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I am submitting herewith a dissertation written by Rebecca Carol Craven entitled "Chemotaxis of *Pseudomonas aeruginosa*: The Control of Chemotaxis by Nitrogen and the Role of Methionine in Chemotaxis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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CHEMOTAXIS OF PSEUDOMONAS AERUGINOSA: THE CONTROL OF CHEMOTAXIS BY
NITROGEN AND THE ROLE OF METHIONINE IN CHEMOTAXIS

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Rebecca Carol Craven

December 1981

3055607

ACKNOWLEDGMENTS

I wish to thank my committee members Drs. Thomas Montie, Raymond Beck, Jeffrey Becker, Leaf Huang and Robert Miller for their criticisms and helpful suggestions during the course of this work. In addition I want to extend special thanks to Dr. Montie for his continued support and encouragement. The support of many people has made the long road easier. In particular I am grateful to my lab mates who have offered their friendship, helping hands and helpful suggestions, my family for their love and encouragement all along, and John Wills whose friendship and love has made this last year very special.

This project was supported in part by an NIH predoctoral traineeship from the National Institute of Allergy and Infections Disease through training grant number AI07123-04.

ABSTRACT

The regulation of amino acid chemotaxis by nitrogen and the role of protein methylation in chemotaxis of the gram-negative bacterium Pseudomonas aeruginosa were investigated. The quantitative capillary tube technique was used to measure chemotactic responses of bacteria to spatial gradients of amino acids and other attractants. Chemotaxis toward serine, arginine, and α -aminoisobutyrate (AIB) was sharply dependent on the form in which nitrogen was presented to the bacteria. Bacteria grown on mineral salts-succinate (MMS) with potassium nitrate gave responses to amino acids that were 2 to 3 times those of cells grown on ammonium sulfate and 10 to 20 times those of cells grown in MSS with casamino acids (CAA) as the nitrogen source. A combination of ammonium sulfate and glutamate was as effective as CAA in depressing serine taxis. The threshold concentration for AIB taxis was consistently lower in nitrate-grown bacteria than in ammonia-grown bacteria. Responsiveness to sodium succinate, however, was not subject to regulation by nitrogen, and glucose chemotaxis was inhibited, rather than enhanced, in nitrate-grown bacteria. These results indicate that chemotaxis of P. aeruginosa toward amino acids is subject to regulation by nitrogen and that this regulation probably occurs at the level of the chemoreceptors or transducers.

P. aeruginosa was further examined for the presence of a chemotaxis-associated methyltransfer reaction. The cells were labeled with [methyl-³H]-methionine in the presence of chloramphenicol and then stimulated with chemoattractants. Labeled components were analyzed by

fluorography of sodium dodecyl sulfate-polyacrylamide electrophoretic gels. Two bands of approximately 73,000 daltons were methylated in response to stimulation by arginine, serine or AIB. The strength of the methylation response corresponded to the strength of the chemotactic stimulus. The level of methylation decreased in response to attractant dilution. The results of labeling of whole cells with [^{35}S]methionine and in vitro labeling with S-adenosyl-[methyl- ^3H]methionine indicated that the 73,000 dalton band is labeled by a methyltransfer reaction. This methylated species, therefore, appears to be analogous to the methyl-accepting chemotaxis proteins (MCP) of the enteric bacteria.

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LIST OF SYMBOLS

AIB	α -aminoisobutyric acid
CAA	casamino acids
cyclic GMP	guanosine 3':5'-cyclicmonophosphate
EDTA	ethylenediaminetetraacetic acid, disodium salt
LB	Luria broth
MCP	methyl-accepting chemotaxis protein
MSS	mineral salts-succinate medium
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

Taxis is the movement of an organism in response to an external stimulus. Such movement may be in the direction of the stimulus (positive taxis) or away from the stimulus (negative taxis). Motile bacteria exhibit a variety of tactic responses of which the most studied is chemotaxis (the tactic response to chemical stimuli). Other types of tactic phenomena include the phototactic (Hildebrand and Dencher, 1975) and thermotactic (Maeda et al., 1976) responses of bacteria.

Chemotaxis of bacteria was described in the 1880's by the German investigators Engelmann and Pfeffer. Since that time a long list of bacterial genera have been investigated. Weibull (1960) reviewed the early literature regarding bacterial tactic phenomena and listed 23 genera whose members have been studied in terms of chemotactic responses. Those studies prior to the 1960s were mainly qualitative descriptions of bacterial behavior.

During the last fifteen years the mechanisms by which bacteria detect chemical signals and alter behavior in response to those signals have been the subject of intensive investigation (Springer et al., 1979; Koshland, 1981). The bacterium represents a simple, single-celled sensory response system that shares with higher sensory systems the properties of sensing, adaptation and regulation. It is hoped that the elucidation of mechanisms of behavioral control in bacteria will lead to greater understanding of complex organisms.

Chemotactic Stimuli

Attractants. The early chemotaxis studies involved microscopic examinations of responses of a population of bacteria to the presence of a gradient of an attractant chemical. The use of the capillary tube, first introduced by Pfeffer, was adapted by Adler (1969, 1973) to quantitate chemotactic responses of bacteria. In this method a capillary tube containing attractant is placed into a pool of bacterial cells and the number of bacteria accumulating inside the capillary is determined by a plate count of the capillary contents. This method has been widely used to examine the tactic responses of bacteria to a broad range of chemicals. The enteric bacteria Escherichia coli and Salmonella typhimurium have been most thoroughly studied in this regard.

Mesibov and Adler (1972) measured the chemotaxis of E. coli toward a large number of amino acids. Alanine, asparagine, aspartate, cysteine, glutamate, glycine, methionine, proline, serine, and threonine were attractants for E. coli, while arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, phenylalanine, tryptophan, tyrosine and valine were not attractants. Melton et al. (1978) found quite similar responses in S. typhimurium with the exception that glutamine was a very strong attractant for S. typhimurium. Bacillus subtilis (Ordal et al., 1977) was attracted to all those amino acids that attract E. coli and S. typhimurium plus nine more. Among the sugars that act as attractants for E. coli are D-fructose, D-fucose, D-galactose, lactose, maltose, N-acetyl-D-glucosamine, 6-deoxy-D-glucose and methyl- β -D-glucoside (Adler et al., 1973). The range of sugars that

attract S. typhimurium is very similar with the exception that maltose does not attract that organism.

Many types of chemicals in addition to amino acids and sugars are attractants for various bacteria. The nucleotides thymine, adenine, cytosine, guanine, and uracil and the vitamins biotin and thiamine are attractants for Pseudomonas lachrymans (Chet et al., 1973). Oxygen is a powerful attractant for many bacteria. Baracchini and Sherris (1959) tested the aerotactic response of a large number of bacterial species. The aerobic bacteria exhibited positive aerotaxis while the anaerobic bacteria were negatively aerotactic. Adler (1966) demonstrated a strong aerotaxis of E. coli and showed that E. coli migrating through soft agar forms a sharp migrating ring as the bacteria deplete oxygen at the site of inoculation and move outwards toward higher concentrations of oxygen. Finally, some inorganic ions can serve as chemoattractants. Moench and Konetzka (1978) reported that ammonium ions attract Pseudomonas aeruginosa. Their observation has not been supported by the results of this author using a different strain of P. aeruginosa (Moulton, 1977). Divalent cations in the form of metal ion-citrate complexes are attractants for S. typhimurium but not for E. coli (Igolia and Koshland, 1979).

In general, chemoattractants are substances that serve as nutrients for the organism in question. Melton et al. (1978) found that the amino acids which elicited the strongest responses from S. typhimurium were those that can serve as the sole nitrogen and carbon source or sole carbon source for that organism. However, metabolism is not a prerequisite for a chemical to be attractive. This was proven

conclusively by Adler (1969) who demonstrated that some extensively metabolized chemicals do not attract bacteria, while some chemicals that are non-metabolizable do attract bacteria; further, mutant bacteria that can no longer metabolize a chemical may still be attracted to it, and some mutants which fail to respond chemotactically to an attractant are still able to metabolize it.

Repellants. Chemorepellants are chemicals which elicit a negative tactic response from organisms. Tso and Adler (1974) tested a wide variety of substances for a negative tactic effect on E. coli. The following nine classes of repellants were defined on the basis of competition experiments, genetic studies and chemical structure: fatty acids (C2 through C6), aliphatic alcohols, hydrophobic amino acids, indole, aromatic compounds (benzoate, for instance), high pH, low pH, mercaptans, and inorganic ions (Co^{2+} and Ni^{2+}). Tso and Adler concluded that although most of the chemicals with repellant activity are toxic to E. coli, toxicity was neither necessary nor sufficient to cause negative taxis.

Seymour and Doetsch (1973) found negative responses to high pH and to low pH by nine species of bacteria including P. aeruginosa and P. fluorescens. Young and Mitchell (1973) concluded that several hydrocarbons were repellants for marine bacteria based on the observation that sublethal concentrations of the hydrocarbons reversed the positive chemotactic response toward an attractant. However, these results were not convincing since the authors did not test the effects of the

hydrocarbons on the accumulations in buffer capillaries to measure effects on the motility of the cells.

Receptors for Chemotactic Stimuli

The chemotactic receptors that have so far been identified and well characterized fall into two major classes, the sugar binding proteins and the serine and aspartate receptors.

Sugar binding proteins. The first group of receptors includes the ribose, galactose, and maltose receptors of E. coli (Koman et al., 1979; Hazelbauer, 1975; Hazelbauer and Adler, 1971), the ribose and galactose receptors of S. typhimurium (Fahnestock and Koshland, 1979; Aksamit, 1974), and the glucose receptor of P. aeruginosa (Stinson et al., 1976, 1977). These receptors are members of the group of small (30,000 dalton) soluble proteins known as periplasmic binding proteins (Oxender and Quay, 1977). Such proteins are located between the inner and outer membranes of gram-negative bacteria and are soluble or quite loosely bound since they can be easily extracted from the cells by cold osmotic shock treatments which disturb the integrity of the outer membrane. Most interesting is the fact that these periplasmic sugar binding proteins, in addition to acting as chemotactic receptors, also serve as the binding component for the corresponding sugar transport systems. Mutations in the gene for a particular binding protein results in the loss of both chemotaxis toward and transport of that sugar (Hazelbauer and Adler, 1971).

The sugar binding proteins interact with transmembrane proteins, called transducers or signallers, which transmit information concerning chemoeffector binding to the interior of the cell (Koshland, 1981). To date three transducer proteins have been identified. These proteins are covalently modified by methylation and demethylation reactions in response to chemotactic stimulation. Therefore, the transducer proteins have been designated as methyl-accepting chemotaxis proteins or MCPs. The three proteins MCP I, MCP II and MCP III are the products of the tsr, tar, and trg genes, respectively. The role of the methylation of MCP I, II, and III in chemotaxis will be discussed more thoroughly below (pages 13-23).

The transducer for the ribose and galactose receptors is MCP III, while that for the maltose receptor is MCP II (Hazelbauer et al., 1981; Springer et al., 1977b). The binding of a sugar attractant to its receptor causes a conformational change in the binding protein allowing it to interact with the appropriate transducer protein. Zukin et al. (1979), using a variety of physical techniques, demonstrated induced conformational changes in the ribose binding protein and the galactose binding protein upon interaction of the binding proteins with their substrates. Koiwai and Hayashi (1979) demonstrated binding of the maltose binding protein to the MCP II following binding of the attractant maltose to the binding protein. As the result of such stimulation the transducer protein is methylated by a specific methyltransfer reaction (see the discussion of MCPs below).

Serine and aspartate receptors. The serine and aspartate receptors of E. coli and S. typhimurium (Clarke and Koshland, 1979) represent an entirely different class of chemoreceptors. These receptors are transmembrane proteins of approximately 60,000 daltons. Recently the serine and aspartate receptors have been identified as the tsr and tar gene products; these receptors are identical to the methyl-accepting chemotaxis proteins I and II, respectively (Wang and Koshland, 1980; Hedblom and Adler, 1980). These receptors have high affinity for their substrates and are highly specific. Approximately 2500 tsr and 1200 tar receptors are present per cell. Evidence for the identity of the receptors with the tar and tsr methylated proteins is the following. Antibody to fragments of tsr or tar proteins precipitates a protein of 60,000 daltons which is carboxymethylated and retains binding activity. Aspartate binding activity copurifies with the methylated tar protein, and partially purified tar product when incorporated into artificial membranes retains aspartate binding and aspartate-sensitive methylation and demethylation.

The tsr and tar receptors are transducers which upon binding serine and aspartate transmit signals directly to the interior of the cell. These proteins further serve as the focusing system for other receptors. As noted above, the maltose receptor after binding maltose interacts with the tar protein which transmits that information to the interior of the cell. The tar protein is also the transducer for the divalent cation repellants (Ni^{2+} and Co^{2+}). Similarly, the tsr protein, in addition to its role as receptor/transducer for serine, is also the transducer for acetate, benzoate, indole and leucine (Springer et al., 1977 b). No receptors for these substances have been found to date.

The involvement of the methylated proteins (tar, tsr, and trg) in signal transmission will be discussed more thoroughly below.

Other receptors. A number of other receptors have been identified but less thoroughly characterized. Taylor et al. (1979) and Laszlo and Taylor (1981) have described evidence that the receptors for oxygen, nitrate and fumarate in E. coli and S. typhimurium are the terminal components of the respective electron transport pathways--cytochrome o, nitrate reductase, and fumarate reductase. The effects of anaerobiosis and respiratory inhibitors indicate that electron acceptor taxis is mediated by the electron transport system and by changes in proton motive force. The response to these attractants is independent of the tsr, tar, and trg proteins. Laszlo and Taylor (1981) have proposed the involvement of a separate signalling protein for oxygen, blue light, nitrate and fumarate. Whether or not methylation is involved has not yet been determined.

The phosphotransferase system (PTS) sugars glucose, mannitol, mannose and sorbitol are also chemoattractants for E. coli, and the chemotactic receptors for these substances are the proteins of the PTS-system (Adler and Epstein, 1974). Like the electron acceptors these attractants do not act through the tsr, tar and trg proteins. Existence of a PTS transducer has been hypothesized (Hazelbauer and Engström, 1980).

It is interesting that Koiwai et al. (1980) and Minoshima and Hayashi (1980) have identified a fourth species of MCP separate from

the MCPs I, II, and III; the level of methylation in MCP IV, like that in the first three species of MCP, is affected by mutations in the che X and che B genes which code for the chemotaxis methyltransferase and methylesterase, respectively. However, there is no evidence to indicate which attractants, if any, function through MCP IV. It is tempting to speculate that the PTS sugar receptors might use MCP IV as transducer.

Finally, a number of lipophilic weak acids such as benzoate, acetate, and salicylate are repellants while lipophilic weak bases such as ammonium, ethanolamine, and methylamine are attractants for E. coli. Repaske and Adler (1981) and Kihara and Macnab (1981) have presented clear evidence that these substances are not detected by specific receptors, but rather that they act by causing a change in the intracellular pH. The cells detect this change and respond accordingly. The response to changes in intracellular pH appears to be mediated by MCP I, the tsr protein (Repaske and Adler, 1981).

Swimming Behavior and Chemotaxis

The flagellum is the bacterial organelle which is responsible for motility. Motile bacteria possess one or more of these structures which are rigid helical filaments and which propel the cell through an aqueous medium by rotating relative to the cell body in a manner somewhat analogous to a propellor (Doetsch and Sjoblad, 1980; Silverman and Simon, 1974).

Runs and tumbles. Microscopic examinations of free swimming bacteria have described a typical pattern of motion (Berg and Brown, 1972). A typical bacterial cell in the absence of a chemotactic stimulus swims in straight or slightly curved lined (runs) punctuated at intervals by brief periods of uncontrolled motion (tumbles). The cell thrashes about for the duration of the tumble, then resumes swimming in a straight line in a new and randomly chosen direction. A given population of bacteria shows a characteristic frequency of tumbles.

Chemotactic stimuli alter the normal, unstimulated tumble frequency. Exposure of the bacteria to an increasing concentration of attractant causes a suppression of tumbles--the tumble frequency decreases and the mean length of the runs increases. A decrease in attractant concentration causes more frequent tumbling and shorter runs (Berg and Brown, 1972; Macnab and Koshland, 1972; Tsang et al., 1973). The effect of this modulation of tumble frequency is to cut short the cell's forward motion when a decrease in attractant concentration is detected and to continue forward motion when an increase is detected. Repellants have the opposite effect of attractants. Increasing repellent concentration causes the bacteria to tumble more frequently, while the decreasing repellent concentration causes a suppression of tumbling (Tsang et al., 1973). As a result forward motion of the bacterium is halted when a repellent is detected. Suppression of tumbling when a decrease in repellent is detected allows the cell to escape from a region containing repellent.

Silverman and Simon (1974) and Larsen et al. (1974) used a clever approach of tethering bacteria to a solid surface by means of anti-flagella antibody to demonstrate that the flagellum rotates relative to the cell body. Since only the flagellum was attached to the solid surface the cell body was free to rotate. An individual cell of E. coli was found to rotate with a speed of two to nine revolutions per second. The cell bodies were observed to rotate mainly counterclockwise (CCW), but occasionally the cell would reverse direction and rotate clockwise (CW) for a few turns before resuming CCW motion (Silverman and Simon, 1974). By examining wild type, as well as constantly tumbling and constantly swimming chemotaxis mutants, Larsen et al. (1974) proved that the CCW rotation mode corresponds to straight swimming while CW rotation corresponds to tumbling behavior. Attractant stimulation caused extended CCW rotation while repellants caused CW rotation. Taylor and Koshland (1974) used a similar technique to prove that the monoflagellated Pseudomonas citronellolis, like the peritrichously flagellated E. coli, alternates between CW and CCW rotation of the flagellum and modulates the frequency of CCW/CW switching in response to chemotactic stimulation in the same manner as does E. coli.

Adaptation. Chemotactic bacteria exhibit the behavioral phenomenon of adaptation (Koshland, 1981). When bacteria such as E. coli or P. citronellolis are exposed to temporal gradients of chemicals by mixing the cells with attractant or repellant, the bacteria respond with the appropriate alteration in tumble frequency. After a period

of time (seconds to minutes), the length of which depends upon the nature of the stimulus, the bacteria return to the prestimulus tumble frequency even though the stimulating chemical is still present (Macnab and Koshland, 1972; Tsang et al., 1973; Larsen et al., 1974). This cessation of the chemotactic response in the continued presence of the stimulus is known as adaptation. Although the adapted cell has ceased to respond, dilution of the attractant will cause a tumbling for a brief period before the unstimulated behavior resumes. This is sometimes referred to as deadaptation. The behavioral phenomena of stimulation, adaptation and deadaptation are characteristic of other types of sensory systems such as those of eucaryotic cells (Koshland, 1981). The biochemical basis of these phenomena is under intensive investigation in the hope of eventually understanding the molecular mechanisms of sensory behavior in bacteria and higher organisms.

Role of Methionine in Bacterial Chemotaxis

A major development in the elucidation of details of the chemotactic response system was the observation by Adler and Dahl (1967) that the amino acid L-methionine is a requirement for chemotaxis. The methionine auxotroph of E. coli studied by Adler and Dahl was incapable of chemotaxis in the absence of methionine although the bacteria were fully motile. Leucine and threonine, also auxotrophic requirements for that strain, were not required for chemotaxis. Armstrong (1972a,b) was the first to show evidence that, rather than methionine per se,

the metabolite S-adenosylmethionine (SAM) was involved in the chemotactic process. This was confirmed in S. typhimurium by Aswad and Koshland (1975).

Microscopic studies of S. typhimurium (Aswad and Koshland, 1974), B. subtilis (Ordal, 1976), and E. coli (Springer et al., 1977a) showed that bacteria deprived of methionine were capable of responding to attractants with a suppression of tumbling but that the period of smooth swimming was abnormally long. The bacteria could not adapt normally to the chemical stimuli. The conclusion from these studies was that methionine is not involved in the detection of chemotactic stimuli or in the alteration of tumble behavior in response to the stimuli, but is involved in adaptation to the stimuli. This conclusion later received support from genetic studies which showed that chemotaxis mutants which are deficient in an enzyme which carries out an S-adenosylmethionine-mediated methyltransfer reaction are defective in adaptation (Parkinson and Revello, 1978; Springer and Koshland, 1977; Goy et al., 1978).

Methyl-Accepting Chemotaxis Proteins (MCPs)

Identification and characterization of MCPs. The identification of a role of methionine in the chemotactic process and the indication that the metabolite SAM is the actual active agent in chemotaxis suggested that a methyltransfer reaction might be involved. This led several investigators to look for such a reaction in the chemotactic

bacteria and nonchemotactic mutants derived from them. Kort et al. (1975) first described a methylated protein in the membrane of E. coli which was altered by several chemotaxis mutations and coreverted with those defects. The level of methylation of that protein was increased by chemoattractant stimulation. This protein was designated the methyl-accepting chemotaxis protein (MCP).

The complexity of the role of methylation in chemotaxis soon became evident when it was discovered the methylated proteins of the sort described by Kort et al. (1975) were coded for by at least three genes: tsr, tar, and trg (Springer et al., 1977b; Silverman and Simon, 1977; Kondoh et al., 1979). Each gene codes for a diversity of forms which run as a set of several, closely spaced bands on SDS-polyacrylamide gel electrophoresis. These three sets of bands have been designated MCP I, II, and III and correspond to the tsr, tar, and trg gene products, respectively. Some overlap of the bands of MCP I with those of MCP II occurs so that it is difficult to distinguish these except by the use of tsr and tar mutants.

The level of methyl group incorporation into the MCPs is controlled by attractants and repellants. As one might expect attractants and repellants have opposite effects: an increase in attractant or decrease in repellant causes an increase in methylation while decrease in attractant or increase in repellant leads to the loss of methyl groups from MCP (Goy et al., 1977). The attractants serine and α -aminoisobutyrate (AIB) and repellants leucine, indole, acetate and benzoate affect the methylation of the tsr protein (MCP I); the tar protein

(MCP II) responds to the attractants aspartate maltose and repellants Co^{2+} and Ni^{2+} (Springer et al., 1977b). Galactose and ribose stimulate methylation of trg protein (MCP III) (Kondoh et al., 1979). The triple mutant (tsr tar trg) is not methylated in response to any of those substances and also is nonchemotactic toward those substances.

Methylation of the MCPs is catalyzed by a protein methyltransferase which is highly specific for MCPs and will not methylate a variety of other proteins that are known to be methylation substrates (Springer and Koshland, 1977). The S. typhimurium MCP methylase is soluble protein of about 38,000 daltons. A similar enzyme has been isolated from B. subtilis (Ullah and Ordal, 1981).

Modification of the MCPs by methylation occurs at a glutamic acid residue (Kleene et al., 1977). This glutamyl methyl ester is an unusual form of methylation since most methylations are through N linkages to lysine or arginine (Paik and Kim, 1980). The ester linkage to glutamate or aspartate, which appears to be rare in procaryotes, has been reported in an E. coli ribosomal protein (Kim et al., 1977). Perhaps one reason so few instances of methyl ester modification of proteins have been reported is the instability of those linkages. The glutamyl and aspartyl methyl esters are especially labile to pH extremes. Upon alkaline hydrolysis the methyl group of MCP is released as methanol (Kleene et al., 1977). Release of methanol from whole cells of E. coli has been detected when [methyl- ^3H]-methionine labeled cells were subjected to chemotactic stimuli that cause demethylation of MCP (Toews

and Adler, 1979). The methylesterase which catalyzes this reaction in vivo has been identified (Stock and Koshland, 1978).

Nature of the multiple bands of MCPs. As described above, the MCPs of E. coli and S. typhimurium are encoded in three genes, and each gene product is seen in several forms. The banding patterns of the MCPs on SDS-polyacrylamide gels show a series of many bands between 55,000 and 65,000 daltons. DeFranco and Koshland (1980) noted that the four bands encoded by the tar gene of S. typhimurium are separated by distances corresponding to a difference of 1500 in molecular weight. The nature of the various forms of the MCP protein was very puzzling since it seemed unlikely that enough methyl groups could be added to the protein backbone to account for the 1500 dalton difference in molecular weight. In 1980 investigators in four different laboratories and that the various forms observed on gels represent the same basic protein and these various forms observed on gels represent the same basic protein with varying levels of methylation. The evidence for these conclusions is outlined below.

Engström and Hazelbauer (1980) demonstrated differential incorporation of methyl label into the various bands of a particular MCP depending upon the strength of the stimulus and the length of time following stimulation. The faster moving bands were more heavily labeled with increasing time after stimulation or with increasing strength of the stimulus. Paoni and Koshland (1979) had noted previously changes in the distribution of label between the various bands during the time course of the methylation response.

Chelsky and Dahlquist (1980) examined tryptic digestion products of MCP I and MCP II and found three distinct methylated peptides in each indicating that multiple methylation sites exist on each protein.

Boyd and Simon (1980) and DeFranco and Koshland (1980) used lambda transducing phages carrying the tar and tsr genes from E. coli and S. typhimurium, respectively, to program the specific synthesis of MCPs in UV-irradiated host bacteria. In this way the MCPs could be labeled in the backbone with [^{35}S]methionine and the behavior of the labeled protein in response to chemotactic stimuli studied. Attractant stimuli caused appearance of new, faster migrating labeled species which disappeared when attractant was removed. The multiple forms comigrated with authentic MCP bands from [methyl- ^3H]methionine labeled cells. If the host cell was defective in methyltransferase the faster migrating forms did not appear upon attractant stimulation. Similarly, a methylesterase defect in the host resulted in mainly faster moving forms. Treatment of the MCPs with alkali to remove methyl esters caused a shift of the labeled protein into the slowest migrating form.

These results taken together provide convincing evidence that the multiple forms of MCP represent the same protein backbone carrying different numbers of methyl groups. On the electrophoretic patterns of [^{35}S]methionine labeled cells the slowest migrating form is the unmethylated MCP protein, and as methyl groups are added to the protein backbone the labeled band shifts to the faster moving forms. The slowest moving band on gels of [methyl- ^3H]methionine labeled cells

represents the singly-methylated form of MCP. It is interesting that simple methylation of an MCP can cause a shift in electrophoretic mobility equal to a change of 1500 daltons (DeFranco and Koshland, 1980). It is probable that the change in the charge of the protein resulting from methylation affects the amount of SDS bound per molecule and causes a change in electrophoretic mobility.

Involvement of Methylation in the Chemotaxis of Other Organisms

Bacteria. Recent reports have described a role for protein methylation in the chemotaxis of two bacterial species which are very distantly related to the enteric bacteria. Ordal (1976) demonstrated that, like E. coli and S. typhimurium, the gram-positive bacterium Bacillus subtilis requires methionine for normal chemotaxis. Methionine starved B. subtilis showed abnormally long periods of smooth swimming after attractant stimulation. Recently Ullah and Ordal (1981) identified two bands in electrophoretic patterns of [methyl-³H]-methionine labeled cells which responded to stimulation with isoleucine with an increase in methylation. Methylation of these components was defective in mutants that display abnormal chemotactic behavior. A methyltransferase, isolated from B. subtilis, catalyzed the in vivo methylation of membrane components with mobility identical to the isoleucine responsive bands of whole cells. The label in these bands was not extracted with chloroform-methanol suggesting the labeled components were protein rather than phospholipid in nature. From this evidence Ullah and Ordal concluded that the two methylated species they have identified are analogous to the MCPs of the enteric bacteria.

Schmiz and Hildebrand (1980) have studied the chemotactic responses of the obligately halophilic Halobacterium halobium. They have identified a membrane component of H. halobium which responds to attractant stimulation with an increase in methylation and to repellent stimulation with a decrease in methylation. It is not clear from their abstract, however, how they determined that this membrane component is a protein.

Eucaryotic cells. Several studies have proven a role of methylation in chemotaxis of eucaryotic cells, but the lack of genetic approaches to the study of chemotaxis in these cells has severely limited the progress in this area. In the slime mold Dictyostelium discoideum stimulation with the chemoattractant cyclic AMP causes changes in the methylation of both protein and phospholipid components of the cell (Mato and Marin-Cao, 1979; Alemany et al., 1980). Protein methylation in response to chemotactic stimulation has been reported in rabbit neutrophils (O'Dea et al., 1978). Although no evidence for phospholipid methylation in bacterial chemotaxis has yet been found, regulation of phospholipid methylation by chemotactic stimuli has been identified by Pike et al. (1979) in human monocytes and by Hirata et al. (1979) in rabbit leukocytes. The roles of protein and phospholipid methylation in chemotaxis of eucaryotic cells are still unknown (Snyderman, 1981).

Chemotactic Mechanism: Adaptation and Excitation

Adaptation. As noted above, several lines of evidence indicate a role for the methylation of the MCPs in chemotactic adaptation. Cells which are starved for methionine are unable to carry out methyltransfer

reactions and show abnormal adaptation to chemotactic stimuli. Also, mutants defective in the enzyme which carries out the methyltransfer reaction show defective adaptation. These results are supported by further observations of the behavior of bacterial cells exposed to chemoeffectors which show that the methylation reaction parallels the adaptation process very closely (Goy et al., 1977). Goy et al. found that the magnitude of the increase in methylation of the MCPs was a function of the size of the stimulus, and the time required to reach the new methylation level was approximately equal to the duration of the behavioral response. Further the methylation response, as well as the behavioral response, show additivity when a combination of stimuli is presented to the cell. It appears that the methylation of MCPs regulates the sensory adaptation process in bacterial chemotaxis. However, how the level of methylation influences the direction of rotation of the flagella is still entirely unknown.

Excitation. What is the nature of the excitation event or signal? There is little evidence but much speculation on this point. Black et al. (1980) suggested that a change in intracellular concentration of cyclic GMP may be the excitation signal. In E. coli they detected a transient rise upon attractant stimulation and a transient drop upon repellent stimulation. Further, they found that adaptation mutants had altered cyclic GMP metabolism, that cyclic GMP inhibited demethylation, and that addition of cyclic GMP to the bacteria caused abnormal swimming behavior. This study was quite thorough and convincing. However, there has not yet been confirmation from other investigators (B. Taylor, personal communication).

Another theory of the regulation of bacterial behavior presented by Ordal and Fields (1977) proposes a role of calcium ion in the regulation of tumble frequency. Ordal proposes that the MCP acts as a calcium binding protein. His theory holds that attractant stimulation causes binding of cytoplasmic Ca^{2+} to MCP and that the resulting drop in internal Ca^{2+} concentration is the excitation signal which causes the flagellum to rotate CCW. Binding of attractant to the cell also stimulates the methylation of MCP. As methylation proceeds Ca^{2+} is released from the MCP until free Ca^{2+} has risen to its original level and flagellum rotation returns to its normal CCW/CW switching frequency (i.e., adaptation).

Direct evidence for the calcium theory is lacking, but several lines of evidence do support the involvement in bacterial chemotaxis. Ordal (1977) used a calcium ionophore to show that calcium concentration can control the swimming behavior of B. subtilis--high calcium caused incessant tumbling while low calcium concentrations caused incessant swimming. Recently Ullah and Ordal (1981) have demonstrated that calcium acts as a competitive inhibitor of MCP methylation with a K_i value of 80 nM. Such competition would be expected if the calcium binds to the site which is methylated by the methyltransferase. Further, it has been known for some time that the presence of functional MCPs in bacterial cells is necessary for normal excitation as well as adaptation (Springer et al., 1977b; Silverman and Simon, 1977) while actual methylation of the MCPs is necessary for adaptation only (Goy et al., 1977; Springer et al., 1977b).

The hypothesis that MCP may act as a calcium binding protein gains

credibility from the recent discovery that the MCPs are multiply methylated (Chelsky and Dahlquist, 1980). One can imagine that the multiple glutamyl residues in the unmethylated form of MCP serve as the binding site(s) for Ca^{2+} and that as methylation removes the negative charges binding activity is lost. There is precedence for this type of mechanism of control of calcium binding in eucaryotic cells. Binding of Ca^{2+} to prothrombin is regulated by a carboxylation reaction which adds carboxyl groups to two glutamic acid residues forming two Ca^{2+} binding sites (Stenflo and Suttie, 1977). Further, a recent study of calmodulin (the eucaryotic calcium binding protein) indicated that calmodulin may be carboxymethylated in vivo and that such methylation may control the activity of phosphodiesterase (Gagon et al., 1981).

Summary of current understanding. The current understanding of the chemotactic process can be summarized in the following way (Springer et al., 1979; Koshland, 1981). Binding of attractant to its receptor causes a conformational change in the receptor which in turn allows the receptor to bind to the appropriate MCP. The MCP acts as a signaller or transducer to transmit information concerning the binding of the attractant at the outside surface of the membrane to the interior of the cell. The interaction of the occupied receptor with the MCP stimulates two activities. First is excitation; a signal of unknown nature interacts with the flagellum rotation apparatus causing tumble suppression and straight swimming. The second effect is the initiation of methylation of the MCPs. The increase in the level of methylation of the MCPs results from the combination of an increased rate of the methylation reaction and decreased rate of demethylation reaction

(Kleene et al., 1979). The methylation level eventually reaches a new plateau and the cell returns to normal tumble frequency (the adapted state). Removal of attractant (or the addition of repellent) causes the opposite effects--a transient period of tumbling and a drop in methylation level of the MCP.

By necessity the information processing system must be focused since there are several thousand receptors but only one or a few flagella. Information from many different types of receptors flows through four or five transducer proteins (the MCPs), and finally information from those several transducers must be focused through some common component(s) so that an integrated signal is delivered to the flagellum. Approximately five genes have been identified whose products are essential to this central signal processing pathway but whose specific functions are still unknown (Koshland, 1981). The nature of the excitation signal and the nature of the "gear shift" which controls the direction of rotation of the flagella are at present the two most exciting questions remaining in the study of chemotaxis.

Chemotaxis by Pseudomonas Species

Most of the studies of bacterial chemotaxis have dealt with E. coli, S. typhimurium, and to a lesser extent, B. subtilis. The studies of chemotaxis in various Pseudomonas species have been few in number and quite limited in scope, describing primarily the types of chemicals that serve as attractants. Seymour and Doetsch (1973) tested responses of Pseudomonas aeruginosa and Pseudomonas fluorescens to a wide variety of chemicals. They recorded taxis toward a few carbohydrates (including glucose, ribose, sorbitol and sedoheptulose), but P. aeruginosa gave

no strong response to any amino acid. P. fluorescens responded to methionine, serine, threonine, and valine. The poor amino acid taxis by P. aeruginosa might simply reflect the particular strain used or, in view of the results described in this manuscript, may be due to the fact that the bacterium was grown on trypticase soy broth.

P. aeruginosa taxis was studied by Moench and Konetzka (1978). Those authors found strong responses to several amino acids (arginine, aspartate, glutamate, leucine) and weaker responses to organic acids (succinate and glutarate). Ammonium chloride was an attractant at a very low threshold, although the total accumulation in the attractant capillaries was very weak. No response to glucose was detected, probably due to the fact that the bacteria were grown on succinate.

P. aeruginosa was further characterized by Moulton and Montie, (1979). This organism was strongly attracted to serine regardless of the carbon source utilized for growth. In contrast, responsiveness to glucose and citrate was dependent on growth on those substances. Further, a peculiar inhibition phenomenon was described. Accumulation in response to an attractant gradient was inhibited by the presence of other attractants. At that time it was not determined whether the inhibition was due to the fact that the inhibiting compound was another attractant or whether metabolism of the second attractant was required to cause inhibition. It may be possible to answer that question by using α -aminoisobutyrate in place of serine.

Only one chemoreceptor of P. aeruginosa has been characterized. Stinson et al. (1977) identified a glucose binding protein and determined

that it functions as the chemoreceptor for glucose. Primose and Dodd (1979) have developed a photoaffinity labeling method which apparently labels the tyrosine chemoreceptor of P. aeruginosa. Exploitation of that approach should allow the isolation and characterization of the tyrosine chemoreceptor.

Among the studies of the chemotaxis of pseudomonads other than P. aeruginosa are the following. P. lachrymans, a cucumber pathogen, was found to be chemotactic toward a variety of amino acids, nucleotides, and vitamins, as well as toward plant extracts and water droplets from the leaf surface of plants (Chet et al., 1973).

P. putida was attracted to β -ketoadipate (Kariman and Ornston, 1981), and characterization of mutants defective in β -ketoadipate taxis indicated that the taxis and transport systems share one or more common components. By analogy with E. coli one might predict that the common component is the receptor.

Lynch (1980) characterized the chemotactic responses of P. fluorescens. He found that, like P. aeruginosa, P. fluorescens showed constitutive taxis to malate, succinate and serine and inducible taxis to citrate. Unlike P. aeruginosa, however, P. fluorescens did not respond to arginine unless the bacteria were grown on arginine.

Chemotaxis and Bacterial Virulence

Several groups of investigators have in recent years begun to question whether the chemotactic capacity of microorganisms might play a role in the pathogenicity of those organisms. A role of chemotaxis in the adhesion of Vibrio cholerae to mucosal surfaces has been suggested. Guentzel and Berry (1975) found that nonmotile mutants of

V. cholerae had lower virulence than the motile parent strains. Freter et al. (1979) found that chemotactic vibrios are more efficient than nonchemotactic mutants in associating with intestinal mucosa in vivo. An involvement of motility in the pathogenesis of P. aeruginosa burn infections has been suggested by McManus et al. (1980) who found reduced virulence in a series of nonmotile mutants of P. aeruginosa. In most cases the nature of the defects in the mutants used in these studies has not been well characterized.

The motility and chemotaxis of several strains of P. aeruginosa which are used in virulence studies have been characterized recently by this author (Craven and Montie, 1981). Strain M2, a highly virulent bacterium in the burned mouse, proved to be strongly chemotactic. Two avirulent strains tested, PA103 and WR5, were defective in motility and chemotaxis, respectively. These strains were isolated independently, and the correlation of virulence with motility and chemotaxis may be fortuitous. However, this observation of heterogeneity among P. aeruginosa strains has stimulated further research in the laboratories of Drs. Thomas Montie (The University of Tennessee, Knoxville) and Ian Alan Holder (Shriner's Burn Hospital, Cincinnati) into the possible role of chemotaxis in pathogenicity of P. aeruginosa. It is very interesting, also, that the nonmotile strain PA103 grows to the same level in burned skin as do the virulent strains, but the nonmotile PA103 does not spread beyond the skin, invade other tissues and kill the host (I. A. Holder, personal communication). This has led to the hypothesis that chemotaxis or random motility facilitates the spread of bacteria from the initial site of infection (Holder, Wheeler and Montie, in press). To approach

such questions of the role of chemotaxis in the pathogenesis of P. aeruginosa infections, a series of well-characterized motility and chemotaxis mutants of a highly virulent and chemotactic strain such as M2 will be essential.

Specific Aims of This Investigation

As described above very little is known about the mechanisms of chemotaxis of P. aeruginosa. No study has examined the behavior and the biochemistry of P. aeruginosa to determine whether the same mechanisms that operate in the enteric bacteria also pertain to this organism. Such knowledge will be necessary if a thorough characterization of chemotaxis mutants is to be undertaken.

The investigation described in this manuscript has focused on two primary questions. The first deals with the regulation of chemotactic responsiveness of P. aeruginosa. Although motility and chemotaxis functions in the enteric bacteria are subject to catabolite repression (Koshland and Anderson, 1980; Dobrogoz and Hamilton, 1971), earlier investigations found (with the possible exception of the glucose receptor) no evidence of catabolite repression of P. aeruginosa chemotaxis (Moulton and Montie, 1979). On the other hand, experiments preliminary to this present investigation indicated that chemotactic responsiveness to some attractants was severely depressed by growth in an amino acid rich medium. The question of nitrogen regulation of chemotaxis is approached in the first half of this study.

The second half of this study deals with the role of methionine in the chemotaxis of P. aeruginosa. It seemed unlikely that P. aeruginosa would possess a chemotactic mechanism entirely different from

the enteric bacteria. In view of the recent evidence of chemotactic methylation systems in B. subtilis (Ullah and Ordal, 1981) and H. halobium (Schmiz and Hildebrand, 1980), it appears that a role for methylation in chemotaxis is widely distributed throughout the bacteria. However, previous investigations (Moulton and Montie, 1979) failed to demonstrate a methionine requirement in P. aeruginosa chemotaxis. This current study was initiated to test for the possible involvement of a methylation system in the chemotaxis of P. aeruginosa.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacteria. The strain RM46 used for these studies, a derivative of Pseudomonas aeruginosa PAO, was obtained from Robert V. Miller (Loyola University, Chicago, Ill.). The strain RM46 is a methionine auxotroph of the Met V transduction group of Calhoun and Feary (1969) and was derived from the parent PAO by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. Strain RM9 is an arginine autotroph derived from P. aeruginosa PAO and was also obtained from Dr. Miller.

Stocks of the bacterial strains were kept at -70°C in Luria broth with 20% glycerol (the various media used are described in the following sections). At intervals of three to four months a sample from the frozen stock was streaked for isolation on tryptone agar. Isolated colonies were tested for auxotrophy in mineral salts medium with and without methonine (or arginine) and for motility on motility agar. The bacteria were stored as a dilute suspension in Luria broth at 4°C . These working stocks were used to inoculate daily cultures.

Growth conditions. The standard growth medium was a mineral salts mixture containing the following: dibasic potassium phosphate 0.04 M, monobasic potassium phosphate 0.02 M, ammonium sulfate 7.6×10^{-3} M, magnesium sulfate 2.0×10^{-4} M, and ferric chloride 9.2×10^{-6} M; for preparation, see the Appendix. Except where otherwise noted, sodium succinate at 0.4% served as the carbon source. In the text this is

referred to as mineral salts-succinate or MSS medium. Methionine or arginine, filter sterilized, was added to autoclaved medium to give 5×10^{-4} M. For some experiments ammonium sulfate was replaced by an equal concentration of potassium nitrate. Amino acids used as nitrogen sources were added to 0.5%.

Cultures were grown in 250 ml Erlenmeyer flasks containing 50 to 75 ml of MSS medium. The inoculum consisted of 0.2 ml of a stock suspension in Luria broth. The cultures were shaken overnight (14 to 16 hours) at 37°C with rotary shaking at 125 rpm. The overnight growth was diluted with fresh medium to give an optical density (A_{590}) of 0.04 to 0.06 on a Bausch and Lomb Spectronic 20 using a 1 cm cuvette. Incubation continued at 37°C with shaking until an A_{590} of 0.03 to 0.04 was reached. This required approximately three hours when the MSS medium was used. At this point the cells were in early logarithmic phase as determined from a plot of absorbance readings on a semi-logarithmic graph.

Amino acid starvation. Overnight cultures of bacteria were subcultured as usual in MSS medium containing methionine or arginine. Exponentially growing cells were centrifuged at room temperature at 3400 g for 15 min. and washed one time with room temperature MSS medium without amino acid. The cells were finally suspended in warm (37°C) amino acid-free medium and shaken at 37°C for up to four hours.

Other media. Luria broth (LB) consisted of 10 g Bacto-tryptone (Difco), 0.5 g yeast extract, 10 g sodium chloride and one liter distilled water. This mixture was neutralized to pH 7.0 before autoclaving.

Motility medium for Pseudomonas, described in the Manual for Clinical Microbiology (2nd edition, American Society for Microbiology), consisted of 10 g Bacto-tryptone (Difco), 3 g yeast extract, 5 g sodium chloride, 3 g agar and one liter distilled water. Final pH was 7.2. After autoclaving, the medium was poured into large 100 mm petri dishes (15 ml each) or small 60 mm dishes (7 ml each). Colonies to be tested for motility and chemotaxis were picked with a sterile inoculating needle and stabbed in the center of a motility plate. Plates were incubated at 37°C and examined periodically over a one to 24 hr. period for swarming and the distinctive rings which are characteristic of chemotactic bacteria.

Tryptone broth (TB) was prepared by mixing 10 g Bacto-tryptone (Difco) with 5 g sodium chloride in one liter distilled water. After autoclaving the medium was dispensed into sterile test tubes.

Tryptone agar was tryptone broth (TB) to which 16 g agar was added per liter of medium before autoclaving. Sterile medium, cooled to 50°C was dispensed 15 ml per 100 mm petri dish. Plates were kept at room temperature overnight to dry, then stored covered at 4°C.

Capillary Assay for Quantitation of Bacterial Chemotaxis

The swarming of motile bacterial cells toward and into the mouth of a capillary tube containing an attractant solution, first described by Pfeffer, was adapted by Adler (1973) for quantitation of chemotaxis. Viable plate counts were used to determine the number of bacterial cells which accumulate in attractant capillaries. For the studies described in this paper Adler's capillary technique was used with a few modifications as described below.

The bacteria were grown to logarithmic phase in MSS medium

(see above). The cultures were chilled on ice for 15 to 45 min., then washed twice with CTX buffer. The CTX buffer, composed of potassium phosphate at 0.05 M and pH 7.0, magnesium chloride at 0.005 M and disodium ethylenediaminetetraacetic acid at 2.5×10^{-5} M, was found in previous studies to give the optimal response of *P. aeruginosa* RM46 to serine in the capillary assay (Moulton, 1977; Moulton and Montie, 1979). After the final wash the bacteria were resuspended in room temperature CTX buffer to an A_{590} of 0.2, approximately 2×10^8 cells/ml and 45 μ g protein/ml. This suspension was used to fill chemotaxis chambers consisting of a slide and coverslip separated by a U-shaped piece of 2 mm glass rod. A flat surface in a 37°C warm room was covered with dampened absorbent paper. The chambers were arranged on this surface. Each chamber was filled with 0.4 ml of cell suspension. Plastic petri-dish lids were placed over the chambers to reduce evaporation. A thermometer on the damp surface registered approximately 32°C.

Chemoattractants dissolved in CTX buffer were used to fill one microliter capillary tubes (Drummond Scientific). One end of the tube was sealed in a flame, the tube warmed gently in the flame and the open end dropped into a beaker of attractant solution. As the tube cooled, the solution was drawn up one-third to one-half the length of the tube. The open ends of the filled capillaries were inserted into the cell pools in the chemotaxis chambers (one capillary per chamber). After 30 min. the capillaries were withdrawn and contents expelled into tubes of cold tryptone broth (TB). Further dilutions were made in TB as necessary. Samples of 0.05 ml were streaked on duplicate tryptone agar

plates. After incubation of the plates overnight at 37°C the colonies were counted. The mean accumulation in triplicate assays was expressed in terms of total cells per capillary.

Calculation of Threshold Concentrations

The capillary assays were performed as described. Capillaries contained the attractant being tested in threefold concentration increments. The average accumulation of bacteria at each concentration was determined and the data were plotted on a double logarithmic graph. A straight line was fitted by linear regression to the points on the rising portion of the concentration-response curve. The concentration at which that line intersected the baseline defined by the background accumulation (in capillaries containing no attractant) was taken as the threshold.

Methyltransfer Experiments

Methyl labeling. Logarithmic phase bacteria were chilled on ice before washing twice with cold (4°C) CTX buffer. The final pellet was resuspended in CTX buffer to an optical density (A_{590}) of 0.3 to 0.6 (3×10^8 to 6×10^8 cells/ml) in warm (37°C) CTX buffer. This cell suspension was first treated with chloramphenicol to halt protein synthesis, then incubated with [methyl- ^3H]methionine before the cells were subjected to a chemotactic stimulus. The details of this procedure follow.

The washed bacteria were added to a flask containing enough chloramphenicol to give a final concentration of 200 $\mu\text{g/ml}$. This mixture was shaken at 37°C for 15 min. to allow for chloramphenicol to exert its

inhibitory effects. The labeling period was begun by the addition of [methyl-³H]methionine at a final concentration of 0.5 μ M and specific activity of 30 C/mole. After incubation with labeled methionine for 15 min., the cells were subjected to an attractant stimulus achieved by a single addition of attractant solution to the cell suspension. Control (unstimulated) cells received an equal volume of CTX buffer. Following a stimulation period of up to 15 min. the cells were pelleted by centrifugation at 12,000 g for 10 min. The pellet was dissolved in electrophoresis S buffer and heated in a boiling water bath for a period sufficient to dissolve the cells. Approximately five minutes was necessary to lower the viscosity of samples prepared from whole cells. The final protein concentration in the prepared samples was approximately 1 mg/ml.

For most experiments a five milliliter volume of cells was placed in a small (25 ml) Erlenmyer flask or a 15 ml centrifuge tube for the labeling procedure. When smaller volumes of cell suspension were used the incubations were carried out in microfuge tubes and the cells pelleted by centrifugation for 90 sec. in a Fisher microfuge.

For some experiments the methylation reactions were halted by the addition of trichloroacetic (TCA) acid to 5%. The cell-TCA mixtures were chilled on ice for at least 15 min., then the precipitates were collected by centrifugation and suspended in S buffer. The presence of TCA caused the bromphenol blue indicator of the S buffer to turn yellow. Small amounts (5-10 μ l) of 0.1 N sodium hydroxide were added to the precipitates just until the indicator turned pale blue; the final pH was approximately 6. Finally, the samples were heated at 100°C for one to two minutes to dissolve. Alternatively formaldehyde (to 0.25%) was

added to halt methylation process, then the cells were pelleted and resuspended in S buffer and boiled as usual.

Polyacrylamide gel electrophoresis. The proteins in S buffer were separated on thin (0.75 mm) vertical slabs of polyacrylamide gel containing sodium dodecyl sulfate (SDS) using the buffer system of Laemmli (1970) as described by Ames (1974). The standard separating gel mixture contained acrylamide in a total concentration of 10% with acrylamide and NN'-methylene-bis-acrylamide in a ratio of 30:0.8. The separating gel buffer consisted of 0.1% SDS in 0.375 M Tris-Cl at pH 8.8 (see Appendix for details). The polymerization reaction was initiated by the addition of ammonium persulfate and tetramethylethylenediamine (TEMED). The stacking gel mixture contained 5% acrylamide and 0.1% SDS in 0.125 M Tris-Cl at pH 6.8 and was polymerized with ammonium persulfate and TEMED. Samples were loaded with a Hamilton 50 μ l syringe that was flushed with 1% SDS between samples. Thirty micrograms (30 μ g) of protein per well gave the sharpest electrophoretic patterns. Electrophoresis was carried out at a constant current of 10 mA for one slab or 20 mA when two were run concurrently. During electrophoresis the voltage increased from 100 volts to approximately 300 volts. A typical 18 cm gel required a 5 to 6 hour run time.

At completion of the electrophoretic run the polyacrylamide slabs were stained with Coomassie Blue R250 according to the method of Fairbanks et al. (1971). Details of this method can be found in the Appendix of this paper. After destaining, the gels were dried onto filter paper under vacuum on a slab gel drier (Hoeffer Scientific Instruments) or were prepared for fluorography according to the following procedure.

Fluorography. After destaining the polyacrylamide gels were soaked in the autoradiography enhancer, EnHance (New England Nuclear), according to the manufacturer's instructions. The treated gels were dried on to filter paper. At this step care was taken to avoid keeping the gel on the heater under vacuum for an unnecessarily long time. Generally one hour drying time was sufficient for a 10% acrylamide gel. Higher percentage gels which required longer to dry frequently developed cracks during the drying process. The dried gels were placed on Kodak X-Omat R film which was subjected to a sensitizing light flash (Laskey and Mills, 1975) immediately prior to exposure to the gel. Films were exposed at -70°C for four days to two weeks depending on the amount of radioactivity in the samples. Autoradiography rather than fluorography was used with one gel containing ^{14}C labeled material. The gel was not treated with EnHance and the film was not presensitized. Due to the lower specific activity of the samples in this experiment the exposure was continued for 114 days. Exposed films were developed according to the manufacture's instructions. Density of the radioactivity in bands on the fluorograms was determined by densitometric scanning using a Quick Scan densitometer (Helena Laboratories).

Molecular weight determinations. The molecular weights of radioactive bands were estimated by a comparison of the mobility of the bands with the mobilities of a series of proteins of known molecular weights (Weber and Osborn, 1969). The following proteins were used as standards: egg white lysozyme (14,300 daltons), bovine milk β -lactoglobulin (18,400), porcine pancreas trypsinogen (24,000), ovalbumin (45,000), bovine plasma albumin (34,700), rabbit muscle phosphorylase a (94,000).

Isolation of Membrane Fraction

Whole cells of P. aeruginosa were washed as usual for a methylation experiment and resuspended in CTX buffer to an optical density (A_{590}) of 0.66 in a final volume of 100 ml. This suspension was labeled with [methyl- ^{14}C]methionine for 15 min. according to the standard methylation procedure. The cells were then stimulated with 1 mM serine for 15 min. Formaldehyde was added to give 0.25% and the cells were chilled on ice. The chilled cells were centrifuged at 12,000 g for 10 min. at 4°C and resuspended in 20 ml cold 0.0625 M Tris-Cl, pH 6.8. The bacteria were broken by two passages through the French pressure cell at 15,000 psi. After a low speed centrifugation (12,000 g for 10 min. at 4°C) to remove unbroken cells, the membrane fraction was pelleted by centrifugation at 150,000 g on a 42.1 rotor (Beckman) for 30 min. The supernatant was carefully removed and both fractions were stored at -20°C until used. The membrane and soluble fractions were dissolved in electrophoresis S buffer to a concentration of 1 mg protein/ml. The membrane and supernatant fractions together with unstimulated and serine stimulated whole cell preparations were separated on a 16 cm long 10% polyacrylamide gel. The dried gel was exposed to Kodak X-Omat R film for 114 days.

Measurement of Carbon Dioxide Release from AIB

Radioactive CO_2 released from a suspension of P. aeruginosa incubated with α -amino[1- ^{14}C]isobutyric acid was determined by scintillation counting of radioactive material trapped in sodium hydroxide. Small (25 ml) side arm flasks fitted with rubber stoppers were filled with a suspension of bacterial cells in CTX buffer (A_{590} 0.6, 150 μg protein/ml). Attached to the top stopper was a small plastic dipper in

which was placed a folded piece of filter paper and 0.4 ml of 0.2 N NaOH. The reaction was started by the addition of α -[1- ^{14}C] amino-isobutyric acid (50 mC/mole, 4×10^{-6} M final concentration) to the cell suspension. The flasks were sealed and incubated at 37°C without shaking for six hours. At two, four and six hours the reactions in two flasks were stopped by the addition of 1.0 ml 0.3 M sulfuric acid injected through the side arm stopper. This lowered the pH of the cell suspension to about 2.0. The flasks were then shaken for two hours to overnight. The acidification step allowed volatilization of CO_2 so that it could be trapped in the alkali-soaked filter paper. The plastic well, filter paper and NaOH were placed in a scintillation vial with Bray's solution (Research Products, Inc.) and counted.

Protein Determination

A modified Lowry protein assay was used as described by Markwell et al. (1978) to determine the protein content of samples dissolved in electrophoresis sample (S) buffer. Twenty microliter (20 μl) volumes of the cell extracts in S buffer were diluted to one milliliter with 1% SDS. Bovine serum albumin (Pentex, Inc., Kankakee, Ill.), prepared in a solution of 1% SDS with one part S buffer to 50 parts SDS, was used as the standard protein. Each unknown sample and BSA standard (1 ml each with 10 to 100 μg protein) was added to 3 ml Lowry solution C (see Appendix), mixed thoroughly and incubated 10 to 60 min. at room temperature. Finally, 0.3 ml of diluted Folin-Ciocalteu reagent (Fisher) was added. After 45 min. further incubation the blue color was read at 600 nm on a Bausch and Lomb Spectronic 20.

Amino Acid Uptake

Logarithmic phase cells of P. aeruginosa grown in MSS medium were washed twice with cold (4°C) CTX buffer and resuspended to a final optical density of 0.6 in CTX buffer with 1 mM succinate. Flasks of this suspension were placed on a reciprocal shaking water bath at 37°C . Chloramphenicol (200 $\mu\text{g/ml}$) was added 15 min. prior to the start of the uptake experiment. The experiment was begun by the addition of AIB (50 mC/mmol for ^{14}C -labeled AIB, 200 mC/mmol for ^3H -AIB; final concentration $1 \times 10^{-5} \text{ M}$) or proline (125 mC/mmol, $5 \times 10^{-6} \text{ M}$). At various times thereafter, duplicate 0.5 ml samples were removed and filtered through 0.45 μm Millipore filters on an Amicon filter manifold. Each filter was rinsed with two 4 ml volumes of ice cold CTX buffer. The filters were dried and placed in vials with Bray's cocktail (Research Products, Inc.) for scintillation counting. A background count, determined by filtering a buffer solution containing labeled amino acid with no bacteria, was subtracted from each count.

Incorporation of Methionine into Protein

P. aeruginosa RM46 was grown to logarithmic phase, washed twice with cold (4°C) CTX buffer and resuspended in room temperature buffer. This suspension was added to flasks of CTX buffer at 37°C containing from zero to 600 $\mu\text{g/ml}$ of chloramphenicol. The final cell density was approximately 3×10^8 cells/ml (A_{590} 0.3). These flasks were incubated in a 37°C water bath with shaking for 15 min. before [methyl- ^3H]-methionine was added to give a final concentration of $5 \times 10^{-7} \text{ M}$ (1.56 C/mmol). At various times after addition of methionine, 0.5 ml samples were transferred to tubes containing 0.5 ml ice cold 10% trichloroacetic

acid (TCA). These tubes were vortexed thoroughly and put on ice for 15 min. or longer. Precipitated material was then collected on Whatman No. 3 filter paper circles in an Amicon filter manifold. Each filter was washed with two 1 ml volumes of cold 5% TCA, followed by two 1 ml volumes of absolute ethanol-ether (1:1) and finally two 0.5 ml volumes ether. The filters were air dried and placed in vials with Bray's cocktail for scintillation counting.

Thin Layer Chromatography of AIB

Logarithmic phase bacterial cells were washed and suspended in CTX buffer to an optical density of 0.8 (180 μ g protein/ml). ^3H -AIB was added to a final concentration of 1×10^{-5} M, 196 mC/mmol. This mixture was incubated with shaking for one hour at 37°C . The cells were then removed by centrifugation. Ten microliter samples of the supernatant were spotted onto Eastman cellulose thin layer plates without fluorescent indicator. A standard solution of AIB containing 3 mg and $1 \mu\text{C}$ ^3H -AIB per milliliter was spotted on the cellulose sheets adjacent to the cell supernatant. Chromatograms were developed in the following solvents: solvent 2, butanol-acetic acid-water 12:3:4; solvent 3, ethanol-ammonia-water 80:4:16; and solvent 55, butanol-pyridine-water 1:1:1. After air drying the AIB standard strips were sprayed with ninhydrin (0.25% in acetone) to visualize the AIB spot. The chromatograms were cut into 0.5 cm strips which were placed in vials with Bray's cocktail for scintillation counting.

Chemicals

The organic acids (pyruvic and succinic) were obtained from Sigma Chemical Co. (St. Louis, Mo.) and were used as the sodium salt. The amino acids L-methionine, L-ethionine, L-serine, L-arginine, α -aminoisobutyric acid (sodium salt) and L-glutamic acid (sodium salt) were also purchased from Sigma at the highest purity available and were used without further purification.

The electrophoresis chemicals acrylamide, methylene bisacrylamide, tris(hydroxymethane)aminomethane, sodium dodecyl sulfate and glycine were all of electrophoresis purity from Bio Rad Laboratories (Rockville, Md.). The protein molecular weight standards were purchased from Sigma, as was the protein stain Coomassie blue R250.

α -[methyl-³H]aminoisobutyric acid (10 C/mmol) was obtained from New England Nuclear. The following radiochemicals were purchased from Amersham: L-[methyl-³H]methionine (7 to 93 C/mmol), L-[³⁵S]methionine (1300 C/mmol), S-adenosyl-L-[methyl-³H]methionine (74 C/mmol), 2-amino[1-¹⁴C]isobutyric acid (58 mC/mmol), L-[³H(G)]proline (2.5 C/mmol), and L-[methyl-¹⁴C]methionine (60.2 mC/mmol).

Chemical purity of the radiolabeled amino acids was assessed at intervals by chromatography on Eastman cellulose thin layer plates developed in Solvent 2 (butanol-acetic acid-water 12:3:4).

CHAPTER III

RESULTS

Effect of Nitrogen Source on the Chemotaxis of Pseudomonas aeruginosa

Earlier investigations by this author involved the application of the quantitative capillary assay to the study of the chemotaxis of P. aeruginosa including a characterization of the types of chemicals that serve as attractants (Moulton, 1977; Moulton and Montie, 1979) and a comparison of the chemotactic capacity of several strains of P. aeruginosa of various origins (Craven and Montie, 1981). The amino acids serine, arginine, leucine and glutamate were very strong attractants for P. aeruginosa. Glucose, succinate, malate and citrate were also attractants, but the amino acids were considerably stronger attractants than were glucose or the organic acids. This greater effectiveness of amino acids has been noted for other species, especially E. coli (Koman et al., 1979). It seems quite reasonable that substances which serve both the carbon and nitrogen needs of the bacterium would be of greater survival value to the organism than a substance which supplied only carbon. In view of these results it was hypothesized that the tactic responses of bacteria toward amino acids might be influenced by the availability of nitrogen in the environment. As part of the present study, this idea was tested by examining the chemotaxis of bacteria which had been grown in media containing different sources of nitrogen.

Effect of Growth Medium

Using the capillary technique to quantitate the responses, bacteria grown on the standard MSS medium and on the complex medium Luria broth (LB) were compared. Figure 1 shows concentration-response curves for two strong attractants, arginine and serine. Bacteria grown in MSS responded to serine at concentrations in excess of about 1×10^{-6} M; the peak response occurred at 1×10^{-3} M. Smaller accumulations were seen in capillaries containing more than 1×10^{-3} M serine. This profile is typical of those previously described for E. coli (Adler, 1973). In contrast the LB cells were very poorly responsive to serine. The maximum accumulation occurred in the 1×10^{-1} M capillaries and was only 44,000 cells per capillary, or 11% of the peak accumulation of the MSS cells. Chemotaxis toward arginine (Figure 1B) was similarly affected by the type of growth medium. The maximum response by LB cells was only 15% of that of the bacteria grown in the mineral salts medium.

Growth of P. aeruginosa in any one of several peptone-containing media caused a severe depression of chemotactic responsiveness of the bacteria toward serine (Table 1). Cells grown in Luria broth and in tryptone broth gave 3% and 11%, respectively, of the response of cells grown in MSS. Also, when CAA was added to MSS medium with or without succinate, serine taxis was reduced to only 3 to 4% of that in cells from MSS without CAA.

These results suggested the possibility that motility and/or chemotaxis functions are "repressed" by high levels of amino acids in the peptone-containing media or more generally by high levels of nitrogen. Several P. aeruginosa enzymes which are involved in the utilization

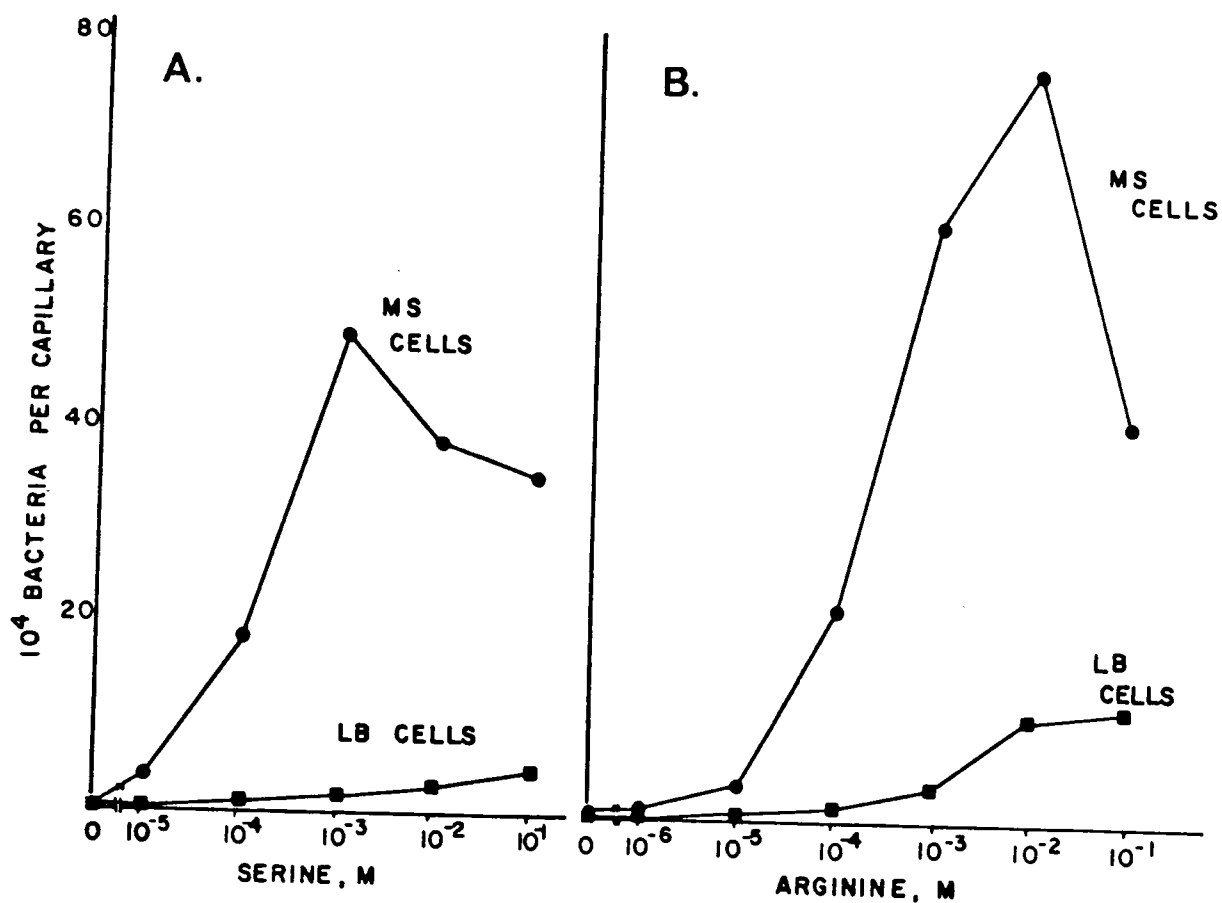


Figure 1. Chemotaxis toward serine and arginine by Pseudomonas aeruginosa grown on MSS medium and on Luria broth.

Table 1. Chemotaxis toward serine by Pseudomonas aeruginosa grown on peptone-containing media.

Growth Medium ^c	Accumulation (10^4 bacteria/capillary) ^a			
	Buffer	Capillaries	Serine	Capillaries ^b
Mineral Salts-Succinate (MSS)	0.34	(± 0.18)	36.3	(± 2.8)
Luria Broth (LB)	0.12	(± 0.04)	1.42	(± 0.29)
Tryptone Broth (TB)	0.68	(± 0.24)	4.17	(± 0.37)
MSS with CAA	0.12	(± 0.06)	0.96	(± 0.48)
MS with CAA (no succinate)	0.13	(± 0.10)	1.46	(± 0.64)

^aStandard deviations are shown in parenthesis.

^bAttractant capillaries contained 1 mM serine.

^cLB and TB are described in the Materials and Methods. Vitamin-free casamino acids (Difco) was added to MSS and to MS media at 1.0%.

of nitrogen compounds are known to be regulated by the nitrogen levels in the culture medium. In particular, the regulation of enzymes of histidine catabolism (Potts and Clarke, 1976) has been well demonstrated as has the regulation of urease and enzymes of ammonia assimilation (Janssen et al., 1980, 1981). Also, the regulation of protease production by ammonia has been described (Liu and Hsieh, 1969; Jensen et al., 1980).

Alternate Nitrogen Sources in the MSS Medium

The complex nature of the peptones which served as the sources of nitrogen in the above experiments makes it impossible to answer questions about the nature of the regulatory effect. Therefore, it was necessary to develop defined media which have the same effect as the CAA or tryptone. P. aeruginosa was grown in MSS medium in which the standard nitrogen source (ammonium sulfate) was replaced by alternate nitrogen sources. Logarithmic phase cells from each culture were prepared as usual for the capillary assay. Table 2 illustrates the chemotactic responses toward serine. As noted above the addition of CAA to the MSS medium containing ammonium sulfate caused a dramatic loss of responsiveness; serine taxis was less than 10% of that by cells grown in MSS with ammonium sulfate. Interestingly, addition of sodium glutamate to the MSS with ammonium sulfate was as effective as CAA in repressing chemotaxis. Growth on glutamate alone without ammonium sulfate produced bacteria that were half as responsive as cells grown in MSS with ammonium sulfate. In sharp contrast to CAA and glutamate, growth on urea and on potassium nitrate yielded cells that were 1.8 and 1.4 times as responsive as cells grown on ammonium sulfate. Addition of glutamate to MSS with potassium nitrate reduced serine taxis to a level

Table 2. Effect of alternate nitrogen sources on the motility and serine taxis of Pseudomonas aeruginosa.

Nitrogen Source in MSS ^a	Accumulation (10^4 bacteria/capillary) ^b			
	Buffer	Capillaries	Serine	Capillaries ^c
KNO ₃	0.93	(±0.28)	83.3	(±8.3) ^d
Urea	1.00	(±0.23)	63.3	(±19.7)
(NH ₄) ₂ SO ₄	0.73	(±0.85)	45.2	(±7.8)
KNO ₃ with Glutamate	0.31	(±0.15)	25.6	(±14.0)
Glutamate	0.26	(±0.10)	21.1	(±7.7) ^d
(NH ₄) ₂ SO ₄ with Glutamate	0.25	(±0.02)	3.24	(±0.30) ^d
(NH ₄) ₂ SO ₄ with CAA	0.17	(±0.12)	4.20	(±0.76) ^d

^aAmmonia, nitrate and urea were used at 7.6 mM, glutamate and CAA at 0.5%.

^bStandard deviations are in parenthesis.

^cCapillaries contained 1 mM serine in CTX buffer.

^dSignificantly different from (NH₄) SO₄ cells, $p < 0.05$.

approximately the same as glutamate alone and 70% lower than potassium nitrate alone.

It is very interesting that the magnitude of the response to serine (Table 2) correlates roughly with the rate of growth of the bacteria. The following doubling times were estimated from the optical density measurements: nitrate, 94 min.; urea, 60 min.; ammonia, 60 min.; glutamate, 77 min.; nitrate-glutamate, 78 min.; ammonia-glutamate, 54 min., CAA, 51 min. The slow growing nitrate cells were most responsive to serine, while the fast growing CAA and ammonia-glutamate were least responsive. The glutamate and glutamate-nitrate cells, however, were less chemotactic than the ammonia cells, but showed doubling times somewhat longer than ammonia-grown cells.

Various Amino Acids as Nitrogen Sources

In the experiment above glutamate was the only amino acid tested. Glutamate was chosen for this test because it is the predominant amino acid in those peptones which are derived from casein as are CAA and tryptone. Glutamate proved to be as effective as CAA in reducing chemotaxis of P. aeruginosa and, for this reason, was used in many of the following experiments. However, glutamate was not the only effective amino acid. Table 3 shows the serine response of bacteria grown with glutamine and with glycine. When added to ammonium sulfate glutamine caused a loss of responsiveness similar to that caused by glutamate (84% and 88%, respectively). In contrast addition of glycine to ammonium sulfate had almost no effect (9% reduction).

In two additional experiments the serine response of CAA-grown bacteria was compared to cells grown on either proline, aspartate, arginine or alanine in place of ammonia. Alanine, like glycine in Table 3,

Table 3. Motility and serine taxis of *Pseudomonas aeruginosa* grown in MSS medium supplemented with glutamate, glutamine, or glycine.

Nitrogen Source	Accumulation (10^4 bacteria/capillary) ^a			
	Buffer	Capillaries	Serine	Capillaries
$(\text{NH}_4)_2\text{SO}_4$	0.99	(± 0.79)	87.5	(± 6.9)
$(\text{NH}_4)_2\text{SO}_4$ + Glutamate ^d	0.37	(± 0.19)	10.4	(± 3.9)
$(\text{NH}_4)_2\text{SO}_4$ + Glutamine	0.56	(± 0.12)	13.9	(± 3.0)
$(\text{NH}_4)_2\text{SO}_4$ + Glycine	0.60 ^c		79.8	(± 0.1)
Glutamate	2.36	(± 2.0)	47.1	(± 14.5)

^aStandard deviations are shown in parenthesis.

^bCapillaries contained 1 mM serine in CTX buffer or buffer alone.

^cOnly one capillary was used to obtain this value; replicate capillaries were lost or broken.

^dAmino acids were added at 0.5%.

had little effect; serine taxis was 94% of that of ammonium sulfate bacteria. CAA caused an 80% loss of serine taxis. The other three amino acids fell in an intermediate range (proline 37%, aspartate 58%, and arginine 40% reduction in serine taxis).

Chemotaxis toward AIB and Succinate

The effect of alternate nitrogen sources on chemotaxis to other attractants was also examined. α -aminoisobutyric acid (AIB) is an amino acid analog which is not metabolized by P. aeruginosa (see pages 105-111). Nevertheless, this chemical serves as a strong chemoattractant for P. aeruginosa. Figure 2 shows the response toward AIB of bacteria grown on MSS containing various sources of nitrogen. Chemotaxis to AIB was found to parallel that of serine in all cases. Growth in nitrate-containing medium caused a three-fold stimulation of chemotaxis toward serine and AIB. This was true whether 7.6 mM or 15 mM nitrate was present in the MSS medium suggesting that the observed differences between nitrate and ammonia cells are due to the chemical form of the nitrogen source and not simply to the number of nitrogen atoms available. Cells grown in MSS with CAA or MSS with ammonia and glutamate gave chemotactic responses to AIB that were 11% and 7%, respectively, of those of the standard ammonium sulfate cells.

The chemoreceptors for attractants in P. aeruginosa have not been characterized. There is some possibility that serine and AIB, both small uncharged amino acids, might be recognized by the same receptor. If this were the case, one would expect responsiveness toward these two chemicals to change concomitantly when growth conditions are altered. Experiments with the strong attractant arginine, which due to its very different

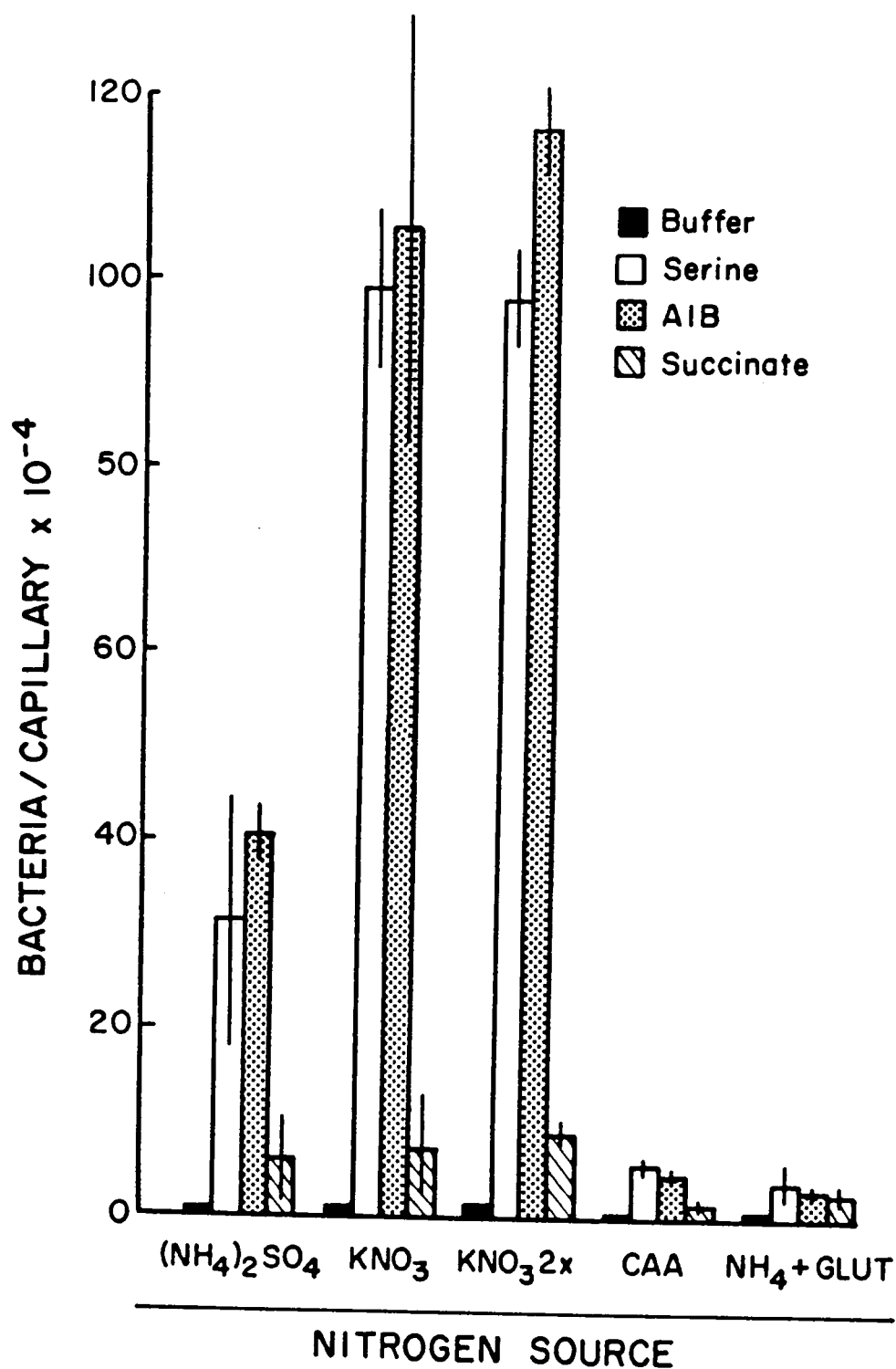


Figure 2. Chemotaxis toward serine, AIB, and succinate by *Pseudomonas aeruginosa* grown in MSS containing various nitrogen sources. The capillaries contained either 1 mM serine, 10 mM AIB, or 1 mM succinate or buffer alone.

chemical structure is very likely detected by a receptor other than the AIB receptor, have shown that the dependence of chemotactic response on nitrogen source is not limited to serine and AIB taxis. Figure 1 (page 44) shows the loss of arginine responsiveness in cells grown in nitrogen rich medium. Further, arginine taxis was stimulated two- to five-fold by growth in nitrate-containing medium (see page 56 and page 68).

In contrast to the amino acid responses, chemotaxis toward succinate was not influenced in the same manner by alternate nitrogen sources (Figure 2). Growth in nitrate did not stimulate succinate taxis. However, the response to succinate was reduced 18% and 44% of the response of ammonia-grown cells in CAA and ammonia-glutamate cultures, respectively.

Alternate Carbon Sources

In the enteric bacteria E. coli and S. typhimurium motility and chemotaxis are subject to catabolite repression. In particular glucose causes repression of the synthesis of the flagellum of E. coli (Adler and Templeton, 1967) and of the chemoreceptors for certain attractants in S. typhimurium (Fahnestock and Koshland, 1979). In P. aeruginosa certain organic acids, rather than glucose, exert a regulatory effect similar to catabolite repression of the enteric bacteria on the synthesis of several catabolic enzymes (Potts and Clarke, 1976). In view of the apparent regulation of amino acid taxis by nitrogen compounds, it was especially pertinent to determine whether amino acid taxis might also be regulated by carbon compounds. Utilization of carbon compounds

known to be less repressive than succinate might yield cells of increased chemotactic ability.

Table 4 shows the results of three experiments in which either glucose or pyruvate was substituted for succinate. The bacteria in pyruvate medium grew much more slowly than the cells in glucose or succinate medium; the approximate doubling times were 180, 95 and 60 min. for pyruvate, glucose and succinate, respectively. In all cases the bacteria were grown to early logarithmic phase prior to the experiment. The tactic responses toward AIB was determined by the capillary method. In each experiment the bacteria grown on glucose or pyruvate were slightly more responsive (by 20 to 48%) than were succinate grown cells. Pyruvate-grown cells were 20% and 33% more responsive than succinate cells while glucose-grown cells were 48% and 28% more responsive than succinate cells. The differences observed were statistically significant ($p < 0.05$) in one experiment but not in the other two experiments. These differences were small compared to the two- to three-fold differences between nitrate- and ammonia-grown bacteria or the ten-fold differences between ammonia- and ammonia-glutamate cells (Figure 2). These experiments do not exclude the possibility that catabolite repression might regulate some components of the chemotaxis system. However, it appears that catabolite repression does not play a major role in taxis toward AIB. Utilization of a pyruvate, a growth-limiting carbon source, did not have an effect similar to growth on a limiting nitrogen source.

The results of the third experiment of Table 4 were part of a larger experiment in which the effect of nitrogen source on the chemotaxis of glucose-grown cells to several attractants was tested (Figure

Table 4. Motility and AIB taxis of Pseudomonas aeruginosa grown on pyruvate or glucose in place of succinate.

Experiment number	Carbon Source in MSS	Accumulation (10^4 bacteria/capillary) ^a			
		Buffer	Capillaries	AIB	Capillaries ^b
1	Succinate	0.50	(± 0.22)	25.0	(± 9.97)
	Pyruvate	0.56	(± 0.43)	30.2	(± 10.4)
2	Succinate	2.00	(± 1.29)	38.6	(± 6.60) ^c
	Pyruvate	1.75	(± 1.11)	51.2	(± 6.61) ^c
	Glucose	1.46	(± 0.77)	57.1	(± 3.36) ^c
3	Succinate	1.43	(± 0.87)	37.5	(± 17.9)
	Glucose	0.24	(± 0.07)	48.1	(± 10.4)

^a Standard deviations are in parenthesis.

^b Capillaries contained 10 mM AIB in CTX buffer.

^c AIB responses of pyruvate and glucose cells were significantly greater ($p < 0.05$) than that of succinate cells (Students t-test).

3). P. aeruginosa was grown in MS medium containing either glucose or succinate as the carbon source and either nitrate or ammonia as the nitrogen source. As described above, the chemotactic responses to AIB and arginine were two-fold higher in bacteria grown in succinate-nitrate as compared to succinate-ammonia cells. The same pattern was evident when glucose served as carbon source; arginine and AIB taxis were twice as strong in nitrate cells compared to ammonia cells. Succinate, which is a moderately effective attractant for succinate-ammonia cells, also attracted bacteria grown in glucose-ammonia. In contrast to the responses to the amino acids, chemotaxis toward succinate was not stimulated in nitrate-grown cells regardless of the carbon source employed. In fact, for glucose-grown cells and succinate-grown cells the succinate response was reduced approximately 30% when nitrate replaced ammonia as the source of nitrogen. Chemotaxis toward glucose was also measured. A barely detectable response was seen in both succinate-grown cultures, while glucose-ammonia cells gave a much stronger response. This is in agreement with previous reports of the inducibility of glucose taxis (Moulton and Montie, 1979; Moench and Konetzka, 1978). In sharp contrast to the glucose-ammonia cells, however, bacteria grown in glucose-nitrate gave no response at all to glucose in spite of the fact that the cells are highly responsive to amino acids.

A concentration-response curve was constructed by testing the response of glucose-nitrate and glucose-ammonia cells to various concentrations of glucose (Figure 4). P. aeruginosa grown in glucose-ammonia gave a peak response of 77,000 cells/capillary when the attractant

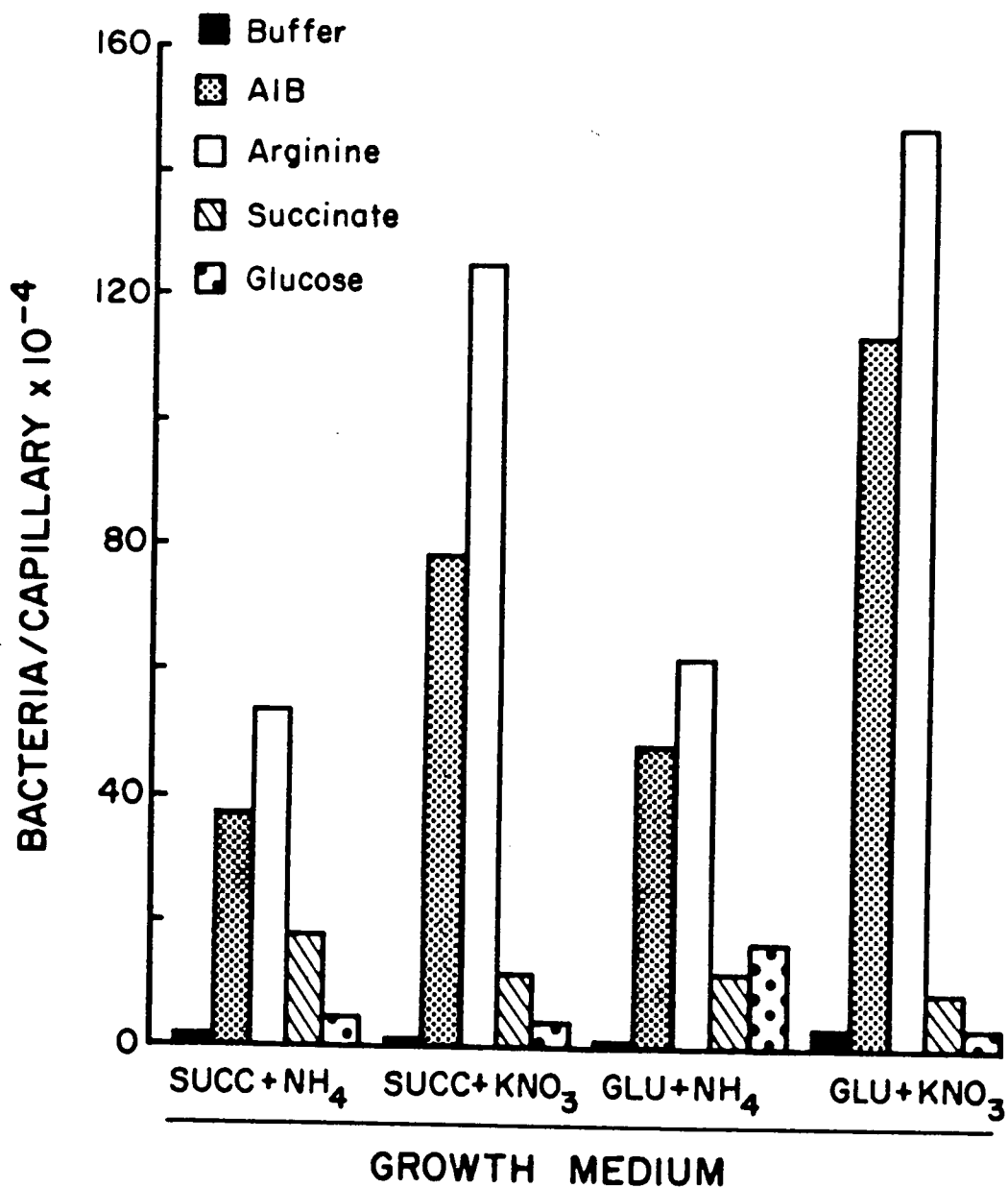


Figure 3. Effect of nitrogen source on chemotaxis of bacteria grown on either succinate or glucose as the carbon source. The capillaries contained either 10 mM AIB, 10 mM arginine, 1 mM succinate, 1 mM glucose, or buffer alone.

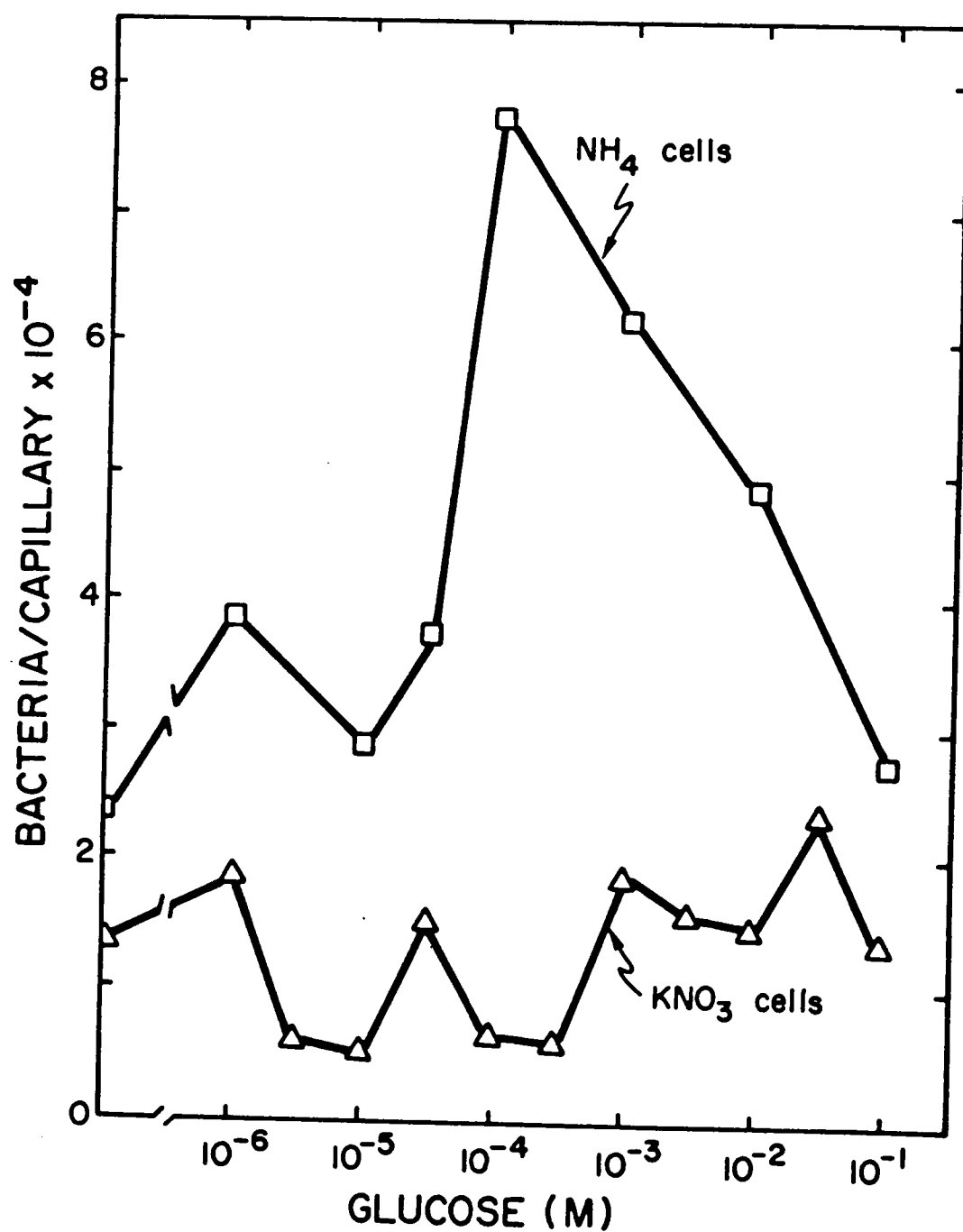


Figure 4. Glucose chemotaxis by *Pseudomonas aeruginosa* grown on ammonia or nitrate as the nitrogen source.

concentration was 1×10^{-4} M. In contrast, bacteria grown in glucose-nitrate gave no response to glucose at any concentration tested. These results support the observations above (Figure 3) that glucose-nitrate bacteria did not respond to glucose.

The results described above indicate that a dichotomy exists with regard to the effect of the source of nitrogen on the chemotactic responsiveness of P. aeruginosa. Taxis toward the amino acids serine, arginine, and AIB is subject to regulation by the nitrogen source of the growth medium. Growth in amino acid-rich media severely suppresses the response toward amino acids while growth in medium containing a relatively poor nitrogen source causes stimulation of those same responses. In contrast, chemotaxis toward the carbon compounds succinate and glucose is not stimulated, but rather appears to be inhibited by growth in nitrogen poor medium.

Background Motility

In the course of these various experiments using cultures grown under different conditions it was frequently noted that the bacteria grown in MSS with CAA or MSS with ammonia-glutamate showed background accumulations considerably lower than those of bacteria grown in MSS with ammonia alone. This would suggest that the motility of these bacteria is reduced in some manner by growth in these media. If this were the case the reduced chemotactic responsiveness of those bacteria could be explained in part or in whole by the loss of motility. To assess the validity of these observations the background values from the large number of experiments performed were tabulated and analyzed. The results of this analysis are shown in Table 5. Bacteria from

Table 5. Analysis of the background accumulations of *Pseudomonas aeruginosa* grown on different sources of nitrogen.

Growth Medium	Number of Experiments	Average Background Accumulation (10^4 Bacteria/Capillary) ^a		Relative to MSS with (NH ₄) ₂ SO ₄ Cells
MSS with KNO ₃	17	2.22	(±1.26)	1.20
MSS with (NH ₄) ₂ SO ₄	12	1.82	(±1.17)	1.00
MSS with (NH ₄) ₂ SO ₄ and Glutamate	8	0.96	(±1.03) ^b	0.53
MSS with CAA	6	0.62	(±0.38) ^b	0.34

^aThe average background accumulation was determined by averaging the background values from many experiments; the standard deviation is shown in parenthesis. Capillaries contained only buffer.

^bSignificantly different from (NH₄)₂SO₄ cells; $p < 0.05$.

ammonia-glutamate medium showed an average background that was only 53% of the ammonia-grown cells; this difference was significant at the $p < 0.05$ level. The background motility of CAA-grown bacteria was only one-third that of ammonia-grown bacteria ($p < 0.005$). Nitrate-grown cells, however, were not significantly different from the ammonia-grown cells.

These results do indicate that statistically significant differences in background accumulations exist. Such differences could result from actual differences in the motility of the bacteria (either in the proportion of motile cells or in swimming speed). This would explain in part the poor chemoresponses of cells grown in nitrogen rich media. On the other hand, the different background accumulations might simply reflect differences in the viability of the bacteria. To test this bacteria grown on the different media were prepared as usual for a chemotaxis experiment. Four suspensions of each culture were prepared. Viable cell counts were performed to determine the number of viable cells per milliliter of buffer (Table 6). Significant differences in viable bacteria per milliliter were seen. The suspensions of nitrate-grown bacteria contained 60% more cells than did the ammonia cell suspensions. Ammonia-glutamate and CAA cell suspensions contained 20% and 32% fewer viable cells than did the ammonia cell suspensions. These results demonstrated that at least some of the observed differences in background accumulations (Table 5) are due to variations in the density of viable cells in the suspensions. Whether this results from different densities of total cells (viable and nonviable) in the various suspensions or whether there are actual differences in viability

Table 6. Density of viable cells in suspensions of Pseudomonas aeruginosa which had been grown on various nitrogen sources.

Growth Medium	Suspension Density ^a (10 ⁸ bacteria/ml)		Relative to MSS with (NH ₄) ₂ SO ₄ cells
MSS with KNO ₃	4.0	(±0.46) ^b	1.6
MSS with (NH ₄) ₂ SO ₄	2.5	(±0.48)	1.0
MSS with (NH ₄) ₂ SO ₄ and Glutamate	2.0	(±0.36)	0.80
MSS with CAA	1.7	(±0.15) ^b	0.68

^aSuspension density was determined by viable plate count of the suspensions of bacteria in CTX buffer.

^bSignificantly different from MSS with (NH₄)₂SO₄ cells; p<0.05.

of the bacteria in the CTX buffer is not known. In future experiments this factor should be taken into account either by correcting the accumulation values to account for different densities in the different suspensions or by adjusting cell densities of each test suspension before the experiment.

A very important question arises. Are the observed effects of nitrogen on chemotactic responses simply an artifact arising from the varying densities of viable cells in the bacterial suspensions used for the taxis assay? In an attempt to address this question the data of Figure 2 (page 51) are illustrated in Figure 5 expressed as relative responses rather than absolute accumulations. For each type of bacteria the accumulations in attractant capillaries are divided by the background accumulation to attempt to normalize for differences in motility and viability of the different suspensions. Figure 5 shows that the relative response to the amino acids serine and AIB are affected by nitrogen source as described previously. Chemotaxis is stimulated in nitrate-grown bacteria and inhibited in bacteria grown in CAA or ammonia-glutamate. In contrast, the relative response to succinate is quite constant regardless of nitrogen source.

The reduced accumulations of the nitrogen-rich (CAA and ammonia-glutamate) bacteria in succinate capillaries noted in Figure 2, then, probably is due to lower numbers of motile and viable bacteria in the suspensions prepared from these bacteria. On the other hand the effect of nitrogen source on the accumulation in amino acid capillaries seems to reflect actual differences in chemotactic responsiveness to these substances. The following points are offered in support of this

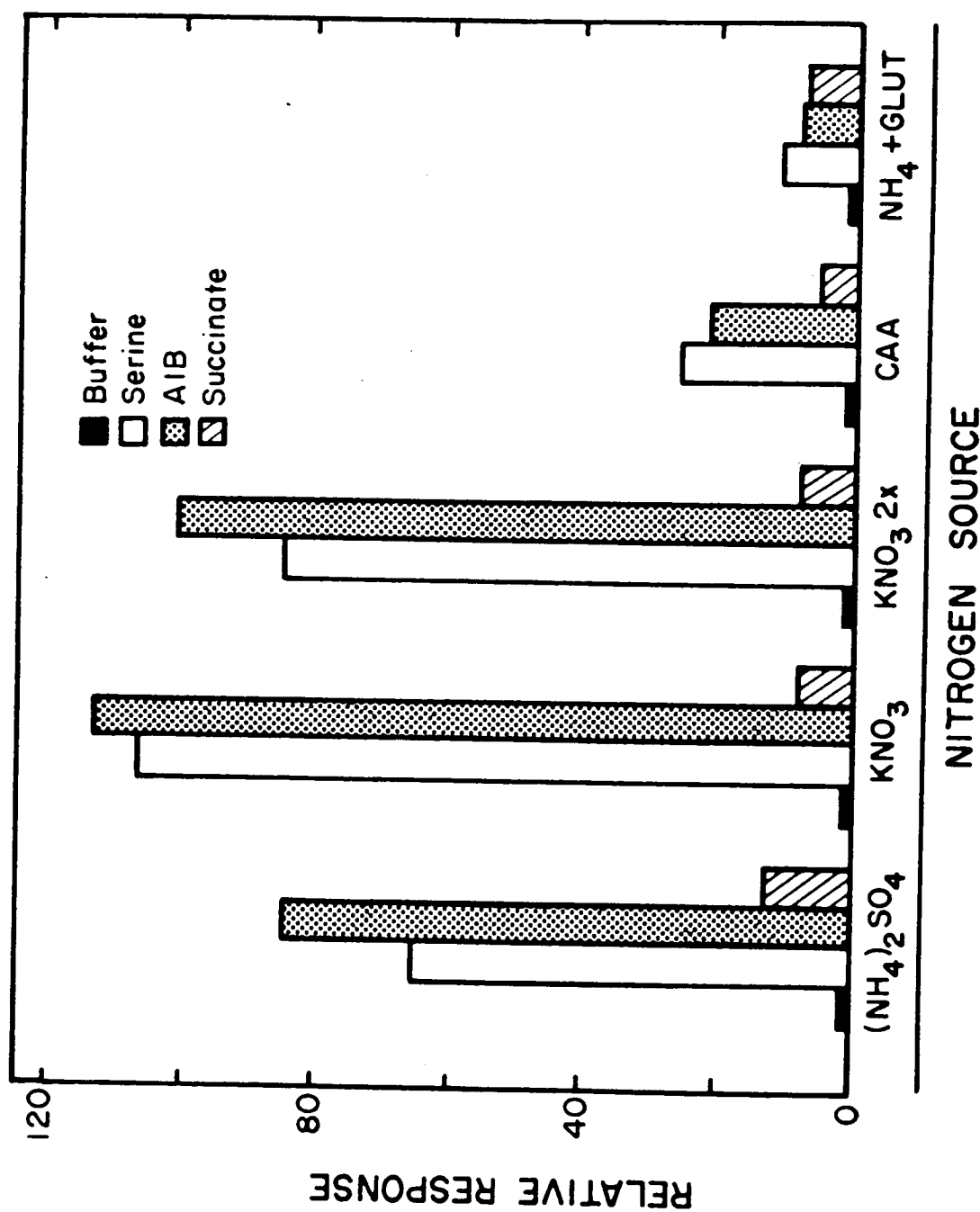


Figure 5. Chemotaxis of *Pseudomonas aeruginosa* expressed as a relative response. The capillaries contained either 1 mM serine, 10 mM AIB, 1 mM succinate, or buffer alone.

conclusion. The average background accumulations of nitrate and ammonia-grown bacteria were not significantly different (Table 5, page 59), however accumulations of nitrate cells in amino acid capillaries were consistently higher than the accumulations of ammonia-grown bacteria. Further, a number of individual experiments (for example, see page 68) show sharp stimulation of amino acid taxis in nitrate cells although no difference in background was seen. The accumulation of nitrate cells was approximately 20 times that of CAA or ammonia-glutamate cells (Figure 2, page 51 and Table 2, page 47) while the average background accumulations (Table 5, page 59) and density of viable cells (Table 6) were only three times those of the nitrogen rich bacteria. Amino acid taxis was stimulated in nitrate cells without an accompanying stimulation of succinate taxis (Figure 2, page 51). Finally, the AIB response of ammonia-grown and nitrate-grown bacteria show different dependence on AIB concentration (see the following section). Such an effect cannot be explained by differences in motility or viability.

Analysis of Concentration-Response Curves

Concentration-response (C-R) curves were constructed for several attractants to determine whether the dependence of the chemotactic response on attractant concentration was altered by the nitrogen source of the growth medium. Figure 6 shows the C-R curves for AIB taxis by P. aeruginosa grown on MSS with either nitrate, ammonia, or ammonia-glutamate as the source of nitrogen. These curves do show different concentration dependences. The nitrate-grown bacteria, as noted above, accumulated to higher levels in AIB capillaries; the response of nitrate cells to 1×10^{-2} M AIB was three times that of the glutamate cells. In

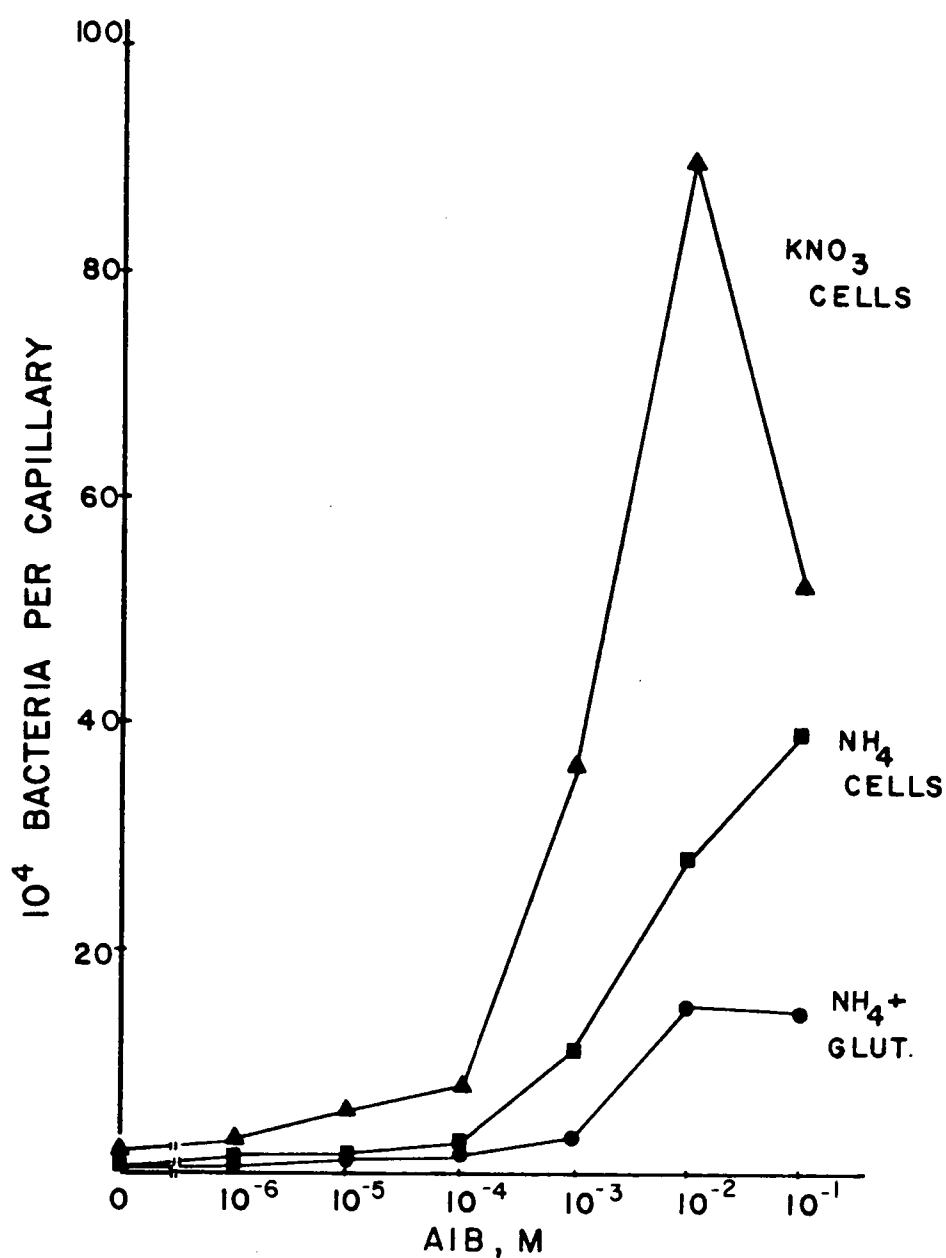


Figure 6. Chemotaxis toward AIB by *Pseudomonas aeruginosa* grown on ammonia, nitrate or ammonia-glutamate as the source of nitrogen.

addition the nitrate grown bacteria respond to AIB in lower concentrations than do either ammonia or ammonia-glutamate cells. Nitrate cells responded to AIB in excess of 1.9×10^{-6} M and gave a peak response at 1×10^{-2} M (the calculation of the threshold values is discussed in the following section). The response range for ammonia-grown bacteria extends from 5.8×10^{-6} M to 1×10^{-1} M. Cells grown on glutamate-ammonia responded to AIB in excess of 2.7×10^{-5} M; this detection threshold was 14-fold higher than the threshold for nitrate-grown bacteria.

Concentration-response curves for succinate taxis show a very different pattern (Figure 7). The concentration dependence appears to be virtually identical, regardless of the nitrogen source utilized for growth. The bacteria responded to succinate at 1×10^{-4} M or greater and reached a peak response at 1×10^{-3} M. Furthermore, as noted above (Figure 2, page 51), succinate taxis was not enhanced in nitrate cells; the nitrate-grown cells as well as ammonia-glutamate bacteria were considerably less responsive than bacteria grown on ammonia.

A comparison of arginine C-R curves for ammonia grown bacteria and nitrogen-rich (LB) bacteria was discussed above (Figure 1, page 44). Figure 8 illustrates C-R curves for nitrate- and ammonia-grown bacteria. In spite of the dramatic differences between the two cultures in the number of bacteria per capillary, the concentration dependence of the curves appears to be similar.

Calculation of Threshold Concentrations

The threshold concentration is defined (Mesibov et al., 1973) as the lowest concentration of attractant that gives an accumulation in the

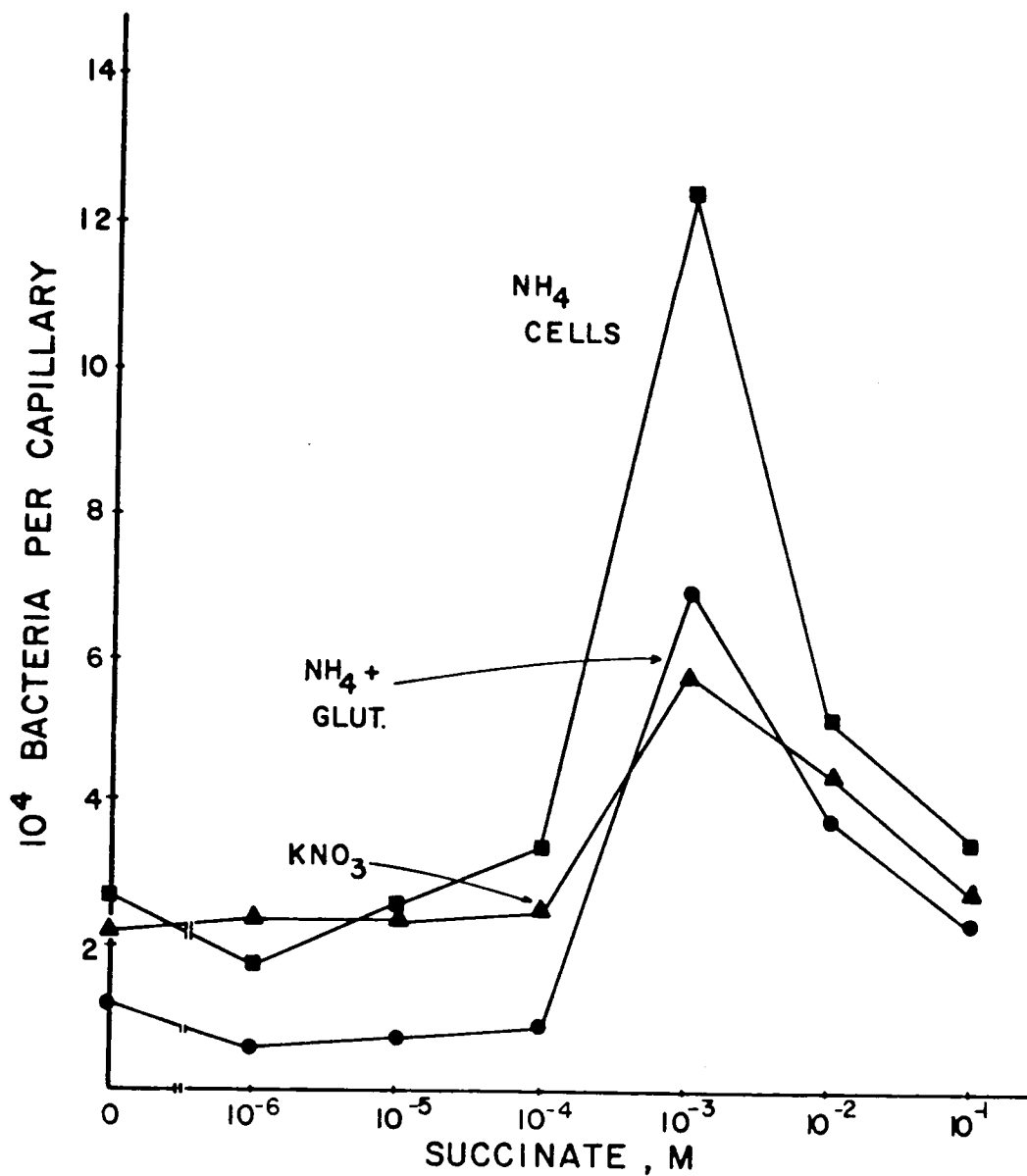


Figure 7. Chemotaxis toward succinate by bacteria grown on ammonia, nitrate, or ammonia-glutamate as the source of nitrogen.

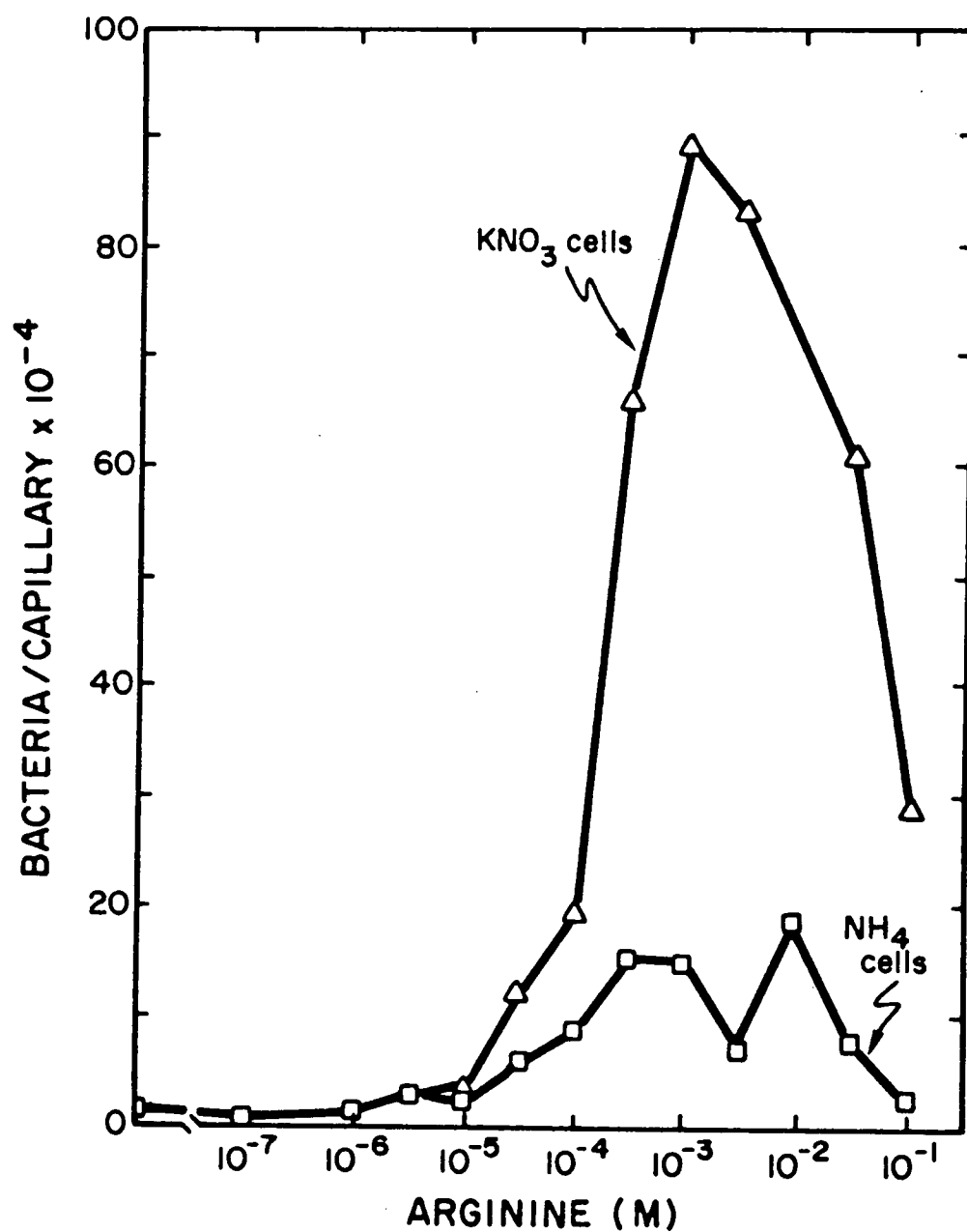


Figure 8. Chemotaxis of ammonia- and nitrate-grown Pseudomonas aeruginosa toward arginine.

accumulation in the capillary greater than that obtained in the absence of attractant. In practice the threshold concentration can be estimated from C-R data. This is illustrated in Figure 9. When the data are plotted on a double-logarithmic graph the points of the rising part of the C-R curve fall on a near-straight line. The concentration of attractant at which this line intersects the baseline, defined by the accumulation of cells in capillaries containing no attractant, is taken as the threshold concentration. Threshold concentrations of nitrate and ammonia bacteria were determined for chemotaxis toward arginine, AIB and succinate. In each case comparisons were made between nitrate- and ammonium-grown cultures prepared on the same day under identical conditions to minimize problems of interpretation caused by day-to-day variability.

The determination of threshold concentrations for AIB is found in Figure 9. A value of 2.1×10^{-5} M was obtained for ammonia-grown cells and 6.7×10^{-6} M for nitrate cells; this is a three-fold increase in sensitivity to AIB in nitrate-grown bacteria.

Table 7 summarizes the results of AIB threshold concentration determinations from four experiments. The first experiment listed is that of Figure 9. Nitrate-grown bacteria responded to AIB at a concentration three-fold lower than ammonia-grown cells and 14-fold lower than the glutamate-grown cells. In three of the four experiments the threshold concentration for nitrate cells was three-fold lower than for ammonia cells. In the fourth experiment a 30-fold difference was seen due to the extremely high threshold for ammonia-grown bacteria in that experiment. No explanation for this large discrepancy is evident.

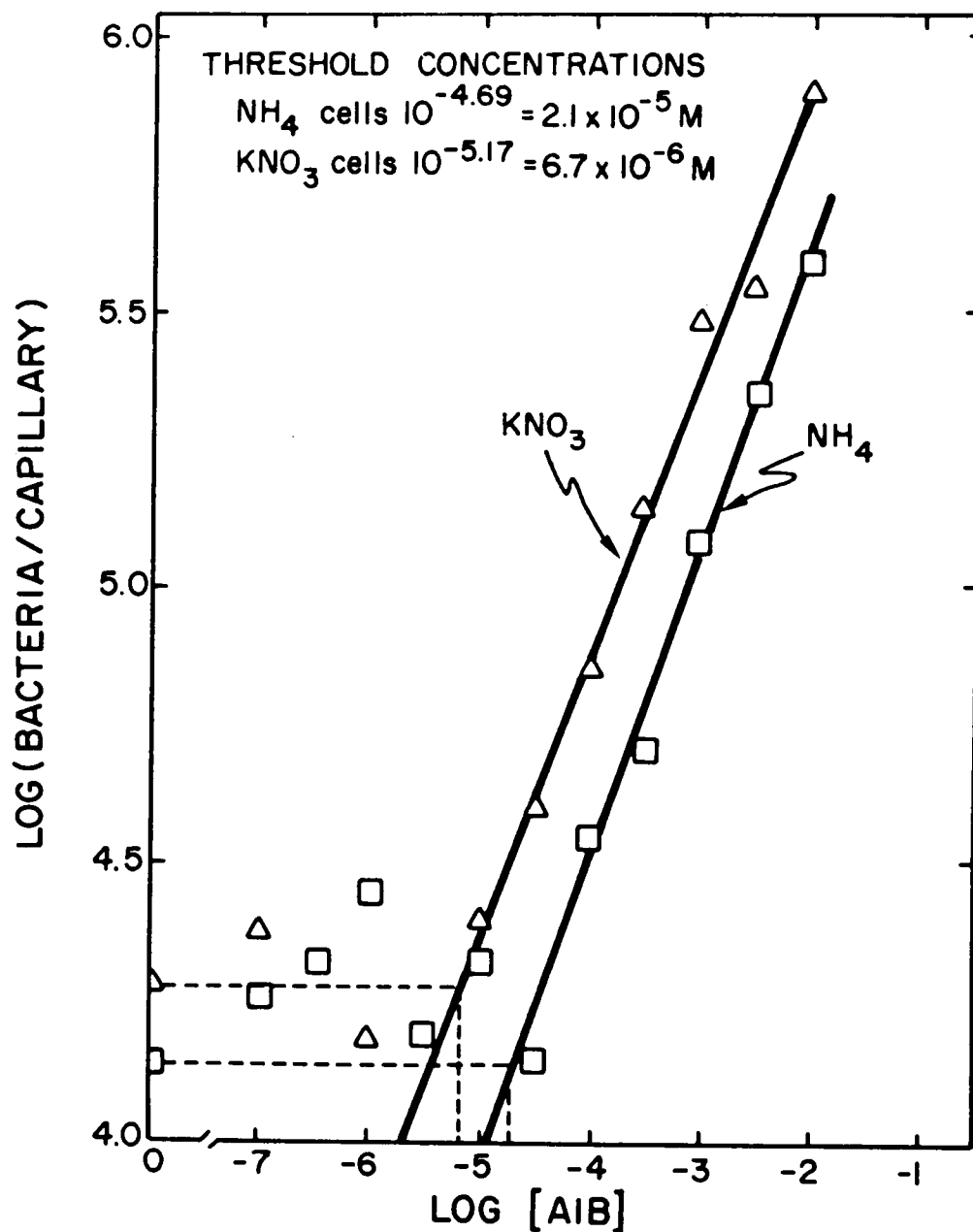


Figure 9. Calculation of threshold concentrations. Each straight line was fitted to the points on the rising portion of the C-R curve. The concentration at which that line crosses the baseline (dotted line) is the threshold concentration.

Table 7. Threshold concentrations for chemotaxis of Pseudomonas aeruginosa toward AIB, arginine and succinate.

Experiment Number	Attractant	Ammonia-grown Bacteria	Nitrate-grown Bacteria	Ammonia-glutamate Bacteria	Ratio	
					Ammonia: Nitrate	Ammonia-glutamate: Nitrate
1	AIB	5.8×10^{-6}	1.9×10^{-6}	2.7×10^{-5}	3.1	14.2
2	AIB	2.1×10^{-5}	6.7×10^{-6}	-	3.1	-
3 ^a	AIB	1.3×10^{-4}	4.3×10^{-6}	-	30	-
4 ^a	AIB	5.7×10^{-6}	1.5×10^{-6}	-	3.8	-
5	Arginine	5.0×10^{-7}	6.0×10^{-7}	-	0.8	-
6	Succinate	4.7×10^{-5}	3.6×10^{-5}	-	1.3	-

^aOvernight cultures for these two experiments were begun with a very small inoculum so that at 14 to 16 hr. the bacteria were still in exponential phase when they were diluted into fresh medium. This treatment should avoid influences of nitrogen deprivation during the stationary phase of the overnight cultures.

Nevertheless, even if that experiment is eliminated from consideration, it is evident that nitrate-grown bacteria are three-fold more sensitive to AIB than are ammonia-grown bacteria. Averaging the results of the first three experiments yields a value of 3.4×10^{-6} M for nitrate cells compared to 1.1×10^{-5} M for ammonia-grown cells.

A considerable degree of uncertainty is inherent in the values determined for threshold concentrations due to experimental error in the determination of the background accumulation (the baseline) and in the placement of the straight line through the points on the rising part of the curve. The error due to the latter can be minimized by testing as many attractant concentrations and using as many capillaries at each concentration as is feasible and by using statistical methods to draw the best straight line to fit those points. The experimental error in the position of the base line is inherent in the capillary method. To assess the degree of uncertainty due to this factor, the threshold concentrations for the data of Figure 9 were recalculated using as baseline the lines defined by one standard deviation above and below the background accumulation. For the ammonia-grown cells a threshold concentration range of 2.5×10^{-5} to 1.6×10^{-5} M was found; for nitrate cells the threshold range was 7.5×10^{-6} to 6.0×10^{-6} M. In view of these results the conclusion stated above that nitrate cells are three-fold more sensitive to AIB than are ammonia cells appears quite justified.

Threshold concentrations were also determined for arginine and succinate chemotaxis (Table 7). For both attractants the threshold concentration for nitrate cells was virtually identical to the corresponding threshold for ammonia-grown cells.

Role of Methionine in Chemotaxis of P. aeruginosa

Effect of Methionine Deprivation on Chemotaxis

Previous studies by this author (Moulton, 1977; Moulton and Montie, 1979) demonstrated that P. aeruginosa strain RM46, a methionine auxotroph, was chemotactic toward various attractants when suspended in a phosphate buffer without exogenous methionine. The same results were obtained whether the bacteria tested were taken from a batch culture containing an excess of methionine or from a culture in which growth had ceased due to lack of methionine. This suggested that the chemotactic mechanism might be independent of methionine. However, such a possibility seemed unlikely in view of the occurrence of methionine-mediated chemotactic systems in a wide variety of organisms (Chapter I, page 18). Therefore, studies were initiated to test the methionine dependence of P. aeruginosa under more stringent conditions of methionine depletion than those used in the previous studies and, then, to look for the presence of a chemotaxis-associated methyl transfer reaction in P. aeruginosa.

Methionine starvation. Starvation conditions were imposed by washing logarithmic phase bacteria with methionine-free MSS medium to remove exogenous amino acid. The bacteria were then incubated at 37°C without methionine for periods of one to four hours. During this time no growth was observed in the absence of methionine and, in fact, the optical density of the culture declined considerably (Figure 10). Serine taxis of the bacteria subjected to starvation periods of various lengths was measured by the capillary method. Figure 11 shows the

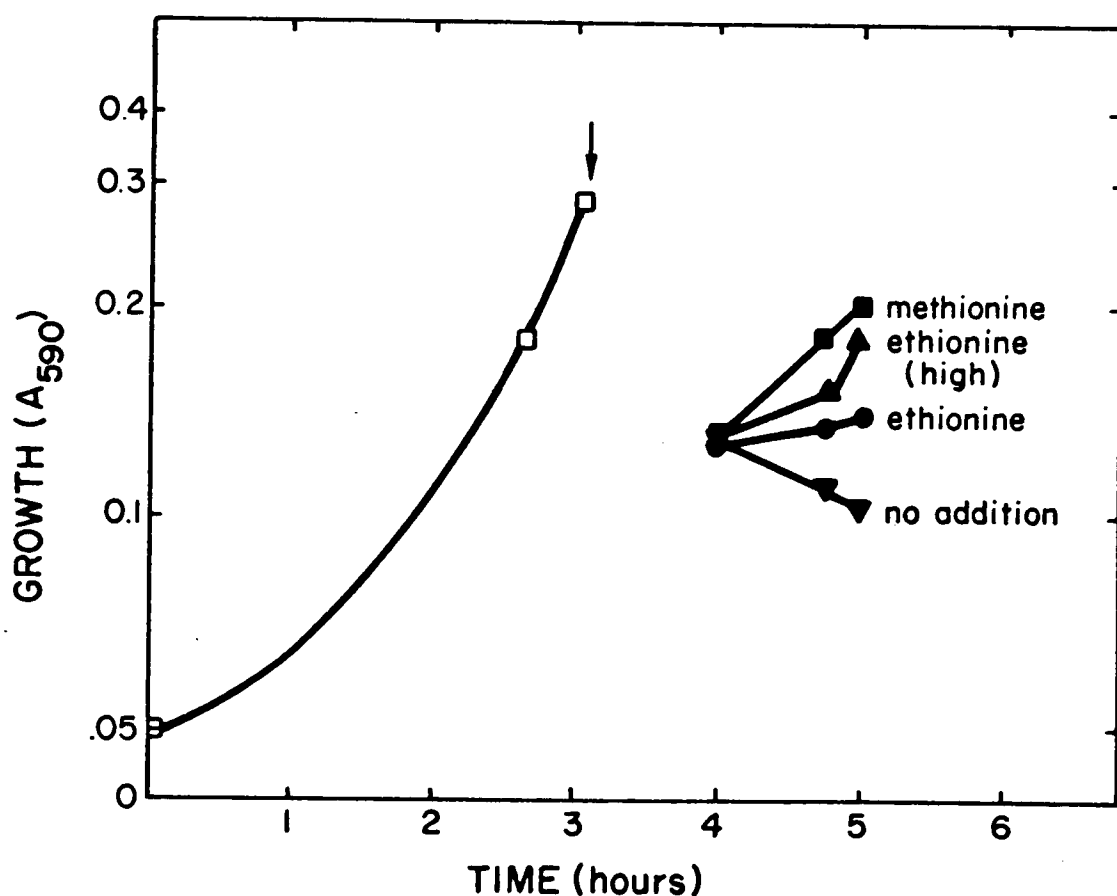


Figure 10. Methionine starvation of *Pseudomonas aeruginosa*. At three hours (indicated by arrow) cells growing in MSS with methionine were washed and resuspended in methionine-free medium. The washed cells were then supplemented with either methionine (0.5 mM), ethionine (5 mM) or ethionine (0.5 mM).

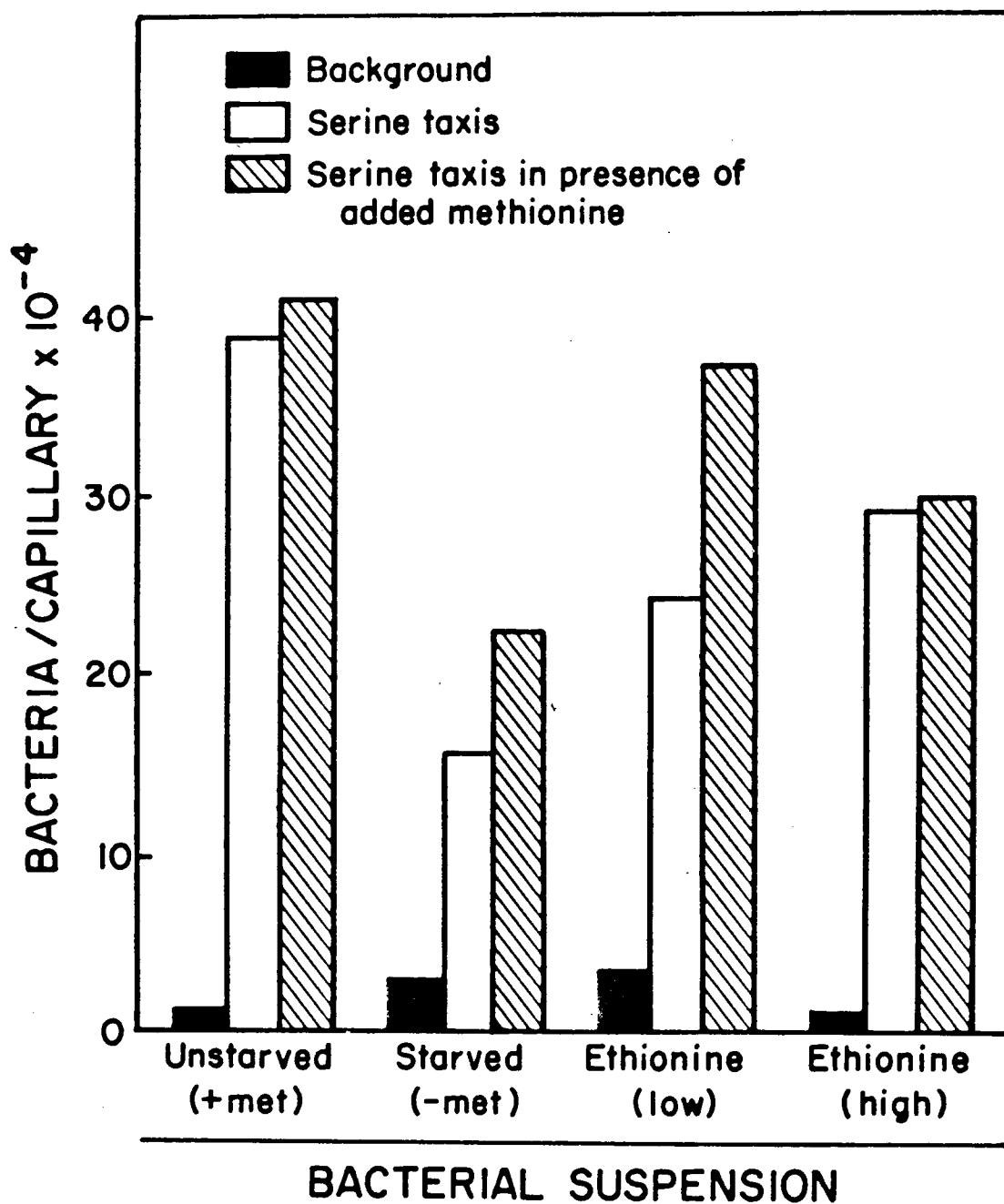


Figure 11. Serine taxis of methionine-starved and ethionine-grown *Pseudomonas aeruginosa*. The bacteria were either unstarved, starved for methionine for one hour or were grown for one hour with ethionine (0.5 or 5 mM) in place of methionine. Taxis toward 1 mM serine was measured in the presence and absence of 0.1 μ M methionine.

results of experiments performed with bacteria which had been starved for one hour. Chemotaxis, measured as the accumulation of bacteria in capillaries containing 1 mM serine, was reduced in the starved cells to a level that was only 40% of that of unstarved cells. In contrast, the accumulation of bacteria in control capillaries containing only CTX buffer was increased by 2.4-fold relative to the unstarved cells. Addition of exogenous methionine at 1×10^{-7} M to the starved bacteria prior to the chemotaxis experiment caused a slight stimulation of serine taxis while no effect on unstarved cells was seen.

Two additional batches of bacteria were grown in medium containing ethionine in place of methionine. It was reasoned that although ethionine substitutes for methionine for growth (Figure 10) and, hence, presumably for protein synthesis, ethionine might be a poor analog of methionine for methyltransfer reactions. If this were true then growth of P. aeruginosa on ethionine should lead to depletion of intracellular levels of the methyl donor SAM and subsequent loss of chemotactic capacity.

P. aeruginosa in MSS medium containing 5×10^{-4} M ethionine in place of methionine grew very slowly (Figure 10) while cells in 5×10^{-3} M grew at a rate closer to that of cells in methionine-containing medium. Like the methionine-starved cells, the low ethionine bacteria exhibited reduced responsiveness toward serine (Figure 11). These bacteria showed only 62% of the tactic response of unstarved cells in spite of the fact that the cells were highly motile. Further, when methionine was added to the suspension of cells, the level of serine taxis increased to 96% of the level in unstarved cells.

In four experiments in which the motility and serine taxis of one hour starved cells was compared to that of unstarved cells the motility of starved cells averaged 292% that of unstarved cells while serine taxis was only 56% that of unstarved cells. With longer starvation periods (1.5 and 4 hours) motility declined to 89% and 62% of the unstarved values, respectively, while serine taxis was 29% and 9% of the unstarved level. From these results it is clear that chemotactic responsiveness is more sensitive to methionine depletion than is motility. This suggests that either some protein component of the chemotactic response mechanism is lost rather quickly during methionine starvation or that methionine itself has a role in chemotactic response independent of its role as a constituent of proteins.

Arginine starvation. *P. aeruginosa* RM9, an arginine auxotroph derived from RM1, was subject to arginine starvation for one hour. Motility and serine taxis of the arginine-starved bacteria was compared to that of methionine-starved RM46 (Table 8). Although methionine starvation had no effect on motility of RM46, serine taxis of RM46 was reduced by 80%. In comparison arginine starved RM9 showed a 28% loss of motility and a 22% loss of serine taxis.

Effect of exogenous methionine. Figure 12 shows the effect of methionine in the CTX buffer on the responsiveness of starved and unstarved cells to a gradient of serine. Methionine in various concentrations was added to suspensions of unstarved and methionine-starved bacteria; the same concentrations of methionine were also included in the buffer and serine solutions used to fill the capillary tubes. Methionine in concentrations of 1×10^{-7} M or greater caused inhibition of the accumulation

Table 8. Effect of arginine starvation on motility and serine taxis of Pseudomonas aeruginosa strain RM9.

Bacterial Suspension	Accumulation (10^4 Bacteria/Capillary) ^b			
	Buffer	Capillaries	Serine	Capillaries ^a
RM46 unstarved	1.04	(± 0.64)	35.3	(± 8.3)
RM46 methionine-starved	1.16	(± 0.19)	7.3	(± 7.1)
RM9 unstarved	0.81	(± 0.45)	20.2	(± 7.5)
RM9 arginine-starved	0.58	(± 0.10)	15.8	(± 6.2)

^aAttractant capillaries contained 1 mM serine.

^bStandard deviations are shown in parenthesis.

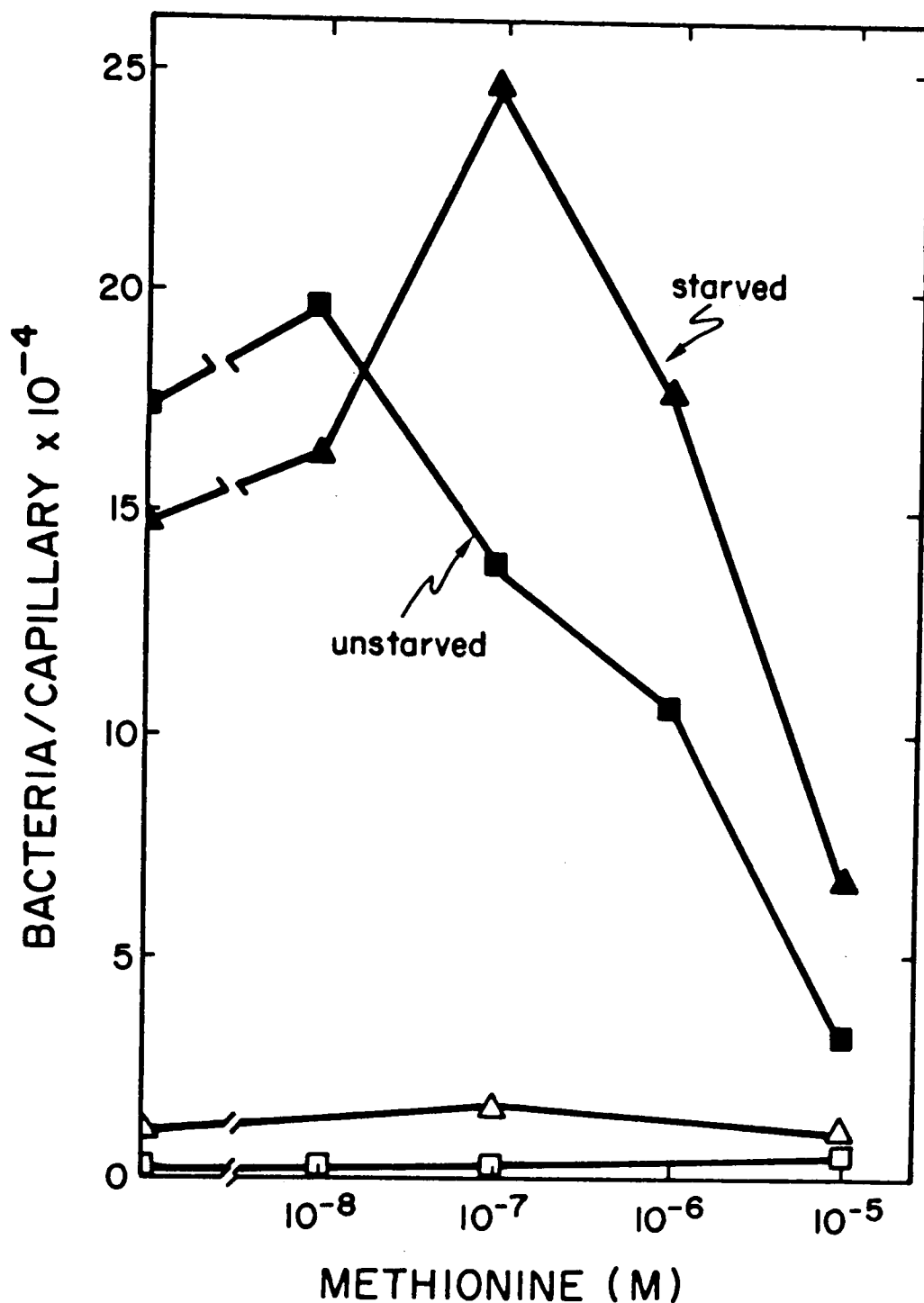


Figure 12. Effect of exogenous methionine on the motility and serine taxis of unstarved and methionine-starved *P. aeruginosa*. Triangles represent cells that had been starved for methionine for one hour; squares, unstarved cells: ▲, ■ 1 mM serine taxis; △, □ background motility.

of unstarved cells in response to a serine gradient; at 1×10^{-5} M only 19% of the normal response (that with no methionine in the buffer) remained. This inhibitory effect of exogenous methionine on unstarved cells has been noted previously by this author (Moulton, 1977; Moulton and Montie, 1979). A number of the other substances will similarly inhibit the accumulation of P. aeruginosa in attractant capillaries. However, the mechanism of this effect has not yet been explained.

The response of starved cells to serine was also inhibited, although less severely, by the highest concentration of methionine tested; the accumulation of starved cells was reduced by approximately one-half by the presence of 1×10^{-5} M methionine. However, at the lower level of 1×10^{-7} M methionine serine taxis was strongly stimulated. Finally, exogenous methionine had very little effect on background motility. As in the previous experiment (Figure 11), the motility of the starved cells was in all cases greater than that of unstarved cells.

The data in Figure 12 verified the observations of Figure 11 above that in spite of vigorous motility of the methionine starved bacteria chemotaxis toward serine is reduced (although in this experiment the magnitude of that reduction is less than the previous experiment). Secondly, the chemotactic responses of the starved cells is stimulated by exogenous methionine at 1×10^{-7} M. However, it is very difficult to interpret the effects exerted by exogenous methionine on starved cells due to the fact that methionine itself blocks migration of bacteria into the capillaries by some mechanism apparently unrelated to methionine starvation.

Chemotaxis-Associated Methyltransfer Reaction in *P. aeruginosa*:

Experimental Design

The experimental strategy used to test for the presence of methyl-accepting chemotaxis proteins (MCPs) in *P. aeruginosa* was similar to that described by Kort et al. (1975) and Goy et al. (1977, 1978). The bacterial cells were treated with a protein synthesis inhibitor, chloramphenicol, to block the incorporation of methionine into proteins by translation. Presumably, the presence of the inhibitor does not interfere with other biochemical reactions involving methionine, in particular the formation of SAM and the utilization of SAM in post-translational modification of proteins. Methionine, radiolabeled with either ^3H or ^{14}C in the methyl position, was added to the cells. After a period of time to allow some uptake and metabolism of methionine to occur, the cells were subjected to a temporal gradient of attractant achieved by a single addition of an attractant solution. Finally, stimulated and control (unstimulated) cells were solubilized in SDS, and the proteins were analyzed for the presence of a component which responds to an attractant stimulus with a change in the level of methyl group incorporation.

In performing experiments in a previously untested organism it was necessary to consider a number of points. For instance what substance should be used as the chemotactic stimulus? Secondly, the composition of the assay mixture was very important. In particular the buffer, the presence or absence of an energy source, the concentration and specific activity of the methionine, and the effectiveness of the protein synthesis inhibitor would all affect the transport and

metabolism of methionine as well as the chemotactic responsiveness of the cells and the visualization of MCP on the gels. Other considerations were the length of exposure of the cells to methionine prior to stimulation and the length of the response period after stimulation. These points are discussed more thoroughly below.

Serine, arginine, and α -aminoisobutyrate (AIB), all strong attractants for P. aeruginosa, were tested as chemotactic stimuli in the methylation experiments. AIB was preferred to the others since it is not metabolized by P. aeruginosa (see page 105).

Chemotaxis (CTX) buffer, which supports vigorous motility and chemotaxis in the capillary experiments, was used for suspending the bacterial cells for the methylation experiments.

Chloramphenicol was tested for effectiveness as an inhibitor of protein synthesis in P. aeruginosa RM46 (Figure 13). The antibiotic effectively blocked incorporation of labeled methionine into acid precipitable material for at least one hour. A concentration of 200 $\mu\text{g/ml}$ was chosen for routine use in these experiments. At this concentration chloramphenicol appeared to have no adverse effect on chemotactic capacity. The number of bacteria accumulating in serine (1 mM) capillaries was reduced 30% if chloramphenicol was added to the cells 15 min. prior to the capillary assay. However, exposure of bacterial cells in CTX buffer to chloramphenicol for one hour in the absence of attractant caused a 33% loss of viability as determined by plate counts on tryptone agar. Thus one can conclude that the apparently lower serine response of chloramphenicol-treated cells was due to reduced plating efficiency of the cells that accumulate in the

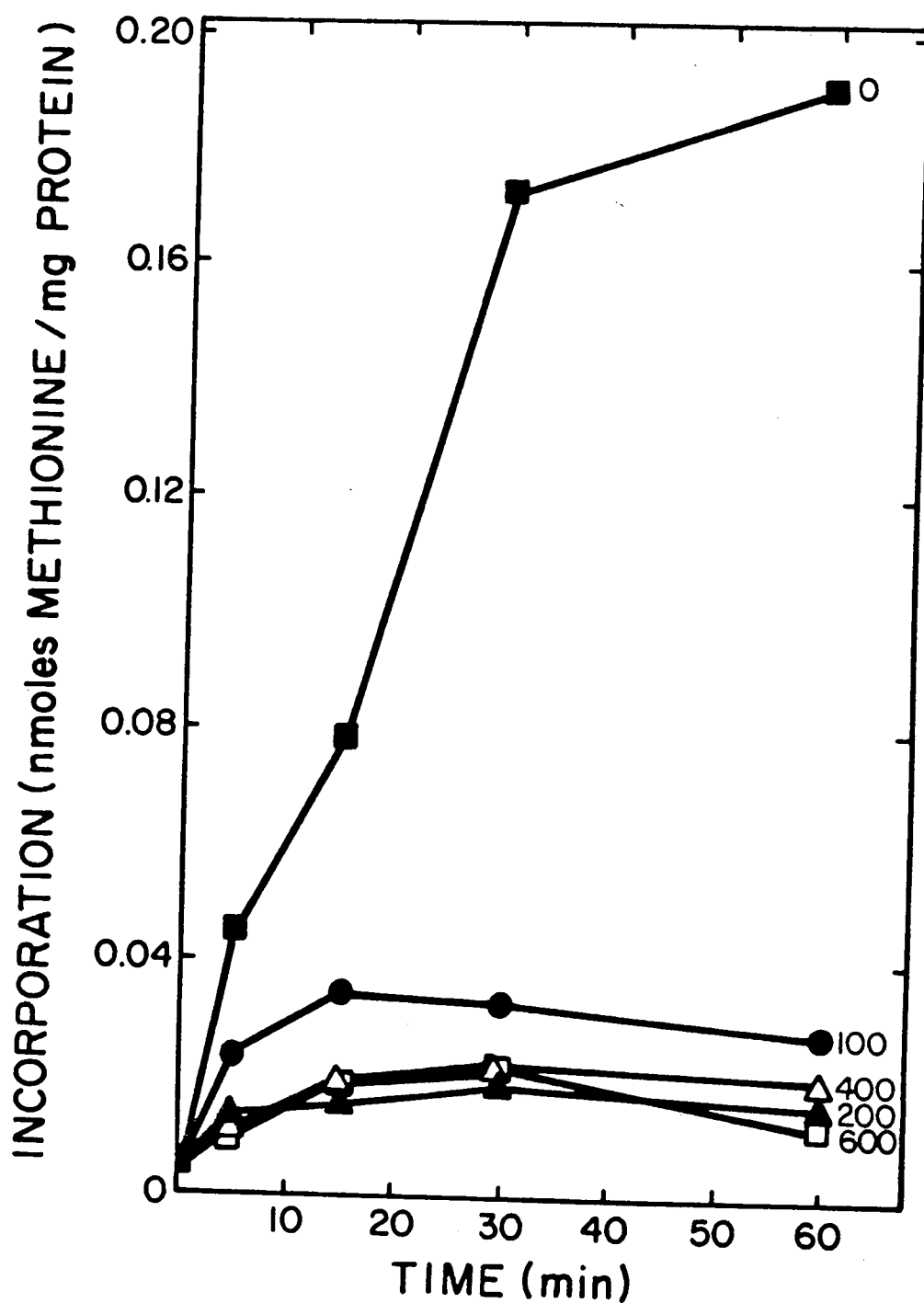


Figure 13. Chloramphenicol inhibition of methionine incorporation into acid-precipitable material. ■, no chloramphenicol; other symbols, chloramphenicol at 100 to 600 µg/ml.

capillary and not to reduced ability of the bacteria to respond to serine. Gentamycin and streptomycin were examined as alternatives to cholamphenicol. However, these antibiotics proved to be less effective as inhibitors of methionine incorporation.

The presence of an energy source in the CTX buffer is not necessary to stimulate the chemotactic responses of P. aeruginosa. In fact the presence of succinate inhibited the accumulation of the bacteria in attractant capillaries (Moulton, 1977). The inclusion of an energy source in the methylation assay mixture might be helpful, however, by stimulating the uptake of methionine and the formation of SAM. It would be necessary to choose a concentration which stimulates methionine uptake with minimal inhibition of chemotaxis. The effects of succinate on uptake of methionine and chemotaxis toward serine are compared in Figure 14. Succinate in excess of 1×10^{-5} M was required to significantly stimulate methionine transport. However, in that concentration range chemotaxis was severely inhibited. In the earliest methylation experiments succinate at 1×10^{-5} M (which gave a slight stimulation of taxis) did not stimulate labeling of macromolecular species by methionine. For these reasons it was decided to eliminate succinate from the methylation assay mixture. No energy sources other than succinate were tested for use in the methylation assay.

As described earlier (Figure 12, page 79) exogenous methionine also inhibits chemotactic migration of P. aeruginosa. Therefore, it was necessary to choose a concentration of methionine for the methylation assay that was high enough to allow effective uptake with a minimal effect on chemotactic responsiveness. A concentration of 5×10^{-7} M

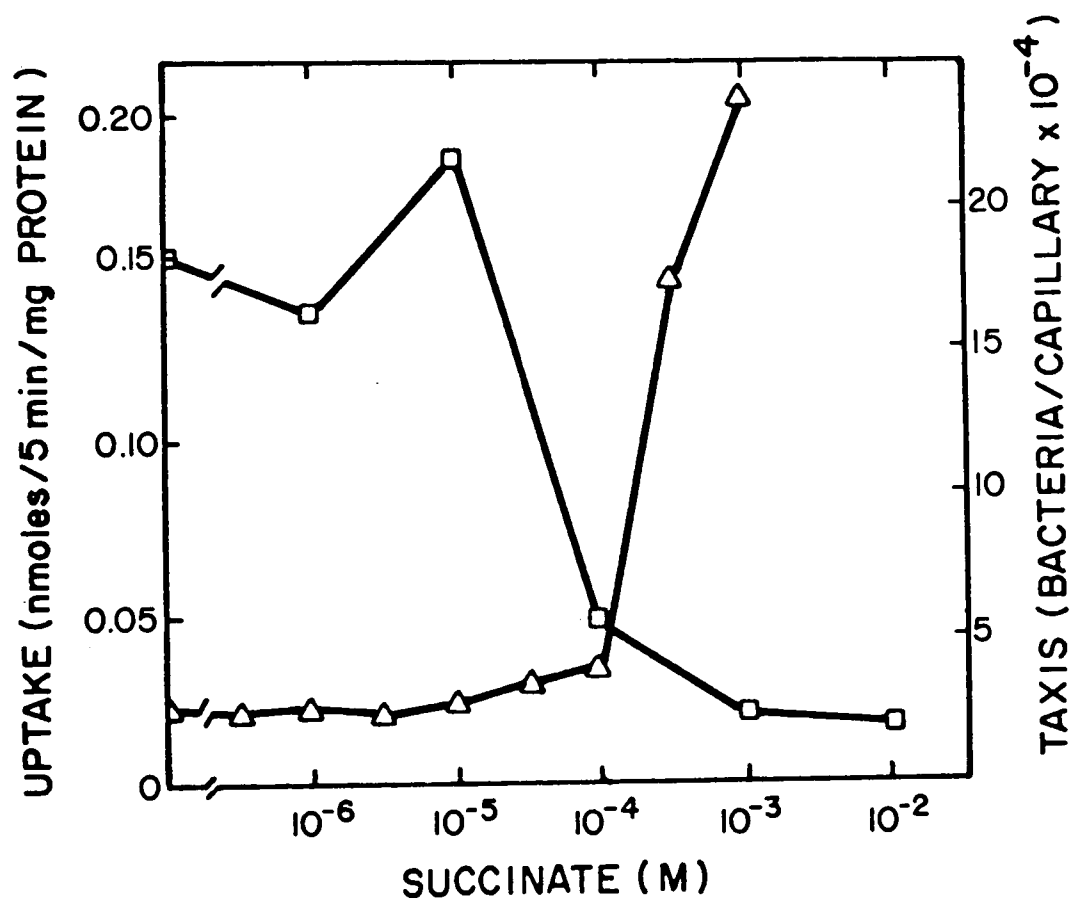


Figure 14. Effect of succinate on the uptake of methionine and on chemotaxis toward serine. $\square$, 1 mM serine taxis; Δ , methionine uptake.

MCPs reached a new plateau within 10 min. after stimulation with a strong attractant. Microscopic observations of the behavior of P. aeruginosa in a spatial gradient of AIB showed that cells began accumulating in capillaries in less than five minutes. Quantitative capillary experiments showed P. aeruginosa accumulating in AIB capillaries reached a maximum in as little as 10 to 15 min. A standard stimulation period of 15 min. was adopted for the methylation assay. This time should be more than sufficient for the methylation response to AIB to occur. As the result of time course experiments (page 96), the stimulation period was shortened to 10 min. in later experiments.

Identification of the Putative MCP

P. aeruginosa RM46 was labeled with [methyl-³H]methionine (60 mC/mole) according to the procedure described in Chapter II (Materials and Methods). After the initial incubation of the cells with radioactive methionine, half of the cells were stimulated by the addition of 1 mM serine (a 0 → 1 mM stimulus). The other half of the cells received only buffer. After 15 min. exposure to serine the cells were solubilized in S buffer and proteins separated on a 16 cm 10% polyacrylamide gel. The labeling patterns of stimulated and unstimulated cells were compared after autoradiography of the dried gels on X-ray film for 114 days. The Coomassie blue stained bands of the original gel are shown in Figure 15 along with the autoradiogram and densitometer tracings of the autoradiogram.

An array of labeled bands was visualized on the autoradiogram. The most heavily labeled band (designated MB1) ran with an apparent molecular weight of 47,000. In several later experiments the MB1 band

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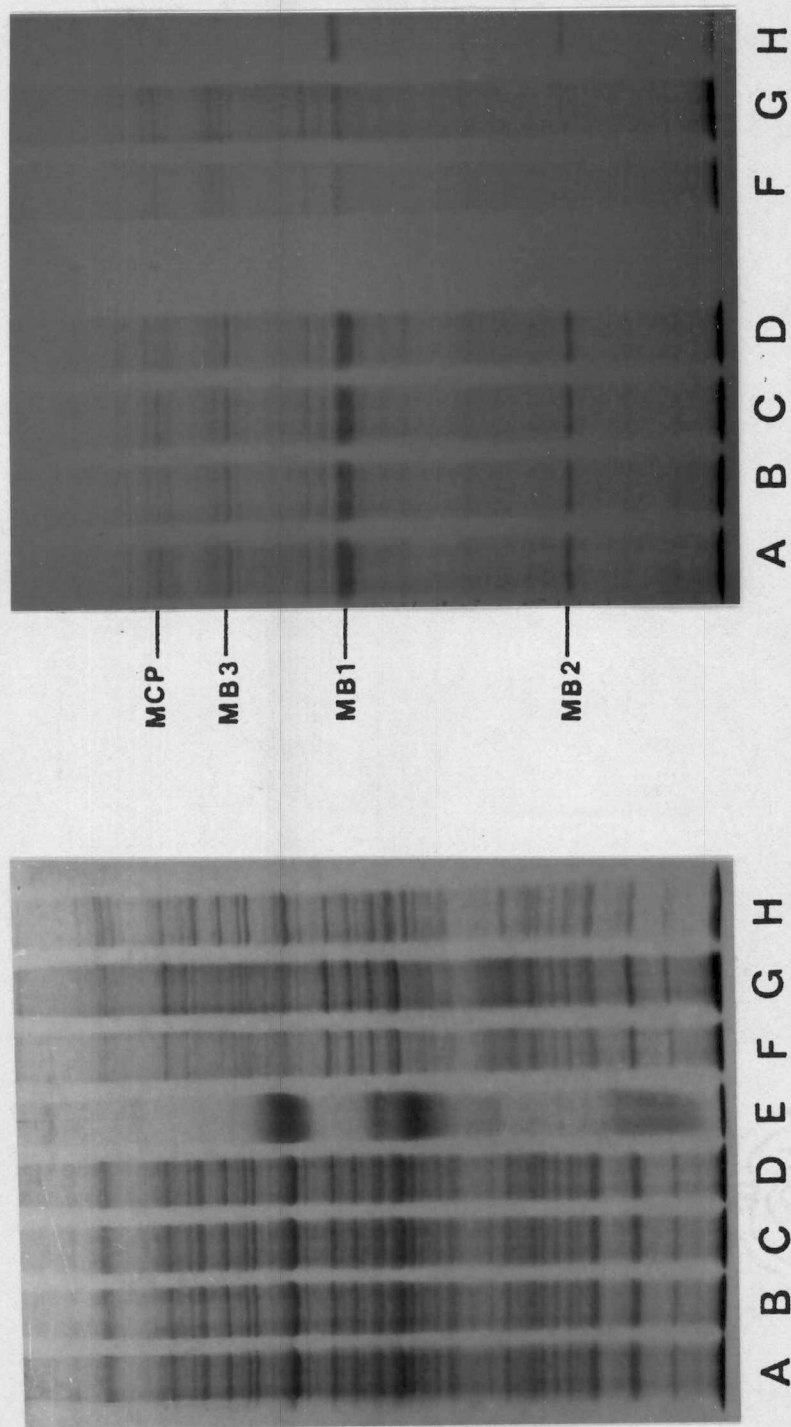


Figure 15. [Methyl- ^3H]methionine labeled *Pseudomonas aeruginosa*. The stained gel is shown on the left and the autoradiogram of that gel on the right. Lanes A and C, serine stimulated cells (1 mM serine for 15 min); lanes B and D, unstimulated cells; lane E, molecular weight markers; lanes F and G, membrane fraction; lane H, soluble fraction. Densitometer scans of lane B and C are shown on the next page.

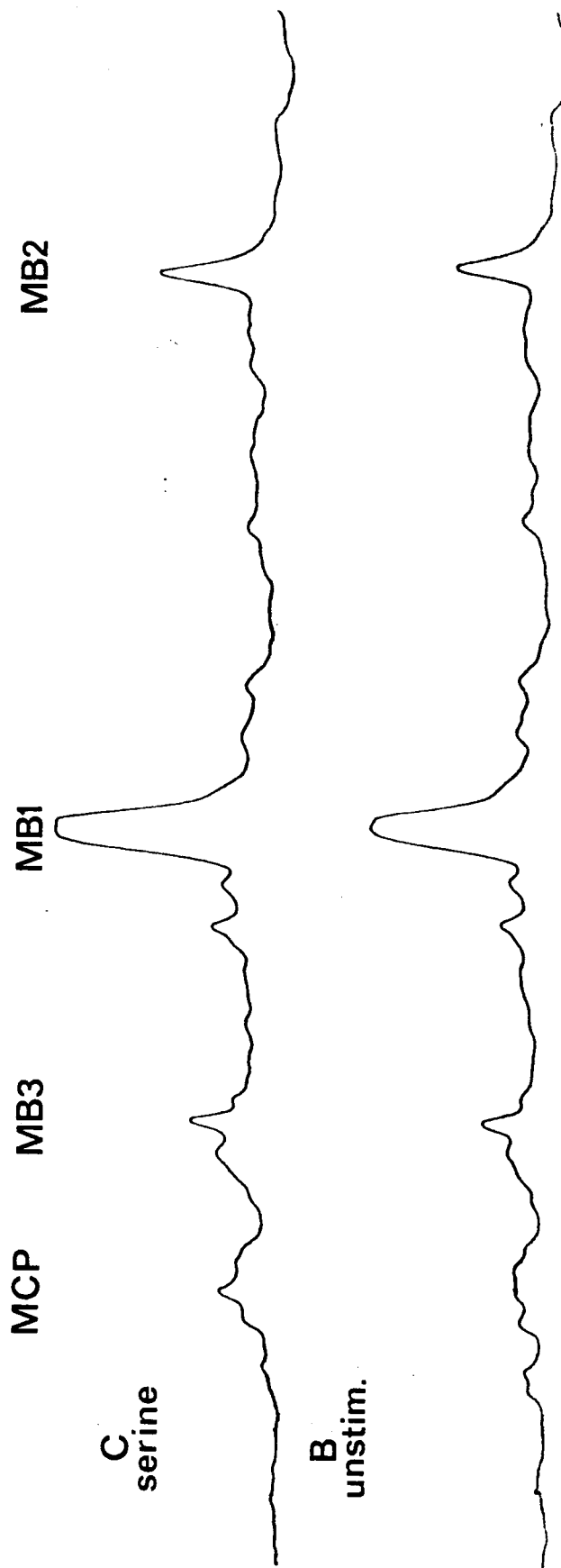


Figure 15 (continued)

was resolved into two components. MB2 and MB3 were less heavily labeled than MB1. Like MB1, MB3 was resolved into two components on later gels. A number of weakly labeled bands, about 20 in all, were seen on this autoradiogram. No apparent differences in the patterns of the stimulated and unstimulated cells were evident except for one band which ran more slowly than MB3. This band was considerably darker in the stimulated cells than in the unstimulated cells. This band was designated as the presumptive MCP or "MCP."

The relative amount of radioactivity associated with the "MCP" band was determined by integrating the area under the peaks on the densitometer tracing. The methylation level of "MCP" was defined as the ratio of the area of the "MCP" peak to the area of the MB3 peak. Values of 0.15 and 0.27 were obtained for unstimulated and stimulated cells, respectively. Thus, the radioactivity associated with the "MCP" band is increased by 80% as the result of serine stimulation.

In later experiments two approaches were taken to improve the resolution of the "MCP" from other labeled components in the same region of the gel. The 10% gels were run for longer periods of time; the resulting gels were 18 to 22 cm long. Secondly, a higher gel concentration (12.5%) was tried.

Autoradiograms of the ^{14}C -labeled cell extracts (Figure 15) showed very sharp banding patterns. However, the low specific activity of [methyl- ^{14}C]methionine required excessively long exposure times. Tritium-labeled methionine, which was available at much higher specific activity, was used for all subsequent experiments. Although the bands of tritium fluorograms were considerably more fuzzy than those on ^{14}C

autoradiograms, the exposure time could be reduced to approximately one week.

Estimation of the Molecular Weight of "MCP"

The molecular weight of the "MCP" was determined by electrophoresis of the labeled and solubilized cells in 10% polyacrylamide with SDS (Figure 16). The electrophoretic mobility of the MCP band was compared to that of the proteins of known molecular weights. The following proteins were used as standards: phosphorylase a (94,000), bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000) and β -lactoglobulin (18,400). From this analysis a molecular weight of 73,000 to 74,000 was estimated. This value is slightly greater than the values reported for E. coli (Engström and Hazelbauer, 1980) and S. tryphimurium (DeFranco, 1980). In those organisms the various MCP bands ran with apparent molecular weights of between 55,000 and 65,000.

Stimulation of Methylation by Arginine and AIB

Labeling of "MCP" in cells stimulated by arginine and by AIB was compared with that in serine stimulated cells. The labeled and stimulated cells were solubilized and separated on a 12.5% polyacrylamide gel. Figure 17 shows the resulting fluorogram. Two components which ran in the 74,000 dalton region and responded to the three attractants were resolved by electrophoresis in the higher percentage gel. Both bands appeared to respond to all three attractants. However, the strength of the response varied depending on the attractant. Arginine stimulated cells showed considerably darker bands than either AIB or serine stimulated cells. Quantitative data are seen in Table 9. Both

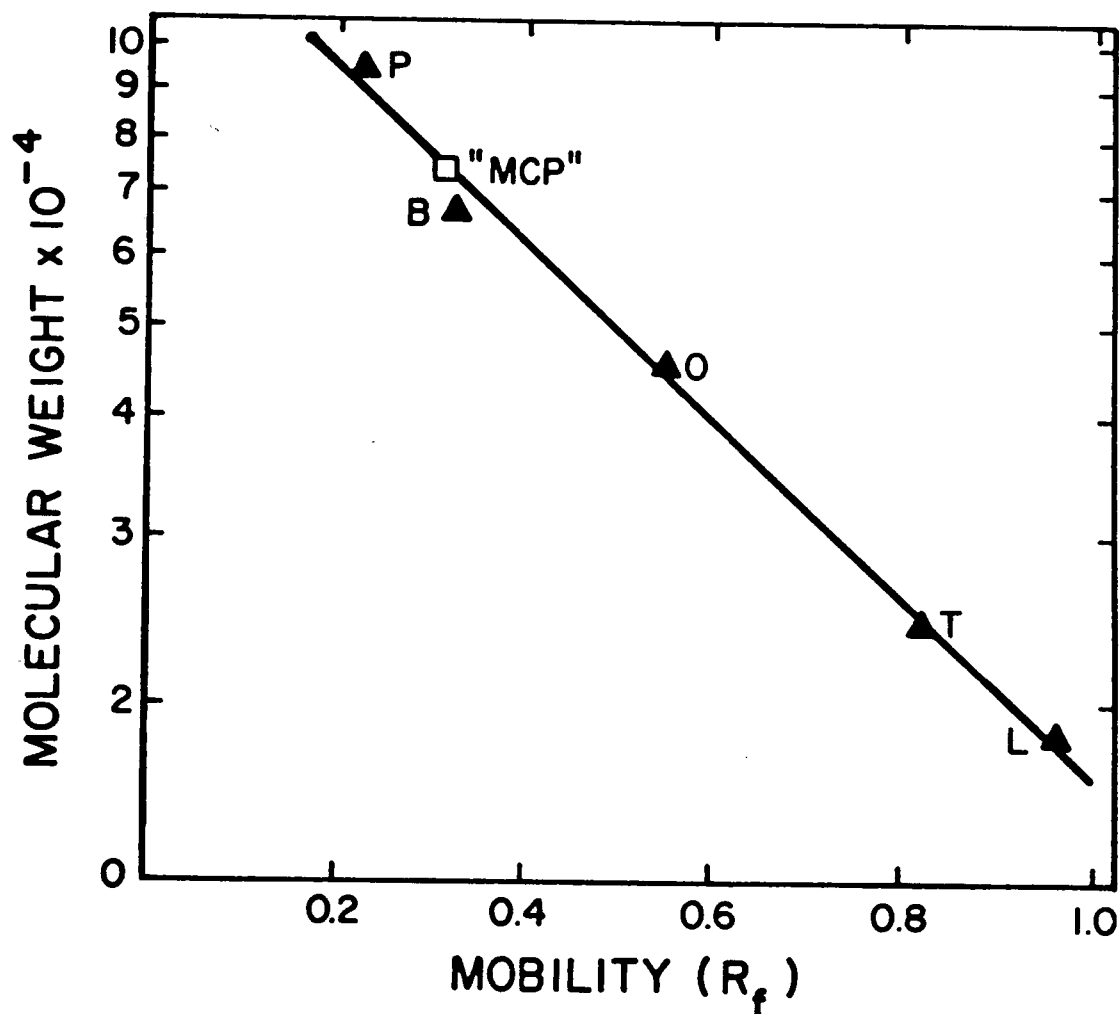


Figure 16. Estimation of the molecular weight of the putative MCP of *Pseudomonas aeruginosa*. P, phosphorylase a; B, bovine serum albumin; O, ovalbumin; T, trypsinogen; L, β -lactoglobulin.

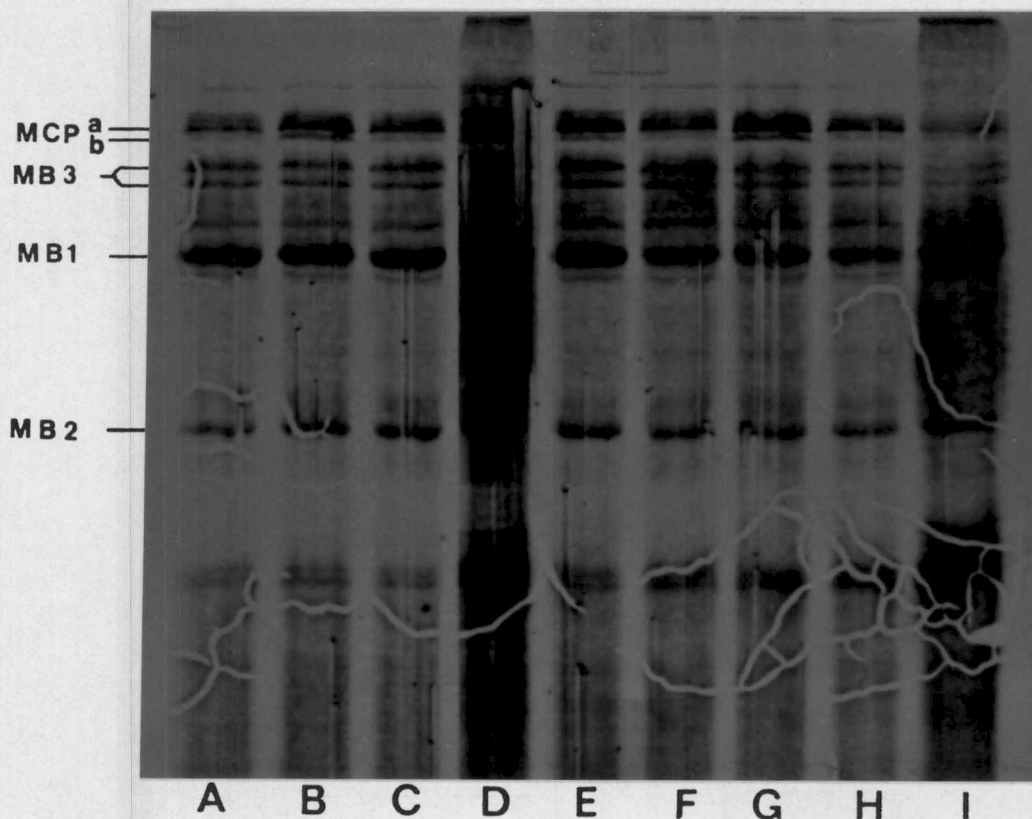


Figure 17. Methylation of MCP in response to arginine, AIB, and serine. Lanes A-E are ammonia grown cells; F-I, nitrate grown cells; A, F, unstimulated; B, G, arginine stimulated; C, H, AIB stimulated; D, I, succinate stimulated; E, serine stimulated. All stimuli were 10 mM for 10 min. Polyacrylamide concentration was 12.5%.

Table 9. Methylation of "MCP" of *Pseudomonas aeruginosa* in response to arginine, AIB and serine stimulation.

Bacteria ^a	Methylation Level of "MCP" ^b		
	"MCP" Band a	"MCP" Band b	Total
Ammonia-grown cells			
Unstimulated	0.605 (1.0)	0.042 (1.0)	0.648 (1.0)
Arginine stimulated	1.126 (1.9)	0.269 (6.4)	1.395 (2.2)
AIB stimulated	0.829 (1.4)	0.085 (2.0)	0.915 (1.4)
Serine stimulated	0.755 (1.2)	0.097 (2.3)	0.852 (1.3)
Nitrate-grown cells			
Unstimulated	0.460 (1.0)	0.040 (1.0)	0.500 (1.0)
Arginine stimulated	0.951 (2.1)	0.192 (4.8)	1.142 (2.3)
AIB stimulated	0.878 (1.9)	0.076 (1.9)	0.954 (1.9)

^aBacteria were grown in MSS medium containing either ammonium sulfate or potassium nitrate, then stimulated with 10 mM arginine, AIB or serine for 10 min.

^bElectrophoresis was carried out on a 12.5% polyacrylamide gel, 16 cm long. The methylation level is a ratio of the radioactivity in "MCP" Band a or Band b to that in MB3. The values in parenthesis show the methylation level of attractant stimulated cells relative to that of unstimulated cells.

bands responded to arginine, AIB, and serine with an increase in label, although serine caused only a 20% increase in band a. Arginine, which is a stronger attractant than AIB and serine in the capillary assay, elicited the strongest response also in the labeling experiment. This is particularly evident in band b where labeling was stimulated six-fold by arginine. For all three attractants the percentage increase in labeling was greater for b than for a. Again, this is most evident for arginine stimulated cells where radioactivity in band a was increased 1.9-fold while that in band b went up 6.4-fold.

Methylation in Nitrate-grown Cells

Bacteria which had been grown on MSS medium containing nitrate instead of ammonia were labeled with [methyl-³H]methionine and stimulated with arginine and AIB (Figure 17 and Table 9). A pattern similar to that of the ammonia-grown cells (described above) was seen. Both arginine and AIB stimulated the labeling of band a and band b; the response to arginine was considerably stronger than that to AIB, particularly in band b. The total response to arginine was of the same magnitude (2.3 times the unstimulated level) in nitrate cells as in ammonia-grown cells. The total response to AIB was somewhat stronger in nitrate cells (1.9 times the unstimulated level in nitrate compared to 1.4 in ammonia cells). The significance of these differences is uncertain until further studies can be carried out on nitrate-grown bacteria.

Distribution of Radioactivity Throughout the Gel

The radioactivity associated with the major labeled species was determined by integration of the densitometer tracings of the fluorogram

and expressed as a percentage of the total activity. Table 10 shows the distribution of radioactivity throughout the gel pattern obtained from unstimulated cells and from AIB stimulated cells. The tritium label of the MCP peak was more than tripled by AIB stimulation while the radioactivity in no other fraction increased or decreased by more than 30%. These results verify that upon attractant stimulation the label of the "MCP" band is actually increased relative to the label in the other components of the cell. A similar result was obtained when serine was used as the stimulus.

In this paper the amount of methyl label in "MCP" is expressed as the ratio of the area of the "MCP" peak to that of the MB3 peak (including components a and b). By the use of this type of internal standardization concerns about variations in the amount of protein loaded in each well can be eliminated.

Time Course of Methylation

Bacteria were prepared as usual for labeling with [methyl-³H]-methionine. After 15 min. exposure to the methionine, samples were removed to determine the methylation level before attractant stimulation. Two minutes later the cells were divided and half were stimulated with AIB (0 → 1 mM); the other half received only buffer. At intervals thereafter samples of stimulated and unstimulated cells were removed and solubilized in S buffer for determination of radioactivity in "MCP." As soon as one minute after addition of AIB, the methylation level of "MCP" in stimulated cells was markedly higher than that of unstimulated cells (Figure 18A). Radioactivity in the "MCP" band reached a peak of 1.6-times the prestimulus level at 10 min., then declined somewhat by

Table 10. Distribution of radioactivity throughout the electrophoretic patterns of unstimulated and AIB-stimulated *P. aeruginosa*.

Fraction	Percentage of Total Radioactivity	
	Unstimulated	AIB-Stimulated ^b
1	0	0
2 "MCP"	1.5	5.0
3	2.4	2.1
4 MB3a	7.8	9.8
5 MB3b	10.2	9.8
6	8.0	8.2
7 MB1	35.9	36.6
8	1.3	0.9
9	2.2	1.6
10	4.3	3.7
11	2.8	2.3
12	5.4	3.9
13 MB2	16.5	14.6
14	1.5	1.4

^aElectrophoresis was carried out on 10% polyacrylamide gel, 18 cm long.

^bBacteria were subjected to a 0→10 mM AIB stimulus, then solubilized 10 min. later.

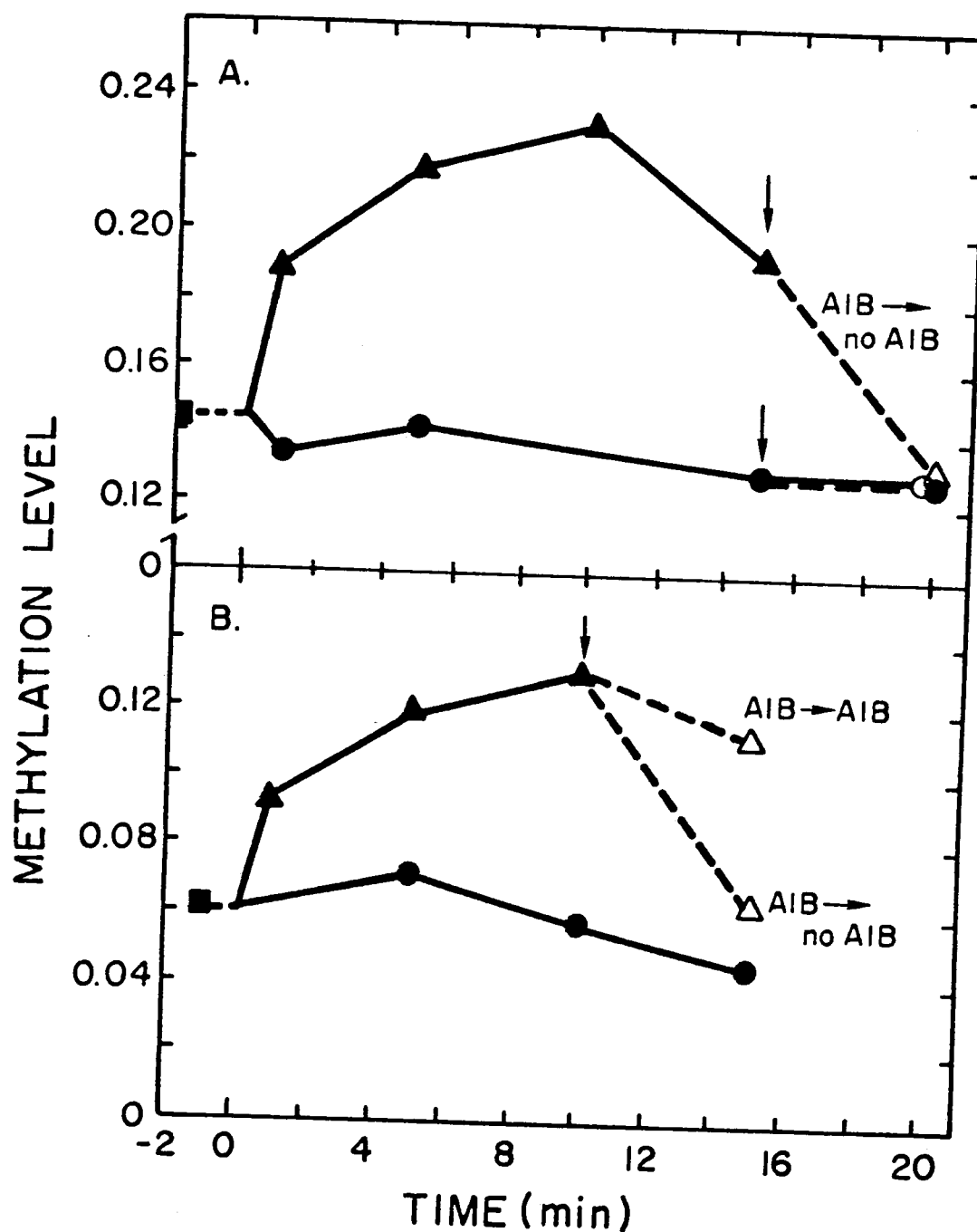


Figure 18. Time course of methylation. *P. aeruginosa* cells were labeled with [methyl- ^3H]methionine. At time zero the cells were divided and AIB (10 mM) was added to one half. Panel A: at 15 min a sample of AIB-stimulated cells and another of unstimulated cells were centrifuged and resuspended in AIB-free buffer. Panel B: at 10 min samples of AIB-stimulated cells were centrifuged and resuspended in buffer with and without AIB. ▲, AIB stimulated; ●, unstimulated.

15 min. In contrast, the level of radioactivity in "MCP" of unstimulated cells remained quite constant for the duration of the experiment.

Removal of the Stimulus

The effect of removal of the attractant on methylation level of "MCP" is also shown in Figure 18A. Samples of stimulated and unstimulated cells at 15 min. were centrifuged in microfuge tubes and the pellets were suspended in AIB-free buffer (CTX buffer containing labeled methionine and chloramphenicol but no AIB) for 5 min. before solubilizing in S buffer as usual. The centrifugation had no effect on the radioactivity level of unstimulated cells; however, removal of AIB caused the radioactivity of "MCP" in stimulated cells to drop to the level of unstimulated cells. In a similar experiment (Figure 18B) AIB was removed after a 10 min. stimulation period. Resuspension of the stimulated cells in AIB-free buffer caused a drop of 50% in the methylation level of "MCP," down to the pre-stimulus level. Centrifugation and resuspension of the same cells in AIB-containing buffer resulted in only a slight reduction (18%) in "MCP" label, indicating that the removal of AIB rather than the centrifugation procedure itself caused the return of methylation to the unstimulated level following removal of AIB. The drop in methylation of the "MCP" band following the removal of the AIB stimulus is similar to that seen in E. coli upon dilution of a chemoattractant (Kort et al., 1975).

Labeling with [³⁵S]Methionine

The preceding experiments have identified a radioactive component in P. aeruginosa which is labeled by [methyl-³H]methionine in response

to attractant stimulation. The response to various attractants, the time course of labeling, and the effect of attractant dilution on the labeling of the component in question are all consistent with the hypothesis that this component is analogous to the methyl-accepting chemotaxis protein of other chemotactic bacteria. A critical question is whether the attractant-responsive band of *P. aeruginosa* is labeled by a methyltransfer reaction mediated by S-adenosylmethionine. This question was first approached by labeling cells with [^{35}S]methionine and comparing the resulting pattern of radioactive bands with the pattern of [methyl- ^3H]methionine labeled cells. If the putative MCP band is labeled by methyltransfer, labeling in response to attractant stimulation should be evident in the tritium-labeled cell preparation but not in sulfur-labeled cells. On the other hand, if the entire methionine molecule (rather than the methyl moiety only) is incorporated into "MCP," the same response to attractant should be observed regardless of the isotope used.

Bacteria were prepared as usual for labeling experiments except that half were incubated with [methyl- ^3H]methionine while the remainder were incubated with [^{35}S]methionine. In both cases the final methionine concentration was $0.5\ \mu\text{M}$ with specific activity of 30 C/nmole. The bacteria were stimulated with 10 mM AIB for 15 min., then solubilized and separated on a 10% polyacrylamide gel. Figure 19 shows the resulting fluorogram. The typical pattern of radioactive bands was seen in the tritium-labeled cell preparation (lanes A and B); radioactivity in the "MCP" band increased 3-fold (from 0.08 to 0.26 relative to MB3) in response to AIB stimulation. No comparable AIB-responsive component was seen in the

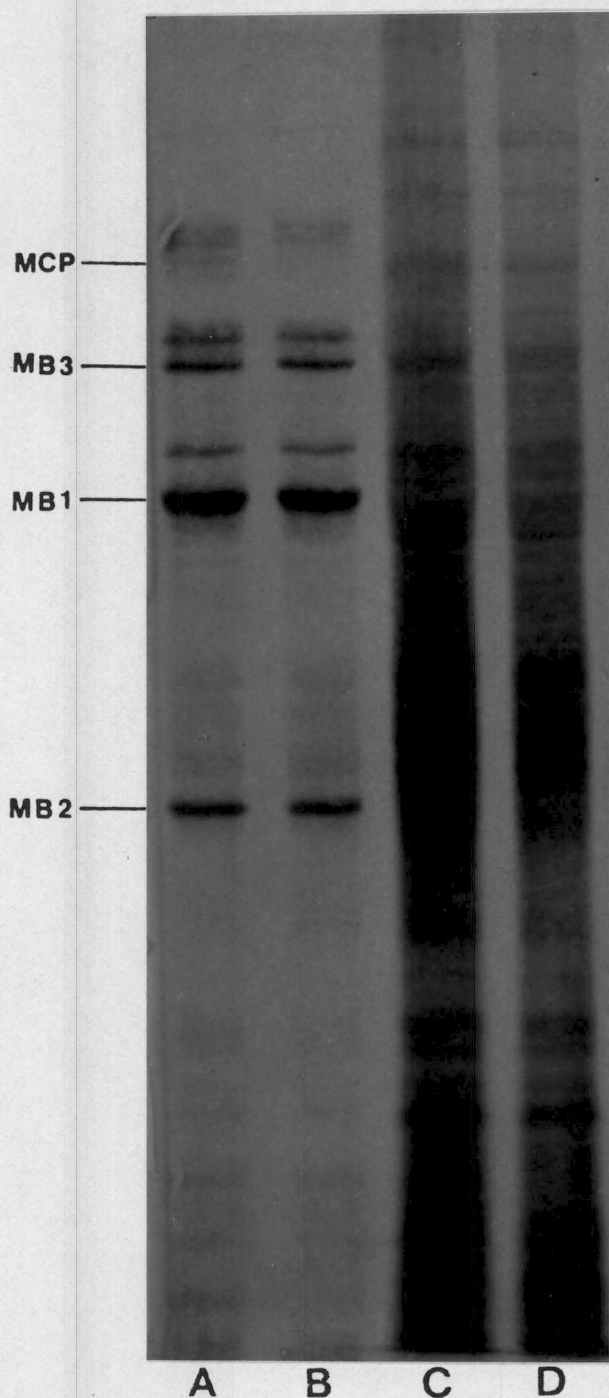


Figure 19. Comparison of [methyl- ^3H]methionine and [^{35}S]methionine labeling. A, B, [methyl- ^3H]methionine; C, D, [^{35}S]methionine. A and C were stimulated with 10 mM AIB for 10 min; B and D are unstimulated.

[^{35}S]methionine-labeled cell preparations (lanes C and D). The radioactivity of the regions of the gel which correspond to the "MCP" and MB3 regions of lanes A and B (the tritium-label patterns) was measured by densitometry. The ratio of the two was 0.26 in both stimulated and unstimulated (lane D) cell preparations. The [^{35}S]methionine label of the "MCP" region when expressed as a percentage of the total radioactivity also showed no difference between stimulated and unstimulated cells. These results indicate that the methyl moiety of methionine, but not the rest of the molecule, is incorporated into the "MCP" band following attractant stimulation.

A dual label experiment was attempted in which the bacteria were incubated with a mixture of [^{35}S] and [methyl- ^3H]methionine prior to AIB stimulation. Labeled and solubilized cells were subjected to electrophoresis on cylindrical gels. The gels were cut into 2 mm and treated to elute radioactivity from the slices for scintillation counting. Tritium-labeled peaks corresponding to MCP, MB3 and MB1 were seen. No ^{35}S was seen in the region of the MCP. However, the results were inconclusive due to the fact that no increase in tritium in the MCP of AIB-stimulated cells compared to unstimulated cells was seen. This may be partly the result of insufficient resolution of MCP from neighboring bands under the conditions employed. The resolution may be improved by running longer gels and cutting thinner slices. This type of experiment could provide the most convincing evidence of a methyltransfer reaction since both isotopes are present in the same cell preparation.

In vitro Labeling with S-adenosyl-[methyl-³H]methionine

The results of the [³⁵S]methionine experiment indicate that "MCP" is labeled by a methyltransfer reaction rather than by incorporation of the entire methionine molecule. S-adenosylmethionine (SAM) serves as the methyl donor in most methyltransfer reactions including those involving methylation of the MCPs of several bacteria (Paik and Kim, 1980). Therefore the following experiment was performed to identify methylated species in P. aeruginosa which are methylated by SAM.

P. aeruginosa was suspended in phosphate-EDTA buffer and then broken in the French pressure cell. SAM carrying tritium in the methyl group was added to a final concentration of 10 μ M and specific activity of 70 C/mole. To half the cell extract was added 10 mM AIB as a chemotactic stimulus. After one hour total labeling time the resultant radioactive species were analyzed by electrophoresis on a 10% polyacrylamide gel (Figure 20). Radioactive species of 47,000, 50,000, 62,000, 73,000, 77,000, and 83,000 daltons were evident (lanes E and F). The 47,000 and 50,000 daltons bands probably represent the major methylated species (MB1) of tritium labeled cells, while the 62,000 dalton species is probably MB3. The mobility of these SAM-labeled species was compared to the mobility of authentic MB1 and MB3 on a tritium label pattern run concurrently on an identical gel. The 72,000 dalton species shows electrophoretic mobility identical to "MCP." The intensity of labeling by SAM was not increased by the presence of AIB, however. The labeling of the 72,000 dalton band, as well as the other bands, was inhibited by S-adenosylhomocysteine (lanes G and H), a known competitive inhibitor of

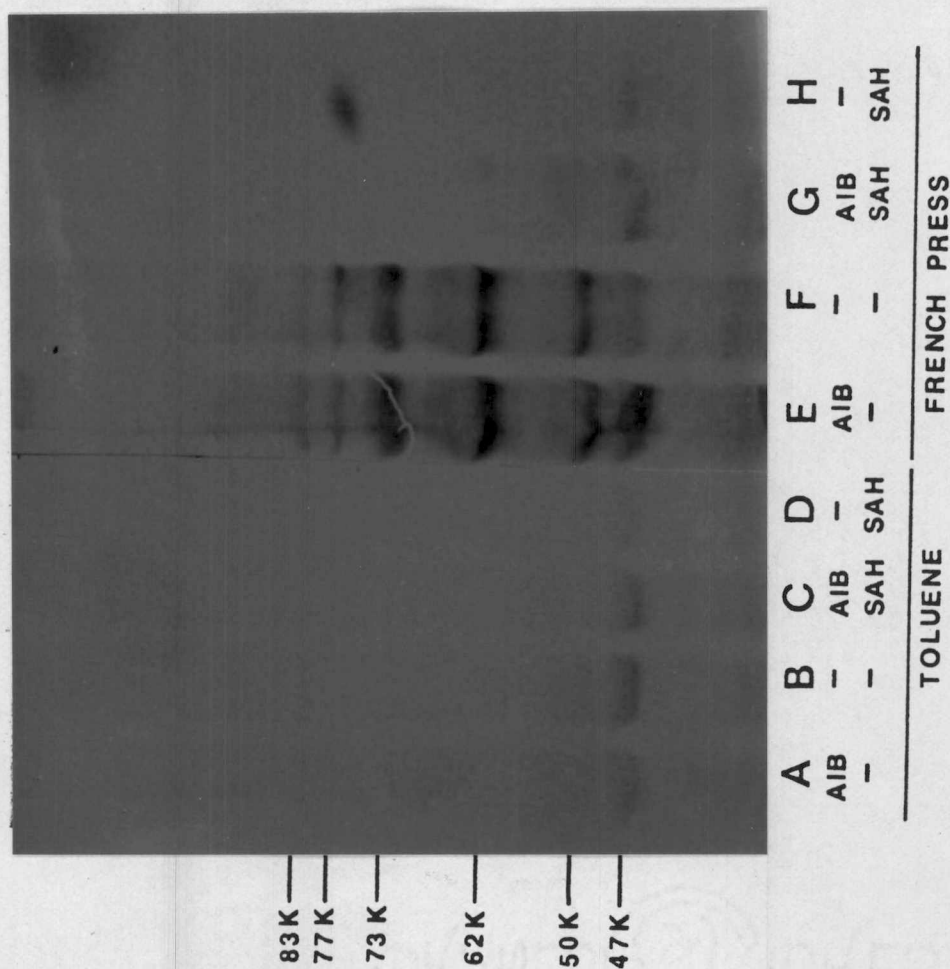


Figure 20. S-adenosyl-[methyl- ^3H]methionine labeling of *Pseudomonas aeruginosa*. Cells were either treated with toluene or broken in the French pressure cell before labeling. S-adenosyl-[methyl- ^3H]methionine was added to the cells preparations in the presence or absence of S-adenosylhomocysteine.

SAM-mediated methyltransfer reactions (Kim and Pail, 1980; Paoni and Koshland, 1979).

In a second procedure for labeling with [^3H]SAM, P. aeruginosa cells were treated with toluene (Montie and Montie, 1975) to make them permeable to small molecules such as SAM and SAH. Such an approach has been successful with S. typhimurium (Paoni and Koshland, 1979). However, this attempt with P. aeruginosa was unsuccessful. Only a very faint labeling of the 47,000 dalton and 50,000 dalton bands was seen. The poor labeling of all six bands suggests that the permeabilization procedure was insufficient to allow entry of SAM. It may be possible to optimize the tolueinization procedure to improve labeling efficiency.

Presence of "MCP" in the Membrane Fraction

P. aeruginosa cells which had been labeled with [methyl- ^{14}C]-methionine and stimulated with serine for 15 min. were ruptured in the French pressure cell and a membrane fraction collected by centrifugation at 130,000 g. The membrane fraction and the supernatant were analyzed by electrophoresis for the presence of a radioactive species comparable to the "MCP" band (Figure 15, page 88, and Figure 21). A radioactive species was seen in the membrane fraction (Lane G of Figure 15, page 88) which comigrated with the serine-responsive band of serine-stimulated whole cell preparation (Lanes A and C of Figure 15, page 88). The densitometer scans of the fluorogram can be seen in Figure 21.

Metabolism of α -Aminoisobutyrate by Pseudomonas aeruginosa

The amino acid α -aminoisobutyrate (AIB) has been widely used as a non-metabolizable analog of glycine in studies of amino acid transport

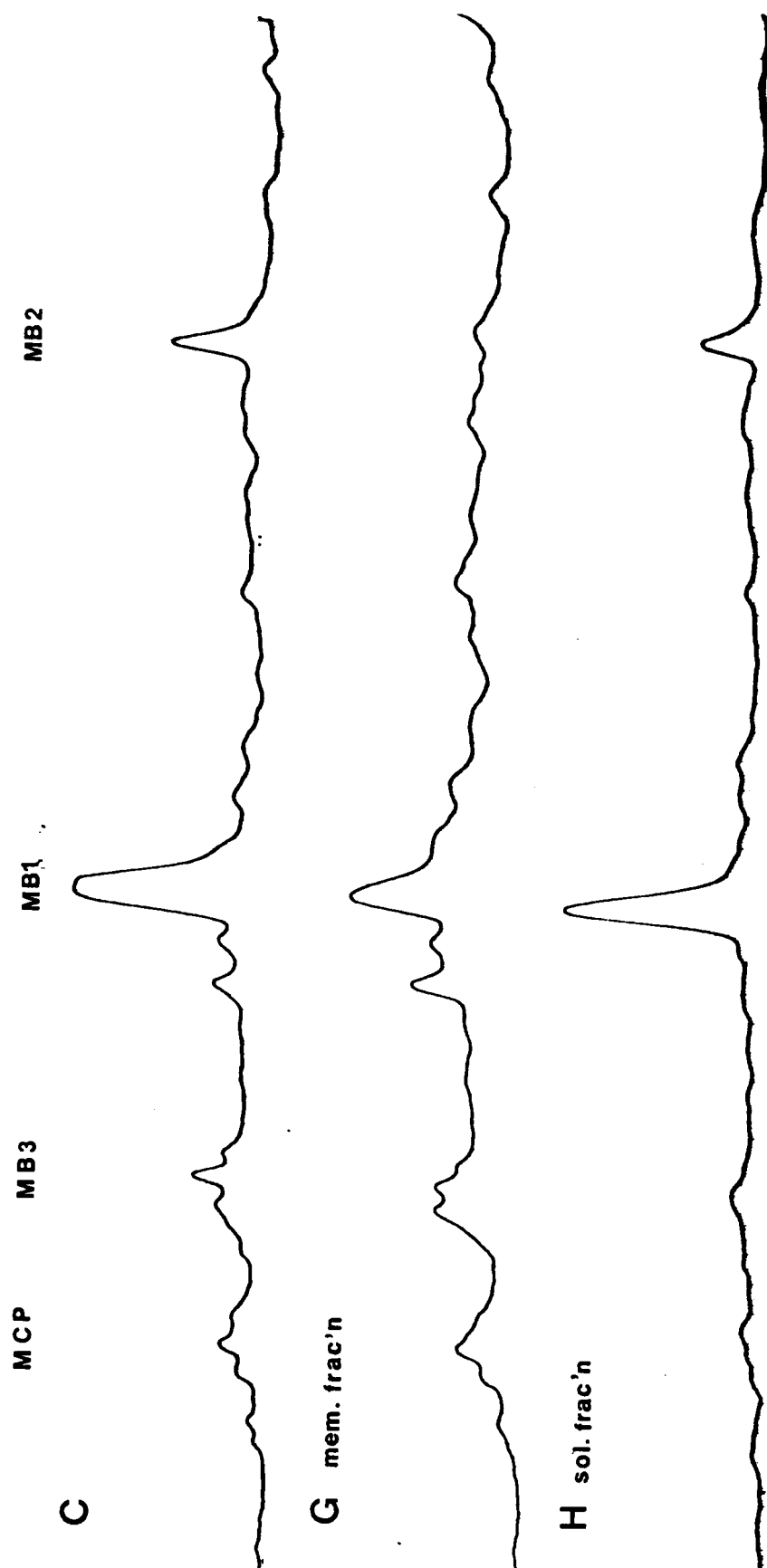


Figure 21. Membrane and soluble fractions of serine stimulated *Pseudomonas aeruginosa*. Densitometer scans of the fluorogram of Figure 15 are shown. Sample C, whole cells stimulated with serine; G, membrane fraction of serine stimulated cells; H, soluble fraction of serine stimulated cells.

(Christensen, 1975; Stephenson et al., 1978) and chemotaxis (Kort et al., 1975; Goy et al., 1977). Clearly, the use of a nonmetabolizable chemotactic stimulus has important advantages. If the attractant is not metabolized, the chemotactic gradient can be more accurately defined. Secondly, the biochemical mechanisms of chemotaxis can be examined without interference from effects caused by metabolism of the attractant.

AIB was investigated as a chemoattractant for P. aeruginosa. As is the case for E. coli and S. typhimurium, AIB proved to be a strong attractant for P. aeruginosa (Figure 6, page 65). Therefore AIB was an excellent candidate for use as a non-metabolizable attractant in these current studies. Due to the well known metabolic diversity of Pseudomonas it was important to determine whether or not P. aeruginosa could in fact metabolize AIB. The following studies were initiated to test this possibility.

Growth of P. aeruginosa on AIB

AIB was tested as a nitrogen source and as a carbon source by substituting the ammonium sulfate and succinate, respectively, of MSS medium with 0.5% AIB. No growth was evident for as long as 7 days in medium containing AIB in place of ammonia or succinate. This fact indicates that P. aeruginosa does not utilize AIB as either a sole carbon or sole nitrogen source. AIB was also added to MSS with a limiting amount of other carbon or nitrogen source; under these conditions also AIB did not support the growth of the bacteria after the primary carbon or nitrogen source ran out.

Uptake of AIB

Bacteria were prepared in CTX buffer as for a capillary experiment and the accumulation of radiolabeled amino acids was determined. Uptake of AIB labeled either with [^{14}C] in the 1-C position or with ^3H in the methyl group was tested and compared to the uptake of proline. As might be expected uptake of proline was stimulated by succinate (Figure 22A). However, no accumulation of [^{14}C]AIB was seen regardless of the presence or absence of succinate. The radioactivity on filters containing bacteria fell entirely within the range of background counts which measured nonspecific sticking of the label to the filters. The same results were obtained whether or not the cells were treated with chloramphenicol before the uptake experiment. Further, when a 50-fold higher concentration of AIB was used, the results were exactly the same as those of Figure 23A.

[^3H]AIB also was not accumulated by P. aeruginosa (Figure 22B). The same results were obtained regardless of the presence or absence of EDTA in the CTX buffer. Thus, from these results no accumulation of AIB was demonstrated under the conditions used in the chemotaxis experiments.

Release of CO_2 from AIB

$^{14}\text{CO}_2$ released from [^{14}C]AIB during six hours of incubation with P. aeruginosa cells in CTX buffer was measured by counting the ^{14}C trapped in sodium hydroxide (Figure 23). A control which measured accumulation of ^{14}C in the alkali trap in the absence of bacteria was subtracted from each measurement. Under the conditions employed, $^{14}\text{CO}_2$

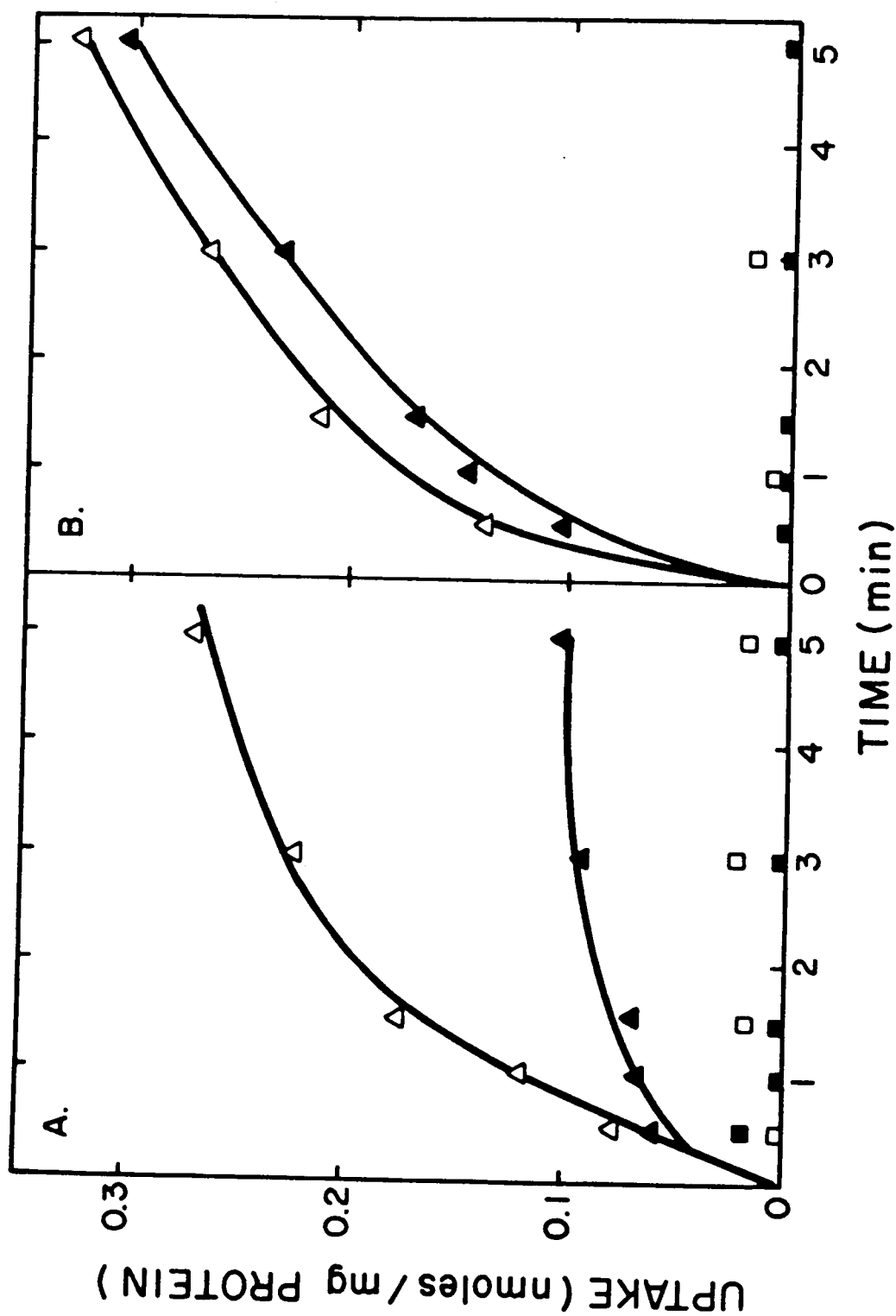


Figure 22. Amino acid uptake by *Pseudomonas aeruginosa*. A. triangles, $[^3\text{H}]$ proline; squares, $[^{14}\text{C}]\text{AIB}$ uptake; Δ, □ with succinate; ▲, ■ no succinate. B. triangles, $[^3\text{H}]$ proline; squares, $[^3\text{H}]\text{AIB}$; ▲, ■ with EDTA; Δ, □ no EDTA.

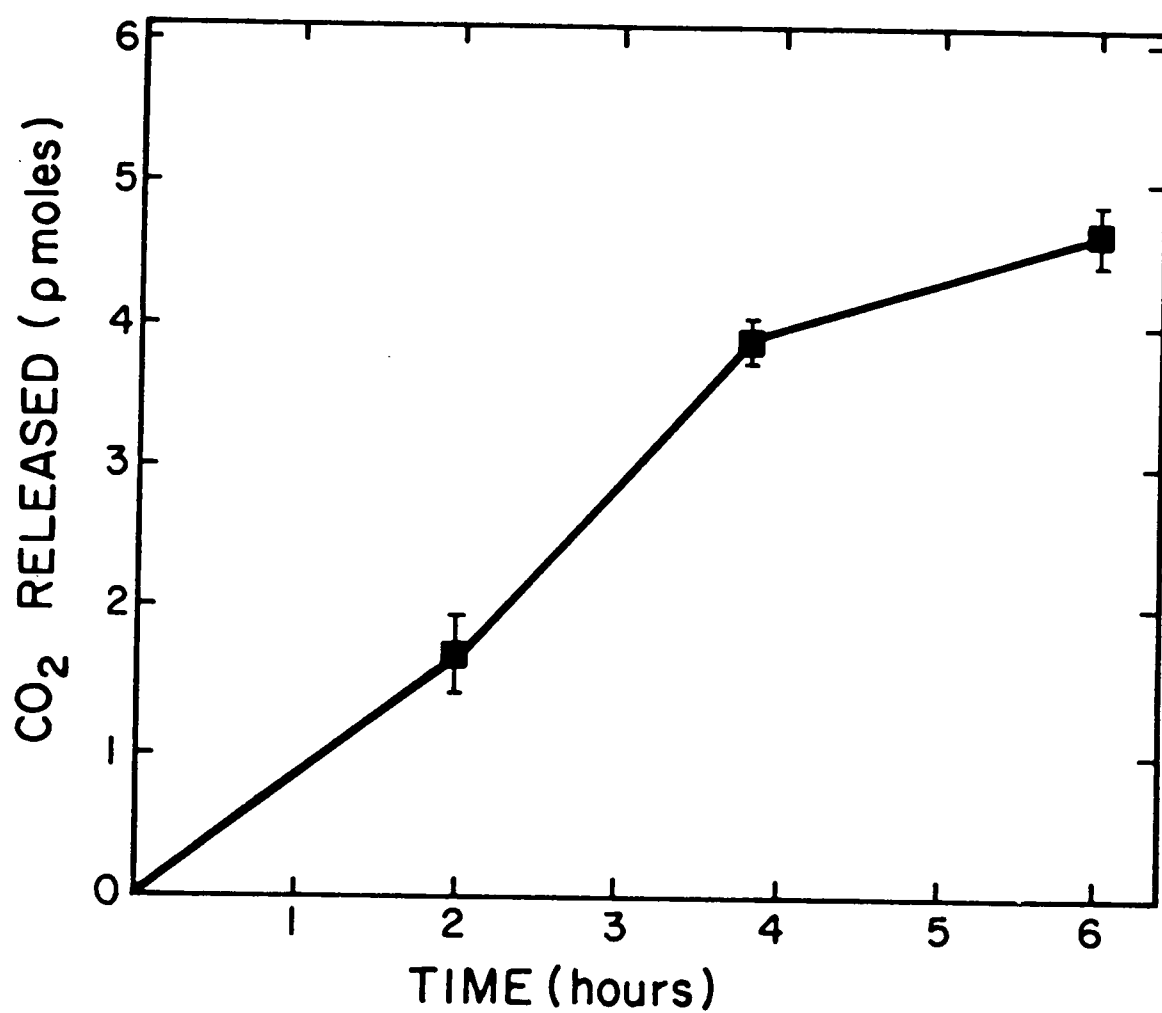


Figure 23. Release of carbon dioxide from α -aminoisobutyrate in the presence of *Pseudomonas aeruginosa*.

was produced at a rate of 0.5 pmoles CO_2 per hour per mg protein. This corresponds to the release of 0.01% of the total available ^{14}C from AIB during six hours.

Chromatographic Analysis of Metabolites of AIB

Since no accumulation of AIB in cells of P. aeruginosa was demonstrated above, the possibility that metabolites of AIB might accumulate in the medium was tested. The buffer supernatant from a suspension of P. aeruginosa which had been incubated [^3H]AIB for one hour was analyzed by thin layer chromatography for the presence of metabolites derived from AIB (Figure 24). In all three solvent systems all radioactivity in the buffer supernatant ran with the mobility of authentic AIB suggesting that extensive metabolism of AIB is not occurring under these conditions. This conclusion is, however, subject to two limitations. First, any metabolites present in levels less than about 0.5% of the total radioactivity would not be detected. Secondly, volatile metabolites would not be detected.

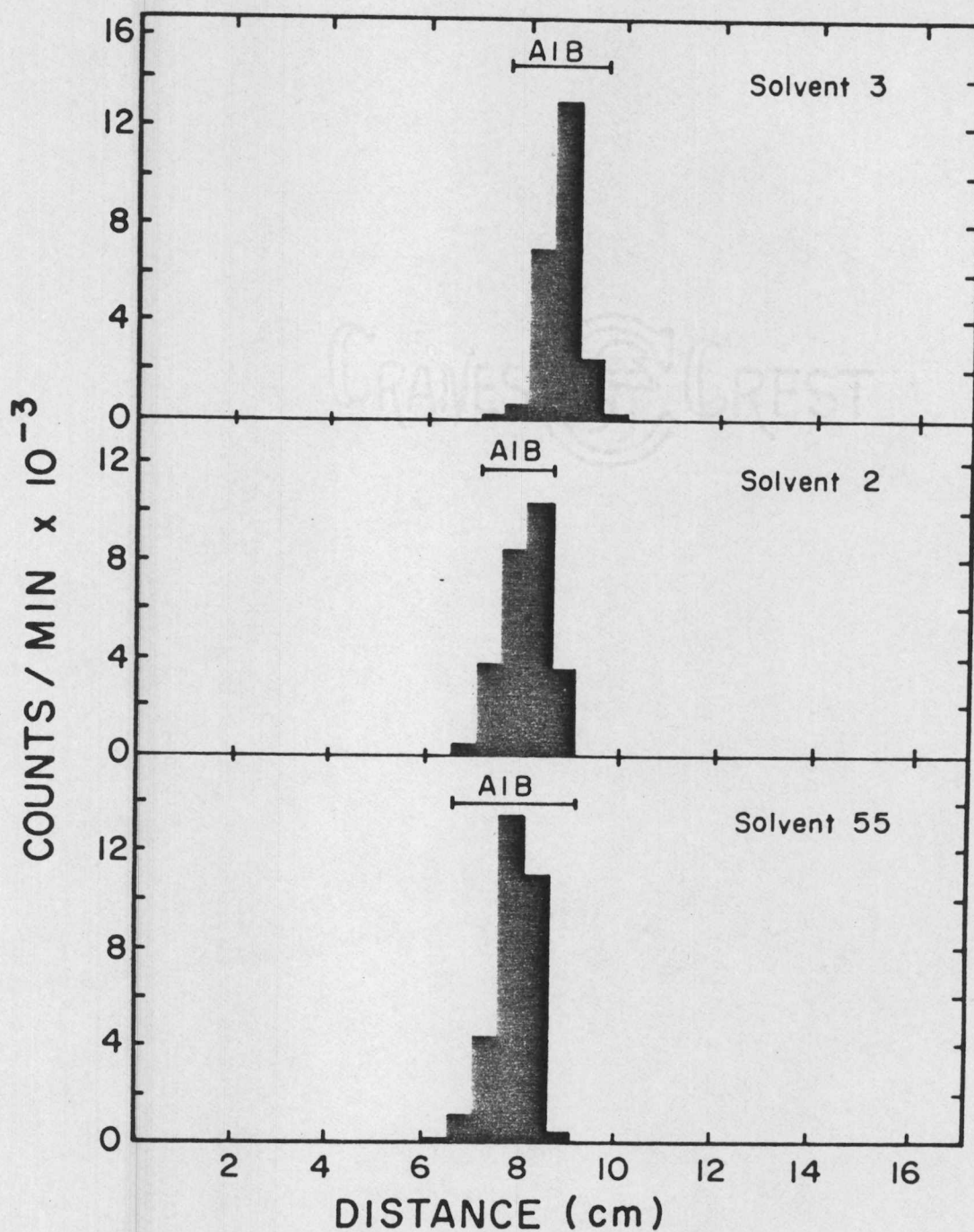


Figure 24. Chromatographic analysis of AIB from suspensions of *Pseudomonas aeruginosa*. The position of authentic AIB is indicated by a bar in each panel.

CHAPTER IV

DISCUSSION

Nitrogen Regulation of Amino Acid Chemotaxis

The results of Chapter III demonstrated that the chemotaxis of Pseudomonas aeruginosa toward several amino acids (serine, arginine and AIB) is strongly influenced by the form in which nitrogen is presented to the bacteria. P. aeruginosa grown on nitrate as the sole source of nitrogen were highly responsive to amino acids and accumulated in attractant capillaries to a level that was 2 to 3 times that of ammonia-grown bacteria and 10 to 20 times that of cells grown in CAA or ammonia-glutamate.

Nitrate is a poor nitrogen source for P. aeruginosa (Brown et al., 1973, 1977). The growth rate of the bacteria was limited when nitrate rather than ammonia served as the source of nitrogen. Further Brown et al. (1973) found that intracellular ammonia pools are very low during growth on nitrate. In contrast, glutamate is an excellent source of nitrogen for P. aeruginosa as are casamino acids (Janssen et al., 1981). Thus it appears that the differences in chemotactic responsiveness of cells grown on various nitrogen sources correlate with the availability of the nitrogen.

In contrast to amino acid taxis, responsiveness to the carbon compounds succinate and glucose was not subject to control by nitrogen availability. Succinate taxis appeared not to be affected while glucose taxis was completely inhibited by growth on nitrate.

The apparent nitrogen repression of chemotaxis may actually consist of more than one regulatory phenomenon. Bacteria grown under conditions of high nitrogen showed lower background accumulations in buffer capillaries than did those cells grown in ammonia or nitrate medium. That was in part due to the lower density of viable cells in the suspensions used for those experiments. It is also possible that the lower background accumulations actually reflect lower motility of these cells. Reduced motility of the cell population either due to a reduced proportion of motile cells in the population or to a change in the average velocity of the cells would be expected to result in lower background accumulations. A thorough microscopic evaluation including determination of the number of motile cells in the population, the velocity of forward motion and the tumble frequency could provide answers for this problem.

One or more components of the chemotactic mechanism must be subject to regulation by nitrogen. This conclusion derives from the observation that taxis of P. aeruginosa toward the amino acids was enhanced by growth under nitrogen limitation while the responsiveness to succinate was not affected. Further, the sensitivity of the bacteria to at least one attractant (AIB) was increased by growth under conditions of nitrogen limitation. Neither of these points can be explained simply by an increase in the motility of the bacteria. Indeed, no significant difference in background accumulation was detected between nitrate and ammonia cells (Table 5, page 59). Rather, the selective effect on amino acid taxis implies that regulation acts on the receptor or transducer level and not in the central response mechanism.

In attempting to draw conclusions about possible regulatory target(s) in the chemotactic mechanism, attention was focused for several reasons on the comparison of AIB taxis in nitrate and ammonia-grown cells. First, the repression of taxis in cells grown in nitrogen excess (CAA or ammonia-glutamate) might involve a combination of effects on motility and on chemotaxis, rather than regulation of chemotaxis alone. Further, although no contribution of catabolite repression has as yet been found, it is not known what effect, if any, the additional carbon supplied in the form of added amino acids has on chemotaxis. Finally, the use of AIB as an attractant eliminates complications due to metabolism of the attractant during the course of the chemotaxis assay.

Concentration-response curves for AIB taxis showed a different profile for nitrate grown bacteria compared to ammonia grown cells. The nitrate cells responded to AIB at a three-fold lower concentration; the threshold concentrations were 1.1×10^{-5} M and 3.4×10^{-6} M for ammonia and nitrate cells, respectively. Also, the peak response increased two- to three-fold. Determination of threshold concentrations can be complicated by metabolism of the attractant which alters the shape of the attractant gradient during the experiment. In the case of AIB this should not be a problem due to the fact that AIB is not metabolized. Given this fact and given the reproducibility of the difference in threshold (Table 7, page 71), the three-fold increase is strong evidence of an actual increase in sensitivity of nitrate cells to AIB.

Possible Targets of the Nitrogen Regulation

The experiments described above demonstrated clearly that AIB and arginine responses were stimulated while the succinate response was unchanged. This fact strongly suggests that regulation is occurring not at a point in the common response pathway which integrates information from all receptors, but rather at the level of the receptors or the transducers responsible for the response to AIB and arginine. In attempting to interpret the experimental results in terms of possible sites of regulation it is necessary to consider two facets of the nitrogen effect--the greater accumulations of nitrate cells in AIB and arginine capillaries and the increased sensitivity of nitrate cells to AIB.

Regulation of the number of AIB or arginine chemoreceptors per cell might result in a comparable effect on the responsiveness of the cells to AIB and arginine. Several investigations have demonstrated that the magnitude of the chemotactic response to a particular chemo-effector is a function of the number of receptors per cell. This relationship has been verified for the galactose and ribose receptors of S. typhimurium and the galactose, maltose and ribose receptors of E. coli (Fahnestock and Koshland, 1979; Koshland and Anderson, 1980; Koman et al., 1979). The increased accumulation of nitrate-grown P. aeruginosa in arginine and AIB capillaries can be easily explained as an increase in the total number of receptors for those attractants. Although no evidence is yet available concerning the number and specificity of different amino acid receptors, it seems reasonable that AIB and arginine would not be detected by the same receptor. Therefore,

this hypothesis predicts that the synthesis of several different chemoreceptors is regulated coordinately.

Alternatively, one could hypothesize that regulation occurs at the level of the transducer(s) (although if the receptor and transducer are the same protein then this distinction becomes unnecessary). The transducer protein itself may be altered in some way during growth on limited nitrogen so that it functions more efficiently to stimulate the response. The affinity of the transducer for the receptors or the effectiveness of the interactions with other components of the response mechanism might be increased. If the transducer protein were the target of the regulation, this would explain how responses to some attractants are stimulated while others are not. This presumes, then, that those responses which are not stimulated (succinate, for example) function through an independent transducer.

Interpretation of the altered threshold concentrations is somewhat more complicated. The sensitivity of bacteria to a chemoeffector is a reflection of the affinity of the receptor for the effector. The response of bacterial cells measured either in spatial gradients (Mesibov et al., 1973) or temporal gradients (Spudich and Koshland, 1975) is consistent with a simple model of receptor-effector binding described by the law of mass action:

$$K_D = \frac{[R][E]}{[RE]}$$

where $[R]$ and $[E]$ and $[RE]$ are the concentrations, respectively, of the receptor, effector and the receptor-effector complex, and K_D is the dissociation constant of the RE complex. The strength of the chemotactic

response is proportional to the change in the number of receptors occupied (Mesibov, et al., 1973; Spudich and Koshland, 1975; Aksamit, 1974). The concentration dependence of chemotaxis (threshold and peak) is defined by K_D . In other words, the affinity of the receptor for the stimulus determines the sensitivity of the cells to the stimulus.

The increased sensitivity of nitrate-grown P. aeruginosa toward AIB, therefore, suggests that the affinity of the chemoreceptors for AIB is increased by growth on nitrate. The simplest explanation for such an effect is that nitrogen availability controls the expression of receptors of different affinities. One can imagine the following situation. Growth of the bacteria in excess nitrogen (such as CAA) yields cells which possess a low affinity receptor for AIB and which respond to AIB only in a millimolar range (Figure 6, page 65). Bacteria grown under limited nitrogen conditions synthesize a different receptor of higher affinity or modify existing receptors resulting in higher affinity. As a result the bacteria are capable of responding to AIB at lower concentrations.

Receptors

It is not unusual for bacteria to possess more than one receptor for a particular amino acid. Leucine transport in E. coli is an example (Oxender and Quay, 1977). Similarly, in Pseudomonas putida arginine uptake is mediated by two permeases of different affinities and specificities (Fan et al., 1972). α -Aminoisobutyrate is transported by high and low affinity systems in Pseudomonas fluorescens (Stephenson et al., 1978). Such a multiplicity of receptor systems for chemotaxis has been identified in at least one case. Serine taxis in E. coli and S. typhimurium is mediated by more than one receptor. A high

affinity receptor specific for serine and homoserine is found in the membrane of both organisms. The contribution of one or more additional low affinity receptors has been identified by several studies but these additional receptors have not been characterized (Clarke and Koshland, 1979; Mesibov and Adler, 1972).

Very little information is available concerning the chemotaxis receptors in any Pseudomonas species. The glucose binding protein (GBP) of P. aeruginosa is the only chemoreceptor that has been clearly identified and isolated (Stinson et al., 1977). Kariman and Ornston (1981) have gathered genetic evidence that the β -ketoadipate receptor of P. putida also has a role in the transport of that substance. Both glucose and β -ketoadipate receptors are induced by their respective substrates. In addition the GBP is subject to catabolite repression by succinate (Stinson, et al., 1976). As yet no study has focused on amino acid chemoreceptors of Pseudomonas, and no evidence for regulation of any amino acid chemoreceptor has been previously presented. The evidence presented in this study shows that some amino acid receptors of P. aeruginosa may be regulated by nitrogen. A careful examination of the receptors involved, their regulation and their possible involvement in amino acid transport may yield interesting results.

Glucose Chemotaxis

The apparent loss of responsiveness of P. aeruginosa to glucose (Figure 4, page 57) deserves comment. Glucose is a weak attractant even under the best conditions. However, when the bacteria were grown on nitrate as the source of nitrogen, glucose taxis was abolished. An interesting question is whether the absence of chemotaxis is due to

the lack of receptors. Glucose taxis is mediated by the glucose binding protein whose synthesis is induced by glucose (Stinson et al., 1977). At high glucose concentrations synthesis of GBP is inhibited (Whiting et al., 1976). Even if one assumes that the loss of chemotaxis is due to the lack of receptor, it is not clear whether this reflects an actual regulation of GBP synthesis by nitrogen or whether, due to the lower growth rate of cells growing in nitrate, the concentration of glucose (intracellular or extracellular) is higher in nitrate cultures than in ammonia cultures resulting in the inhibition of synthesis of GBP.

The Molecular Basis of Nitrogen Regulation

From the data presented one can only speculate about the molecular basis of the regulatory event. It is not yet clear whether regulation of protein synthesis is involved or whether the activity of one or more components is regulated in some manner (perhaps by a mechanism similar to feedback inhibition or by covalent modification). Nevertheless, the effect of the various nitrogen sources on amino acid taxis parallels the nitrogen regulation of a number of *P. aeruginosa* enzymes involved in nitrogen metabolism.

Two enzymes of histidine catabolism, histidase and urocanase, are subject to catabolite repression by succinate, but those enzymes are derepressed under conditions of nitrogen limitation (Potts and Clarke, 1976). A similar pattern of regulation has been reported for agmatine hydrolase of the arginine catabolic pathway (Mercenier et al., 1980). Urease and the assimilatory nitrate reductase (Sias and Ingraham, 1979) are also controlled by nitrogen. Two investigators (Liu and Hsieh, 1969; Jensen et al., 1980) have indicated that the levels of proteolytic

enzymes produced by P. aeruginosa are subject to regulation by high ammonia.

Evidence is accumulating which suggests that the synthesis of several of these enzymes is linked to glutamine metabolism. Urease and histidase are regulated coordinately with glutamine synthetase (GS) (Janssen et al., 1981). Further, Janssen et al. (1981) examined the regulation of NADP-dependent glutamate dehydrogenase (GDH), urease and histidase in a glutamine-requiring mutant which had no detectable GS activity. In that mutant ammonia, glutamate and casamino acids all failed to exert the normal regulatory effects. However, excess glutamine did cause repression of urease and histidase and derepression of GDH. These results suggest that glutamine or some metabolite of glutamine plays a role in the regulation of these enzymes either directly or indirectly through the regulation of the synthesis of another regulatory protein.

Chemotaxis may be considered to be a mechanism of nitrogen acquisition. Certainly the amino acids are very strong chemoattractants for P. aeruginosa. It is interesting that arginine is a very potent attractant for P. aeruginosa since it attracts neither E. coli (Mesibov and Adler, 1972) nor S. typhimurium (Melton et al., 1978). Of all the attractants used in this study arginine is by far the strongest attractant for RM46, showing the largest accumulations (Figure 3, page 56) and the lowest threshold (Table 7, page 71). It also appears that this amino acid is detected by more than one system (Figure 3, page 56). Arginine proved to be a strong attractant for two other strains of P. aeruginosa (Craven and Montie, 1980). In fact it was the only attractant

which elicited a strong response from strain WR5 which is deficient in chemotaxis toward several attractants.

Experiments with strain RM46 by this author verified the observations of other investigators (Kay and Gronlund, 1969) that P. aeruginosa grows very well on arginine as the sole source of nitrogen. In P. aeruginosa the primary route of catabolism is the arginine deiminase pathway which in three steps yields two moles of ammonia and one mole of ATP per mole of arginine (Rahman et al., 1980). Therefore, arginine chemotaxis and transport provide an efficient mechanism of nitrogen acquisition.

When viewed in this manner, it seems reasonable that amino acid taxis could be subject to the same (or similar) control mechanisms that regulate other aspects of nitrogen acquisition and metabolism. The possibility that the control of amino acid taxis described in this manuscript might be related to glutamine metabolism is intriguing. The effect of the various nitrogen compounds on amino acid taxis is consistent with this hypothesis. The relative effectiveness of different nitrogen sources in allowing chemotaxis is as follows: nitrate > ammonia > glutamate > CAA or ammonia-glutamate. This relationship is the same as that found for urease and histidase synthesis and also closely parallels that for the effects on glutamine synthetase activity (Janssen, 1981).

Role of Methionine in Chemotaxis

This study was undertaken to clarify the role of methionine in P. aeruginosa chemotaxis. Initial attempts to demonstrate a methionine

requirement in P. aeruginosa were unsuccessful; the bacteria were strongly chemotactic in the absence of exogenous methionine (Moulton, 1977; Moulton and Montie, 1979). In view of the fact that methionine has been implicated in the mechanism of chemotaxis of a variety of bacteria as well as eucaryotic cells (see Section I), it seemed quite unlikely that an entirely different mechanism had developed in Pseudomonas.

Effect of Methionine on Chemotaxis

P. aeruginosa RM46, a methionine auxotroph, was subjected to methionine starvation by washing the growing cells and resuspending in methionine-free MSS medium. One hour of starvation caused a loss of serine responsiveness of 15 to 80% (Figure 11, page 75, Table 8, page 78). In contrast, background accumulations were higher for starved cells than for unstarved cells, indicating that the bacteria are quite motile. Transfer of growing bacteria to MSS containing ethionine instead of methionine mimicked the effect of methionine starvation. The ethionine-grown bacteria exhibited reduced responsiveness to serine although the background motility of the cells was three times that of unstarved cells (Figure 11, page 75). These results suggest that some factor that functions in chemotaxis of P. aeruginosa is altered or depleted during methionine starvation. In the case of the enteric bacteria and B. subtilis that factor is known to be methionine and its metabolite, S-adenosylmethionine.

If the reduced chemotaxis of methionine starved P. aeruginosa was due to depletion of cellular pools of methionine and SAM, then it should be possible to restore chemotaxis by replenishing those pools. The addition of methionine to starved P. aeruginosa in CTX buffer prior

to the taxis assay gave some stimulation of the serine response (Figure 11, page 75). However, in many experiments the magnitude of the stimulation was small and only occasionally did exogenous methionine stimulate taxis to the level of unstarved cells. In view of the fact that methionine uptake by P. aeruginosa is relatively inefficient in the absence of an energy source (Figure 14, page 85; also, Montie and Montie, 1979), an attempt was made to load the starved bacteria with methionine by incubating the cells in CTX buffer with methionine and succinate under conditions known to allow methionine uptake, then washing the cells again prior to the taxis assay. Although cells treated in this way should have substantial methionine pools, this procedure did not increase the serine taxis of the starved cells. Thus, the failure of exogenous methionine to stimulate taxis of starved cells was probably not due simply to the failure to transport methionine. Together these results seem to suggest that methionine starvation is causing not only SAM depletion, but perhaps also turnover of some protein components of the chemotaxis system.

On the other hand, the poor stimulation of taxis by added methionine may be merely a reflection of the complex interactions of this amino acid with the bacterium. Methionine is required as a growth factor for the auxotrophic strain RM46, and methionine can itself serve as an attractant. Also methionine may function in the chemotactic mechanism by acting as a methyl donor. Finally the presence of exogenous methionine is known to inhibit the chemotactic migration of P. aeruginosa in response to a spatial gradient of another attractant (Figure 12, page 79). The mechanism of this inhibition is still unclear. This

inhibition phenomenon greatly complicates the interpretation of those experiments which measure the tactic response of starved cells in the presence of exogenous methionine. Any stimulatory effect caused by replenishment of the cellular SAM pools may be masked by the simultaneous inhibition by the added methionine.

The increased background counts of starved bacteria (Figure 11, page 75) deserve a more thorough investigation. Does the high background really reflect increased motility of the starved cells? If that were true, it would have interesting and important implications for the role of motility and chemotaxis as a survival mechanism in natural environments. A complete microscopic evaluation of starved and unstarved cells will answer this question. It is also possible that the differences in background counts might result from variable densities of cell suspensions of the starved and unstarved bacteria.

Chemotaxis-Associated Methyltransfer Reaction

The potential involvement of methionine in chemotaxis of P. aeruginosa was further investigated by testing for the presence of an attractant-responsive methylation reaction. Experiments comparing the methyl labeling of stimulated and unstimulated P. aeruginosa led to the identification of a methylated species which appears to be comparable to the methyl-accepting chemotaxis proteins (MCPs) of E. coli and S. typhimurium. By analogy to the enteric bacteria the P. aeruginosa methylated species will be referred to in this paper as MCP although the protein nature of this component has not yet been verified.

The P. aeruginosa MCP was visualized as a single radioactive band of approximately 73,000 daltons on 10% polyacrylamide gels. On

12.5% gels two components were resolved. The MCP apparently is methylated by an S-adenosylmethionine-mediated methyltransfer reaction. As in E. coli (Springer et al., 1977b) and S. typhimurium (DeFranco and Koshland, 1980), the methylation of MCP was influenced by the presence of attractants. The attractants arginine, serine, and AIB caused increased incorporation of methyl group from [methyl-³H] methionine into MCP, and removal of attractant caused loss of methyl label from the MCP band. Taken together these experiments provide strong supportive evidence that the observed methylated component is analogous to the MCPs of enteric bacteria. The isolation of chemotaxis mutants, currently underway in Dr. T. Montie's laboratory, should provide definitive proof of the involvement of this methylated component in chemotaxis.

Particularly interesting is the observation on the 12.5% gels of two components which respond to chemotactic stimulation (Figure 17, page 93). Two bands are evident which run with an apparent difference in molecular weight of approximately 1000. The MCPs of the enteric bacteria are known to exist in multiply methylated forms which on SDS-polyacrylamide gels run as distinct bands corresponding to the basic MCP protein with varying degrees of methylation. Attractant stimulation causes a shift in the intensity of methyl label from the singly methylated, faster migrating form to the more highly methylated slower migrating forms (DeFranco and Koshland, 1980). In view of this, the two labeled components observed in P. aeruginosa may represent the singly and doubly methylated forms of MCP.

Arginine, which is a stronger attractant than either serine or AIB in the capillary assay, elicited a much stronger methylation

response than did either serine or AIB. Except for the differences in intensity no obvious differences in methylation patterns were seen between arginine , serine and AIB stimulated cells. The same two bands appear to respond to each of the three attractants. One cannot discern from these results whether the three different attractants are causing the methylation of the same species of MCP (that is, the identical protein backbone) or whether more than one MCP with similar electrophoretic mobilities are present. Although further refinements in the electrophoretic techniques may help to distinguish those possibilities, the answer to this question must ultimately be sought through a genetic analysis.

The apparent decline of methylation level in AIB stimulated cells after 10 min. deserves comment. This decline in radioactivity may indicate that demethylation is occurring after longer periods of stimulation. Such is not the case in E. coli, however, since Goy et al. (1977) demonstrated that the MCP methylation remains at the stimulated level until the attractant is removed. Alternatively, the decline in methylation level in P. aeruginosa "MCP" Figure 18, page 98) might be due to the limitation of some component of the methylation system--methionine, attractant, endogenous energy supply or oxygen. It is also possible that the apparent decline in methylation level is actually the result of an increase in radioactivity in MB3 rather than a decline in radioactivity in "MCP."

Evidence that the attractant-responsive band of P. aeruginosa is methylated by a methyltransfer reaction mediated by SAM comes from two experiments. The first demonstrated that the methyl moiety of methionine, but not the sulfur, was incorporated into MCP in response

to attractant stimulation. The second piece of evidence comes from in vitro labeling with S-adenosyl-[methyl-³H]methionine. Among the species labeled by SAM was one with electrophoretic mobility identical to the putative MCP. The intensity of methyl label in that band was not increased by the addition of AIB. This result is perhaps not surprising. It is quite possible that the severe forces involved in rupturing the bacterial cells in the French press caused disturbances of the normal arrangement of chemotaxis proteins in the membrane. The chemoreceptor and methyl-accepting proteins, if not preserved in their normal configuration, may fail to interact as in whole cells upon attractant stimulation.

Paoni and Koshland (1979) were successful in using a toluene permeabilization method to make cells of S. typhimurium permeable to SAM. Their leaky cells retained normal methylation responses to aspartate and serine. Aspartate and serine, however, represent a special case in that the tar and tsr methylated proteins are the receptors for aspartate and serine, respectively (Hedblom and Adler, 1980; Wang and Koshland, 1980). If the receptors were distinct from the MCPs, the chances of retaining attractant responsiveness following permeabilization would very likely be much lower. In the case of P. aeruginosa there is yet no evidence to indicate whether AIB binds directly to the MCP or to another receptor which then interacts with the MCP. Certainly the success of experiments designed to measure methylation responses to attractant stimulation in vitro would be affected by the relationship of receptor and MCP.

From the information generated in this study it is possible to make an estimate of the number of MCPs per cell. The number of

disintegrations per minute in the MCP of P. aeruginosa was not determined directly but can be estimated. In some preliminary experiments in which the gels were sliced and solubilized, MB1 was found to contain 11,200 dpm in a typical experiment. If the radioactivity incorporated into MCP in response to serine or AIB is equal to 10 to 20% of the label in MB1, this corresponds to 1100 to 2200 dpm in MCP. Assuming (1) that an amount of protein representing 1.3×10^8 cells was loaded onto each well, (2) the specific activity of methionine was 15 Ci/mmol with no dilution by unlabeled intracellular methionine, and (3) that an average of one methyl group was added per MCP molecule, this represents 150 to 300 copies of MCP per cell. This range should be considered as a minimum number per cell since it is quite likely that there is significant dilution of the radioactive methionine in the bacterial cells. Approximately 2500 tsr and 1200 tar proteins are known to be present in the membranes of E. coli and S. typhimurium (Clarke and Koshland, 1979).

In one experiment (Table 9, page 94) the methylation of MCP in both ammonium-grown and nitrate-grown bacteria was determined. Nitrate cells exhibited a stronger methylation response to AIB than did ammonia-grown bacteria. Arginine elicited the largest methylation response in both ammonia- and nitrate-grown cells, but the response to arginine was no greater than in ammonia cells. It is tempting to speculate that this increased AIB-induced methylation reflects the increased responsiveness of nitrate cells to AIB demonstrated in the capillary assays. A study of the effect of attractant concentration on the methylation response and of the time course of the methylation response may help answer this question.

α -Aminoisobutyrate as a Non-Metabolizable Attractant
for Pseudomonas aeruginosa

Several attempts were made to detect metabolism of AIB by P. aeruginosa. Under no conditions tested was AIB found to support the growth of P. aeruginosa strain RM46 either as the sole nitrogen or sole carbon source or when added with a growth-limiting amount of another nitrogen or carbon source.

No uptake of AIB was detected under conditions which supported vigorous uptake of proline. The presence or absence of sodium succinate, chloramphenicol, or EDTA had no effect on AIB accumulation. No uptake was detected under any conditions tested. The failure of P. aeruginosa to accumulate AIB is particularly interesting in view of the fact that AIB is an excellent attractant for P. aeruginosa (Figure 6, page 65). AIB transport in P. fluorescens has been reported by two investigators (Romano et al., 1980; Stephenson et al., 1978). Using assay conditions very similar to the ones utilized for this study, Stephenson et al. (1978) identified two active transport systems with apparent K_M values of 3 and 105 μM in P. fluorescens. This author is unaware, however, of any studies of the transport of AIB by P. aeruginosa.

Some Pseudomonas species are capable of metabolizing AIB. Pseudomonas cepacia (Lamartiniere et al., 1971) and another Pseudomonas species (Honma and Shimomura, 1974) are able to grow by utilizing AIB as the sole nitrogen source. The reaction catalyzed by the AIB-decomposing enzyme is a decarboxylation-dependent transamination which yields acetone, carbon dioxide and alanine from AIB and pyruvate.

In addition this enzyme catalyzes the ordinary transamination between alanine and α -ketobutyrate (Sato et al., 1978).

The release of CO_2 from AIB (Figure 23, page 110) suggests that a similar reaction may occur in P. aeruginosa. However, the rate of CO_2 release was very low. Further, the fact that no growth of P. aeruginosa on AIB was seen indicates that the amount of nitrogen available to P. aeruginosa from AIB must be very small.

Chromatographic analysis of AIB in the buffer supernatants showed no evidence of metabolites derived from AIB. No metabolites were found. However, if the [methyl- ^3H]AIB were degraded by the type of reaction found in P. cepacia (Lamartiniere et al., 1971), the radioactivity from AIB would be in the form of acetone and so would not be detected by this method.

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APPENDIX

APPENDIX

Mineral Salts Medium (MSS) For *Pseudomonas aeruginosa*

To make one liter of MSS mix the following components:

<u>Component</u>	<u>Concentration of stock solution</u>	<u>Volume required to make one liter</u>	<u>Final concentration (M)</u>
K_2HPO_4	70 g/l	100 ml	4.0×10^{-2} M
KH_2PO_4	30 g/l	100 ml	2.0×10^{-2} M
$(NH_4)_2SO_4$	10 g/100 ml	10 ml	7.6×10^{-3} M
$MgSO_4 \cdot 7H_2O$	2.5 g/100 ml	2 ml	2.0×10^{-4} M
$FeCl_3 \cdot 6H_2O$	250 mg/100 ml	1 ml	9.2×10^{-6} M

Add distilled water to make 980 ml, adjust pH to 7.0. Carbon sources should be separately autoclaved as 20% solutions in distilled water; 10 ml added per 500 ml of medium after autoclaving. Amino acid supplements such as methionine should be filter sterilized and added after autoclaving to the MSS to give 5×10^{-4} M.

Modified Lowry Method for Determination of Protein (Markwell et al., 1978)

Lowry Solution A: 20 g Na_2CO_3
(per liter)

4 g NaOH

1.6 g sodium tartrate

10 g SDS

Lowry Solution B: 40 g $CuSO_4 \cdot 5H_2O$ per liter

Lowry Solution C: Mix together 100 parts A and 1 part B; prepare Solution C fresh each time.

Folin-Ciocalteu Reagent (FC): Dilute 1:1 with distilled water just prior to use.

Use a sample volume of 1 ml containing 10 to 100 μ g protein. Add 3 ml Solution C, mix and incubate 10 to 60 min. at room temperature. Add 0.3 ml FC reagent, mix well and incubate 45 min. Read absorbance at 660 nm.

Chemotaxis (CTX) buffer

Mix together 30.5 ml of a 1.0 M stock solution of K_2HPO_4 and 19.5 ml of a 1.0 M stock solution of KH_2PO_4 . Add about 900 ml of distilled water, then add 1.0 g $MgCl_2 \cdot H_2O$ and 10 ml of a 2.5 mM solution of disodium EDTA. Adjust pH to 7.0 and the volume to one liter.

SDS-Polyacrylamide Gel Electrophoresis

Stock I. Acrylamide: BIS (30: 0.8)

30.0 g acrylamide
0.8 g methylene bis-acrylamide
distilled water to make 100 ml

Filter and store at 4 C in the dark.

Stock II. 1.5 M Tris-Cl, pH 8.8
18.17 g Tris(hydroxymethyl)Aminomethane (Tris base)
50 ml distilled water

Adjust pH to pH 8.8 with HCl, then bring volume to 100 ml with distilled water.

Stock III. 0.5 M Tris-Cl, pH 6.8
6.06 g Tris base
50 ml distilled water

Adjust pH to 6.8 with HCl, then bring volume to 100 ml with distilled water.

Stock IV. Electrode buffer

6.06 g Tris base	(final concentration 0.023 M)
28.8 g glycine	(0.192 M)
2.0 g SDS	(0.1%)

Add distilled water, adjust pH to 8.3, then bring volume to 2 liters.

Stock V. Sample buffer (S buffer)

21.7 ml distilled water	
7.5 ml 0.5 M Tris-Cl,	(final concentration
pH 6.8 (Stock III)	0.0625 M)
6.0 g glycerol	(10%)
12.0 ml 10% SDS	(2%)
0.6 ml 0.1% bromophenol blue	(0.001%)
0.6 ml 2-mercaptoethanol	(1%)

This makes a total of 48 ml. If mixing with liquid sample, allow 4 parts S buffer to 1 part protein sample. Dilute 4:1 with distilled water before use when dissolving solid samples.

Preparation of Gel Mixtures

Separation Gel Mixture (10% acrylamide):

18.2 ml distilled water
11.3 ml 1.5 M Tris-Cl, pH 8.8
0.45 ml 10% SDS
15.0 ml Acrylamide:BIS (30:0.8)
150 μ l 10% ammonium persulfate
11.3 μ l TEMED

Stacking Gel Mixture (5% acrylamide):

5.6 ml distilled water
2.5 ml 0.5 M Tris-Cl, pH 6.8
0.1 ml 10% SDS
1.7 ml Acrylamide:BIS (30:0.8)
100 μ l 10% ammonium persulfate
5.0 μ l TEMED

Protein Stain for Polyacrylamide Gels

Solution 1: 25% isopropanol
10% acetic acid
0.05% Coomassie Blue R250 (0.5 g/l)

Stain overnight.

Solution 2: 10% isopropanol
10% acetic acid
0.0025 to 0.005 % Coomassie Blue R250 (50-250 mg/l)

One to two hours.

Solution 3: 10% acetic acid
0.0005% Coomassie Blue R250 (5 mg/l)

One hour.

Solution 4: 10% acetic acid

Several changes, until completely destained.

For thin slab gels (0.75 mm), approximately one hour at each step is sufficient.

VITA

Rebecca Carol Craven was born to Carolyn and John Craven on October 11, 1952, in Cleveland, Ohio. She attended public schools in Knoxville, Tennessee, and was graduated from West High School in June 1970. The following September she entered Vanderbilt University in Nashville, Tennessee, where she majored in molecular biology. After receiving the Bachelor of Arts degree from Vanderbilt in May 1974, she began graduate study in the Department of Microbiology at The University of Tennessee, Knoxville, and was awarded the Master of Science degree in 1977 and the Doctor of Philosophy degree in 1981.