To the Graduate Council:

I am submitting herewith a thesis written by Melanie Haas Fox entitled "The Localization of a Tri-Methionine Peptidase within the Cell Wall Envelope of Pseudomonas aeruginosa." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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We have read this thesis and recommend its acceptance:

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Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

THE LOCALIZATION OF A TRI-METHIONINE PEPTIDASE WITHIN THE CELL WALL ENVELOPE OF PSEUDOMONAS AERUGINOSA

A Thesis

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Master of Science

Degree

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Melanie Haas Fox

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ABSTRACT

The location of peptidase activity within the cell envelope structure of Pseudomonas aeruginosa has been studied. Inner and outer membrane fractions were separated on the basis of buoyant density using two consecutive sucrose gradients and identified on the basis of known markers. The majority of peptidase activity associated with the particulate fraction was found within the inner membrane fraction. The Km of tri-methionine peptidase activity in this fraction was determined to be 9.4×10^{-4} M as compared to the Km of 8.0×10^{-4} M in crude lysates. The optimum pH for inner membrane peptidase activity is between 7.0 and 8.0; the optimum pH for crude lysate activity is 7.5. Enhancement of enzymatic activity occurs with the addition of 17 mM $MgCl_2$, or the addition of 33 mM $MnCl_2$. Solubilization attempts with certain detergents and glucosides are discussed as are the possibilities involving future isolation of the tri-methionine peptidase. Some phospholipid analysis on the membranes of the cell wall structure is presented. Ramifications, for peptide transport, of the enzyme as an inner membrane component are considered. The possibility is presented that the tri-methionine peptidase functions as the peptidase activity of the signal hypothesis for protein export.

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CHAPTER I

INTRODUCTION

In recent years it has been possible to utilize the peptide transport system to introduce deleterious and otherwise impermeable molecules into bacterial and fungal cells (3, 23). This process has been termed "illicit transport" and has important ramifications for the design of new antimicrobial agents (3, 23, 82). The need for a new approach towards antibiotic construction has arisen with the development of multiple resistant strains of bacteria. These strains have become resistant to conventional therapeutic tools in response to prolonged drug use. Pseudomonas aeruginosa, a plasmid bearing organism, is notorious for its ability to acquire resistance factors.

P. aeruginosa does not cause disease in normal healthy individuals. It is, however, an opportunistic pathogen given one of several debilitating conditions. Cystic fibrosis is perhaps the most serious of these conditions. Patients with this disease develop chronic bronchopulmonary infections that demand aggressive therapeutic measures. To circumvent staphylococcal infection physicians have advocated continuous use of antibiotics (41). This leads directly to the development of drug resistant flora among which Pseudomonas is the predominant genus (33). Gentamycin, carbenicillin, colistin,

and occasionally tobramycin are the drugs most commonly employed under these circumstances. Gentamycin and colistin dosages are as high as 250 mg/kg/24 hours. Carbenicillin is given between 300 and 1000 mg/kg/24 hours. Despite high dosages significant levels of these antibiotics are difficult to achieve in the bronchi (52). The resulting low drug level over a period of time makes the location an excellent environment for the development of resistant strains.

There are at least two other major conditions under which P. aeruginosa becomes classifiable as a pathogen. Studies have shown that in patients with burns over 20% of their body surface area, 75% of observed morbidity is attributable to the incidence of infection (2). The major pathogen in these cases is P. aeruginosa. Patients with leukopenia from antineoplastic chemotherapy and other forms of immunosuppressive treatment share a similar problem. The major cause of death is not from leukemia or lymphoma but bacterial septicemia. P. aeruginosa is the single most important organism that causes death in the immunosuppressed host (72). Recent evidence indicates that these are nosicomial infections. Hospitals are well known to harbor resistant strains of bacteria; and for the compromised host this is an acute problem.

The successful use of the peptide transport system to satisfy the need for a specific antibiotic for Pseudomonas

depends on the elucidation of transport requirements and the characterization of the process itself. Recent studies of peptide utilization by RM46, a methionine auxotroph of P. aeruginosa strain PAO, demonstrate considerable trimethionine peptidase activity within the particulate fraction of cell lysates (59). Membrane associated peptidase activity suggests the possibility that peptides are not transported intact but are broken down into constituent amino acids for conveyance across the membrane barrier. This occurrence would preclude the use of the peptide transport mechanism for illicit transport in Pseudomonas. The localization of the peptidase, therefore, is a question of prime importance. Should the peptidase be located in the outer membrane peptide transport probably would not occur. If found within the inner membrane the molecule may remain intact until introduction into the cytosol. Once inside cleavage would occur by an enzyme localized in the vicinity by the cytoplasmic membrane. It is possible, however, that breakdown occurs simultaneously with transport. While technically not considered true peptide transport this process may none-theless be exploited. On the condition that the recognition mechanism permits uptake of the molecule in its entirety, the stage at which cleavage occurs is relatively immaterial.

It has been suggested that the peptidase may be located on the outer surface of the inner membrane bilayer. Such a

model necessitates peptide breakdown in the periplasmic space. Given the following information about the system this is an unlikely possibility. Acetylated tri-methionine, tri-methionine blocked at the amino terminus by an acetyl group, will not support the growth of a methionine auxotroph. Breakdown of the modified peptide occurs, however, if it is incubated with membrane fragments in vitro (59). Were the cleavage effected by an enzyme that faced the periplasmic space, with subsequent amino acid transport, one would assume that the specificity of the enzyme did not extend to the modified peptide. This assumption can be made due to the fact that acetylated tri-methionine does not fulfill the methionine nutritional requirement. Since the potential for breakdown can be demonstrated the active site of the enzyme must face the interior of the cell.

Evidence is presented in this thesis that the enzyme is located within the inner membrane facing the cytosol which indicates that peptide transport in fact occurs. The tri-methionine peptidase has been characterized both within the gram-negative envelope and purified inner membrane. This characterization includes studies of enzyme kinetics, optimum pH, and the effects of certain divalent cations on peptidase activity. Some phospholipid analysis on the membranes of the cell wall structure is presented. Solubilization attempts with certain detergents and glucosides are

discussed as are the possibilities involving future isolation of the tri-methionine peptidase.

CHAPTER II

LITERATURE REVIEW

Evidence for Peptide Transport: Microorganisms

There are only twenty amino acids that occur naturally in protein, and it is not difficult to accept the hypothesis that an individual transport mechanism for each exists. The large number of structural possibilities for peptides, 4×10^2 for di-peptides, 8×10^3 for tri-peptides etc., make a similar peptide transport model implausible (67). To overcome this conceptual problem investigators proposed that peptides and amino acids utilize the same transport system. Cohen and Rickenberg (18) were the first to demonstrate that this was not necessarily true—that independent systems do exist. They showed that uptake of radioactively labelled valine in Escherichia coli was not inhibited competitively by peptides containing valine, leucine, or isoleucine. These peptides were shown to support growth of a leucine auxotroph; therefore transport and degradation to free amino acid must have occurred. Analogous studies with unlabelled valine demonstrated competitive inhibition.

Leach and Snell also reported evidence for distinct transport systems (43). Their investigations showed that the accumulation rate of L-alanylglycine in Lactobacillus casei

was ten times greater than that of free glycine. The disparity in kinetics between peptide and amino acid uptake was taken as an indication of separate permease mechanisms. Subsequent work (44) on competition in this system confirmed this hypothesis. Many other bacterial systems have been investigated with similar conclusions (11, 30, 38, 45, 46, 73, 79, 89).

Peptide transport has been clearly demonstrated in Saccharomyces cerevisiae by Becker et al. (7) and Naider et al. (50). In these studies growth response served as an indicator of peptide uptake. The supernatant culture medium was assayed for exopeptidase activity to eliminate the possibility of amino acid transport. Several peptides were found to be readily cleaved by cell extracts without being used by the auxotroph as an amino acid source. This indicated that the lesion in the mutant strain effected transport ability rather than the capacity to metabolize the substrate. They indirectly concluded that amino acid and peptide transport must be by different systems in this organism.

Further evidence has been published by these same investigators that distinguishes between the two permease systems in yeast. Becker and Naider have demonstrated that methionine is not a competitive inhibitor of radioactively labelled tri-methionine in Saccharomyces cerevisiae (7). Marder (50) has shown that growth is inhibited in a leucine lysine auxotroph of S. cerevisiae when grown on leucine and

phenylalanine. When grown on leucine containing peptides growth inhibition by phenylalanine is absent. Phenyalanine is thought to prevent leucine from entering the cell (87). More evidence for the existence of distinct transport systems is that phenylalanine affects amino acid uptake but not uptake of peptides. In addition to these reports a mutant has recently been generated in S. cerevisiae (51) that can grow on leucine but has lost the ability to grow on leucine containing peptides. Cell lysates of this strain have the necessary peptidase to hydrolyze di-leucine and tri-leucine. The mutant strain's failure to grow on these peptides strongly indicates a lack of peptide transport and therefore further distinguishes between the two transport systems.

Similar mutant strains have been found in many other systems (37, 45, 71). In these cases the organisms are unable to concentrate a specific amino acid but are able to transport peptides containing that amino acid into the cytosol. The genus Bacteroides exhibits this phenomenon in the wild type. It is unable to accumulate most free amino acids and obtains them exclusively in the form of peptides (73). While this is an unusual occurrence, several studies have shown that peptides can serve as better amino acid sources than equivalent concentrations of free amino acids (25, 39, 56, 58, 71, 76). Ze'ev Barak and Charles Gilvarg (5) explain these findings by postulating distinct amino acid and peptide transport

systems with similar kinetic properties. Hydrolysis of peptides provides more amino acids per mole of absorbed compound, hence less energy is expended in transport for the same amount of nutrient.

Despite the considerable evidence for separate transport mechanisms there have been reports in the literature supporting common uptake. Yoder et al. (89) have found a stimulatory effect of unlabelled valine dipeptides on the transport of labelled valine into Leuconostoc mesenteroides. Payne states that even though distinct systems have been demonstrated we may yet be able to find interactions between the two (64). This hypothesis is conjecture; and there is little supporting evidence available.

Evidence for Peptide Transport: Intestinal System

The evidence for peptide transport in the brush border membranes of small intestinal mucosa is particularly relevant to this thesis because of the existence of a membrane bound peptidase (90). Despite this peptidase considerable evidence exists that true peptide transport occurs. Many of the approaches to answering this question have been similar to those used in the microorganism systems. Competition studies give some of the most convincing data that transport of intact peptides occur. Numerous reports have shown an absence of competition between the transport of peptides and

amino acids and distinct competition between the transport of peptides (14, 19, 53, 54, 77). Several investigators have been able to show that poorly hydrolyzed peptides are accumulated within the cells against an electrochemical gradient indicating that active transport of peptides occurs (1, 53, 54, 55).

Active transport in mammalian gut membranes can be disrupted by the use of dinitrophenol (DNP) or the production of anionic conditions. Either one of these conditions concomittantly inhibits the hydrolysis of dipeptides seen using intact mucosa. Homogenized mucosa under anoxic condition or in the presence of DNP is not affected and hydrolysis of dipeptides still occurs (15, 80). The inference is that the hydrolysis takes place intracellularly and therefore peptide transport must have occurred. Direct evidence of peptide transport thru the peptidase bearing mammalian gut membranes has been seen with the intracellular accumulation of poorly hydrolyzed peptides in enterocytes (1, 53, 54). Intact peptides in enterocytes have been demonstrated in the serosal fluid of everted sacs and in the blood (22, 77, 80). Further circumstantial evidence of distinct transport systems is based on the observation of Hartnup's disease. This condition is a genetically determined disorder of amino acid transport, with pellagra-like skin leisons, transient cerebellar atoxia, constant renal amino-aciduria and other

biochemical abnormalities. Normal peptide transport occurs in these amino acid transport deficient patients (4). Patients with cystinuria, another disease involving deficient amino acid transport, also have normal peptide uptake in the intestine (32). In fact, investigators have reported different sites along the intestine for amino acid and peptide transport in normal intestine (53, 54). In addition to this evidence is the fact that the dipeptidase activities in the brush border of enterocytes is quite low compared to activity seen in the cytosal (47, 69, 70, 80).

Illicit Transport

The cell membrane has always posed a formidable problem for investigators trying to internalize charged molecules. Passive diffusion seldom leads to considerable levels of compound within the cytosol. Attempts in circumventing this problem lead to chemical modification of the compound making it more membrane soluble. Cyclic AMP was internalized in this manner by modification to $N^6,2^1$-0-dibutyryl cyclic AMP (75). Since structural integrity of the molecule being transported was interrupted to a great extent this method had a considerable drawback. Investigators then proposed designing compounds that were covalently linked to a molecule that would be actively conveyed across the membrane by a preexisting transport mechanism. In this way maximum structural integrity

can be maintained and the anionic and cationic charges left unmasked.

Once the existence of peptide transport was verified the mechanism became a prime candidate for such illicit use. Two reports in 1973 demonstrated that illicit transport was in fact plausible (3, 23). One group, Ames and his co-workers, were able to smuggle a biosynthetic intermediate of histidine, histidinal phosphate ester, into Salmonella typhimurium. This was accomplished by conjugating the intermediate to diglycine and transporting the product into the cell by way of the oligopeptide permease system. It was one of the first demonstrations of illicit transport via peptide transport. Earlier investigations had proved successful in utilizing the ferrichrome and glucose-6-phosphate transport mechanisms for the irregular uptake of albomycin and phosphonomycin respectively. An analogous study to the Ames report by Fickel and Gilvarg presented evidence that dilysylhomoserine phosphate also enters Escherichia coli through the oligopeptide transport system (23).

More recently this approach to molecular conveyance across the cell membrane has been attempted in eukaryotic systems. Steinfeld, Naider, and Becker employed 5-Fluorocytosine (5-FC) peptide conjugates to obtain antiyeast activity against S. cerevisiae, C. albicans, and C. krusei (81). Unfortunately the stabilities of the 5-FC conjugates within

the growth medium suggested that extracellular hydrolysis takes place. These studies have been extended recently and peptide conjugates with 5-fluoroorotic acid have been theorized to enter yeast intact via the peptide transport system (81).

Membrane Bound Peptidases

While the reports have been few in number, membrane bound peptidases have been found over a wide range of systems. Among the prokaryotes the first membrane bound peptidases were found while researching peptidoglycan synthesis. These peptidase were generally noted in the particulate fraction of cell lysates. Further substantiation of true membrane association was not always made. Izaki et al. in 1966 (34) reported such a particulate peptidase activity in Escherichia coli. The peptidase was found to have specific carboxy peptidase activity. Lawrence et al. (40) later in 1970 reported the existence of a D-alanine carboxypeptidase activity within the particulate fraction of Bacillus subtilis cell lysates. This carboxypeptidase was found to be involved with the last stages of peptidoglycan synthesis. While demonstrated within the particulate fraction it was never actually shown to be membrane associated. Diaz-Mauriño et al. (21) reported carboxypeptidase activity in the membrane fraction of Bacillus megatarium cell lysates. This group took the argument involving membrane association several steps farther by demonstrating vesicularization in electron micrographs of that fraction called the particulate fraction.

Dias-Mauriño also reported that successive washes of this particulate fraction removed only 0.4% of the carboxypeptidase activity.

Choules et al. (17) in 1971 reported aminopeptidase, carboxypeptidase, and dipeptidase activity in membranes from Mycoplasma laidlawii. The existence of these peptidase activities was found accidentally. These investigators were attempting to do peptide maps of the mycoplasma membranes and found that much of the protein had been hydrolyzed to free amino acid. These peptidase activities be they amino peptidase or carboxypeptidase were always exopeptidases. Endopeptidases were not found. In 1972 Pecht et al. (68) reported the existence of an alanine oligopeptidase activity in the membrane fraction of Mycoplasma laidlawii. Direct evidence of membrane association was given in this report as it was demonstrated that phospholipase C also caused peptidase inactivation. These investigations suggested that this peptidase had an external location. Mycoplasma laidlawii can be rendered impermeable to alanine by adjustment of salt concentration. $(Ala)_4$ under these nonpermissive conditions is still acted upon by the oligopeptidase of whole cells. If the peptidase had not been located externally the activity would not have been seen.

As mentioned previously membrane bound peptidase's have been located in intestinal epithelial cells. The report by Ugolev in 1965 (83) deals with this peptidase activity. This

particular peptidase activity was found when studies of protein absorbtion in the gut demonstrated that unmodified yeast protein and yeast hydrolysate were both absorbed at the same rate in the intestine. This observation seemed to be an anomaly. Ugolev as early as 1960 had suggested, without experimental evidence, that enzymes at the membrane as well as in the lumen of the intestine were responsible for the anomaly seen in absorption rates. Holt and Miller in 1962 (31) tested Ugolev's hypothesis. Brush border of intestinal epithelial cells was isolated by differential centrifugation. This fraction was then demonstrated to have leucine amino peptidase activity. Many techniques have been developed for use in the brush border membrane system that have been useful in determining peptidase activity of other types of membranes. Fujita et al. (24) described a coupled enzyme reaction which will be discussed in detail later.

Strong evidence for a membrane associated tri-methionine peptidase in Pseudomonas aeruginosa has been given by Miller and Becker (59). These investigators demonstrated that the peptidase activity of the particulate fraction could not be shocked from the periplasmic space under conditions that removed the majority of alkaline phosphatase. Alkaline phosphatase has long been known to be a periplasmic enzyme and may be released from its environment by the use of a Mg^{++} buffer (16). Miller and Becker also demonstrated in this

same report that successive washes of the particulate fraction did not remove significant amounts of the peptidase from the membrane indicating the association of peptidase to membrane was more than ephemeral. No intracellular peptidase was found in the culture medium of this organism. Peptidase activity was shown to break down the modified peptide $Ac(Met)_3$ as well as $(Met)_3$, however $(Met)_3$ supported growth of the methionine auxotroph while $Ac(Met)_3$ did not. This was taken to be evidence that the peptidase was located internally.

While the reports of membrane bound peptidases have been few the reports of peptidases located in the cytosol have been considerable. The evidence for free intracellular peptidases generally depends on its presence in the supernatant fraction of cellular lysates. Good reviews of this subject have been written by Payne (65, 66). David et al. (20), Gilvarg and Sussman (26), Vogt (85), Wolfinbarger and Marzluf (88), Cascieri and Mallette (13) and Simmonds (78) are some of the investigators working with intracellular peptidases and their purification. Simmonds has shown that peptidases in Escherichia coli were not located in the periplasmic space. This was determined after osmotic shock procedures and spheroplast formation did not release peptidase activity. Peptidase activity generally has not been found in the periplasmic space although considerable work has been done to find them. In 1975 Lazdunski (42) reported the existence of an inducible aminopeptidase in the periplasmic space in

E. coli. This activity was evaluated later by Payne (66) who reported that the peptidase Lazdunski found was in fact only active in p-nitroanilide and other β napthylamide derivatives. True peptides such as ALA-GLY-GLY, ALA-VAL, ALA-PRO, or ALA-SER were not cleaved by the peptidase. Studies of peptide utilization in yeast led to the suggestion that intracellular peptidases may be compartmentalized within lysosome-like vesicles (66). This has not been established experimentally.

CHAPTER III

MATERIALS AND METHODS

Organism

Pseudomonas aeruginosa strain RM46 was used without exception in the following studies. This organism is a methionine auxotroph of Pseudomonas aeruginosa strain PAO (R. V. Miller, The University of Tennessee, Knoxville). The lesion that prevents methionine biosynthesis is found in the metIV transduction group as described by Calhoun and Feary (12).

Cultivation Conditions

Stock cultures were maintained as dilute suspensions in Luria Broth at 4°C. This medium is composed of 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 0.1 g NaOH in 1 liter of distilled water. Overnight growth at 37°C was permitted before inoculation of larger flasks. Fresh stock cultures were prepared periodically. Methionine auxotrophy was verified for each new set of stock cultures.

Cells used for the isolation of whole cell wall structures were grown on Pseudomonas Minimal Media (PMM). PMM contained potassium phosphate buffer (60 mM, pH 7.2), sodium citrate (1.7 mM), $MgSO_4 \cdot 7H_2O$ (0.4 mM), and $(NH_4)_2SO_4$ (7.5 mM). Filter sterilized methionine (50 µg/ml) and glucose

(0.4%) were added to the autoclaved medium. Cultures were grown at 37°C with aeration.

Cells used for the preparation of separated inner and outer membranes were grown on Luria Broth as described above. These cultures were also grown at 37°C with aeration.

Phage neutralization experiments were carred out in R medium which contained 10 g tryptone, 1 g yeast extract, and 8 g NaCl in 1 L of distilled water. Two ml of 1 M $CaCl_2$ and 5 ml of 20% glucose were filter sterilized and added to the autoclaved salts solution.

Chemicals and Enzymes

L-methionine was purchased from Sigma Chemical Co., St. Louis, Mo. Tri-methionine and acetylated tri-methionine were the kind gift of F. Naider, Department of Chemistry, College of Staten Island of the City University of New York. Tri-methionine peptides were judged homogeneous after analysis by high voltage electrophoresis and thin layer chromotography. Lysozyme and DNase were purchased from Sigma Chemical Co., as were L-amino acid oxidase and horseradish peroxidase. RNase was purchased from Worthington Biochemical Corp., Freehold, N.J.

Growth of RM46 on Modified Tri-Peptides

Pseudomonas aeruginosa RM46 was grown in PMM medium as described above. PMM was supplemented with acetylated

tri-methionine ($Ac(met)_3$). $Ac(met)_3$ is tri-methionine that has been blocked at the amino terminal by an acetyl group. These growth curves were compared to the growth of similar cultures with tri-methionine or methionine used as a supplement for the RM46 methionine auxotroph.

Isolation of Cell Wall Structure

The method used to lyse cells for the isolation of the entire cell wall structure was a modification of the procedure described by Miller and Clark (60). Cultures were grown to late log phase and harvested by centrifugation at 10,000 X g for 20 min. The cells were washed in a solution of 0.85% NaCl and resuspended in 10% sucrose in 50 mM Tris-hydrochloride buffer (pH 8.0), at a concentration of 0.5 g (wet weight) cells/ml. The cell suspension was quick frozen in an ethanol dry-ice bath and allowed to thaw slowly in an ice bath. For each milliliter of cell suspension, 0.1 ml of a solution of lysozyme (2 mg/ml) and 0.1 ml 1 M NaCl was added. The suspension was incubated at room temperature for 1 h until lysis had occurred. Magnesium sulfate was then added to the lysate at a final concentration of 10 mM. Ten microlitiers of a deoxyribonuclease I solution (10 mg/ml) was added per milliliter of cell lysate to digest DNA and reduce the viscosity of the solution. The lysate was then centrifuged at 20,000 X g for 1 h at 4°C and the membrane fraction pelleted. Membranes were resuspended in a small amount of

distilled water and dialyzed overnight against a 50 mM Tris-hydrochloride buffer (pH 8.0). Aliquots were made of the membrane fraction and stored at -20°C until needed.

Separation of Inner and Outer Membranes

A modification of the procedure of Hancock and Nikaido (28) was used to prepare the separated membrane fractions. Four liters of cells were grown overnight to a $Klett_{660}$ of 500 units. The cells were removed by centrifugation at 10,000 X g for 20 min and resuspended in 20 mls of 20% sucrose in 50 mM Tris-hydrochloride buffer (pH 8.0). DNase and RNase were added to give a final concentration of 1 mg/ml each. The cell suspension was then lysed by two passages through a French Pressure cell at 15,000 psi. Two milliliters of a solution of lysozyme (1 mg/ml) dissolved in water was then added to the lysate. After this mixture was incubated for 10 min at room temperature, a protease inhibitor, p-toluenesulfonylfluoride, was added to a final concentration of 1 mM. Cellular debris was removed by centrifugation at 1,000 X g for 10 min. Five to six mls of the membrane suspension was then applied to each of the six sucrose step gradients. The gradients contained 1 ml 70% sucrose in water (w/v), and 6 mls 15% sucrose (w/v). Each of the tubes were then placed in a Beckman type SW41 rotor and centrifuged for 2 hrs at 200,000 X g. After centrifugation the bottom 2 mls of each gradient were removed by pipetting from above and

applied to a second sucrose step gradient. The second gradient contained 1 ml 70% sucrose in water (w/v), 3 ml 64% sucrose (w/v), 3 ml 58% sucrose (w/v), and 3 ml 52% sucrose (w/v). These tubes were then centrifuged overnight (16 hrs) at 200,000 X g in the Beckman type SW41 rotor. Twenty-two fractions, 0.6 ml each, were collected from the top of the gradient with an ISCO density gradient fractionator (model 640). After fractionation the membranes were stored at -20°C until needed.

Peptidase Assay

Quantitative analysis of peptidase activity was accomplished by a modification of the procedure described by Fujita et al. (24). Breakdown of $(Met)_3$ by the peptidase was followed by determining the amount of free methionine produced. Membrane fraction was incubated with the $(Met)_3$ substrate. The reaction was stopped at various times by heating the reaction mixture to 100°C for 3 min. Two milliliters of a solution containing 0.6 mg L-amino acid oxidase, 1.0 mg horseradish peroxidase, and 1 mg o-dianisidine [5 mg/ml in 50 mM Tris-hydrochloride pH (8.0)] per 100 mls distilled water, were added to the stopped reaction mixture. After 1 h incubation at room temperature the absorbancy of the solution at 440 nm was determined in a Gilford spectrophotometer (Model 250). The increase in absorbancy was brought about by a coupled enzyme reaction. L-amino acid

oxidase (cobra venom) catalyzes the formation of keto acids from amino acids with the concomitant evolution of hydrogen peroxide. H_2O_2 in the presence of horseradish peroxidase leads to the formation of H_2O and O_2. The endogenous O_2 evolved in this reaction oxidizes the o-dianisidine producing a brown color change. The amount of O_2 formed and hence the color change is directly proportional to the amount of peptidase initially present. L-methionine was used as a standard.

Succinate Dehydrogenase Assay

The method described by Kasahara (36) was used to assay for succinate dehydrogenase (SDH). Five hundred µl of membrane fraction were added to 1.25 ml of a solution containing 35 mM succinate and 4.7 mM KCN in 58.3 mM Tris-hydrochloride (pH 8.0). This mixture was allowed to incubate at room temperature for 5 min. The rate of the reaction was determined using 2,6 dichlorophenol-indophenol (DCPIP) as an indicator. DCPIP has an extinction coefficient of 2.2×10^4 at 600 nm. A solution of 0.047 mM DCPIP and 0.23 mM phenylmethylsulfonyl-fluoride was added to the reaction mixture. The decrease in absorbance was followed on a Gilford spectrophotometer (Model 250) with a light path of 1 cm. The reaction was followed at a wavelength of 600 nm.

2-Keto 3-Deoxyoctonate Assay

A modification of the method used by Weissbach et al. (86) to identify 2-keto 3-deoxyheptonic acid (KDH) was used

to assay for KDO. One milliliter of each fraction to be assayed was precipitated with an equal volume of 10% Trichloroacetic acid. After 10 min in an ice bath the solution was centrifuged at 10,000 X g for 30 min and the precipitate resuspended in 0.5 ml of 50 mM Tris-hydrochloride buffer (pH 8.0). The saccharide portion of the lipopolysaccharide was then acid hydrolysed from the lipid portion of the membrane by the addition of H_2SO_4 to a final concentration of 0.2 N. The mixture was then heated to 100°C for 20 min. Two tenths ml of this fraction and 0.25 µl of a solution containing 10 µM HIO_4 in 0.125 N H_2SO_4 were then incubated for 20 min at room temperature. One half ml of 100 µM sodium arsenate in 0.5 N HCl was added and the mixture allowed to stand, at room temperature, for two min. Two ml of a solution of 0.3% thiobarbituric acid (pH 2.0) were added, and the mixture heated for 30 min at 100°C. A red chromophore directly proportional to the amount of 2-keto 3-deoxyoctonate developed during this time as well as a flocculate precipitate. Some reports indicate that this turbidity may be removed by reheating the reaction mixture after it has cooled to room temperature (35). This procedure decomposes the red chromophore however, and for this reason the reaction mixture was centrifuged in a clinical centrifuge at top speed for ten minutes to remove the flocculate precipitate. The color change was then read on a Gilford spectrophotometer (Model 250) at a wavelength of 548 nm.

Bacteriophage Neutralizing Assay

P. aeruginosa strain RM46 was determined to be sensitive to phage E79. The receptor of this phage is known to be in the outer membrane (62). The presence of this phage receptor was detected by a method described by Mizuno (62). The neutralization activity of the membrane fractions towards phage was estimated by the inhibition of plaque formation. Phage and membrane fractions were incubated for 5 min prior to the addition of the *P. aeruginosa* strain RM46. The amount of membrane fraction added was based on protein content. The portion of infective phage remaining was counted by an agar overlay technique.

Optimum pH Buffers

Buffers of varying pH were prepared with 100 mM Tris-hydrochloride. These buffers were added to tri-methionine substrate in 0.1 ml volumes. Peptidase was then added and the coupled reaction assay completed as described above.

Effects of Cations

Various concentrations of $MgCl_2$ and $ZnCl_2$ in 50 mM Tris-hydrochloride buffer (pH 8.0) were added to the tri-methionine substrate in 0.1 ml volumes. $MnCl_2$ was added in distilled water because of its tendency to react with Tris-hydrochloride forming a brown precipitate. The peptidase assay was then done according to the method described above.

Protein Assay

Protein determination was made by the method of Lowry et al. (48), or the Bio-rad protein assay (10). Bovine serum albumin was used as a standard.

Lipid Extraction

Qualitative analysis of the lipids making up the membranes of Pseudomonas aeruginosa strain RM46 was done using techniques described by Bligh and Dyer (8). All glass materials in this experiment were acid cleaned prior to use. A 1 ml suspension of 50 mg cells (dry weight) was made with distilled water. Methanol and chloroform (2:1 v/v) in a volume of 3.75 ml was added to the suspension. The mixture was homogenized and allowed to incubate for 4 h at room temperature. The mixture was centrifuged at 10,000 X g for 1 h in a Beckman SW41 type rotor and the supernatant fluid decanted and set aside. Methanol chloroform was added to the resulting pellet and the above procedure repeated. The supernatants were combined and to this mixture 2.5 ml chloroform and 2.5 ml water were added. This mixture was centrifuged at 10,000 X g for 10 min and the were added to remove any water in the lower phase. This mixture was placed in a rotary evaporator at 35°C and the resulting residue immediately redissolved in a chloroform-methanol solution (1:1 v/v). The methanol was removed by

centrifugation at 10,000 X g for 10 min and the lipids collected in the lower chloroform phase. This solution was brought to two ml with chloroform.

Analysis of the extracted lipids was then made by two-dimensional thin layer chromotography. A volume of 5 µl was spotted onto a silica gel plate (Eastman Co.) 20 cm by 20 cm. The first dimension was run in a solvent system containing chloroform, methanol and ammonium hydroxide (28% concentrated) in a ratio of 15:5:1 v/v. After complete saturation the plate was allowed to dry in a hood for 15 min. It was then rotated 90° and run in a second solvent system that contained chloroform, methanol, acetone, glacial aceteic acid, and water in a ratio of 6:2:8:1 v/v. Again, after complete saturation, the plate was removed and dried thoroughly for 30 min. The dried silica gel plate was then placed in a chamber, that had been previously saturated with iodine, until the phospholipid spots developed. The plate was removed and the R_f index of the spots measured and compared to known standards.

Triton-X Solubilization of Peptidase

Attempts were made to isolate the peptidase from the cell wall structure. Membrane fraction was incubated with various concentrations of Triton-X detergent for 20 min and gently homogenized. The fraction was then centrifuged at 250,000 X g for 1 h. Supernatant fluid was decanted and the

precipitate resuspended in 50 mM Tris-hydrochloride (pH 7.8). All fraction were then assayed for peptidase activity using the coupled reaction assay described above.

Experiments analogous to those with Triton-X detergent were done using C-8-glucopyranoside as the solubilizing agent.

Solubilization with Chaotropes

These substances, usually the halogenated acetates, interact with water in such a way that the environment is made less hydrophilic. The polarity of the water molecule is of import to the integrity of the membrane lipid bilayer. The drop in polarity of the water molecule due to chaotropic interactions allows the gentle dismemberment of the membrane (29). Hydrophobic-hydrophobic interactions between the inner portion of the membrane "sandwich" and the environment occur. Membrane proteins may be extracted as a result. Trichloracetic acid and thiocyanate were used to disrupt the membranes. Various concentrations of chaotrope were used. The chaotrope membrane mixture was kept in an ice bath for 10 min and then subjected to centrifugation at 10,000 X g for 1 h. Both pellet, resuspended in 50 MM Tris-hydrochloride (pH 7.8), and supernatant were assayed for peptidase activity by the coupled enzyme assay. The pellet was washed with chaotrope a second time and the centrifugation process and peptidase assay repeated.

Peptidase Activity on $Ac(Met)_3$ Substrate

Membrane fraction was allowed to react with $Ac(Met)_3$ as a substrate. At different time points 5 µl were taken and applied to a cellulose thin layer chromotography plate (Eastman Co.). The plate was placed into a solvent system that contained isopropanol, methyl ethyl ketone, sodium hydroxide (12:3:5). After complete saturation the plate was thoroughly dried and placed in a saturated iodine chamber. R_f values of the breakdown products were measured and compared to known standards.

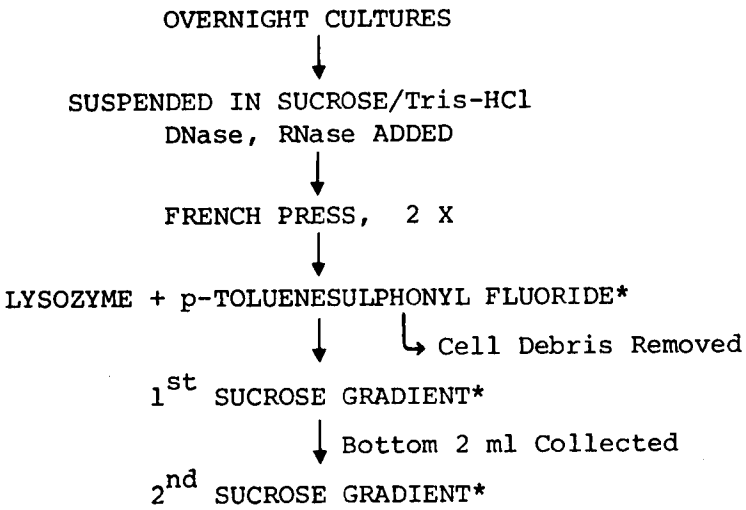
CHAPTER IV

RESULTS

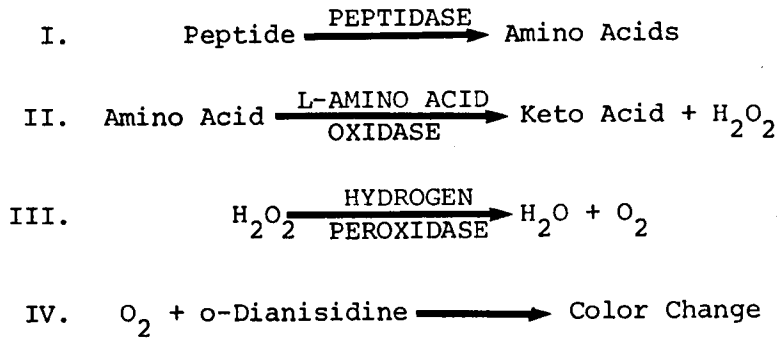
Separation of Inner and Outer Membrane

The procedure for the separation of inner and outer membranes is described in detail elsewhere (28). Figure 1A is an outline of this procedure. The cell lysate, immediately prior to being placed on the first sucrose step gradient, was treated with P-toluenesulphonyl fluoride (PMS). PMS is a protease inhibitor. This measure was taken to prevent membrane protein from being broken down by cellular proteases. Intact membrane protein helps to insure separation of inner and outer membrane. Each of these membranes has a different protein to lipid ratio that is reflected in the sucrose gradient by different buoyant densities. The first sucrose step gradient was used to isolate the combined inner and outer membrane fragments from other cellular constituents. After centrifugation membrane was found at the interface between the 15% (w/v) sucrose and the 70% (w/v) sucrose. This fraction was collected as previously described and placed on the second sucrose gradient which was used to separate the inner and outer membrane from one another.

Figure 1B is an outline of the assay used to detect peptidase activity in both the first and second sucrose



A. Separation Procedure



B. Peptidase Assay

Figure 1. Separation procedure for inner and outer membranes and the assay used to detect peptidase activity.

step gradients. This procedure has also been described in detail elsewhere (24).

Peptidase Assay on the First Sucrose Gradient

The first sucrose step gradient contained both membrane and cytosol constituents. Peptidase activity was determined after the gradient was fractionated into twenty-two parts. Approximately 70% of the total observed activity was located at the interface between the two concentrations of sucrose. Tri-methionine peptidase activity was therefore found predominantly within the region of the membrane fraction that constituted the bottom 2.5 ml of the first sucrose gradient. Table 1 indicates the total peptidase activity of the twenty-two aliquots in addition to the specific activities and protein content of each fraction. Presumably 30% of the cells tri-methionine peptidase activity has an intracellular location. This estimate may in fact be too high as the majority of this activity is located relatively near the membrane in the gradient. This peptidase activity may be due to small membrane pieces that have not sufficient density to band with the larger membrane fragments. Those fractions marked with an asterisk were pooled and placed on the second sucrose step gradient.

TABLE 1

ANALYSIS OF PEPTIDASE ACTIVITY ON THE FIRST SUCROSE STEP GRADIENT

Fraction (from top to bottom)	Protein Conc. (mg/ml)	Specific Activity (μmet released) min/mg prot.)	Total Activity (μmoles met/ min/sample)
1	6.4	1	4
2	9.0	0	0
3	8.4	1	5
4	9.3	0	0
5	8.0	0	0
6	9.2	1	6
7	9.9	1	6
8	10.9	1	7
9	8.8	1	5
10	9.4	1	6
11	9.5	1	6
12	8.2	1	5
13	7.2	24	104
14	7.1	22	94
15	6.4	23	88
16	6.1	23	84
17	6.6	22	87
18	8.0	24	115
19	38.0	27	616†
20	28.0	9	151†
21	35.0	7	147
22	25.0	9	135

*Fractions applied to second sucrose step gradient. This represents 70% of the total peptidase activity on the gradient.

†The interface between sucrose concentrations is located between bands 19 and 20.

Peptidase Assay on the Second Sucrose Step Gradient

The resultant bands of the second sucrose gradient after centrifugation may be seen in Figure 2. The difference in buoyant density between the two membranes is due, in part, to the different protein to lipid ratios as previously mentioned and also to lipopolysaccharide (LPS) content. LPS, a component of the outer membrane in gram-negative bacteria, is a relatively dense substance. The outer membrane is thus found within the high density region of the gradient. Between the purified inner and outer membranes two additional bands appear. These bands are labelled "Mixed 1" and "Mixed 2" respectively and have been shown to contain mixed inner- and outer-membrane markers by Hancock and Nikaido. The inner membrane band bears a pink pigment the other three bands are relatively white. The results of assaying the second sucrose step gradient after fractionation for peptidase activity are displayed in Figure 3. The vast majority of peptidase activity is found within the region known to contain inner membrane. Mixed 1 and Mixed 2 fractions have smaller amounts of peptidase activity and the region that contains outer membrane has little or no peptidase activity. It is of interest to note that a two-fold increase in activity is seen between the first and second gradients. While the reason for this increase is not completely

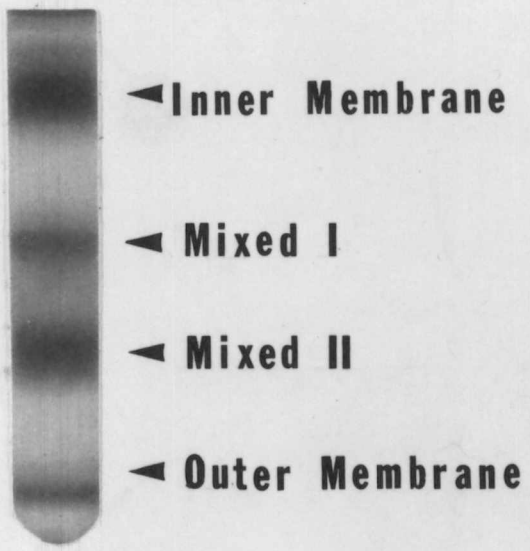


Figure 2. Second sucrose gradient. This gradient is composed of 1 ml 70%, 3 ml 64%, 3 ml 58%, and 3 ml 52% sucrose (w/v). Inner and outer membrane are separated on the basis of buoyant density. The lipopolysaccharide (LPS) containing membrane is found in the high density region of the gradient. The inner membrane is less dense and found at the top of the gradient. Mixed 1 and Mixed 2 bands contain mixtures of inner and outer membrane markers. Twenty-two 0.6 ml fractions were collected from the top of the gradient and assayed for enzymatic activities and LPS content as described in the text.

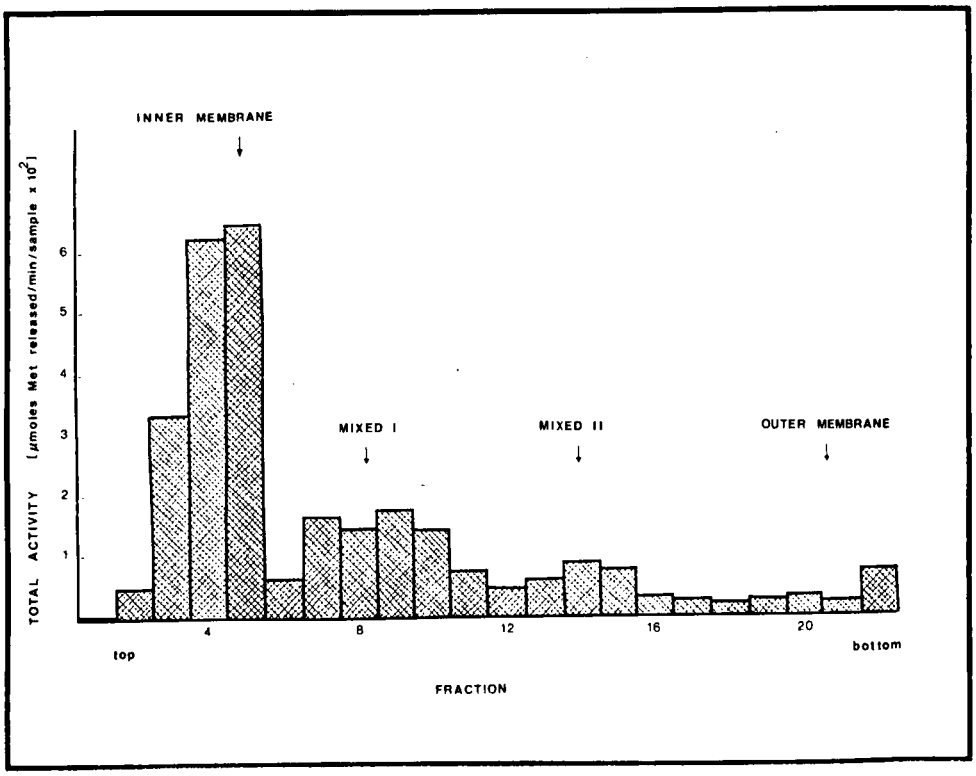


Figure 3. Peptidase activity on tri-methionine in the second sucrose step gradient fractions. Each fraction was assayed for peptidase activity as described in the text.

understood, it is possible that an inhibitor, present in the cytosol, is removed during isolation of the inner membrane.

Distribution of Succinate Dehydrogenase (An Inner-Membrane Protein)

Succinate dehydrogenase (SDH) has been shown to be membrane associated and since the Krebs cycle is an intracellular phenomenon SDH must be associated with the inner membrane (36). Figure 4 illustrates the distribution of SDH activity in the fractions collected from the second sucrose gradient. SDH is associated predominantly with the first band. (The region containing inner membrane.) Mixed 1 and Mixed 2 bands show relatively little SDH activity as they had shown little peptidase activity. The outer membrane has little or no SDH activity.

Bacteriophage Neutralization

Another marker that identifies the outer membrane is phage E79 receptor protein (62). Table 2 shows the % neutralization of phage E79 when incubated with inner or outer membrane fraction at a concentration of 1 mg protein/ml. Outer membrane preparations cause a decrease in viable phage titer of 25%. Inner membrane preparations do not cause a decrease in phage titer; therefore the outer membrane marker phage receptor protein is presumed absent.

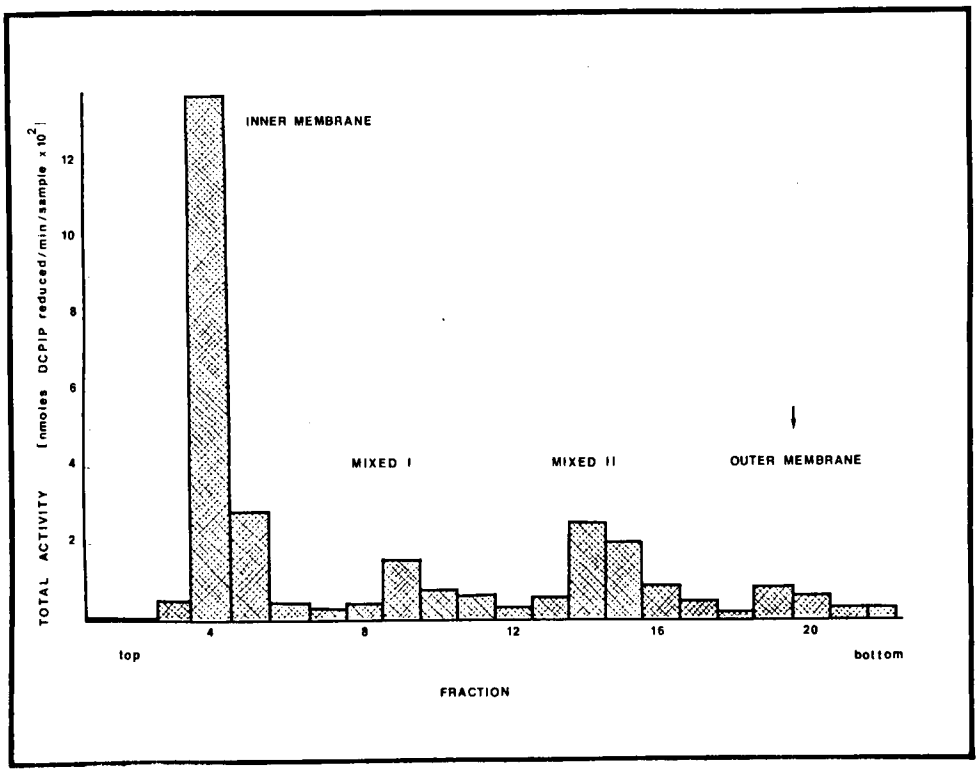


Figure 4. Succinate dehydrogenase activity. Succinate dehydrogenase is an inner membrane component. The reduction of dichlorophenol indophenol by this enzyme is determined spectrophotometrically at a wavelength of 600 nm. The receptor for phage E79 serves as an outer membrane marker. The membrane fraction that lowers E79 titer upon incubation is indicated by the arrow.

TABLE 2

PEPTIDASE ACTIVITY COMPARED WITH INNER AND OUTER MEMBRANE MARKERS

Fraction	Peptidase Activity µmole met/ min/sample	Protein Conc. (mg/ml)	SDH nmole DCPIP/ min/sample	KDO µmole	ϕ Neutralization (%)
Inner Membrane	248	1.65	1800	000	0
Mixed 1	21	0.41	1600	930	n.d.*
Mixed 2	17	0.22	0	1730	n.d.*
Outer Membrane	17	0.25	0	350	25

*Not determined.

2-Keto 3-Deoxyoctonate (An Outer-Membrane Component)

2-keto 3-deoxyoctonate (KDO) is the polysaccharide component of the lipopolysaccharide moiety of the outer membrane. Each of the four bands seen in the second sucrose gradient was assayed for KDO. Table 2 shows the amount of KDO in the four membrane bands. The majority of KDO was in the fraction labeled Mixed 2. These results differ somewhat from the reports of Hancock and Nikaido (28) who found the largest amount of KDO in the band labelled outer membrane.

Sensitivity to P-Toluenesulphonyl-fluoride

P-toluenesulphonylfluoride (PMS), a protease inhibitor, was added to the lysate during the inner and outer membrane separation procedure (Fig. 1A, page 31). It was therefore prudent to determine that peptidase activity was not affected by PMS. The whole cell envelope structure was isolated as described above and treated with PMS at a concentration comparable to that used in the inner and outer membrane separation procedure. This activity was compared to the activity of untreated cell envelope structure as shown in Table 3. Tri-methionine peptidase activity was not affected by the protease inhibitor.

Stability at -20°C

Table 3 also shows the percent of peptidase activity of a cell envelope fraction which remained after storage at -20°C

TABLE 3

PEPTIDASE SENSITIVITY TO P-TOLUENESULPHONYLFLUORIDE (PMS) AND STABILITY AT -20°C

Fraction	% Peptidase Activity of Cell Envelope Structure	
	PMS treated	PMS untreated
Cell envelope structure	96	100[a]
Cell envelope stored at -20°C 1 month (approx.)	n.d.[b]	49

[a]Taken to be 100%.

[b]Not determined.

for approximately one month. After this period of time only one half of the activity seen in fresh preparations remained. Because of these results, samples were assayed for peptidase activity within one week of the separation procedure.

Characterization of the Tri-Methionine Peptidase

The kinetics of the peptidase activity associated with the inner membrane were investigated as well as the response of the peptidase activity to several divalent cations. These results were compared with peptidase activity associated with the unfractionated cell envelope under the same conditions.

The Km of tri-methionine peptidase in the unfractionated cell envelope was determined to be 8×10^{-4} M. The V_{max} for the peptidase at this point in membrane isolation was 2.5 μM/min. These data are determined from the Lineweaver-Burke Plot (Figure 5). This figure also gives the data for tri-methionine peptidase activity within the isolated inner membrane. The Km for inner membrane enzymatic activity is 9.4×10^{-4} M and the V_{max} is 2.7 μM/min. The Km and V_{max} of peptidase activity seen in the isolated inner membrane is essentially the same as the Km and V_{max} before membrane separation.

The optimum pH for peptidase activity associated with the inner membrane was determined and compared to the

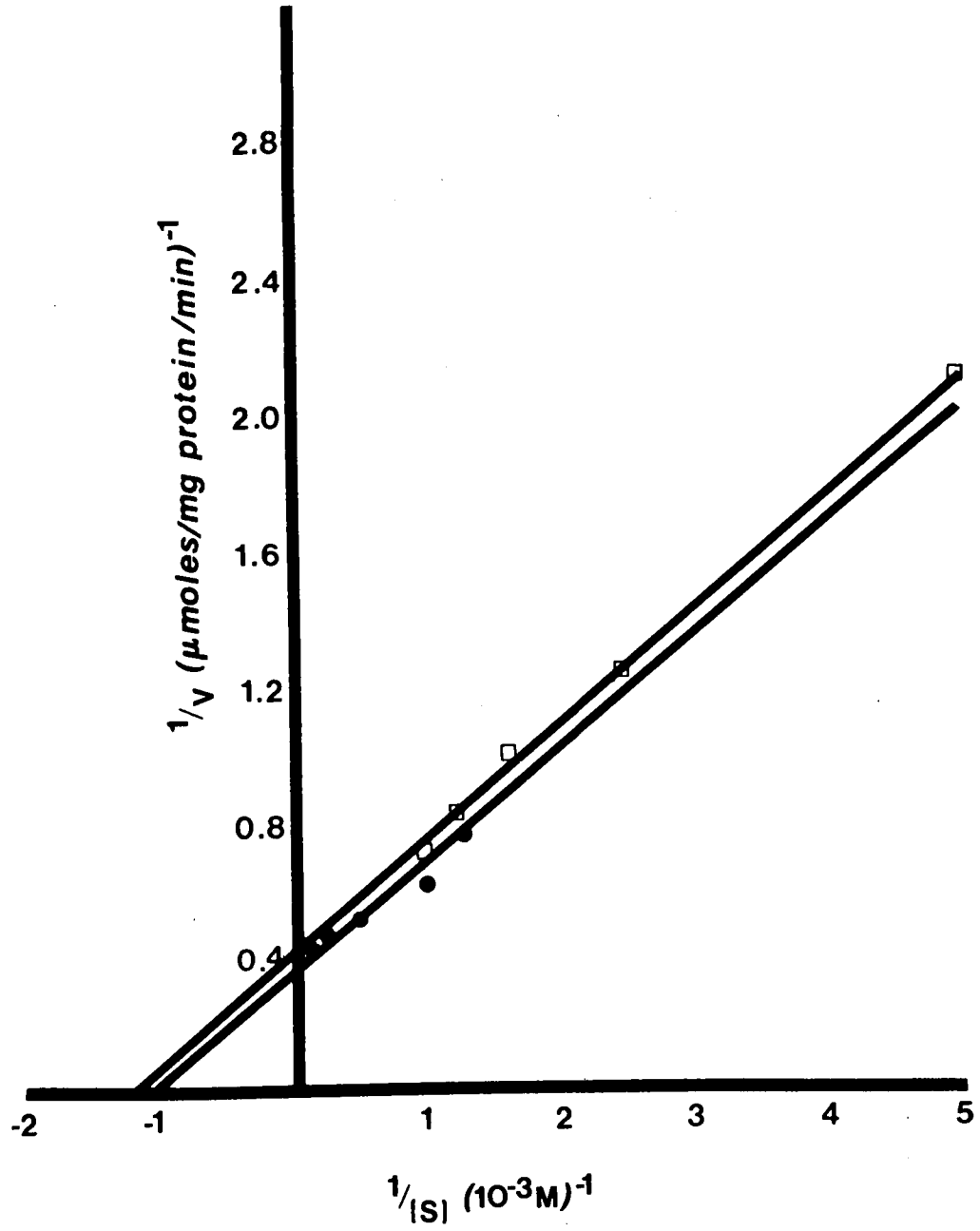


Figure 5. Lineweaver-Burke plot of tri-methionine peptidase activity. The kinetics of the peptidase activity in the inner membrane compared to the peptidase activity before separation. The peptidase activity of the separated inner membrane has a Km of 9.4×10^{-4} M and a V_{max} of 2.7 μM/min (closed circles). The peptidase activity of the unfractionated cell envelope has a Km of 8.0×10^{-4} M and a V_{max} of 2.5 μM/min (open squares).

activity associated with the unfractionated cell envelope. The results are given in Figure 6. The peptidase activity of the inner membrane was maximal at pH between 7.5 and 8.0. Peptidase activity from the unfractionated cell envelope had an optimum at a pH of 7.5.

Peptidase activities from both isolated inner membrane and unfractionated cell envelope had similar responses to various concentrations of $MgCl_2$ as seen in Figure 7. Peptidase activity under both conditions was maximal when Mg^{++} was present at 17 m$\underline{M}$. The higher percent of maximum activity for peptidase activity within the inner membrane without additional $MgCl_2$ may reflect different initial residual concentrations of $MgCl_2$ within the samples tested.

Figure 8 illustrates the effects of Mn^{++} on peptidase activity. The same fractions used in previous studies to determine optimum pH (Figure 6) and response to $MgCl_2$ (Figure 7) were used to determine $MnCl_2$ effect. The peptidase activity associated with isolated inner membrane was maximal at 17 m$\underline{M}$ $MnCl_2$. There is little stimulation over the activity seen in the absence of added $MnCl_2$. This, again, may be a reflection of initial $MnCl_2$ content of the isolated inner membrane fraction. The percent of maximum peptidase activity in unfractionated cell envelope is slightly higher in the presence of 33 m$\underline{M}$ $MnCl_2$ than it is with 17 m$\underline{M}$ $MnCl_2$. There is considerable increase in activity observed upon addition of 17 to 33 mM $MnCl_2$.

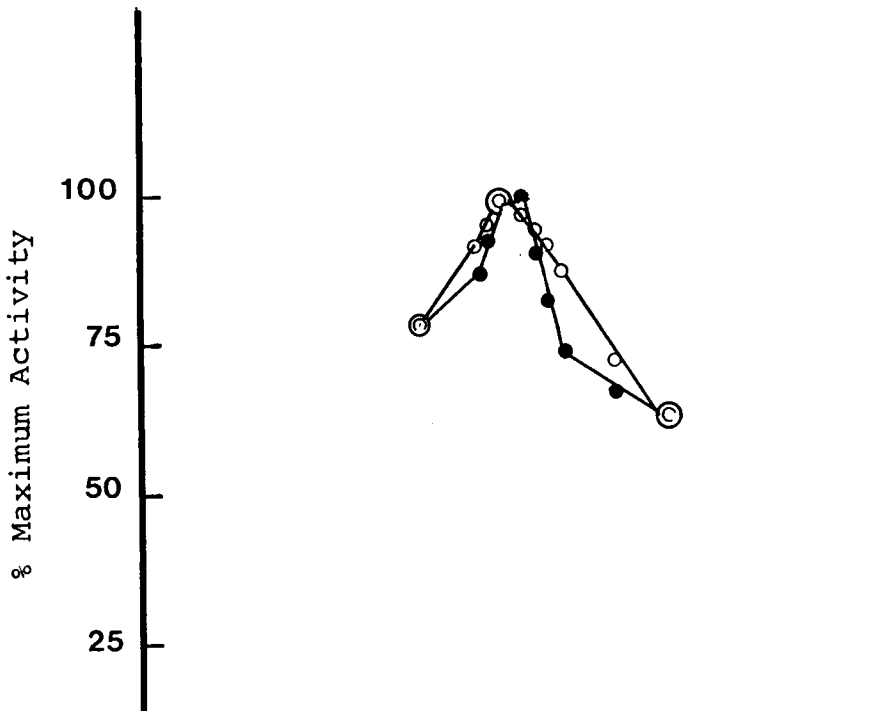


Figure 6. Optimum pH for tri-methionine peptidase activity. The peptidase activity in the inner membrane (closed circles) and in the unfractionated cell envelope (open circles) was determined at various pHs as described in the text.

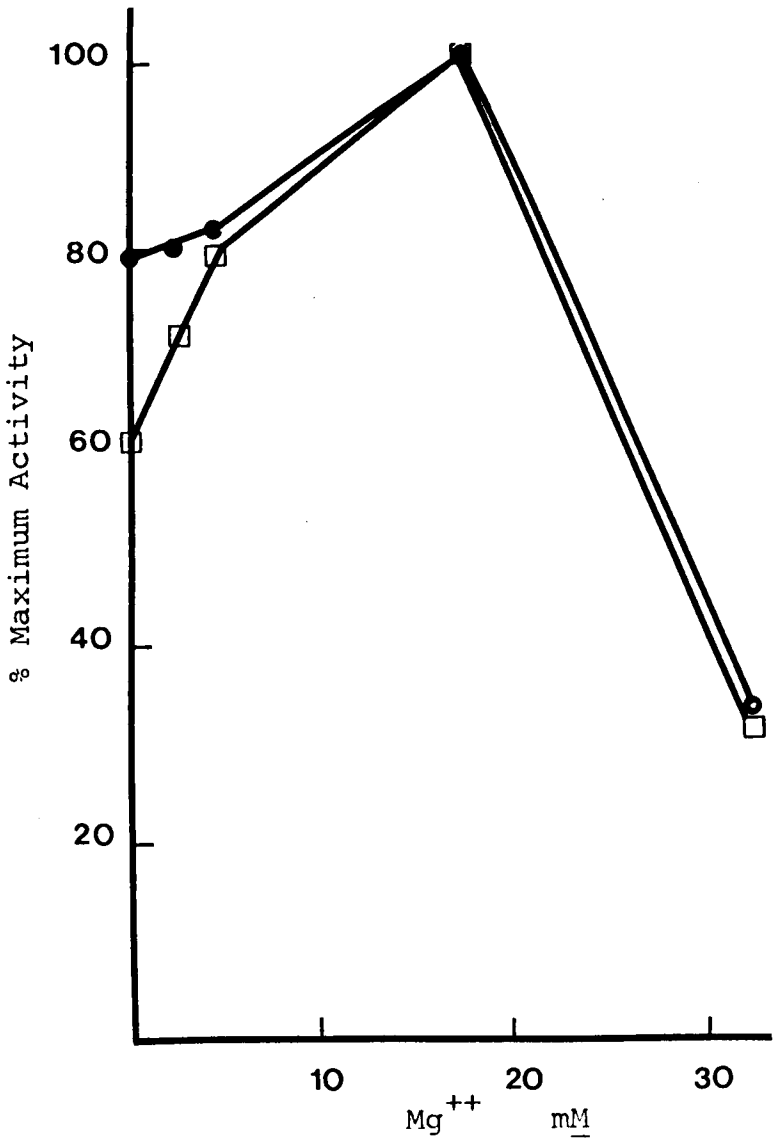


Figure 7. Effect of Mg^{++} on tri-methionine peptidase activity. Stimulation of inner membrane peptidase activity (closed circles) by $MgCl_2$ was compared with the stimulation of the activity of the unfractionated cell envelope (open squares). Assays were performed as described in the text.

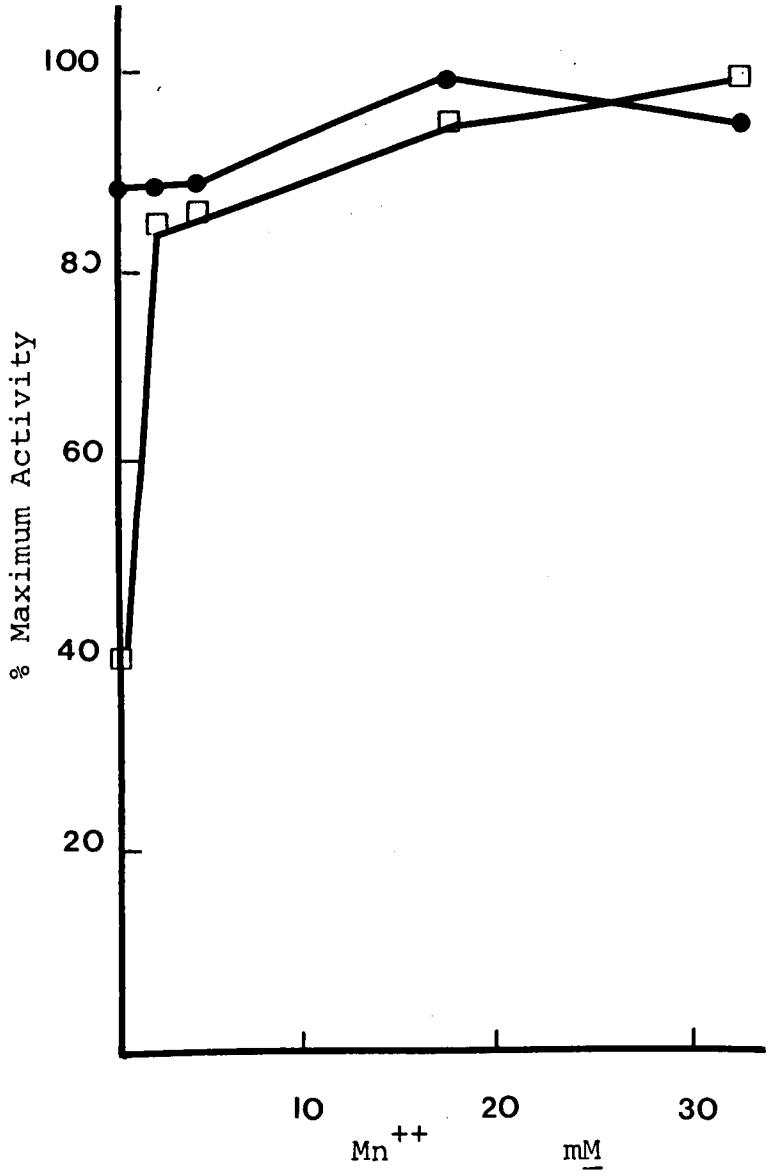


Figure 8. Effect of Mn^{++} on tri-methionine peptidase activity. Activity of the inner membrane (closed circles) and that of the unfractionated cell envelope (open squares) was determined in the presence of various concentrations of $MnCl_2$ as described in the text.

Phospholipid Analysis on Pseudomonas aeruginosa RM46

Lipids of P. aeruginosa strain RM46 were solubilized by methods previously described and placed on a two-dimensional thin layer chromatography system. Figure 9 is a representation of a thin layer chromatography plate showing the relationship of solubilized phospholipids and standard preparations of phospholipids. P. aeruginosa strain RM46 membranes contained phosphatidyl inositol and phosphatidyl glycerol. Four other unidentified phospholipids were observed. Phospholipid analysis has shown phosphatidyl ethanolamine to be a major component of P. aeruginosa membranes with smaller amounts of phosphatidyl glycerol, diphosphatidyl glycerol and phosphatidyl glycerol phosphate (56). It is likely that the unidentified phospholipids presented in Figure 9 are in fact one or more of these constituents. There may have been some modification during the extraction process that caused them to move in a slightly different pattern on the thin layer chromotography plate.

Growth of P. aeruginosa Strain RM46 on Various Methionine Sources

P. aeruginosa RM46 was grown on $(Met)_3$ and the modified peptide $Ac(Met)_3$ and compared to cultures grown with and without methionine. Figure 10 demonstrates that growth of the methionine auxotroph is possible using $(Met)_3$ to supply the nutritional requirement. $Ac(Met)_3$ will not supply the methionine necessary for the auxotroph.

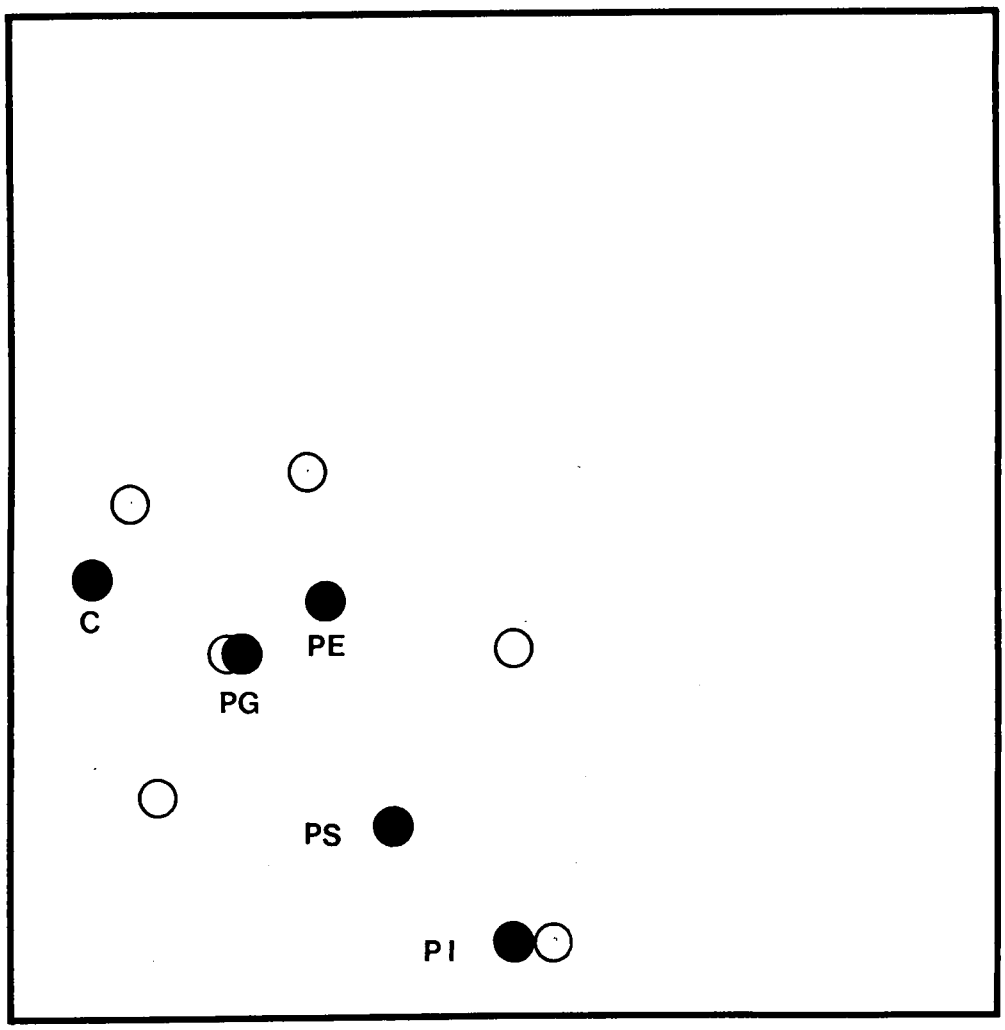


Figure 9. Phospholipid analysis of Pseudomonas aeruginosa strain RM46 membranes. Lipids were solubilized according to the method of Bligh and Dyer (8). They were then placed on a two-dimensional thin layer chromotography plate. The first solvent system contained chloroform, methanol, and ammonium hydroxide (28% concentrated) (13:5:1 v/v). The second solvent system contained chloroform, methanol, acetone, glacial acetic acid, and water (6:2:8:1 v/v). Closed circles represent standard preparations of phospholipid. C = Cardiolipin, PG = Phosphotidyl glycerol, PE = Phosphotidyl ethanol, PS = Phosphotidyl serine, PI = Phosphotidyl inositol. The phospholipids found within the membranes of P. aeruginosa strain RM46 are indicated by open circles.

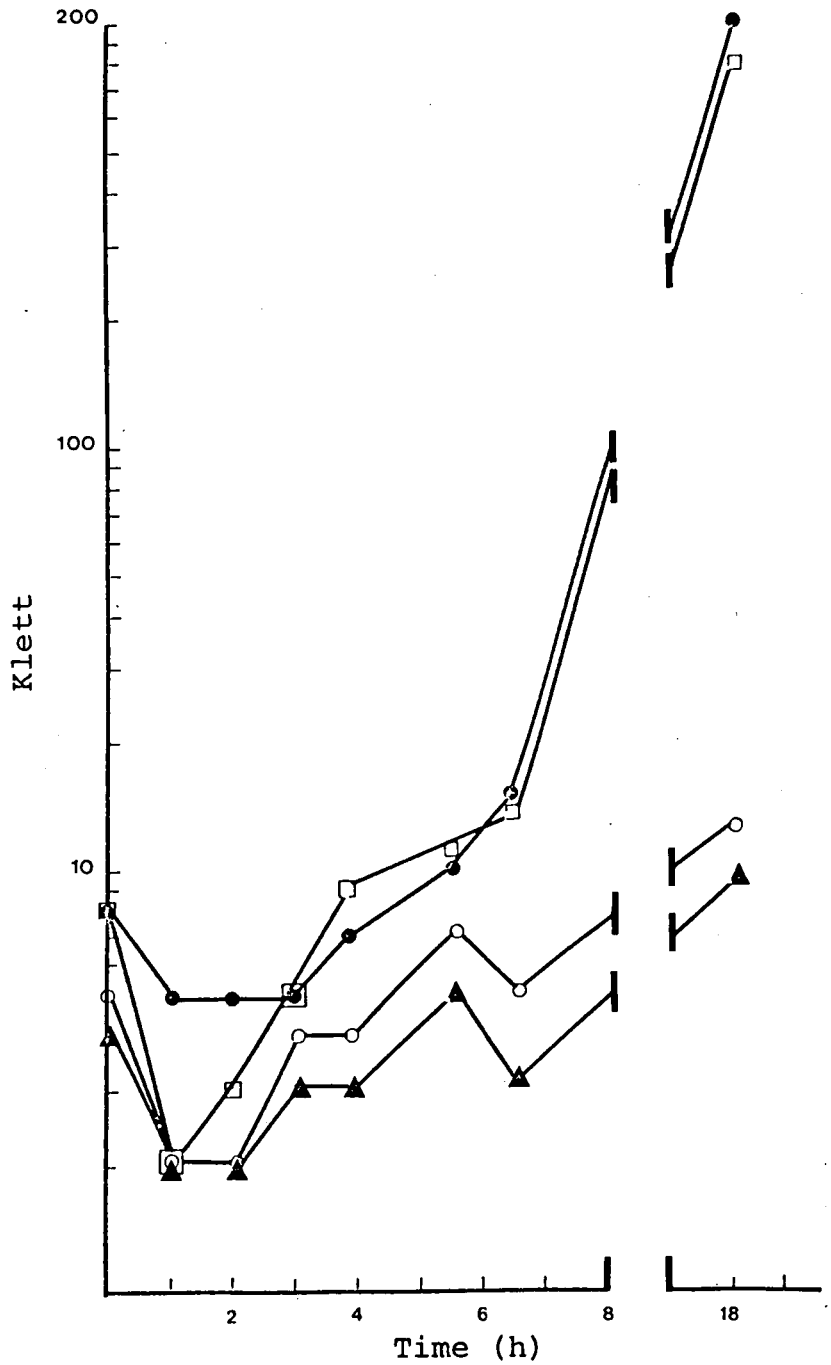


Figure 10: Growth of P. aeruginosa strain RM46 on $Ac(Met)_3$ and other methionine sources.

: growth on methionine (closed circles)
: growth on tri-methionine (open squares)
: growth on $Ac(Met)_3$ (open circles)
: no methionine added (closed triangles)

Thin layer chromatography plates used to detect breakdown of peptides upon incubation with membrane fraction showed that the peptidase was active on both the $(Met)_3$ and $Ac(Met)_3$ substrates.

Solubilization Attempts

SDS. Disruption of the membrane by 10% sodium dodecyl sulfate (SDS) caused total inactivation of tri-methionine peptidase activity and therefore was not a good method to solubilize this protein.

Triton-X. Triton X was used at various concentrations (0.01 mg detergent/mg protein to 0.24 mg detergent/mg protein) to solubilize tri-methionine peptidase. At best 5-6% of the enzymatic activity was recovered in the supernatant fraction while 94-5% of the activity remained in the pellet. The small amount of activity in the supernatant fraction was observed only if the peptidase assay was done immediately after the solubilization procedure. If the assay was done 24 h later no activity in the supernatant was found. This may be an indication of lipid requirement for peptidase activity.

C-8 glucopyranoside. Solubilization attempts using C-8 glucopyranoside (1 mg detergent/0.5 mg protein) allowed

up to 11% recovery of peptidase activity within the supernatant fraction. Again, trimethionine assays were performed immediately after solubilization.

Chaotropes. Trichloroacetic acid (2M) was unacceptable as a solubilizing agent because of it's effect on enzymatic activity. Thiocyanate (2M, 4M) did not inhibit enzymatic activity; however, protein did not appear to be solubilized.

CHAPTER V

DISCUSSION

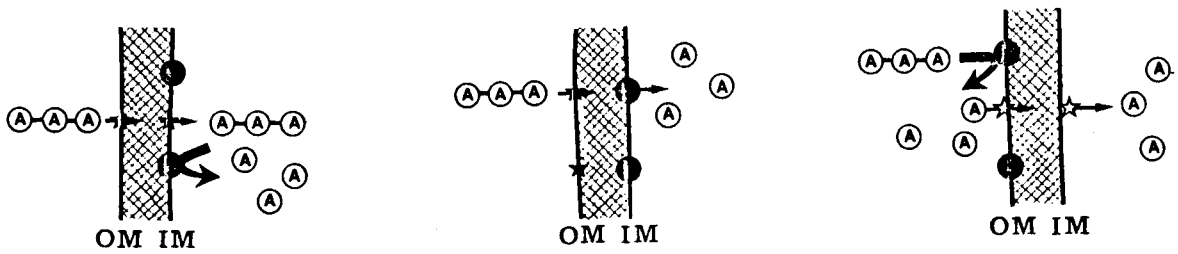
The tri-methionine peptidase activity of the cell envelope structure of Pseudomonas aeruginasa strain RM46 has been further localized after separation of inner and outer membrane components. The peptidase is found associated with the inner membrane. Succinate dehydrogenase and 2-keto 3-deoxyoctonate, inner and outer membrane components, respectively, mark the region of the two membranes. The majority of 2-keto 3-deoxyoctonate is found in the "Mixed 2" fraction rather than the "outer membrane" fraction. The outer membrane fraction, however, does contain phage receptor protein and for the purposes of this thesis further discrimination between the Mixed 2 and outer membrane fraction is unnecessary.

In addition to the localization of the peptidase within the inner membrane further analysis of the results presented here indicate that the active site of the enzyme faces the cytosol. This conclusion is based on the fact that P. aeruginasa strain RM46 will not grow on the modified peptide $Ac(Met)_3$. This peptide is broken into its constituent amino acids, however, by the action of the inner membrane bound peptidase. Were the active site of the enzyme facing

the periplasmic space $Ac(Met)_3$ would be broken down into methionine residues and transported as such. This model would allow for the growth of the organism using $Ac(Met)_3$ as a methionine source. Since this does not happen the active site of the enzyme must face away from the periplasm towards the cytosol. This model is based on the assumption that $Ac(Met)_3$ has free access to the inner membrane. It is likely that this assumption is fact. The outer membrane of P. aeruginosa strain PA01 (the strain from which RM46 was derived) has been shown to contain four major proteins. One of these proteins when placed into reconstituted vesicles renders the vesicle permeable to saccharides less than 9,000 molecular weight (27). Vesicles without this protein are impermeable to these saccharides. The outer membrane is thus thought of as a porous membrane barrier through which molecules having molecular weight of 9,000 or less pass by faciliated diffusion. Facilitated diffusion seems more likely than active transport under these circumstances since a source of ATP, necessary for most active transport systems, is not readily available at the outer membrane level. The molecular exclusion limit, 9,000 MW, for Pseudomonas aeruginosa is in fact quite high. $Ac(Met)_3$, with an approximate molecular weight of 450 would have no problem passing through the outer membrane. Even if the molecular exclusion limit proposed is somewhat too high, $Ac(Met)_3$ would be well within range for

facilitated diffusion. The exclusion limit for certain enteric bacteria for instance is 500 to 600 daltons.

Three models of peptide transport through the cell envelope have been proposed. (See Figure 11.)



A. True peptide transport

B. Modification during transport

C. Amino acid transport

Figure 11. Proposed models for transport of peptide across cell envelope.

The first model is illustrated in Figure 11A. In this model the peptide is broken down, after transport as an intact molecule, by a peptidase located in the inner membrane. In the second proposed transport process (Figure 11B) the peptide is cleaved simultaneously to transport, again by a peptidase located in the inner membrane. These models are still plausible given the data presented in this thesis. While technically not true peptide transport, the mechanism illustrated in Figure 11B could still be used to transport an antimicrobial substance conjugated with the peptide. Recognition and transport of the modified peptide would have

to leave the antimicrobial substance intact. The third model represented in Figure 11C is no longer considered a possibility. In this model the peptidase located in the outer membrane would break down peptides into constituent amino acids for transport as such. Since the peptidase has been demonstrated to be in the inner membrane this breakdown could not occur extracellularly.

The localization of the peptidase within the inner membrane invites one to speculate on its natural function. That function may involve the export of proteins. The trimethionine peptidase of the inner membrane of Pseudomonas aeruginasa strain RM46 may be the "signalase" proposed by Blobel in his "signal hypothesis" (9). This hypothesis concerns the export and integration of certain proteins within membranes. P. aeruginosa is known to excrete cellular proteases that some investigators believe cause much of the damage to the lung seen in cystic fibrosis patients. The serum of these patients also is known to contain a characteristic short peptide of unknown origin.

Much work is currently being done on protein excretion. This is an area of interest in part because many hormones have been demonstrated to be simple peptides. In Blobel's Signal Hypothesis the "signal" is a unique sequence of 10-40 amino acids coded for by the mRNA's of the proteins to be taken across the membrane (or integrated into it). This sequence

thought to be cleaved from the protein at the membrane by a "signalase"—a peptidase localized by the membrane that provides the signal removing function. Millstein et al. (61) were the first to propose that a unique amino acid sequence at the N terminus of the protein interacted specifically with another protein at the membrane. This interaction is thought to occur at the same time the rest of the mRNA is being translated. The net result is the binding of the ribosome to the membrane. Figure 12 is a schematic representation of the Signal Hypothesis.

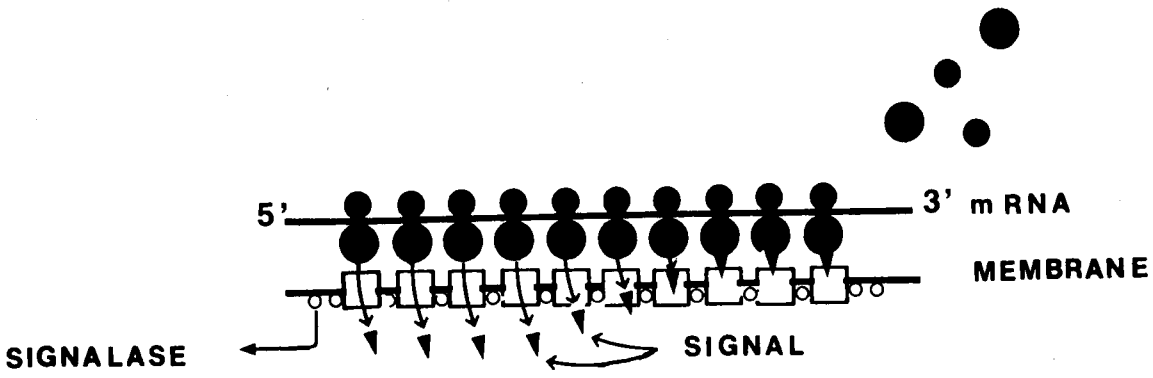


Figure 12. The signal hypothesis.

The initiation complex is formed at the "AUG" codon of the mRNA. There is, therefore, no specific requirement for unique ribosomes. The signal sequence is translated and associated with the membrane bound protein. By virtue of the translated protein the ribosome is transiently associated

with the membrane. Due to the signal sequence the rest of the protein is pulled through the tunnel created by the alignment of the ribosome and membrane protein. Once through the membrane the signalase removes the initial signal sequence. The signalase by nature must be localized in the vicinity by attachment to a cell envelope structure. Once the message has been completely translated and the protein taken through the membrane, the ribosomes dissociate into their component subunits.

There have been reports of the existence of a "signalase" in some systems. Blobel (9) in 1975 noted peptidase activity of rough microsomes from dog pancreas that cleaved presecretory proteins. He was able to solubilize this peptidase from the membrane with sodium deoxycholate and demonstrate peptidase activity on prolactin and pre-growth hormone. It may be that the peptidase activity of the membrane in P. aeruginasa strain RM46 is a "signalase." If this is true, its function would be involved in the excretion of proteins through the membrane.

It would be worthwhile to continue attempts at solubilization of the peptidase in order to ask further questions about its function. Although the preliminary results indicate that peptidase activity is not possible without the membrane it may be possible to restore enzymatic activity with the addition of long chain hydrocarbons, or by

reconstitution into membrane vesicles with similar lipid content to the inner membrane.

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