200 mM). NaCl concentrations were (mM): Curve 1, 200; Curve 2, 50; Curve 3, 25; Curve 4, 10.

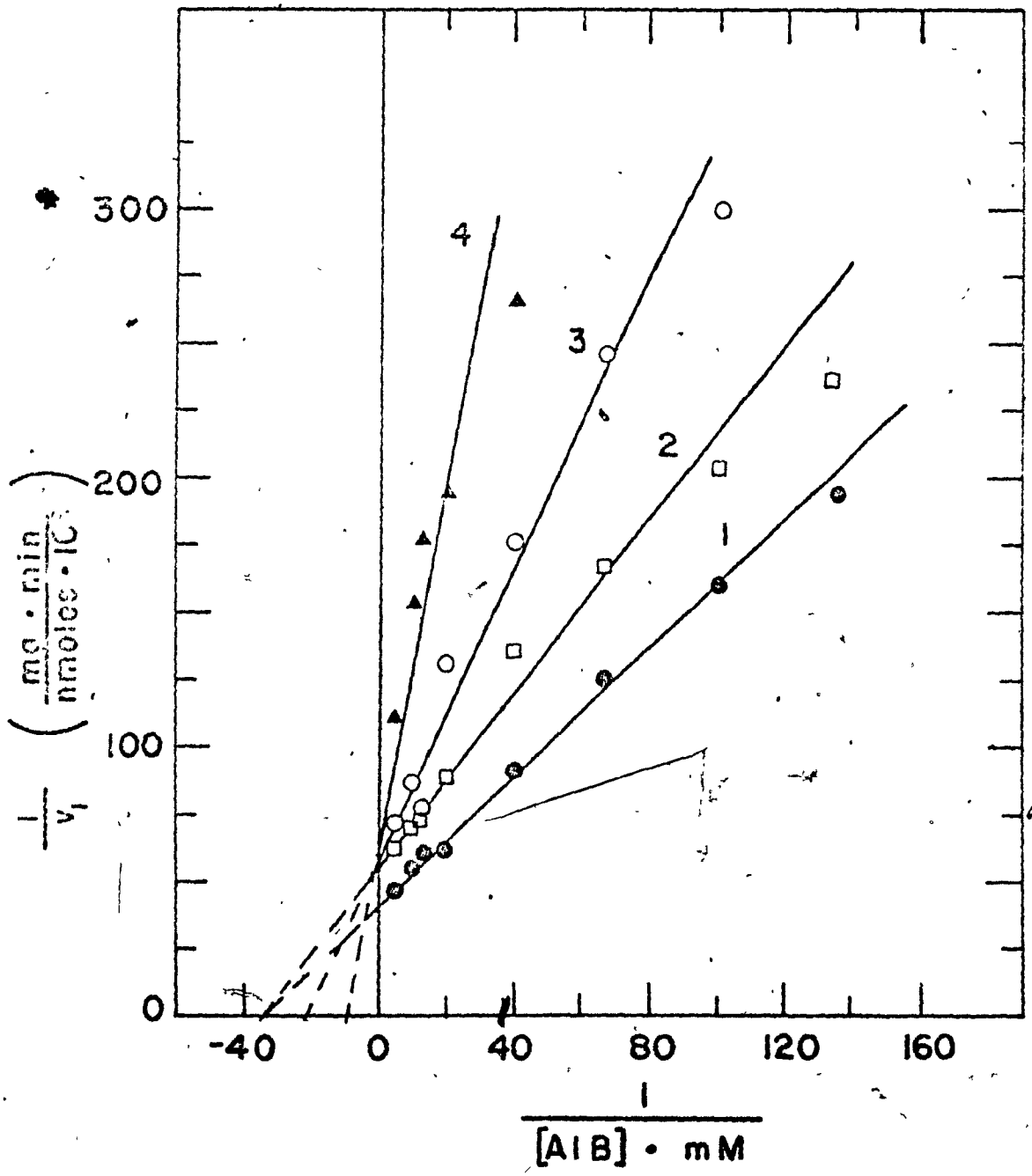

300
200
100
0
4
3
2
1
$\frac{1}{v_i}$ $\left(\frac{mg \cdot min}{nmoles \cdot 10^3}\right)$
-40
0
40
80
120
160
$\frac{1}{[AIB] \cdot mM}$

Fig. 39. Second primary Lineweaver-Burk plot for AIB uptake into cells using 0.5 M KCl in the uptake medium.

AIB concentrations were (μM): Curve 1, 25; Curve 2, 50; Curve 3, 75; Curve 4, 100; Curve 5, 150.

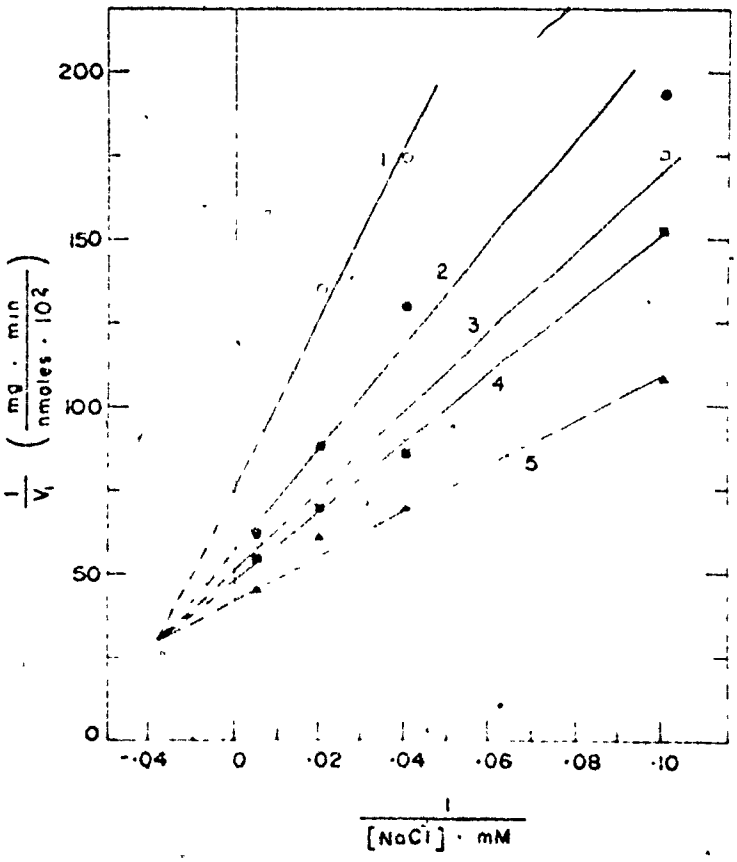

200
150
100
50
0
1
2
3
4
5
-·04
0
·02
·04
·06
·08
·10
$\frac{1}{V_i}\left(\frac{mg \cdot min}{nmoles \cdot 10^2}\right)$
$\frac{1}{[NaCl] \cdot mM}$

Fig. 40: Secondary plots of the intercepts of the Lineweaver-Burk plots shown in Fig. 39 against the reciprocals of AIB concentration, or the intercepts from Fig. 38, against the reciprocals of NaCl concentration.

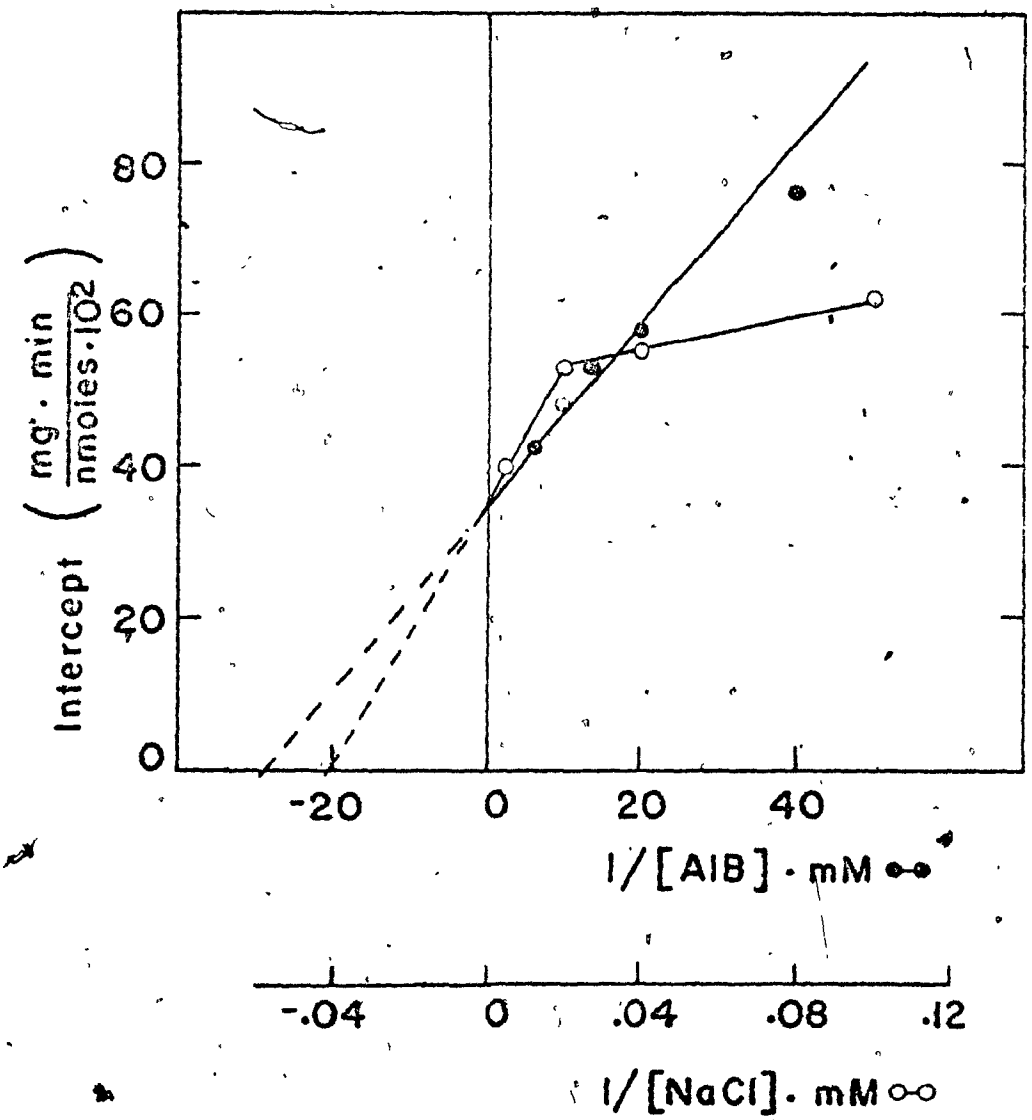
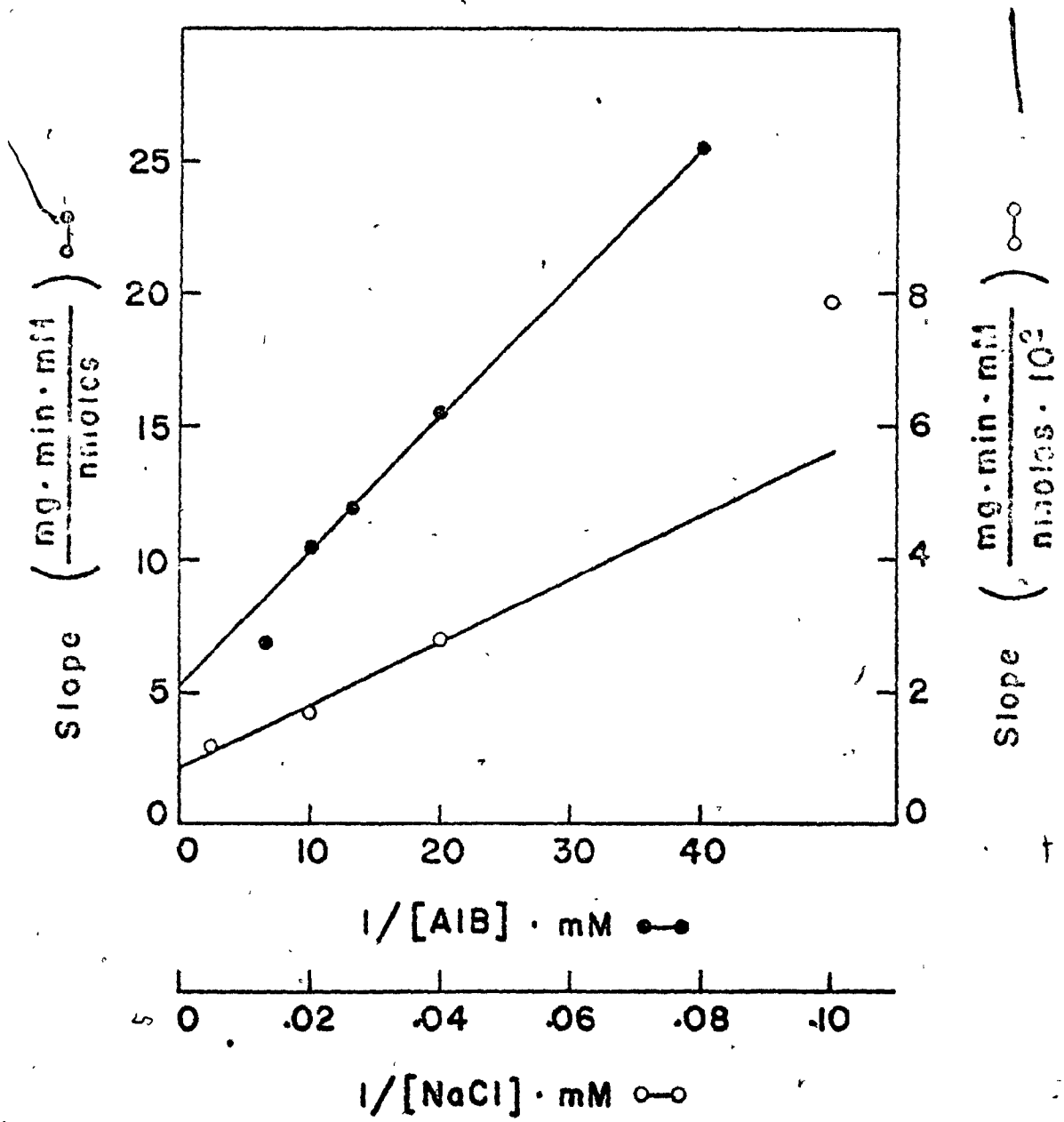

Intercept ( mg · min / nmoles · 10² )
80
60
40
20
0
-20
0
20
40
1/[AIB] · mM
-.04
0
.04
.08
.12
1/[NaCl] · mM

Fig. 41. Secondary plots of the slopes of the Lineweaver-Burk plots shown in Fig. 38 against the reciprocals of NaCl concentration, or the slopes from Fig. 39 against the reciprocals of AIB concentration.

Slopes were 0.50 (solid circles) and 0.48 (open circles).


Slope $\left(\frac{mg \cdot min \cdot mM}{nmoles}\right)$
25
20
15
10
5
0
0
10
20
30
40
1/[AIB] · mM
0
.02
.04
.06
.08
.10
1/[NaCl] · mM
Slope $\left(\frac{mg \cdot min \cdot mM}{nmoles \cdot 10^2}\right)$
8
6
4
2
0

T A B L E 26.

*Kinetic parameters for the transport of L-alanine and AIB into vesicles or cells of the marine pseudomonad.*

| Transporter | Amino Acid | V pmoles/mg/min | $K_m^A$ mM | $K_m^B$ mM | $K_s^A$ mM |
|---|---|---|---|---|---|
| Vesicles | L-alanine | 385 | 18 | 0.091 | 17 |
| Vesicles | AIB | 28 | 27 | 0.033 | 36 |
| Cells | AIB | 4800 | 29 | 0.035 | 67 |

TABLE 27.

*Effect of KCl concentration in the uptake medium on AIB transport into cells of the marine pseudomonad.*

Uptakes were performed in 10 ml volumes. One ml volumes were removed for filtering at appropriate intervals. NaCl, 200 mM; [$^{14}$C] AIB, 100 µM, 0.78 µCi/µmole.

| Uptake Time (min) | KCl (M) 0.01 | 0.25 | 0.45 | 0.50 | 0.75 | 1.0 |
|---|---|---|---|---|---|---|
| | nmoles/mg | | | | | |
| 1 | 11 | 9 | 13 | 9 | 7 | 4 |
| 5 | 35 | 38 | 38 | 33 | 21 | 6 |
| 10 | 69 | 65 | 66 | 56 | 46 | 20 |
| 20 | 95 | 107 | 112 | 105 | 89 | 46 |
| 30 | 135 | 135 | 151 | 132 | 118 | 67 |

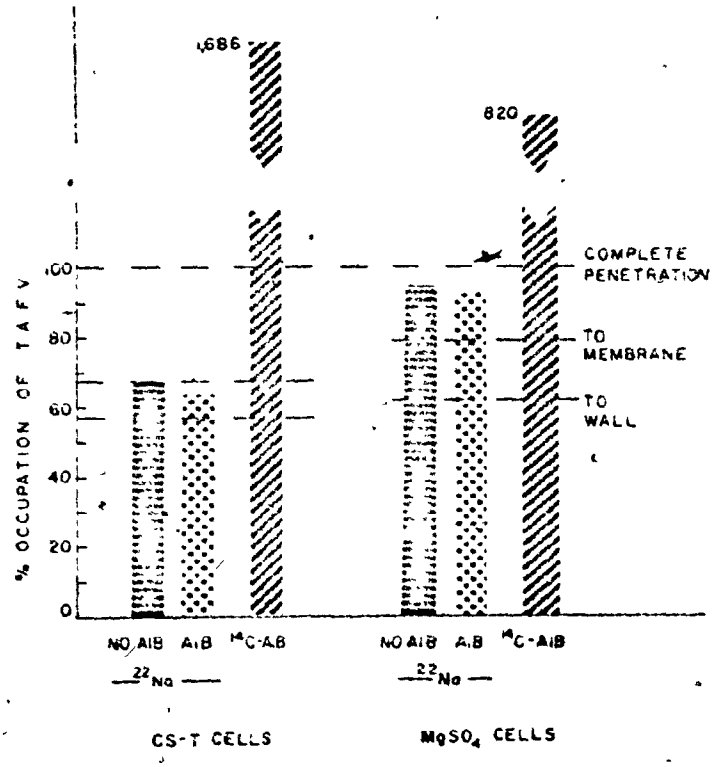
TABLE 28.

*An attempt to demonstrate cotransport of AIB and $^{22}Na$ into cells using the filter assay.*

| Time min | AIB Uptake nmoles/mg | $^{22}Na$ Uptake | |
|---|---|---|---|
| | | No AIB | Plus AIB |
| | | nmoles/mg | |
| 0.16 | 0.2 | 0.02 | 0.03 |
| 0.5 | 0.6 | 0.03 | 0.03 |
| 1.0 | 1.3 | 0.04 | 0.05 |
| 1.5 | 3.1 | 0.05 | 0.06 |
| 2.0 | 6.1 | 0.05 | 0.04 |
| 3.0 | 11.8 | 0.06 | 0.07 |
| 4.0 | --- | 0.03 | 0.03 |
| 5.0 | 25.1 | 0.03 | 0.03 |

Fig. 42. Comparison of $^{22}Na$ penetration into cells washed in CS-T or in 50 mM $MgSO_4$ using the thick cell suspension technique.

Each cell type was added at zero time to the assay fluids containing 200 mM NaCl. Centrifugation was begun 5 min later. It is assumed that $^3H_2O$ represents complete penetration, [$^{14}$C]sucrose penetrates to the cytoplasmic membrane, and [$^{14}$C]inulin penetrates to the cell wall. These penetrations are represented by dashed lines.

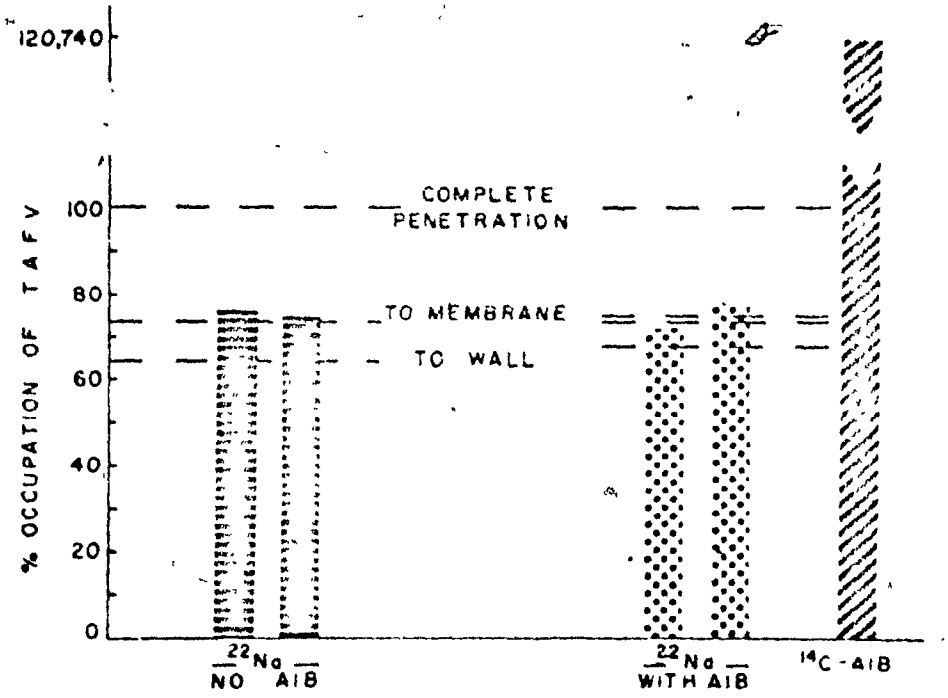

1686
820
COMPLETE PENETRATION
TO MEMBRANE
TO WALL
% OCCUPATION OF T A F V
100
80
60
40
20
0
NO AIB
AIB
14C-AIB
22Na
CS-T CELLS
MgSO4 CELLS

$MgSO_4$ washed cells. In CS-T washed cells, AIB concentrated within the cells yet the $^{22}Na$ penetrated only to the extent of [$^{14}C$]sucrose. This observation, however, is not necessarily inconsistent with a cotransport theory, since the experimental variation is approximately ± 3% penetration and 1% penetration corresponds to approximately 10 mM intracellular $Na^+$. Washing these cells in 50 mM $MgSO_4$ is known to alter the permeability of the cytoplasmic membrane (Thompson *et al.*, 1970). This procedure allowed $^{22}Na$ to enter the cytoplasm although no difference was noted in penetration when AIB was present or absent. The penetration was not to 100%, suggesting that either $Na^+$ does not completely penetrate, or as the membrane was returned to normal the $Na^+$ was being extruded over the 10 min period required for centrifuging.

A further investigation of cotransport using $^{22}Na$ is shown in Fig. 43. In this case CS-T washed cells were incubated for 30 min in the presence or absence of AIB and $^{22}Na$. To ensure that cell swelling was not a factor, the controls ($^3H_2O$, inulin and sucrose) were performed with and without AIB. Duplicate sucrose assays are shown in the case where AIB was included. It is obvious that in spite of the large concentration of AIB in the cells cotransport was not demonstrable by this method. It was calculated that the cell pellet took up 1.78 µmoles of AIB. Assuming an equal amount of $Na^+$ uptake and no efflux of $Na^+$, the difference in $^{22}Na$ occupation of the total available fluid volume (TAFV) due to cotransport would

Fig. 43. Penetration of $^{22}Na$ into CS-T washed cells in the presence or absence of AIB.

Penetration of $^{22}Na$ was measured in duplicate assays after 30 min incubation with shaking. Each assay contained 52 mg dry weight cells. The difference in $^{3}H_2O$ and [$^{14}C$] sucrose penetrations gave internal spaces of 0.94 and 0.84 µl/mg cells for cells incubated without and with AIB, respectively.


120,740
% OCCUPATION OF TAFV
100
80
60
40
20
0
COMPLETE PENETRATION
TO MEMBRANE
TO WALL
22Na NO AIB
22Na WITH AIB
14C-AIB

be only 4%. This small difference would scarcely be detected by this method, and certainly not at all if a $Na^+$ extrusion mechanism is functioning.

Extrusion of $^{22}Na$ from cells equilibrated with the isotope after $MgSO_4$ washing was observed (Fig. 44). With time the $^{22}Na$ was extruded from the cells to approximately the level of the cytoplasmic membrane.

Rather than using $^{22}Na$ to test the cotransport theory it was decided to measure $Na^+$ directly by flame photometry, thereby bypassing any possible isotope preference effects. Cells were incubated in the presence or absence of AIB. The cells were collected by centrifugation within the initial interval where little $Na^+$ efflux was expected (Fig. 44), and the supernatant fluids and pellets were digested for $Na^+$ analysis by flame photometry. Extracellular $Na^+$ in the pellets was corrected for by determining the $\mu g$ $Na^+$ per $\mu l$ in the supernatant fluid and by determining the $^3H_2O$ and [$^{14}C$] sucrose penetrations. Two such experiments are shown in Table 29. Although the method was very reproducible for the supernatant fluids, considerable variation occurred in the determination of intracellular $Na^+$. This was a result of the low intracellular volume. In experiment 2, for example, the wide variation of 332 ± 46 resulted from a difference in per cent transmittance between the two pellets of only 1.5.

Fig. 44. Loss of intracellular $^{22}Na$ as a function of time after resuspension of $MgSO_4$ washed cells into CS-T (NaCl, 200 mM).

The percentage of the total available fluid volume (T.A.F.V.) occupied by AIB is given above the first bar for each time interval.  , no [$^{12}C$]AIB; ■, 150 μM [$^{12}C$]AIB.

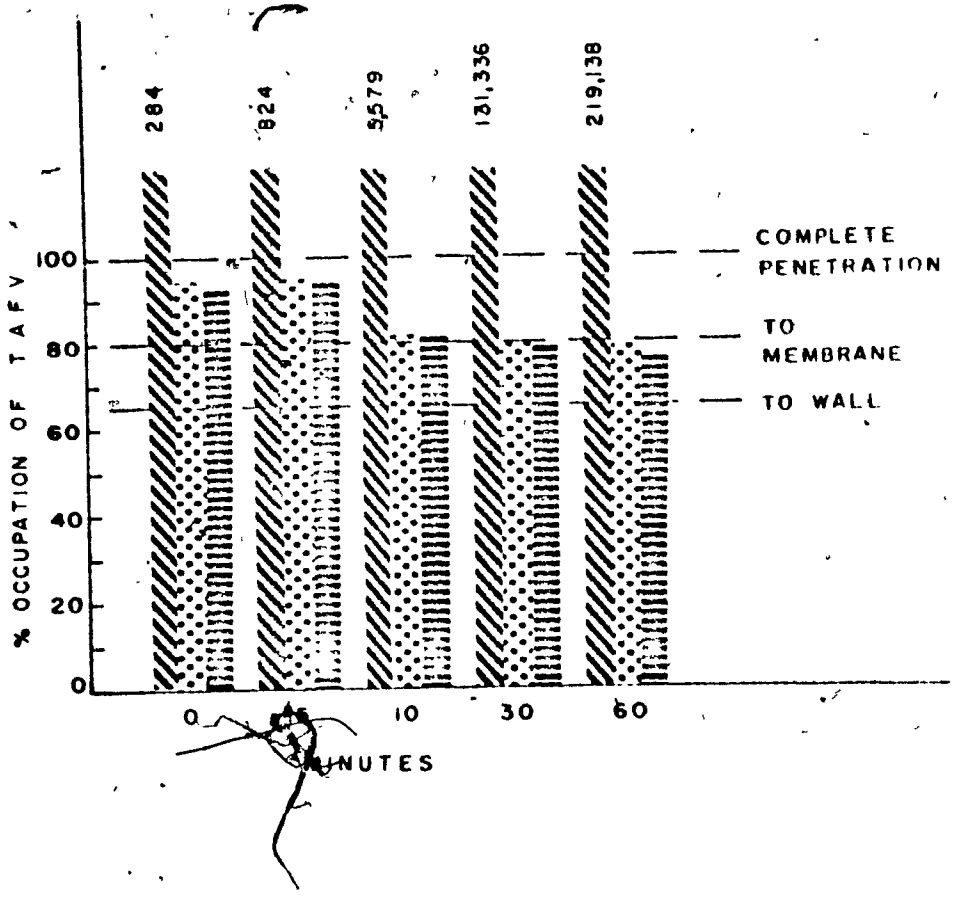

284
824
5,579
131,336
219,138
% OCCUPATION OF TAFV
100
80
60
40
20
0
COMPLETE PENETRATION
TO MEMBRANE
TO WALL
0
10
30
60
MINUTES

TABLE 29.

*Intracellular concentration of $Na^+$ in $MgSO_4$ washed cells after resuspension into CS-T in the presence and absence of [$^{12}$C]AIB.*

Assays were performed in duplicate. As in previous assays AIB was added at 150 µM. After resuspension of $MgSO_4$ washed cells into CS-T, the cells were quickly added to the assay fluids and centrifugation started 3 min later. The dry weight of cells per assay was 99 mg in experiment 1 and 153 mg in experiment 2. Separate assays using [$^{14}$C]AIB showed in experiment 2 that AIB penetrated within the given time interval to 219% of the T.A.F.V. of the packed cell pellet.

| Experiment number | Internal space µl/mg cells | Concentration of $Na^+$ | | | |
|---|---|---|---|---|---|
| | | no AIB | | with AIB | |
| | | Extracellular | Intracellular | Extracellular | Intracellular |
| | | mM | mM | mM | mM |
| 1 | 0.68 | 186 ± 1 | 142 ± 90 | 187 ± 4 | 227 ± 33 |
| 2 | 0.65 | 189 ± 2 | 332 ± 46 | 190 ± 11 | 217 ± 22 |

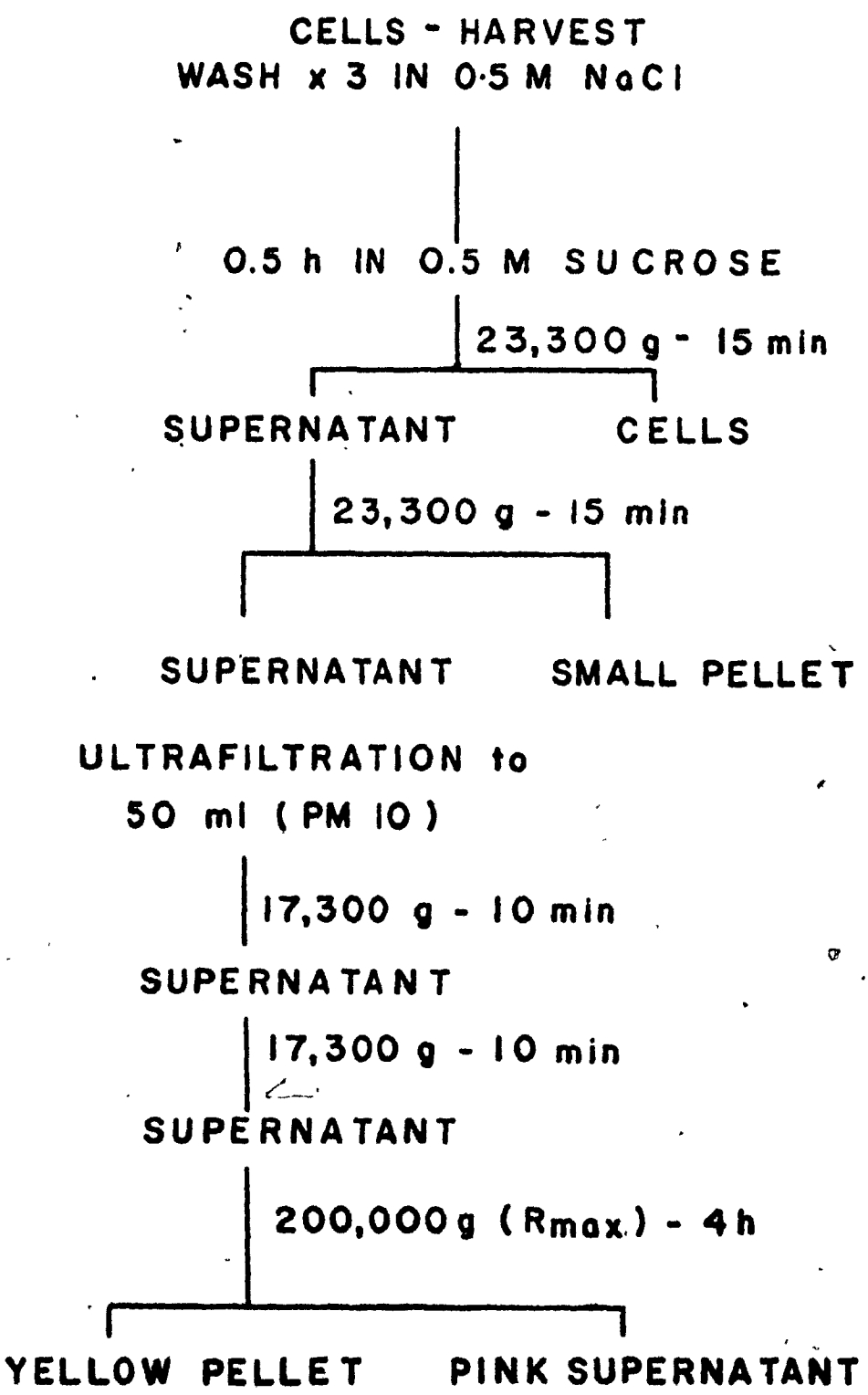
# SECTION III.

## POSSIBLE INVOLVEMENT OF CELL WALL COMPONENTS IN TRANSPORT

In the vesicle studies it was obvious that the activity observed was due to the cytoplasmic membrane. However, in intact cells one cannot be so certain. Many reports of solute binding proteins have been presented (see Literature Review) and evidence for their probable role in transport given. In cells of the marine pseudomonad it was found that various cell forms, including protoplasts, retained equal transport activity (DeVoe *et al.*, 1970). It was concluded that the cell wall did not participate in the transport process but did stabilize the cytoplasmic membrane. Because these studies were conducted only with the non-metabolized compound AIB, it was decided to test directly for binding activity of L-alanine and L-leucine by cell wall layers.

The isolation of outer-double-track (ODT) and underlying layer (ULL) is shown in Fig. 45. The procedure was developed from that of Forsberg (Ph.D. thesis, 1969). An ultrafiltration step was substituted for flash evaporation allowing concentration of the sucrose fraction at 4°C without altering the sucrose molarity. Steps were taken after ultrafiltration (PM-10 filter) to ensure the removal of any possible cell contamination, and the final supernatant fluid was separated

Fig. 45. Isolation of cell wall fractions used for binding studies.


CELLS - HARVEST
WASH x 3 IN 0·5 M NaCl
0.5 h IN 0.5 M SUCROSE
23,300 g - 15 min
SUPERNATANT
CELLS
23,300 g - 15 min
SUPERNATANT
SMALL PELLET
ULTRAFILTRATION to
50 ml (PM 10)
17,300 g - 10 min
SUPERNATANT
17,300 g - 10 min
SUPERNATANT
200,000 g (Rmax.) - 4 h
YELLOW PELLET
PINK SUPERNATANT

by high speed centrifugation into a yellow pellet and a pink supernatant fluid.

The final two fractions were assayed for L-alanine binding activity by equilibrium dialysis techniques (Table 30). The supernatant fluid (ULL) was entirely soluble and was pink, while the pellet when resuspended appeared water insoluble but did not settle out. Binding was observed for the ODT fraction only. Quenching was corrected for by the channels ratio method (Appendix 1), although binding was evident even without this correction.

When the ULL was scanned with a Unicam SP 800 spectrophotometer a result similar to that expected for pure cytochrome c was obtained (Fig. 46). Alpha, beta and Soret peaks were at 550, 522 and 415 nm for the oxidized sample. Cytochrome c has been found in the ULL by Nelson and MacLeod (unpublished observation).

The influence of pH on L-alanine binding by ODT was evaluated in Table 31 using citrate and tris buffers. An approximate pH optimum of 5.6 was found with no binding activity below 3.6 or above 7.5. The relation between pH responses for transport into cells and vesicles (Fig. 2) and for binding by ODT will be discussed in a later section.

An assay to measure binding activity, in addition to the equilibrium dialysis technique, was developed from the method of Briggs and Bourgeois (1968). In this assay the radioactive amino acid and

TABLE 30.

*Binding of L-[$^{14}$C]alanine by isolated cell wall fractions.* Quenching was corrected by the channels ratio method (Wang and Willis, 1965) using chloroform as quenching agent. Each assay was performed using citrate buffer, pH 6.0, and approximately 8 mg protein per assay. L-[$^{14}$C]alanine, 1 µM, 156 µCi/µmole.

| Fraction | Sac Contents DPM | Dialysis Fluid DPM | Ratio |
|---|---|---|---|
| Pink Supernatant Fluid (ULL) | 6,136 | 6,888 | 0.9 |
| Yellow Pellet (ODT) | 12,460 | 4,918 | 2.5 |

Fig. 46. Oxidized spectrum of the "pink supernatant" fraction using the Unicam SP 800.

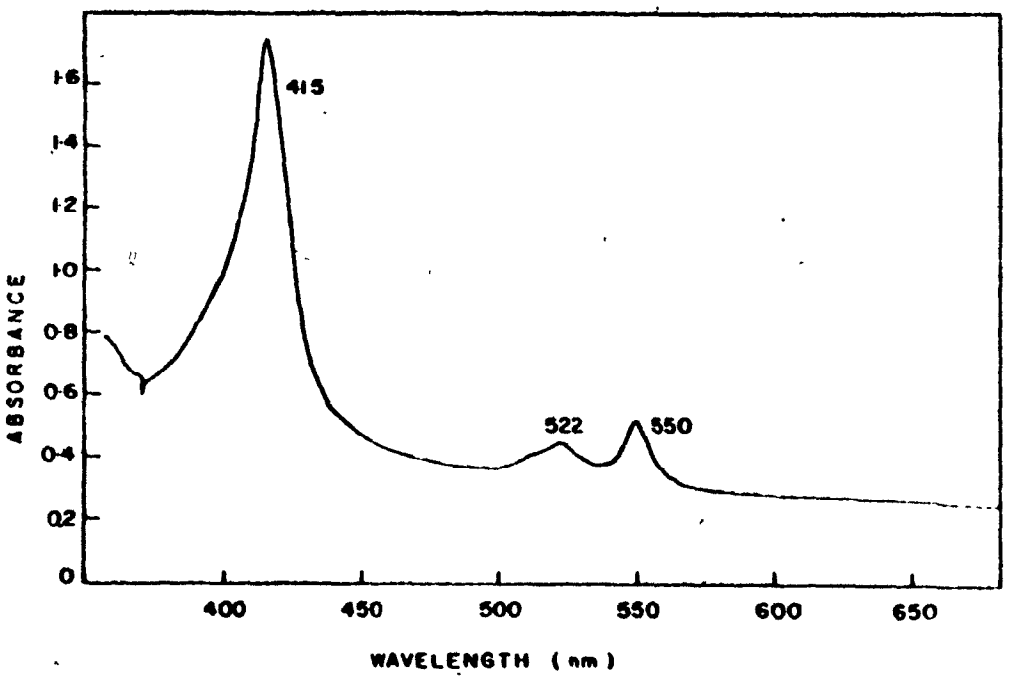

ABSORBANCE
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
415
522
550
400
450
500
550
600
650
WAVELENGTH ( nm )

TABLE 31.

*Effect of pH on L-alanine binding to isolated outer-double-track.* Assays contained 11.9 mg protein. The binding ratio is the ratio of DPM in equal volumes of dialysis sac contents and in dialysis fluids, following 20 hours dialysis at 4 C. L-[$^{14}$C]alanine, 1 µM, 156 µCi/µmole.

| pH | Buffer | Binding ratio |
|---|---|---|
| 2.6 | Citrate | 0.94 |
| 3.6 | Citrate | 0.95 |
| 4.6 | Citrate | 2.27 |
| 5.6 | Citrate | 4.40 |
| 7.5 | Tris-HCl | 1.31 |
| 8.5 | Tris-HCl | 0.99 |

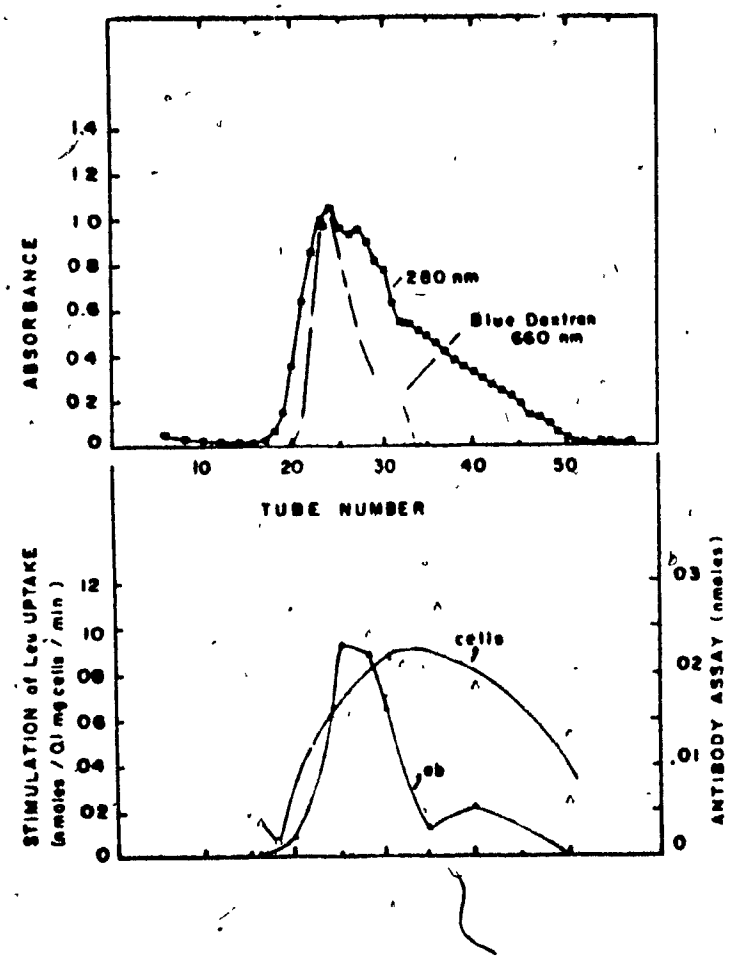
cell wall extract were mixed. Antibody was then added. The antibody had been prepared by injecting whole cells of this organism into rabbits and was kindly supplied by Dr. John Nelson, Jr. After 5.0 min it was assumed that agglutination was complete, and the "antibody-cell wall-amino acid" complex was filtered. Without agglutination the cell wall extract was not retained appreciably by the 0.45 μ filters used.

Another assay was developed in which the extract was added to cells and transport capacity compared to control cells lacking the extract.

Attempts to fractionate the ODT by Sepharose 4B chromatography is shown in Fig. 47. Absorbance at 280 nm was recorded (Zeiss spectrophotometer) and selected fractions assayed for activity. Two assays were used. Firstly, uptake of L-leucine into cells was measured by the filtration technique both in the presence and absence of extract. Uptake was then recorded as nmoles above the control lacking extract. In this assay the results were somewhat erratic, but nevertheless did show very dramatic increases in uptake dependent on the concentration of extract used. Secondly, binding of the radioactive L-leucine was measured using the assay involving agglutination with rabbit B-16 antiserum and collecting the precipitate on Millipore filters. Counts remaining on the filters were found to follow the pattern for 280 nm absorbance, indicating a relation

Fig. 47. Sepharose 4B column chromatography of the outer-double-track cell wall fraction.

Approximately 16 mg protein, (Lowry) was applied to the column. Transport stimulation was measured using cells washed in 0.05 M $MgSO_4$ and deplasmolyzed 1.5 hours in CS-T. Cells and column extracts (0.25 ml of each) were mixed for 15 min, L-[$^{14}$C]leucine added (5 µM, 31.3 µCi/µmole), and uptake measured over 1.0 min. Antibody assays consisted of 0.25 ml of the column fraction plus 0.20 ml CS-T and L-[$^{14}$C]leucine as above. At this time 0.05 ml of antibody was added and incubation continued 5.0 min. Assays were filtered on 0.45 µ Millipore filters and not washed. Only fractions 16 and 50 did not agglutinate.


ABSORBANCE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
280 nm
Blue Dextran
660 nm
10
20
30
40
50
TUBE NUMBER
STIMULATION of Leu UPTAKE
(cmoles / 0.1 mg cells / min)
12
10
08
06
04
02
0
cells
ab
ANTIBODY ASSAY (nmoles)
03
02
.01
0

between concentration of extract and binding. As can be seen, the two assay techniques did not show optimum activities to be present for the same tube number. This may be taken as suggestive evidence that transport stimulating activity and binding activity are contributed to by different components in the ODT extract. However, it is equally possible that transport stimulating activity was lower than expected because of a depression due to the high concentration of extract used in assaying fractions 23-26.

The specific activity for transport stimulation appeared higher in the latter fractions emerging from the column. To see if this was a useful observation the experiment shown in Fig. 48 was conducted. After fractionation of ODT using a Sepharose column (A), the fractions were pooled to give three components (this is the same run shown in Fig. 47). When components I, II or III were subjected to rechromatography (B, C) it was found that this material behaved as did the original unfractionated component (A). It is thus unlikely that a fraction rich in transport stimulating activity can be obtained by this method.

Tubes 18 to 25 were pooled (Fig. 48, B) and this material analyzed for transport stimulating activity using cells (Table 32). Both L-alanine and L-leucine uptake was stimulated by the extract, while AIB uptake was not affected. Similar results were found when the extract was tested by equilibrium dialysis for binding of L-leucine

Fig. 48. Sepharose 4B fractionation of outer-double-track and reaggregation.

Part A represents the same profile as shown in Fig. 47. Column fractions were pooled into the three fractions as shown. Part B represents the profile obtained on repassing Fraction III through the column. Part C represents the profile obtained on repassing Fractions I and II through the column.

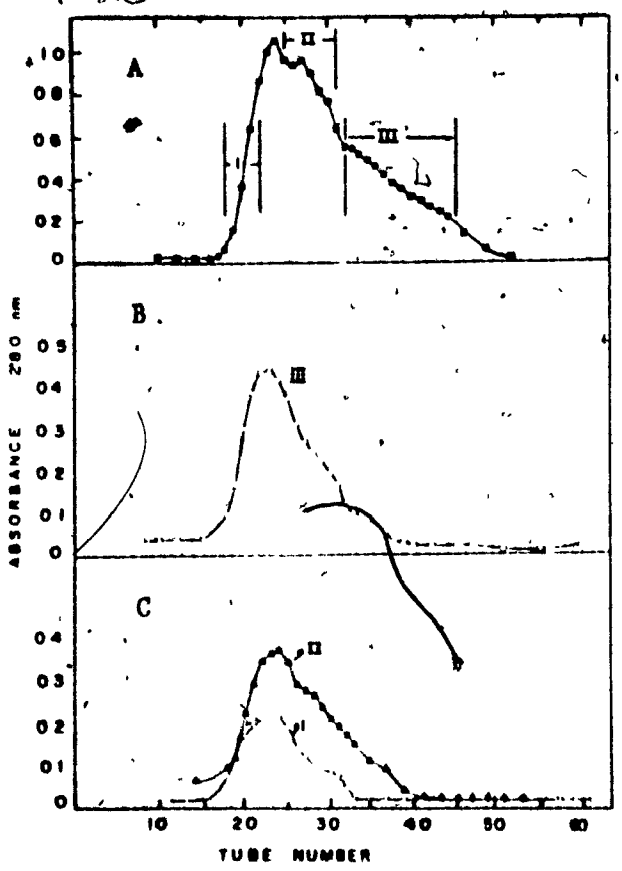

A
B
C
I
II
III
ABSORBANCE 280 nm
TUBE NUMBER
10
20
30
40
50
60

TABLE 32.

*Effect of a Sepharose 4B fraction of outer-double-track on uptake of amino acids in cells.*

Each assay consisted of 100 µg cells with or without extract, and [$^{14}$C]amino acids at 10 µM. Specific activities were L-alanine 14.2, L-leucine 31.3, and AIB 0.78 µCi/µmole. Extract added per assay contained 31 µg protein by the method of Lowry *et al.* (1951) using lysozyme as standard, and 16 µg reducing sugar by the phenol-sulphuric method (Dubois *et al.*, 1956) using glucose as standard.

| Amino Acid | Extract | Uptake nmoles/mg | Stimulation % |
|---|---|---|---|
| L-alanine | - | 2.74 | |
| L-alanine | + | 3.53 | 29 |
| L-leucine | - | 1.00 | |
| L-leucine | + | 1.86 | 86 |
| AIB | - | 0.73 | |
| AIB | + | 0.64 | nil |

and AIB (Table 33). Thus, AIB uptake was neither stimulated by the cell wall extract nor was it bound by the extract as measured by equilibrium dialysis.

It is evident from the experiments described, that future work on the nature of the binding material will require solubilization of ODT as a first step in the purification. An equilibrium dialysis apparatus was used to assay ODT and Triton X-100 solubilized ODT for binding of L-alanine. Activity remained after solubilization (Table 34), and exhaustive dialysis did not diminish this binding. Until the cell wall factor(s) possibly involved in transport are solubilized and purified it is not possible to state whether or not the same component is responsible for L-alanine and L-leucine binding and for L-alanine and L-leucine transport stimulation. Further work in this area is obviously required to answer this question.

T A B L E 33.

*Binding of L-leucine and AIB by isolated outer-double-track.*

Equilibrium dialysis assays contained 2 mg protein per ml and either L-[$^{14}$C]leucine (0.5 μM, 344 μCi/μmole) or [$^{14}$C]AIB (3.3 μM, 8.6 μCi/μmole).

| Amino Acid | Binding Ratio |
|---|---|
| L-leucine | 7.1 |
| AIB | 0.7 |

TABLE 34.

*Solubilization of L-alanine binding activity from outer-double-track using Triton X-100.*

The crude extract (8 mg protein per ml) was suspended in citrate buffer (pH 5.5) and 1% Triton X-100 added. After 10 min Triton X-100 soluble and insoluble material were separated by centrifugation 30 min at 58,000 x g ($R_{max}$). The pellet was resuspended to a volume equal to the supernatant fluid using citrate buffer. The equilibrium dialysis apparatus as described by Weiner and Heppel was used. Crude extract 6 mg protein per assay; citrate buffer pH 5.5; L-[$^{14}$C]alanine, 0.45 µM, 156 µCi/µmole. Note: Rabbit B-16 antiserum gave positive slide agglutination tests for all three of the extracts.

| Sample | Binding Ratio at several dialysis times | | |
|---|---|---|---|
| | 20 hours | 28 hours | 40 hours |
| Crude extract | 4.5, 5.7 | | |
| Triton X-100 soluble | 2.9, 2.9 | 2.7, 2.7 | 2.6, 2.5 |
| Triton X-100 insoluble | 3.9, 4.2 | | |

# DISCUSSION

Using a rapid filtration technique it was shown that vesicles of the marine pseudomonad actively accumulated L-alanine against a concentration gradient. The activity observed does represent active transport and not merely binding of radioactivity to the membranes. Support for this conclusion is drawn from the findings that electron donors and oxygen are required for the uptake of alanine, that respiratory chain inhibitors can prevent this uptake, and that these same inhibitors and phenethyl alcohol cause the loss of the amino acid from vesicles preloaded with alanine. The phenethyl alcohol effect was reversible as it is in intact cells (Thompson and DeVoe, 1972), showing that the membrane was not irreversibly damaged. In addition, the activity was observed only if $Na^+$ was added to the uptake medium, with no sparing action by KCl, LiCl, or RbCl, as is the case for intact cells (Drapeau *et al.*, 1966). Also, amino acid competition and counter transport was observed. Most of the vesicle transport activity was, therefore, indicative of the intact cell. It seems most unlikely that the amount of amino acid associated with the vesicles could be a specific binding to the transport carrier without its release to the intravesicular space.

There are several lines of evidence to indicate that the uptake of L-alanine by vesicle preparations is not due to intact cells

present as contaminants. First, the number of cells determined by plate count to be present as contaminants in the vesicle preparations was found to be insufficient to permit the uptake of detectable amounts of alanine. Also, only a fraction of the total cell count was able to grow in a medium containing NaCl but not in its absence, and could be presumed to be cells of the marine pseudomonad (Gow, Ph.D. thesis, 1973). Second, intact cells accumulated $^{42}K$ but vesicles did not. Third, transported alanine was rapidly metabolized by cells contrasting to the slow rate of metabolism of alanine associated with vesicles. Another is that the optimum $Na^+$ concentration for the transport of alanine into vesicles is 75 mM while for cells it is 200 mM. Finally, efflux from preloaded cells (Wong *et al.*, 1969) and preloaded vesicles demonstrated dramatic differences when inhibitors were added.

Using the finding that cytoplasmic membrane represents 12% of the cell dry weight (Martin and MacLeod, 1971), it can be calculated that the $V_{max}$ for L-alanine uptake into the intact cell was nearly 1,000 times greater than into vesicles. This loss in specific activity is not characteristic of all vesicle transport systems studied, although McKillen *et al.* (1972) observed similar effects for citrate transport in *B. subtilis* vesicles. In the case of *E. coli*, vesicles transport more effectively than intact cells (Kaback, 1972) and for *B. licheniformis* (MacLeod *et al.*, 1973) and *S. aureus* (Short *et al.*,

1972a) vesicles transport equally well to the cells. The low level of transport in vesicles of the marine pseudomonad may be related to the inability of the vesicles to concentrate $K^+$. In intact cells, the intracellular $K^+$ concentration determines the rate and extent of AIB uptake (Thompson and MacLeod, 1971), where at the steady state level the $K^+$ to AIB ratio is 1.6 to 1 (Thompson and MacLeod, unpublished). It can be calculated from the data that vesicles, prepared under conditions to free them as completely as possible of $K^+$, still contained $K^+$ in excess of that required for the amounts of amino acid accumulated. In fact, the $K^+$ to L-alanine ratio was 10 to 1. Thus, a $K^+$-requirement for vesicle transport may exist in spite of the inability to demonstrate it. As compared to cells (Wong *et al.*, 1969), the vesicles dramatically lost intracellular alanine on the addition of metabolic inhibitors. The inhibitor KCN blocks uptake of AIB (Drapeau *et al.*, 1966) into cells, showing that the lack of efflux from cells was not through an inability of KCN to reach the membrane. Unlike results obtained for *E. coli* vesicles (Kaback and Barnes, 1971) inhibition of electron flow, regardless of the point of inhibition, resulted in net efflux. From these observations, it is likely that the low specific activity observed is a consequence of a "pump and leak" effect, electron flow being required for influx of amino acid which is continually effluxing from the vesicles. When influx and efflux rates become the same, no net movement of solute would be observed. This effect

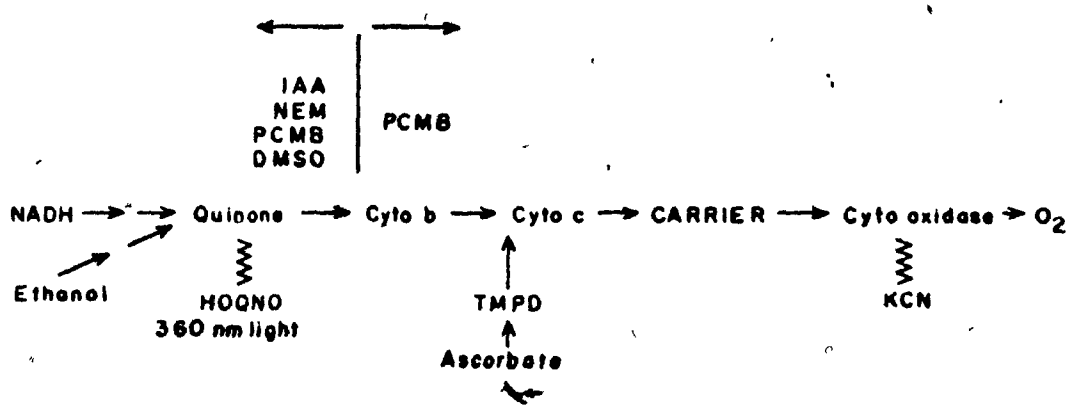
would explain the efflux data, but could not solely account for the low specific activity, since initial rates of transport were used in kinetic experiments for $V_{max}$ calculations. During the initial minute of uptake it can be calculated, using the intravesicular space (0.4 µl/mg) and kinetic data, that little concentration of alanine above the outside level occurs. Thus, one should in this case be measuring influx primarily. The low specific activity may merely reflect the low intravesicular $K^+$ concentration.

Previous to this study transport in cells of this organism was studied using endogenous energy. Endogenous oxygen uptake and transport in vesicles was found to be negligible, thus allowing a definitive study on energy sources. The transport system was activated by NADH, and by the artificial electron donor system ascorbate-TMPD. A group of short chain primary alcohols were found to energize transport, following the original observation by Thompson that AIB uptake into cells was stimulated by ethanol (Thompson and MacLeod, 1973b). Both NADH and ethanol when added to vesicles reduced cytochromes b and c, and a trough in the region of flavoprotein appeared (Jones and Redfearn,1966). Ascorbate-TMPD reduced relatively more cytochrome c than b and gave no evidence of flavoprotein reduction. The point of entry of the electron donors is illustrated in Fig. 49.

Cytochrome scans using ethanol as electron source revealed

Fig. 49. Proposed site of energy coupling, points of inhibition by transport inhibitors, and points of coupling of electron donors, in a respiratory chain coupled to transport.

Cyto, cytochrome.


IAA
NEM
PCMB
DMSO
PCMB
NADH
Quinone
Cyto b
Cyto c
CARRIER
Cyto oxidase
$O_2$
Ethanol
HOQNO
360 nm light
TMPD
Ascorbate
KCN

HOQNO, IAA, NEM and PCMB to inhibit electron flow prior to cytochrome b. The site of HOQNO inhibition did not change when NADH was used in place of ethanol. The site of cyanide inhibition was found to be after cytochrome c. The scheme shown in Fig. 49 is entirely consistent with the transport data. Inhibition of transport by the inhibitors was the same when NADH or ethanol were energy sources. However, ascorbate-TMPD bipassed the sites of IAA, NEM, and HOQNO inhibition since transport was in these cases not inhibited. With ascorbate-TMPD, inhibition by PCMB was about 50%, showing that PCMB inhibited after the site of TMPD coupling as well as before cytochrome c. Inhibition by 366 nm light was partially overcome by ascorbate-TMPD, suggesting a quinone component to lie before cytochrome b. This component has not as yet been characterized. Transport was inhibited with either ethanol or ascorbate-TMPD as donors by flushing the assay tube with nitrogen gas.

By the use of a series of inhibitors and electron donors, results were obtained from which it is concluded that only part of the total respiratory activity in the vesicles is coupled to transport. Total respiratory activity was measured as oxygen uptake induced by either NADH or ascorbate-TMPD. Ethanol oxidation is efficiently coupled to that respiratory activity involved in amino acid transport, while NADH and ascorbate-TMPD in addition to coupling to transport respiratory chains, couple to the total respiratory activity. Experimental results leading to and supporting these conclusions are as follows:

1. Ethanol, NADH and ascorbate-TMPD are equally active in promoting transport yet NADH and ascorbate-TMPD promote more oxygen uptake by the vesicles than ethanol. In fact, based on initial rates, ethanol induced only about 4% as much oxygen uptake as did the other donors (Table 18). Ethanol drove transport via the respiratory chain and required oxygen to act.

2. A lack in correlation between the capacity of a compound to activate transport and to promote $O_2$ uptake has been observed in vesicles of *E. coli* where amino acid and sugar transport is activated most effectively by D-lactate (Kaback and Milner, 1970; Barnes and Kaback, 1970). In vesicles of the marine pseudomonad this was observed in a striking fashion, since ethanol activated transport fully but was slowly oxidized, while succinate showed the reverse effect. Specific coupling of electron donors in the case of ethanol and succinate would explain these effects. Ethanol oxidation is efficiently coupled to transport while succinate oxidation is not.

3. A series of inhibitors showed quantitatively similar effects on transport energized by NADH and by ethanol. This is the expected result if both NADH and ethanol are coupled to respiratory activity involved in transport since NADH and ethanol enter the respiratory chains at the same point (Fig. 49). When the effect of a series of inhibitors were compared for inhibition of transport and oxygen uptake using ethanol, it was found that in every case the

extent of inhibition of oxygen uptake paralleled the inhibition of transport. Thus, the amount of ethanol oxidized is directly related to transport (i.e., efficient coupling). For NADH and ascorbate-TMPD, however, the inhibition of oxygen uptake did not necessarily correspond to transport inhibition, the most likely interpretation being that only part of the NADH or ascorbate-TMPD induced respiratory activity is coupled to transport.

4. Considerable support for the hypothesis that ethanol oxidation, but not that of NADH or ascorbate-TMPD, is very specifically coupled to respiratory activity and transport was obtained. It was found that oxidase activity associated with ethanol could be specifically inhibited by IAA, NEM, and DNP. Importantly, the inhibitors IAA and NEM were equally as effective for NADH driven transport as for ethanol driven transport. Thus, the data available shows that NADH must be oxidized in two ways, only one of which is coupled to transport. Of particular importance, DNP inhibited ethanol oxidation with much less effect on the oxidation of NADH or ascorbate-TMPD, yet DNP equally inhibited transport induced by all three compounds. It is concluded that in vesicles of the marine pseudomonad DNP inhibits transport by specifically blocking electron flow in these respiratory chains involved in transport.

5. Ethanol was found to reduce only 25% of the cytochromes b and c which were dithionite reducible.

6. An important question to be asked is whether the amount of oxygen taken up in the presence of ethanol is sufficient to account for the calculated limiting velocity for the uptake of amino acid. Assuming one molecule of alanine transported per atom of oxygen consumed it can be calculated that 4 ng (0) per mg per min would give arise to 316 pmoles alanine transported per mg per min. The obtained value was about 250 pmoles per mg per min (Fig. 22) with a limiting maximum velocity of 385 pmoles per mg per min (Table 26). Experimental and calculated data agree very well further supporting the concept of efficient coupling of transport to ethanol oxidation.

The next important question is how the energy from respiration is specifically coupled to transport. That electron flow does not act by generating ATP for use in transport seems clear. None of the electron donor systems active in promoting transport served to generate ATP in the vesicles. The production of a phosphorylated high energy intermediate seems unlikely since one would expect more of such an intermediate to be generated by NADH than by ascorbate-TMPD as the latter introduces electrons into the respiratory chain primarily at the level of cytochrome c, yet both NADH and ascorbate-TMPD are equally active in promoting transport. It has been suggested that DNP may uncouple oxidation from phosphorylation or discharge a proton gradient (for review see Harold, 1972a). The possibility that a proton gradient drives transport is unlikely, again on the grounds that on the basis of the chemiosmotic hypothesis (Mitchell, 1966) a

steeper gradient would be expected to result from the oxidation of NADH than from the oxidation of ascorbate-TMPD. Furthermore, neither proton gradient nor high energy intermediate theories explain the specificity for transport of the donors and inhibitors used.

In intact cells ethanol and dithionite rapidly reduce cytochromes b and c to the same extent. Ethanol reduction of cytochromes in cells most probably proceeds via NADH production by cytoplasmic alcohol dehydrogenase.

The data presented here is best explained by assuming, as proposed by Kaback and Barnes (1971), that a carrier protein undergoes redox induced conformational changes and reflects the redox potential of a specific portion of the respiratory chain. A respiratory chain believed to be involved in transport in the marine pseudomonad is shown in Fig. 49. These chains alone are coupled to ethanol oxidation and a carrier protein. Since, ascorbate-TMPD is coupled primarily to cytochrome c, the carrier is shown in the main chain after cytochrome c. It may be, however, that the carrier is located close to cytochrome b, because cytochrome b was partially reduced by ascorbate-TMPD. That the site of energy coupling is not cytochrome oxidase is suggested by studies with intact cells. These show that the inhibition of AIB transport by KCN can be overcome by adding $K_3Fe(CN)_6$ as an electron acceptor (Thompson and MacLeod, 1973b). Inhibition of alanine transport into vesicles by KCN, however, could not be overcome by $K_3Fe(CN)_6$.

Dimethyl sulfoxide is a well known solvent and penetrant (Lovelock and Bishop, 1959). Transport data indicate that in this organism it acts as a respiratory inhibitor at a site prior to cytochrome c (Table 15).

When ascorbate-TMPD was the electron donor, those respiratory chain inhibitors which did not inhibit active transport in vesicles actually stimulated it, often showing an increase in $O_2$ uptake. There is known to be some back flow of electrons in the respiratory chain when ascorbate-TMPD is the electron donor (Klingenberg, 1968). Perhaps inhibitors acting before the site of entry of electrons from reduced TMPD, channel electrons more efficiently through that part of the chain wherein resides the carrier.

For some time it has been known that $Na^+$ is specifically required for transport in cells of the marine pseudomonad (Drapeau and MacLeod, 1963). $Na^+$ is required for facilitated diffusion of AIB into the cells and this process shows amino acid competition (Thompson and MacLeod, 1971). For this reason $Na^+$ was thought to interact with a carrier protein. In the present study, $Na^+$ was shown essential for transport of AIB and L-alanine into vesicles. Because of the requirement for electron flow, and because ethanol oxidation was directly related to transport, the requirement for $Na^+$ for ethanol oxidation was tested. No $Na^+$ requirement was found.

In cells of this organism the $Na^+$ concentration used, when below

50 mM, alters the apparent $K_m$ for AIB transport, while $Na^+$ concentrations above 50 mM alter the maximum velocity (Wong *et al.*, 1969). The latter effect was shown to be an effect of $Na^+$ on the prevention of leakage of AIB from the cells, being partially replaced by LiCl. Recently sucrose has been found to show a similar sparing action to LiCl suggesting an osmotic effect in preventing leakage of accumulated radioactivity (Thompson and MacLeod, unpublished). Such an osmotic effect has been shown in *E. coli* K12, where sucrose reduced the exit rate constant with no effect on entry (Halpern *et al.*, 1973[b]). Vesicles of the marine pseudomonad required less NaCl for full transport capacity than did cells (75 versus 200 mM). Increases of NaCl above 75 mM depressed transport indicating either an inhibition of alanine influx or increased rates of efflux perhaps through membrane damage. Certainly it would be an over simplification to suggest that the second function of NaCl found in cells (i.e., prevention of leakage) was not observed because of lower levels of alanine accumulation in vesicles, since in this case uptake should have remained constant as NaCl was increased from 75 to 200 mM. It is suggested that the difference between the quantities of NaCl required for maximum uptake of amino acid in vesicles and cells is through an increased inhibitory effect of high levels of NaCl in the absence of cell wall or cytoplasmic constituents. Furthermore it is suggested that protoplasts require as much $Na^+$ as do the intact cells for AIB uptake (DeVoe *et al.*, 1970) since appreciable concentrations

of the amino acid occurs resulting in a need to osmotically stabilize these cell forms. In vesicles no such concentrations of the amino acid occurs, thus possibly accounting for the lower $Na^+$ requirement.

Using the data of Wong *et al.* (1969) for cells, plots of the reciprocals of velocity against reciprocals of the $Na^+$ concentrations do not give straight lines (Fig. 37). Rather a plot is obtained showing that as $Na^+$ is decreased below 200 mM the velocity of transport becomes progressively less than expected. Since the concentration of internal $K^+$ determines the extent of AIB transport into cells (Thompson and MacLeod, 1971), it is probable that the effect of $Na^+$ on the maximum velocity may reflect an effect of $Na^+$ on the intracellular $K^+$ concentration. Support for this was found when cells exposed to NaCl below 10 mM appeared plasmolyzed (Table 25), a condition associated with the loss of internal $K^+$ (Thompson *et al.*, 1970). Recently experiments by Gow and Thompson have shown a direct relation between retention of internal $K^+$ and the external NaCl concentration.

In contrast to cells, vesicles do not demonstrate a $K^+$-requirement for transport. In this case an opportunity of studying $Na^+$ effects in the absence of any secondary $K^+$ effects was presented. With the vesicles both primary Lineweaver-Burk plots were linear. Furthermore, kinetic results with cells paralleled the vesicle kinetics if the KCl concentration in the suspending medium was equal to

that inside the cells (Fig. 38). Active transport of glutamate in *E. coli* requires $Na^+$ and $K^+$ ions and kinetic experiments have been interpreted (Halpern *et al.*, 1973a) as was the original work of Wong *et al.* (1969). The results presented here using vesicles and cells, indicate extreme caution must be taken to maintain a constant internal $K^+$ concentration as $Na^+$ is varied. Otherwise, kinetic results on the effects of $Na^+$ may be artifacts due to changes in internal $K^+$ levels.

If $Na^+$ and the amino acid form a ternary complex with a membrane component on the outside surface of the membrane prior to translocation, then one could predict the transport system to exhibit bisubstrate kinetics. Following this study it was found that Thomas and Christensen (1971) have considered $Na^+$ as a substrate in $Na^+$-dependent serine uptake into pigeon erythrocyte. Support for a bisubstrate reaction was obtained from Lineweaver-Burk plots where a common point of intersection was obtained, for alanine and AIB using vesicles, either on or above the abscissa (Figs. 22, 23, 28 and 29). This indicates a sequential rather than a ping pong mechanism, since parallel lines would be expected if the sequence was ping pong (Cleland, 1963). The experimental data agreed extremely well with the theoretical for a bisubstrate mechanism for both amino acids, with the exception of data obtained using a combination of low $Na^+$ and high amino acid concentrations. This fact was demonstrated on secondary plots of the

intercepts, taken from double reciprocal plots of velocity and amino acid concentration, and the reciprocal of $Na^+$ concentration. These plots for vesicles and cells are shown in Figs. 24, 30 and 40. The non-linear plot obtained only deviated from theoretical at the lower $Na^+$ concentrations used. This is a very important observation, since as shown for each of the mentioned figures the two curves must intersect on the ordinate at the same point. In a bisubstrate mechanism, this point of intersection has been called the "limiting intercept" and is equal to $\frac{1}{V}$ (Mahler and Cordes, 1971).

The deviation from theoretical is still a matter for speculation. In the equation derived for a bisubstrate reaction it is assumed that the rate limiting step is the conversion or activation of the ternary complex (Florini and Vestling, 1957). At low $Na^+$ levels deviations from theoretical may occur because of a different step becoming rate limiting. Using the kinetic parameters calculated for the transport systems and the equation for a bisubstrate mechanism, Lineweaver-Burk plots were generated showing that at low $Na^+$ concentrations the obtained data fit the theoretical but for only the lower amino acid concentrations. Rather than a different step becoming rate limiting, it may be that allosterism is involved. Such a possibility has been observed by J. Drozdowski (Project, B.Sc., 1973) using vesicles of this organism, and D-galactose as the solute transported. In the presence of an energy source he observed no $Na^+$-requirement for transport at high levels of galactose. Attempts to demonstrate allosterism for alanine

transport were negative (Fig. 27).

An interpretation of ion effects in mammalian cell transport systems was presented by Crane (1965). This theory requires that $Na^+$ bind first to a permease on the outside of the membrane, thus putting it into a conformation necessary for solute binding. The ternary complex would then translocate across the membrane. On the inside of the membrane where the $Na^+$ concentration is low compared to the medium, the complex would dissociate. A secondary consideration of the hypothesis was that $K^+$ at its high internal concentration could displace the $Na^+$ from the ternary complex, resulting in a further conformational change of the permease with the release of solute to the inside. This hypothesis requires first, the formation of a ternary complex, second, a requirement for $Na^+$ gradients for transport and to a lesser degree $Na^+$ and $K^+$ competition for binding depending on their relative concentrations, and finally, $Na^+$ and solute cotransport. Since transport kinetics do closely follow a bisubstrate mechanism, it is expected that a ternary complex forms. However, $Na^+$ and $K^+$ gradients are not formed in vesicles and furthermore, abolishing the gradients in cells of this organism has no effect on transport (Thompson and MacLeod, 1973a). Nevertheless, $Na^+$ and $K^+$ may be required for the association and dissociation of the ternary complex, and it has been pointed out by Johnstone (1972) that these ions may have different affinities for the transport system on the inside and outside membrane surface. This may account for

the absence of a requirement for $Na^+$ and $K^+$ gradients in the pseudomonad. In support of the suggestion by Johnstone, no $K^+$ interference in transport was observed until $K^+$ was added to the assay medium above the expected internal level (Table 27). The final point concerned $Na^+$ and solute cotransport. Cotransport has been demonstrated in animal cells (see Literature Review) and in *S. typhimurium* (Stock and Roseman, 1971). In the marine pseudomonad attempts were negative to demonstrate cotransport by comparing the percentage penetration of $^{22}Na$ into a packed cell pellet in the presence and absence of AIB. However, in a typical such experiment (Fig. 43) it was calculated that if AIB and $Na^+$ enter the cell at a ratio of 1 to 1 and $Na^+$ does not efflux, then a difference of only 4% penetration due to cotransport would be expected. The error in these experiments was about ± 3 % indicating the inadequacy of the method for the purpose of demonstrating cotransport. Furthermore, $^{22}Na$ when allowed to penetrate by $MgSO_4$ treatment of the cells was found to efflux quite rapidly from the cells when resuspended into CS-T. A second method in which $Na^+$ was measured directly by flame photometry was also used. The procedure adopted was to deplete the cells of $K^+$, since it appears that the internal concentration of $K^+$ is closely related to $Na^+$ efflux (Thompson and MacLeod, 1973a). Then the cells were resuspended into CS-T with or without AIB and immediately centrifuged. When $Na^+$ was assayed by flame emission spectroscopy the error inherent in this

method of measuring intracellular $Na^+$ appeared too large for any conclusion to be drawn. No direct evidence, therefore, has been obtained in proving or in disproving the cotransport theory.

No evidence has been obtained for this organism showing which of the two substrates, $Na^+$ and solute, binds first to the membrane component. Since the intercept plots do not pass through the origin, the presence of one substrate on the active site of the enzyme probably affects the complexing of the second (Florini and Vestling, 1957). Whether $Na^+$ or the amino acid must complex first to the enzyme by an ordered mechanism, or whether the mechanism is random, cannot be determined from initial rate data (Cleland, 1963). To answer this question binding studies using the purified permease may be required.

Introductory studies have been conducted to test the possibility of cell wall components being involved, either directly or indirectly, in solute transport across the cytoplasmic membrane. Binding studies localized the activity to the outer wall, with no activity being observed in the soluble underlying layer (periplasmic space). This contrasts to reports on periplasmic binding proteins (Heppel, 1967 and 1969). The isolated outer-double-track bound both L-alanine and L-leucine but not AIB, and addition of the extract to cells stimulated the uptake of L-alanine and L-leucine but not AIB. It has not yet been proven whether this stimulation represents binding to the

outer parts of the cell or an increase in intracellular amino acid. Additional information on the possible involvement of cell wall in transport was obtained by comparing pH responses in vesicles and cells for transport, and for binding to outer-double-track. Cells were more resistant to acidic conditions than vesicles while binding was optimum at about pH 5.6. Although the interpretation of this data is still open to question, it is clear that the cell wall cannot be ignored at this time if a complete understanding of the transport process is to be achieved.

# BIBLIOGRAPHY


Appleby, C.A.
1969 Electron transport systems of *Rhizobium japonicum*. II. Rhizobium haemoglobin, cytochromes and oxidases in free-living (cultured) cells. Biochim. Biophys. Acta 172:88-105.

Barnes, E.M.
1972 Respiration-coupled glucose transport in membrane vesicles from *Azotobacter vinelandii*. Arch. Biochem. Biophys. 152:795-799.

Barnes, E.M., and H.R. Kaback.
1970 β-galactoside transport in bacterial membrane preparations: energy coupling via membrane-bound D-lactic dehydrogenase. Proc. Nat. Acad. Sci. U.S.A. 66:1190-1198.

Barnes, E.M., and H.R. Kaback.
1971 Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β-galactoside transport in *Escherichia coli* membrane vesicles. J. Biol. Chem. 246:5518-5522.

Baumann, L., P. Baumann, M. Mandel, and R.D. Allen.
1972 Taxonomy of aerobic marine eubacteria. J. Bacteriol. 110:402-429.

Berger, E.A.
1973 Different mechanisms of energy coupling for the active transport of proline and glutamine in *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. 70:1514-1518.

Bhattacharyya, P., W. Epstein, and S. Silver.
1971 Valinomycin-induced uptake of potassium in membrane vesicles from *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. 68:1488-1492.

Boos, W., and A.S. Gordon.
1971 Transport properties of the galactose-binding protein of *Escherichia coli*. Occurrence of two conformational states.
J. Biol. Chem. 246:621-628.

Briggs, A.D., and S. Bourgeois.
1968 On the assay, isolation and characterization of the *lac* repressor.
J. Mol. Biol. 34:361-364.

Brodie, A.F., and J. Ballantine.
1960 Oxidative phosphorylation in fractionated bacterial systems. II. The role of vitamin K.
J. Biol. Chem. 235:226-231.

Bryant, M.P., I.M. Robinson, and H. Chu.
1959 Observations on the nutrition of *Bacteroides succinogenes* - a ruminal cellulolytic bacterium.
J. Dairy Sci. 42:1831-1847.

Buckmire, F.L.A.
1967 Studies on the cell envelope of a marine bacterium.
Ph.D. Thesis, McGill University, Montreal, Canada.

Buckmire, F.L.A., and R.A. MacLeod.
1970 Penetrability of a marine pseudomonad by inulin, sucrose, and glycerol and its relation to the mechanism of lysis.
Can. J. Microbiol. 16:75-81.

Castor, L.N., and B. Chance.
1959 Photochemical determinations of the oxidases of bacteria.
J. Biol. Chem. 234:1587-1592.

Chance, B.
1953 The carbon monoxide compounds of the cytochrome oxidases. I. Difference spectra.
J. Biol. Chem. 202:383-396.

Chance, B., and G.R. Williams.
1955 Respiratory enzymes in oxidative phosphorylation. II. Difference spectra.
J. Biol. Chem. 217:395-407.

Cleland, W.W.
1963 The kinetics of enzyme-catalyzed reactions with two or more substrates or products. I. Nomenclature and rate equations.
Biochim. Biophys. Acta 67:104-137.

Cohen, G.N., and J. Monod.
1957 Bacterial permeases.
Bacteriol. Rev. 21:169-194.

Costerton, J.W., C. Forsberg, T.I. Matula, F.L.A. Buckmire, and R.A. MacLeod.
1967 Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, sphaeroplasts, and related forms from a gram-negative marine bacterium.
J. Bacteriol. 94:1764-1777.

Cox, G.B., N.A. Newton, F. Gibson, A.M. Snoswell, and J.A. Hamilton.
1970 The function of ubiquinone in *Escherichia coli*.
Biochem. J. 117:551-562.

Crane, R.K.
1965 $Na^+$-dependent transport in the intestine and other animal tissues.
Fed. Proc. 24:1000-1006.

Crane, R.K., G. Forstner, and A.Eichholz.
1965 Studies on the mechanism of the intestinal absorption of sugars. X. An effect of $Na^+$ concentration on the apparent Michaelis constants for intestinal sugar transport, *in vitro*.
Biochim. Biophys. Acta 109:467-477.

Dalziel, K.
1957 Initial steady state velocities in the evaluation of enzyme-coenzyme-substrate reaction mechanisms.
Acta Chem. Scand. 11:1706-1723.

DeVoe, I.W., J. Thompson, J.W. Costerton, and R.A. MacLeod.
1970 Stability and comparative transport capacity of cells, mureinoplasts, and true protoplasts of a gram-negative bacterium.
J. Bacteriol. 101:1014-1026.

Dietz, G.W.
1972 Dehydrogenase activity involved in the uptake of glucose 6-phosphate by a bacterial membrane system.
J. Biol. Chem. 247:4561-4565.

Drapeau, G.R., and R.A. MacLeod.
1963a $Na^+$-dependent active transport of α-aminoisobutyric acid into cells of a marine pseudomonad.
Biochem. Biophys. Res. Commun. 12:111-115.

Drapeau, G.R., and R.A. MacLeod.
1963b Nutrition and metabolism of marine bacteria. XII. Ion activation of adenosine triphosphatase in membranes of marine bacterial cells.
J. Bacteriol. 85:1413-1419.

Drapeau, G.R., and R.A. MacLeod.
1965 A role for inorganic ions in the maintenance of intracellular solute concentrations in a marine pseudomonad.
Nature 206:531.

Drapeau, G.R., T.I. Matula, and R.A. MacLeod.
1966 Nutrition and metabolism of marine bacteria. XV. Relation of $Na^+$-activated transport to the $Na^+$ requirement of a marine pseudomonad for growth.
J. Bacteriol. 92:63-71.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith.
1956 Colorimetric method for determination of sugars and related substances.
Anal. Chem. 28:350-356.

Eddy, A.A., and J.A. Nowacki.
1971 Stoicheiometrical proton and potassium ion movements accompanying the absorption of amino acids by the yeast *Saccharomyces carlsbergensis*.
Biochem. J. 122:701-711.

Florini, J.R., and C.S. Vestling.
1957 Graphical determination of the dissociation constants for two-substrate enzyme systems.
Biochim. Biophys. Acta 25:575-578.

Forsberg, C.W.
1969 Studies of the composition of the cell wall of a marine pseudomonad.
Ph.D. Thesis, McGill University, Montreal, Canada.

Forsberg, C.W., J.W. Costerton, and R.A. MacLeod.
1970a Separation and localization of cell wall layers of a gram-negative bacterium.
J. Bacteriol. 104:1338-1353.

Forsberg, C.W., J.W. Costerton, and R.A. MacLeod.
1970b Quantitation, chemical characteristics, and ultrastructure of the three outer cell wall layers of a gram-negative bacterium.
J. Bacteriol. 104:1354-1368.

Fox, C.F., J.R. Carter, and E.P. Kennedy.
1966 Properties of the membrane protein component of the β-galactoside transport system of *E. coli*.
Fed. Proc. 25:591.

Fox, C.F., and E.P. Kennedy.
1965 Specific labelling and partial purification of the M protein, a component of the β-galactoside transport system of *Escherichia coli*.
Proc. Nat. Acad. Sci. U.S.A. 54:891-899.

Frank, L., and I. Hopkins.
1969 Sodium-stimulated transport of glutamate in *Escherichia coli*.
J. Bacteriol. 100:329-336.

Furlong, C.E., and J.H. Weiner.
1970 Purification of a leucine-specific binding protein from *Escherichia coli*.
Biochem. Biophys. Res. Commun. 38:1076-1083.

Gomori, G.
1955 Preparation of buffers for use in enzyme studies, p. 138-146. *In* S.P. Colowick and N.O. Kaplan (eds.), Methods in enzymology, Vol. I, Academic Press, New York.

Gordon, A.S., F.J. Lombardi, and H.R. Kaback.
1972 Solubilization and partial purification of amino acid-specific components of the D-lactate dehydrogenase-coupled amino acid-transport systems. Proc. Nat. Acad. Sci. U.S.A. 69:358-362.

Gow, J.A.
1973 Growth of a marine pseudomonad at suboptimal $Na^+$ concentrations.
Ph.D. Thesis, McGill University, Montreal, Canada.

Gow, J.A., I.W. DeVoe, and R.A. MacLeod.
1973 Dissociation in a marine pseudomonad.
Can. J. Microbiol. 19:695-701.

Halpern, Y.S., H. Barash, S. Dover, and K. Druck.
1973a Sodium and potassium requirements for active transport of glutamate by *Escherichia coli* K-12.
J. Bacteriol. 114:53-58.

Halpern, Y.S., H. Barash, and K. Druck.
1973b Glutamate transport in *Escherichia coli* K-12: nonidentity of carriers mediating entry and exit.
J. Bacteriol. 113:51-57.

Harold, F.M.
1972a Conservation and transformation of energy by bacterial membranes.
Bacteriol. Rev. 36:172-230.

Harold, F.M.
1972b Ion transport and electrogenesis in bacteria.
Biochem. J. 127:49-50 P.

Harold, F.M., and J.R. Baarda.
1968 Inhibition of membrane transport in *Streptococcus faecalis* by uncouplers of oxidative phosphorylation and its relationship to proton conduction.
J. Bacteriol. 96:2025-2034.

Harold, F.M., E. Pavlasova, and J.R. Baarda.
1970 A transmembrane pH gradient in *Streptococcus faecalis:* origin and dissipation by proton conductors and N,N'-dicyclohexylcarbodiimide.
Biochim. Biophys. Acta 196:235-244.

Heppel, L.A.
1967 Selective release of enzymes from bacteria.
Science 156:1451-1455.

Heppel, L.A.
1969 The effect of osmotic shock on release of bacterial proteins and on active transport.
J. Gen. Physiol. 54:95-109.

Hirata, H., A. Asano, and A.F. Brodie.
1971 Respiration dependent transport of proline by electron transport particles from *Mycobacterium phlei.*
Biochem. Biophys. Res. Commun. 44:368-374.

Holm-Hansen, O., and C.R. Booth.
1966 The measurement of adenosine triphosphate in the ocean and its ecological significance.
Limnol. Oceanogr. 11:510-519.

Hong, J.-s., and H.R. Kaback.
1972 Mutants of *Salmonella typhimurium* and *Escherichia coli* pleiotropically defective in active transport.
Proc. Nat. Acad. Sci. U.S.A. 69:3336-3340.

Hudson, R.F., and D.R. Caldwell.
1972 Sodium, a predominant obligate growth requirement for ruminal bacteria.
Abstr. Am. Soc. Microbiol. 72:35.

Hunter, G.R., and G.P. Brierley.
1969 Ion transport by heart mitochondria. XIV. The mannitol-impermeable compartment of the mitochondrion and its relation to ion uptake.
Biochim. Biophys. Acta 180:68-80.

Jacobs, E.E.
1960 Phosphorylation coupled to electron transport initiated by substituted phenylenediamines.
Biochem. Biophys. Res. Commun. 3:536-539.

Johnstone, R.M.
1972 Glycine accumulation in absence of $Na^+$ and $K^+$ gradients in Ehrlich ascites cells.
Biochim. Biophys. Acta 282:366-373.

Jones, C.W., and E.R. Redfearn.
1966 Electron transport in *Azotobacter vinelandii*.
Biochim. Biophys. Acta 113:467-481.

Jones, K., and J.G. Heathcote.
1966 The rapid resolution of naturally occurring amino acids by thin-layer chromatography.
J. Chromatog. 24:106-111.

Kaback, H.R.
1970 Transport.
Ann. Rev. Biochem. 39:561-598.

Kaback, H.R.
1972 Transport mechanisms in isolated bacterial cytoplasmic membrane vesicles, p. 291-319. *In* J.F. Woessner, Jr., and F. Huijing (eds.), The molecular basis of biological transport, Vol. III, Academic Press, New York.

Kaback, H.R., and E.M. Barnes.
1971 Mechanisms of active transport in isolated membrane vesicles. II. The mechanism of energy coupling between D-lactic dehydrogenase and β-galactoside transport in membrane preparations from *Escherichia coli*.
J. Biol. Chem. 246:5523-5531.

Kaback, H.R., and T.F. Deuel.
1969 Proline uptake by disrupted membrane preparations from *Escherichia coli*.
Arch. Biochem. Biophys. 132:118-129.

Kaback, H.R., and L.S. Milner.
1970 Relationship of a membrane bound D-(-)-lactic dehydrogenase to amino acid transport in isolated bacterial membrane preparations.
Proc. Nat. Acad. Sci. U.S.A. 66:1008-1015.

Kaback, H.R., and E.R. Stadtman.
1966 Proline uptake by an isolated cytoplasmic membrane preparation of *Escherichia coli*. Biochemistry 55:920-927.

Kashket, E.R., and A.F. Brodie.
1963 Oxidative phosphorylation in fractionated bacterial systems. X. Different roles for the natural quinones of *Escherichia coli* W in oxidative metabolism. J. Biol. Chem. 238:2564-2570.

Kashket, E.R., and T.H. Wilson.
1972 Galactoside accumulation associated with ion movements in *Streptococcus lactis*. Biochem. Biophys. Res. Commun. 49:615-620.

Kersters, K., and J. DeLey.
1966 Primary and secondary alcohol dehydrogenases from *Gluconobacter*, p. 346-354. *In* W.A. Wood (ed.), Methods in enzymology, Vol IX, Academic Press, New York.

Kerwar, G.K., A.S. Gordon, and H.R. Kaback.
1972 Mechanisms of active transport in isolated membrane vesicles. IV. Galactose transport by isolated membrane vesicles from *Escherichia coli*. J. Biol. Chem. 247:291-297.

Kimmich, G.A.
1970 Active sugar accumulation by isolated intestinal Epithelial cells. A new model for sodium-dependent metabolite transport. Biochemistry 9:3669-3677.

Klein, W.L., and P.D. Boyer.
1972 Energization of active transport by *Escherichia coli*. J. Biol. Chem. 247:7257-7265.

Klingenberg, M.
1968 The respiratory chain, p. 3-54. *In* T.P. Singer (ed), Biological oxidations, Interscience publishers, New York.

Knook, D.L., and R.J. Planta.
1971 Restoration of electron transport in ultraviolet-irradiated membranes of *Aerobacter aerogenes*. FEBS Lett. 14:54-56.

Konings, W.N., E.M. Barnes, Jr., and H.R. Kaback.
1971 Mechanisms of active transport in isolated membrane vesicles. III. The coupling of reduced phenazine methosulfate to the concentrative uptake of β-galactosides and amino acids. J. Biol. Chem. 246:5857-5861.

Konings, W.N., and E. Freese.
1971 L-serine transport in membrane vesicles of *Bacillus subtilis* energized by NADH or reduced phenazine methosulfate. FEBS Lett. 14:65-68.

Konings, W.N., and E.Freese.
1972 Amino acid transport in membrane vesicles of *Bacillus subtilis*. J. Biol. Chem. 247:2408-2418.

Langridge, R., H. Shinagawa, and A.B, Pardee.
1970 Sulfate-binding protein from *Salmonella typhimurium*: physical properties. Science 169:59-61.

Lanyi, J.K.
1969 Studies of the electron transport chain of extremely halophilic bacteria. III. Mechanism of the effect of salt on menadione reductase. J. Biol. Chem. 244:4168-4173.

Lineweaver, H., and D.J. Burk.
1934 The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56:658-666.

Lovelock, J.E., and M.W.H. Bishop.
1959 Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 183:1394-1395.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall.
1951 Protein measurement with the folin phenol reagent.
J. Biol. Chem. 193:265-275.

McKillen, M.N., K. Willecke, and A.B. Pardee.
1972 Citrate transport by *Bacillus subtilis*, p. 249-266.
*In* J.F. Woessner, Jr., and F. Huijing (eds.),
Vol. III, Academic Press, New York.

MacLeod, R.A., P. Thurman, and H.J. Rogers.
1973 Comparative transport activity of intact cells, membrane vesicles, and mesosomes of *Bacillus licheniformis*.
J. Bacteriol. 113:329-340.

Mahler, H.R., and E.H. Cordes.
1971 Enzyme kinetics, p. 267-324. *In* Biological chemistry, 2nd edition, Harper and Row, New York.

Manno, J.A., and D. Schachter.
1970 Energy-coupled influx of thiomethylgalactoside into *Escherichia coli*.
J. Biol. Chem. 245:1217-1223.

Martin, E.L., and R.A. MacLeod.
1971 Isolation and chemical composition of the cytoplasmic membrane of a gram-negative bacterium.
J. Bacteriol. 105:1160-1167.

Matin, A., and W.N. Konings.
1973 Transport of lactate and succinate by membrane vesicles of *Escherichia coli*, *Bacillus subtilis* and a *Pseudomonas* species.
Eur. J. Biochem. 34:58-67.

Mitchell, P.
1963 Molecule, group and electron translocation through natural membranes.
Biochem. Soc. Symp. 22:142-168.

Mitchell, P.
1966 Chemiosmotic coupling in oxidative and photosynthetic phosphorylation.
Biol. Rev. 41:445-502.

Nakayama, T., and J. DeLey.
1965 Localisation and distribution of alcohol-cytochrome 553 reductase in acetic acid bacteria. Antonie Van Leeuwenhoek J. Microbiol. Serol. 31:205-219.

Neu, H.C., and L.A. Heppel.
1964 The release of ribonuclease into the medium when *Escherichia coli* cells are converted to spheroplasts. J. Biol. Chem. 239:3893-3900.

Nossal, N.G., and L.A. Heppel.
1966 The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. 241:3055-3062.

O'Brien, R.A., and J.R. Stern.
1969 Requirement for sodium in the anaerobic growth of *Aerobacter aerogenes* on citrate. J. Bacteriol. 98:388-393.

Parker, R.P., and R.H. Elrick.
1970 Cerenkov counting as a means of assaying β-emitting radionuclides, p. 110-122. *In* E.D. Bransome, Jr. (ed.), The current status of liquid scintillation counting, Grune and Stratton, New York.

Pavlasova, E., and F.M. Harold.
1969 Energy coupling in the transport of β-galactosides by *Escherichia coli:* effect of proton conductors. J. Bacteriol. 98:198-204.

Penrose, W.R., G.E. Nichoalds, J.R. Piperno, and D.L. Oxender.
1968 Purification and properties of a leucine-binding protein from *Escherichia coli*. J. Biol. Chem. 243:5921-5928.

Pratt, D., and F.C. Happold.
1960 Requirements for indole production by cells and extracts of a marine bacterium. J. Bacteriol. 80:232-236.

Rayman, M.K.
1970 Lysis of a marine pseudomonad. Ph.D. Thesis, McGill University, Montreal, Canada.

Rayman, M.K., T.C.Y. Lo, and B.D. Sanwal.
1972 Transport of succinate in *Escherichia coli*. II. Characteristics of uptake and energy coupling with transport in membrane preparations.
J. Biol. Chem. 247:6332-6339.

Reeves, J.P.
1971 Transient pH changes during D-lactate oxidation by membrane vesicles.
Biochem. Biophys. Res. Commun. 45:931-936.

Sachan, D.S., and J.R. Stern.
1971 Studies of citrate transport in *Aerobacter aerogenes*: binding of citrate by a membrane bound oxalacetate decarboxylase.
Biochem. Biophys. Res. Commun. 45:402-408.

Sanui, H., and N. Pace.
1959 Sodium and potassium binding by rat liver cell microsomes.
J. Gen. Physiol. 42:1325-1345.

Scarborough, G.A., M.K. Rumley, and E.P. Kennedy.
1968 The function of adenosine 5'-triphosphate in the lactose transport system of *Escherichia coli*.
Proc. Nat. Acad. Sci. U.S.A. 60:951-958.

Schafer, J.A., and J.A. Jacquez.
1967 Change in $Na^+$ uptake during amino acid transport.
Biochim. Biophys. Acta 135:1081-1083.

Schairer, H.U., and B.A. Haddock.
1972 β-galactoside accumulation in a $Mg^{2+}$-, $Ca^{2+}$- activated ATPase deficient mutant of *E. coli*.
Biochem. Biophys. Res. Commun. 48:544-551.

Schultz, S.G., and P.F. Curran.
1970 Coupled transport of sodium and organic solutes.
Physiol. Rev. 50:637-718.

Schultz, S.G., and R. Zalusky.
1965 Interactions between active sodium transport and active amino acid transport in isolated rabbit ileum.
Nature 205:292-294.

Shen, B.H.P., and W. Boos.
1973 Regulation of the β-methylgalactoside transport system and the galactose-binding protein by the cell cycle of *Escherichia coli*.
Proc. Nat. Acad. Sci. U.S.A. 70:1481-1485.

Short, S.A., D.C. White, and H.R. Kaback.
1972a Active transport in isolated bacterial membrane vesicles. V. The transport of amino acids by membrane vesicles prepared from *Staphylococcus aureus*.
J. Biol. Chem. 247:298-304.

Short, S.A., D.C. White, and H.R. Kaback.
1972b Mechanisms of active transport in isolated membrane vesicles. IX. The kinetics and specificity of amino acid transport in *Staphylococcus aureus* membrane vesicles.
J. Biol. Chem. 247:7452-7458.

Simoni, R.D., and M.K. Shallenberger.
1972 Coupling of energy to active transport of amino acids in *Escherichia coli*.
Proc. Nat. Acad. Sci. U.S.A. 69:2663-2667.

Skou, J.C.
1965 Enzymatic basis for active transport of $Na^+$ and $K^+$ across cell membrane.
Physiol. Rev. 45:596-617.

Stein, W.D.
1967a The coupling of active transport and facilitated diffusion, p. 177-216. *In* J.F. Danielli (consulting ed.), The movement of molecules across cell membranes, Vol. VI, Academic Press, New York.

Stein, W.D.
1967b Facilitated diffusion - the kinetic analysis, p. 126-176. *In* J.F. Danielli (consulting ed.), The movement of molecules across cell membranes, Vol. VI, Academic Press, New York.

Stinnett, J.D., L.F. Guymon, and R.G. Eagon.
1973 A novel technique for the preparation of transport-active membrane vesicles from *Pseudomonas aeruginosa:* observations on gluconate transport.
Biochem. Biophys. Res. Commun. 52:284-290.

Stock, J., and S. Roseman.
1971 A sodium-dependent sugar cotransport system in bacteria.
Biochem. Biophys. Res. Commun. 44:132-138.

Thomas, E.L., and H.N. Christensen.
1971 Nature of the cosubstrate action of $Na^+$ and neutral amino acids in a transport system.
J. Biol. Chem. 246:1682-1688.

Thompson, J., J.W. Costerton, and R.A. MacLeod.
1970 $K^+$-dependent deplasmolysis of a marine pseudomonad plasmolyzed in a hypotonic solution.
J. Bacteriol. 102:843-854.

Thompson, J., and I.W. DeVoe.
1972 Physiological and morphological effects of phenethyl alcohol upon a gram-negative marine pseudomonad.
Can. J. Microbiol. 18:841-852.

Thompson, J., and R.A. MacLeod.
1971 Functions of $Na^+$ and $K^+$ in the active transport of α-aminoisobutyric acid in a marine pseudomonad.
J. Biol. Chem. 246:4066-4074.

Thompson, J., and R.A. MacLeod.
1973a $Na^+$ and $K^+$ gradients and α-aminoisobutyric acid transport in a marine pseudomonad.
J. Biol. Chem. (in press)

Thompson, J., and R.A. MacLeod.
1973b Electron donor energized transport of α-aminoisobutyric acid and $K^+$ into cells of a marine pseudomonad.
J. Biol. Chem. (submitted for publication)

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

Vidaver, G.A.
1964a Transport of glycine by pigeon red cells.
Biochemistry 3:662-667.

Vidaver, G.A.
1964b Some tests of the hypothesis that the sodium-ion gradient furnishes the energy for glycine-active transport by pigeon red cells. Biochemistry 3:803-808.

Wang, C.H., and D.L. Willis.
1965 Measurement of radioactivity by the liquid (internal-sample) scintillating method, p. 104-143. *In* W.D. McElroy and C.P. Swanson (eds.), Radiotracer methodology in biological science, Prentice-Hall, Inc., New Jersey.

West, I.C.
1970 Lactose transport coupled to proton movements in *Escherichia coli*. Biochem. Biophys. Res. Commun. 41:655-661.

West, I.C., and T.H. Wilson.
1973 Galactoside transport dissociated from proton movement in mutants of *Escherichia coli*. Biochem. Biophys. Res. Commun. 50:551-558.

Wilbrandt, W.
1954 Secretion and transport of non-electrolytes. Symp. Soc. Exptl. Biol. 8:136-161.

Winkler, H.H., and T.H. Wilson.
1966 The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. 241:2200-2211.

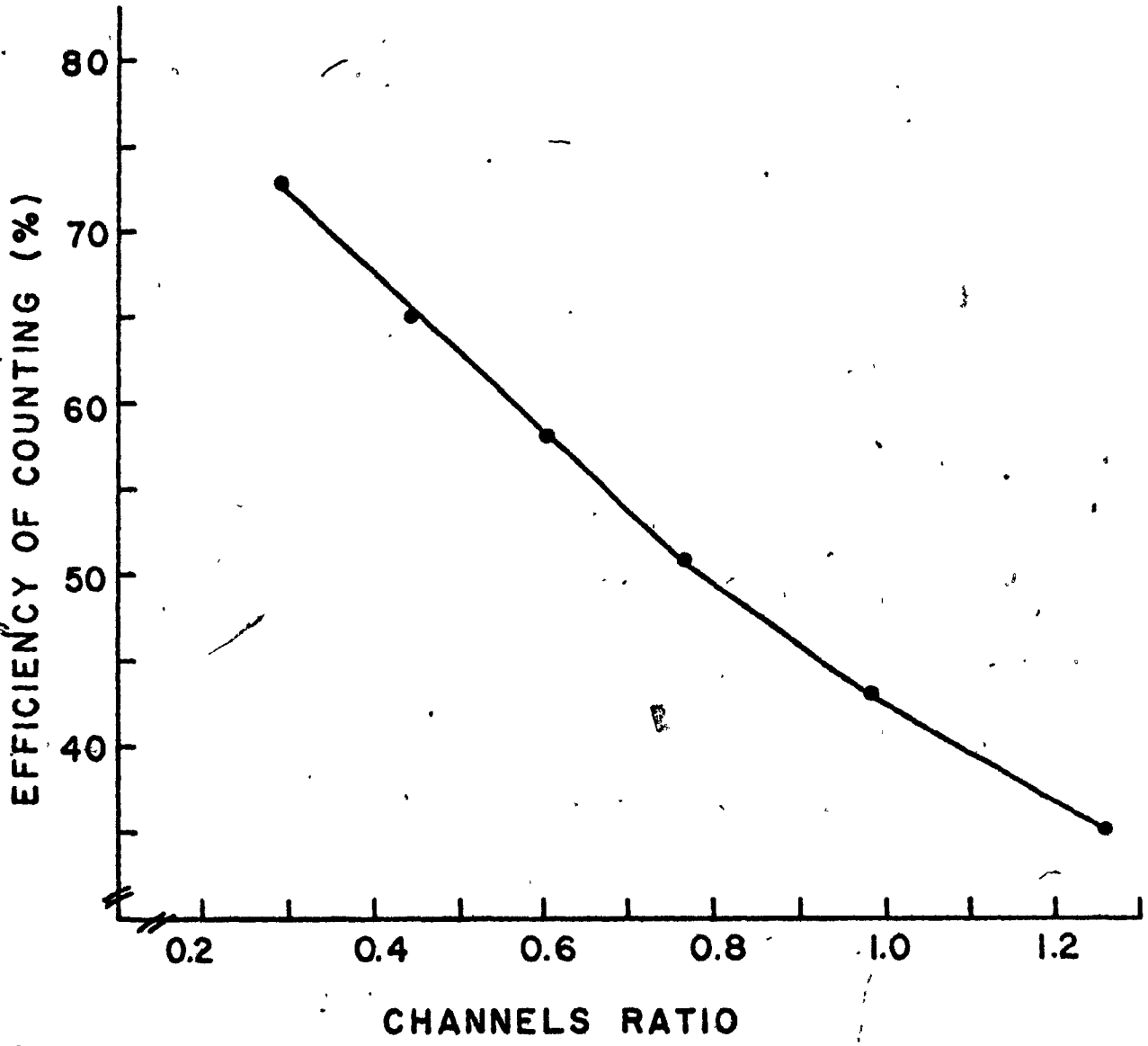
Wong, P.T.S.
1968 Studies on the mechanism of $Na^+$-dependent transport in marine bacteria. Ph.D. Thesis, McGill University, Montreal, Canada.

Wong, P.T.S., J. Thompson, and R.A. MacLeod.
1969 Nutrition and metabolism of marine bacteria. XVII. Ion-dependent retention of α-aminoisobutyric acid and its relation to $Na^+$-dependent transport in a marine pseudomonad. J. Biol. Chem. 244:1016-1025.

# A P P E N D I X  I.

Channels ratio quench correction curve for $^{14}C$.

Counting vials contained 6 ml of aquasol and 1.8 ml water. Equal volumes of an aqueous solution of L-[$^{14}C$]alanine were added to each counting vial. The DPM added in the absence of quenching agent was determined using the [$^{14}C$]toluene spiking technique (Wang and Willis, 1965). Chloroform was used as quenching agent. Samples were counted using a Nuclear Chicago Isocap 300 liquid scintillation spectrometer.


80
70
60
50
40
EFFICIENCY OF COUNTING (%)
0.2
0.4
0.6
0.8
1.0
1.2
CHANNELS RATIO