

AN ABSTRACT OF THE THESIS OF

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Title: THE SYNERGETIC EFFECTS OF STARVATION AND HYDROSTATIC PRESSURE
ON UPTAKE OF ALPHA-AMINOISOBUTYRIC ACID BY A PSYCHROPHILIC
MARINE VIBRIO

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The uptake ability of a psychrophilic marine vibrio, Ant-300, was tested at pressures of 1 to 800 atm during starvation periods ranging from 3.5 hr to 90 days. The substrate used, alpha-aminoisobutyric acid- $1-^{14}\text{C}$ (AIB), was readily taken up by Ant-300, but was not metabolized by the cells. Uptake of AIB exhibited saturation kinetics. This substrate provided a test of relative pressure effects on uptake, while minimizing the contribution of pressure effects on other cellular processes (i.e., protein synthesis) to the observed results.

Rates of AIB uptake by Ant-300 cells starved 1.5 to 14 days were inhibited an average of 11% at 100 atm, 42% at 200 atm, 73% at 300 atm, 89% at 400 atm, and 93% at 500 atm. At 400 atm, the AIB uptake rate remained constant through 30 hr, while uptake was stopped by 14 hr at 500 atm or higher pressures. At 200 atm, a decrease in the V_{max} (average, 40%) and an increase in the K_m (average, 60%) of AIB uptake were observed.

During the first two days of starvation, AIB uptake rates and baro-tolerance of uptake decreased. Uptake ability per viable cell increased again after a low around seven to ten days, then remained fairly

constant through 90 days. Cells starved longer than 20 days also exhibited a recovery in their barotolerance of uptake.

Starving Ant-300 cells survived equally well for 8 wk at 1, 100, 200, and 300 atm, but at 400 and 500 atm increased death rates were observed.

In Ant-300 cells starved 1.5 to 14 days, uptake is apparently more pressure sensitive than in cells starved shorter or longer periods of time. The barotolerance of uptake by Ant-300 cells starved 12 hr or less indicated that the reserve of energy from growth in a rich medium was capable of alleviating some of the detrimental effects of pressure. Cells starved longer than 20 days were capable of cellular adjustments which facilitated recovery of AIB uptake capabilities, including increased barotolerance of uptake, without additional energy input. These observations indicate that Ant-300 may be well adapted to survival at increased pressure under extended starvation, but may not be capable of growth under pressure when nutrients (energy) are limiting.

The Synergetic Effects of
Starvation and Hydrostatic Pressure
on Uptake of Alpha-aminoisobutyric Acid
by a Psychrophilic Marine Vibrio

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THE SYNERGETIC EFFECTS OF STARVATION
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INTRODUCTION

Two major environmental factors which affect microbial activity and survival in the ocean are hydrostatic pressure and nutrient (energy) availability. The deepest known area in the ocean is approximately 10,000 m while the average ocean depth is 3800 m. Since hydrostatic pressure increases about 1 atm for every 10 m in depth, microorganisms in the marine environment may be exposed to pressures up to 1100 atm. In addition, the open ocean and particularly the deep ocean are characterized by extremely low nutrient concentrations (Lee and Bada, 1975, and Menzel and Ryther, 1970).

Increased pressure has been observed to inhibit, stimulate, or have no effect on microbial activity depending on the organism, the pressure employed, and other environmental conditions (such as salinity and temperature). Under simulated or in situ oceanic conditions, the activity of free-living microorganisms generally decreases with increasing pressure, and most surface marine organisms expire at pressures of 600 atm or less.

Numerous researchers (as reviewed by Marquis, 1976, and Marquis and Matsumura, 1978) have attempted to determine which single metabolic process is the limiting factor to microbial growth and survival under pressure. Pressure effects on DNA replication, substrate uptake and respiration, protein synthesis, ATP synthesis and utilization, catabolic pathways, isolated enzymes, and inducible enzyme systems have all been

studied to various degrees. Presently, protein synthesis is considered by most investigators as the most pressure sensitive key cellular process which limits the rate of microbial growth under pressure (Marquis, 1976; Pope and Berger, 1973; and Schwarz and Colwell, 1975).

A recent publication (Masuda and Albright, 1978) however, emphasized that pressure effects on numerous cellular processes, including active uptake, are important in limiting microbial growth. In addition, Matsumura and Marquis (1977) reported that the efficiency of Streptococcus faecalis growth was reduced at increased pressure; hence, the cells required more energy than at one atmosphere for the same amount of metabolic work. If the intracellular energy demand was higher at increased pressure and substrate uptake was inhibited, the energy supply could become the critical limiting factor to cellular growth or survival. The low concentrations of nutrients in the open ocean are evidence for the necessity of a cell to maintain high-affinity uptake systems.

Paul and Morita (1971) characterized the effects of moderate hydrostatic pressures (100 to 500 atm) on the ability of a marine psychrotroph to incorporate amino acids. They observed that incorporation was inhibited by pressure; therefore, the authors suggested that substrate uptake was the limiting factor to cell growth at increased hydrostatic pressure. Masuda and Albright (1978) studied pressure effects on a marine vibrio and found increased pressure decreased (1) rates of substrate uptake, (2) maximum velocity of uptake, and (3) the uptake system's affinity for its substrate. However, their study, as many others, used non-starved cells grown in rich media, while cells in the natural ocean environment, especially in the deep ocean, are under low nutrient or starvation conditions.

The significance of the lack of energy is emphasized by the research of Novitsky and Morita (1976, 1977, and 1978a,b) which detailed some of the physiological changes a marine vibrio exhibited when starved. They also found that starved cells showed increased survival at moderate pressures (100 to 500 atm) as compared to non-starved cells.

Three general questions have been asked as the basis for this study. (1) How pressure sensitive is uptake (kinetics of uptake and uptake rates) by short term (1.5 to 14 days) starved cells and is this significant in limiting microbial growth and survival under pressure? (2) What effect does starvation have on the barotolerance of uptake? (3) How does starvation survival at increased pressures correlate to observed pressure effects on uptake?

To help answer these questions, a study of the combined effects of increased hydrostatic pressure and nutrient starvation on the ability of a psychrophilic marine vibrio to take up a non-metabolizable amino acid (alpha-aminoisobutyric acid) was initiated.

LITERATURE REVIEW

Since Certes (1884) and Regnard (1884) first documented the effects of increased hydrostatic pressure on various micro- and macro-organisms, the limits placed on microbial activity by hydrostatic pressure have been a topic of continued study. ZoBell and Johnson (1949), ZoBell and Oppenheimer (1950), and Oppenheimer and ZoBell (1952) tested the effects of 1 to 600 atm on the growth and survival of numerous terrestrial and marine organisms, and found even 200 atm to be fatal for a few isolates. Increased pressure has also been found to stimulate the growth of some organisms (Marquis, 1976). The most recent discovery has been the isolation and subculturing of a marine barophile with an optimum pressure for growth between 425 and 500 atm at 2 to 4°C (Yayanos et al., 1979).

Growth and survival studies of organisms exposed to increased hydrostatic pressure have shown that the effect of pressure can be dependent on a variety of other environmental conditions, such as available nutrients and temperature. Growth of Ant-300 in a complex medium (Lib-X) at 5°C was unaffected by pressures up to 300 atm (Baross, Hanus, and Morita, 1974). At 400 atm no growth occurred and cells began to die slowly by 100 hr. In a glucose medium, a culture of Ant-300 lost over 99% of its viable cells after seven days at 250 atm and 5°C (Novitsky and Morita, 1978). A culture of glucose grown cells pressurized immediately after suspension under starvation conditions lost over 75% of their viable counts after three days at 100 atm (Novitsky and Morita, 1978). However, glucose grown cells starved one week at 1 atm, then pressurized to 250 atm retained 100% viability through six weeks at pressure, compared to 1 atm controls.

Increased survival of starving Escherichia coli and Streptococcus faecalis was observed at 500 atm as compared to 1 atm when the organisms were suspended in artificial seawater at temperatures (4°C) below their minimum growth temperature (Baross, Hanus, and Morita, 1975). The authors suggested from this and earlier study (Baross, Hanus, and Morita, 1974) that cellular processes, such as macromolecular synthesis, substrate transport, and enzyme activity, may be less sensitive to irreversible pressure damage when inactive, thereby explaining the protective effect of temperatures below the organisms' minimum growth temperatures. Johnson and Lewin (1946) and ZoBell and Cobet (1962) also reported that microbial death rates under pressure were reduced at lower temperatures.

Marquis (1976) reported that E. coli grown at 100 and 200 atm had increasingly greater growth yields as compared to 1 atm grown cells at temperatures from 30°C up to 45°C , while at an incubation temperature of 15°C yields at 100 and 200 atm were below the 1 atm yields. E. coli growth in the same medium at 9°C , which is at the low end of the organism's growth range, was very barosensitive, exhibiting decreased growth rates and yields at 50 and 100 atm (Marquis and Matsumura, 1978).

Observation of the limits hydrostatic pressure places on microbial growth and survival has led researchers to seek an understanding of the mechanisms of its effects. This effort has particularly concentrated on delineating pressure effects on various microbial processes, including DNA replication, substrate uptake and respiration, ATP synthesis and utilization, catabolic pathways, isolated enzymes, inducible enzyme systems, and many aspects of protein synthesis. An example of the detailed understanding that has been achieved in some of these areas is

presented in two papers by Smith, Pope, and Landau (1975) and Landau, Smith, and Pope (1977). Their results clearly implicate the 30S ribosomal subunit as the critical component in determining the pressure sensitivity of protein synthesis.

In an attempt to show that uptake of protein synthesis precursors was not limiting to the rate of protein synthesis under increased hydrostatic pressure, Landau (1970) and Schwarz and Landau (1972a) incubated E. coli cells with ^{14}C -amino acids at 1 and 680 atm for five to ten minutes at 37°C. Each sample was then filtered, rinsed, and treated with 5% trichloroacetic acid (TCA). The TCA insoluble fraction remained associated with the filter and accounted for substrate incorporated into protein, while the TCA soluble fraction accounted for the remainder of the substrate taken up by the cells but not incorporated into protein or respired to $^{14}\text{CO}_2$. The TCA insoluble fraction showed a significantly greater decrease than the soluble fraction at 680 atm, therefore the authors considered it highly improbable that uptake limited the rate of protein synthesis.

Cell-free protein synthesis was also examined in an effort to bypass pressure effects on uptake of protein synthesis precursors. Schwarz and Landau (1972b) reported that their E. coli cell-free system exhibited barotolerance characteristics similar to whole cells. Pseudomonas fluorescens also showed the same patterns of pressure inhibition on protein synthesis in cell-free and whole cell systems, while cell-free systems of Pseudomonas bathycetes exhibited increased pressure sensitivity in comparison to whole cell synthesis (Pope et al., 1975). Subsequent experimentation with Ps. bathycetes showed that effects of hydrostatic pressure on protein synthesis were comparable in cell-free and

whole cell systems, when proper specific ion concentrations were used in the cell-free preparation (Smith, Landau, and Pope, 1976).

Various investigators have studied hydrostatic pressure effects on uptake of ^{14}C organic compounds by acidifying cells with H_2SO_4 to terminate uptake, which allows monitoring of substrate respired to $^{14}\text{CO}_2$ and substrate incorporated into cellular macromolecules. The resulting data are evidence of pressure's effect on uptake but only account for that substrate utilized by the cell and not for total uptake. Paul and Morita (1971) used this technique to examine utilization of four different amino acids by a psychrotrophic marine vibrio. Compared to 1 atm controls, utilization of phenylalanine, glycine, proline, and glutamate at 100 atm and 5°C for 5 hr was reduced approximately 15, 30, 45, and 55%, respectively. At 500 atm, utilization of all four amino acids was reduced about 90%.

Albright (1975) found that glucose incorporation and respiration by E. coli, Vibrio marinus, and natural bacterial populations were much less pressure sensitive than phenylalanine utilization from 1 to 600 atm. The author indicated that the higher pressure sensitivity of amino acid utilization reflected the pressure sensitivity of amino acid incorporation into protein, and concluded that protein synthesis was probably the critical growth limiting factor under increased pressure.

According to Wirsén and Jannasch (1974), incubation of coastal water samples with various substrates (acetate, mannitol, glutamate, and casamino acids) at 3.8 atm resulted in 40 to 60% reductions in microbial utilization compared to samples incubated at 1 atm. Haualand (1978, Ph.D. thesis, Universitetet i Bergen) compared microbial incorporation and respiration at 1 and 10 atm, and found that 10 atm measurably reduced

incorporation when organisms were tested under more natural conditions (i.e., low nutrients), while the effect was less noticeable under ideal laboratory conditions.

Three psychrophilic isolates were shown to have glutamate incorporation reduced 40 to 80% at 20 atm and 8°C (Wirsen and Jannasch, 1975). Results of psychrophilic and mesophilic organisms incubated with glutamate at 1, 50, and 400 atm for 4 hr to 7 days, showed decreases of utilization at both increased pressures.

Schwarz, Yayanos, and Colwell (1976) presented data for rates of ^{14}C -starch utilization by water column and sediment organisms incubated for seven hours at 3°C. Their results showed a greater than 90% decrease in the rate at 750 atm. However, samples from the intestinal microflora of a deep-sea invertebrate gave rates of starch utilization at 750 atm which were only 14% less than the 1 atm rate. Bacterial isolates from the gut flora exhibited utilization rates at 750 atm, 35 to 65% below their respective rates at 1 atm.

Acidifying part of each uptake sample to capture the $^{14}\text{CO}_2$ and filtering the remainder of the sample with no fixation, allowed Jannasch, Wirsen, and Taylor (1976) to account for all the substrate taken up by the cells. Additionally, the water samples used for this study were incubated at in situ pressure and temperature and were undecompressed until each experiment was terminated. Samples taken from 1700 to 3130 m of water were incubated with a mixture of ^{14}C -amino acids or ^{14}C -glutamate. Final substrate concentrations were approximately 5 $\mu\text{g}/\text{ml}$ sample. For all experiments, increased pressure (170 to 313 atm) not only caused slower rates of uptake, but also reduced the maximum amount of substrate the microorganisms were capable of taking up.

Surface water samples incubated at 1 and 190 atm at 4°C exhibited similar results.

Using similar techniques for uptake termination, Schwarz, Walker, and Colwell (1975) tested the effect of in situ conditions on n-hexadecane-1-¹⁴C uptake and respiration by a subcultured mixed microflora from a 4949 m sediment sample. Their non-acidified samples were rinsed with methanol and were assumed to retain only the intracellular pool of hexadecane. The results showed an increased cellular pool of substrate and a decreased rate of ¹⁴CO₂ evolution at 500 atm and 4°C as compared to 1 atm controls. The authors' assumption that the observed uptake was intracellular, led them to conclude that cellular catabolism and assimilation was significantly more pressure sensitive than uptake.

Using three different techniques for terminating ¹⁴C-amino acid uptake, Schwarz and Colwell (1975) reported deliniating four fractions of the total substrate taken up. These fractions consisted of substrate incorporated into macromolecules, that respired to ¹⁴CO₂, and extracellular and intracellular pools. The fraction of substrate considered intracellular was reduced less than incorporation when incubated at in situ pressure, therefore the authors ruled out transport of amino acids into the cells, as the immediate limiting factor to growth under pressure. Their conclusion was that the translational mechanism of protein synthesis was the limiting factor to cellular growth under pressure.

Comparison of pressure effects on glucose and phenylalanine utilization by E. coli, V. marinus, and natural bacterial populations from coastal ocean waters, led Albright (1975) to the similar conclusion that the pressure sensitivity of protein synthesis probably determined the

barotolerance of cellular growth. The author did, however, recognize that the utilization of the substrates reflected the combined effects of pressure on a variety of cellular processes. After studies with V. marinus MP-1 encompassing viability, cellular leakage of protein, RNA, DNA, malic dehydrogenase, and amino acids, and uptake of a non-metabolizable substrate (cycloleucine) at increased pressure, Masuda and Albright (1978) attributed the pressure sensitivity of V. marinus growth to pressure effects on several cellular processes including inhibition of macromolecular synthesis, cell division, catabolic and anabolic enzymatic activity, and active uptake. In addition to the simple inhibition of uptake rates observed in that study, kinetic experiments revealed increasing K_m values as pressure was increased. V_{max} remained constant at 1 and 250 atm, but decreased at 500 atm. The authors suggest that the affinity of the cycloleucine transport system for its substrate decreased as pressure increased. Increased pressure was also found to affect the efflux rate of cycloleucine, causing progressively increased rates at 250 and 500 atm.

Shen and Berger (1974) had earlier used another non-metabolizable substrate, alpha-methylglucoside (α -MG), to assess hydrostatic pressure effects on uptake alone. This substrate is transported via group translocation by E. coli but it is not metabolized by the cells. During the first 60 seconds after the substrate was added and the pressure applied, uptake appeared not to be affected by pressures up to 680 atm, but by 2.5 min uptake was noticeably reduced at 340 atm or above. When incubated for 20 minutes, pressures from 68 to 680 atm caused increasingly greater reductions in α -MG uptake. The authors also found increased

pressure stimulated α -MG efflux from the cells.

For Streptococcus faecalis growth in a lactose medium under pressure, substrate transport was concluded to be the critical limiting factor (Matsumura, 1975, Ph.D. thesis, University of Rochester c.f. Marquis and Matsumura, 1978). Uptake and hydrolysis were rate-limiting at 1 atm and though hydrolysis was not significantly inhibited by increased pressure, uptake was reduced and as a result growth slowed.

The basic nature of pressure effects on biochemical reactions is believed to be an effect on molecular volume. Since increased hydrostatic pressure decreases molecular volume, those reaction steps which involve an increase in volume would be adversely affected by increased pressure, while steps which entailed a decrease in volume would be stimulated. The relationship of the apparent activation volume (ΔV^*) to the specific rate constant for any elementary process can be expressed as

$$k_p = k_o e^{-p\Delta V^*/RT}$$

where k_o is the rate at zero pressure, k_p is the rate at pressure p , ΔV^* is the difference in volume between the "activated complex" and the reactants, R is the gas constant (82.05 cc atm/mole K), and T is the temperature in K (Johnson, Eyring, and Polissar, 1954). A more applicable form of the equation is

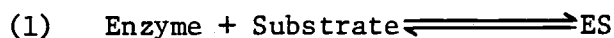
$$\Delta V^* = 2.3 RT \log_{10}(k_{p1}/k_{p2}) / (P_2 - P_1)$$

where k_{p1} and k_{p2} are the reaction rates at pressures P_1 and P_2 , respectively. Numerous researchers have used this formula to calculate ΔV^* values for complex metabolic processes such as protein synthesis,

uptake, and growth (Albright, 1975).

Albright (1975) calculated and compiled ΔV^* values for various organisms' metabolic processes, from his own and other researchers' data. For growth of 30 different species of microorganisms, he listed ΔV^* values of 25 ml/mol at 100 atm, 49 ml/mol at 200 atm, 81 ml/mol at 300 atm, 117 ml/mol at 400 atm, and 129 ml/mol at 500 atm. E. coli protein synthesis was reported to exhibit ΔV^* values ranging from 72 ml/mol at 1 atm to 150 ml/mol at 500 atm. Other data listed indicated mono- and disaccharide utilization was much less pressure sensitive (1 to 55 ml/mol at 400 atm) than amino acid utilization (34 to 185 ml/mol at 400 atm). The apparent activation volume for growth of S. faecalis at 1 and 408 atm was dependent on the carbon source, ranging from about 50 ml/mol for glucose and sucrose to about 90 ml/mol for lactose (Matsumura and Marquis, 1977). For cycloleucine uptake by V. marinus MP-1 at 1, 250, and 500 atm, Masuda and Albright (1978) calculated a ΔV^* of 85.5 ml/mol.

Laidler (1951) emphasized the important role substrate concentration played in determining pressure effects. Using a two stage reaction model



the author showed that at high substrate concentrations the second step was usually the rate limiting step, while at low concentrations step one (binding) could become rate limiting. Since the activation volumes for step one and step two could be very different, the effects of pressure (inhibition, stimulation, or no effect) would be dependent on the substrate concentration. For those data examined by Laidler, reactions run

with substrate concentrations below enzyme K_m values, pressure inhibited reaction rates, but when substrate concentrations were above the enzyme K_m values, pressure enhanced reaction rates.

Matsumura (1975, Ph.D. thesis, University of Rochester c.f. Marquis, 1976) found that pressure stimulated the membrane ATPase in S. faecalis when ATP concentrations were greater than 0.5 mM, but at lower concentrations the enzyme was suppressed by pressure. These data agree with Laidler's theory and observations, suggesting that the substrate binding step (1) had a positive volume change, while subsequent step(s) to the products (2) had a negative volume change. Mohankumar and Berger (1974) reported that increasing pressure from 1 to 333, 666, and 1000 atm facilitated a progressive decrease in the V_{max} and K_m parameters of a malic dehydrogenase when malate concentrations were low, while at high malate concentrations V_{max} increased with increasing pressure.

MATERIALS AND METHODS

Organism

A psychrophilic marine vibrio, designated Ant-300, was used for this study. This organism was isolated from the Antarctic Convergence from a depth of 300 m during cruise #46 of the R/V Eltanin. Ant-300 has a temperature growth range of less than 1°C to 13°C with optimum growth temperature between 4°C and 7°C (Geesey and Morita, 1975).

Media and Reagents

The following media and reagents were used in this study.

LIB-X MEDIUM

Yeast Extract (Difco)	1.2 g
Trypticase (BBL)	2.3
L-Glutamic Acid	0.3
Sodium Citrate	0.3
NaNO ₃	0.05
FeSO ₄	0.005
Rila Marine Mix	35
Distilled H ₂ O	1.0 liter
pH	7.8

Lib-X was adjusted to pH with 2.0 N NaOH. For Lib-X agar, 12 g of agar was added per liter. One-tenth strength Lib-X agar was prepared by adding one-tenth of all Lib-X ingredients except Rila salts and agar which were added in normal amounts.

4% SALT MIXTURE (4% SM)

NaCl	26 g
KCl	0.8
MgCl ₂ ·6H ₂ O	5.6
MgSO ₄ ·7H ₂ O	7.6
Distilled H ₂ O	1.0 liter

Buffered 4% SM for starvation was prepared by adding 1.0 ml of a 5% Na₂HPO₄ solution (filter sterilized) per 100 ml sterile 4% SM (pH = 7.5).

Four percent SM was prepared then filtered through 0.45 µm Type HA Millipore filters. One, two, and three percent SM were prepared by the appropriate dilutions of 4% SM.

Alpha-aminoisobutyric acid-1-¹⁴C (AIB) (51.6 mCi/mmmole) and ¹⁴C(U)-alanine (146 mCi/mmmole) were obtained from New England Nuclear.

Isotopes were prepared for use by diluting them to the desired concentration with distilled water, filter sterilizing the solution, then dispensing the solution into one or two milliliter sterile ampules which were sealed and frozen. Ampules of isotope were then thawed and used as needed.

Growth and Starvation

Ant-300 was grown in Lib-X broth at 5°C on a reciprocating shaker. Medium volumes of 100, 200, 400, and 1500 ml were used for growth in 250, 500, and 1000 ml Erlenmeyer flasks and Fernbach flasks, respectively. Growth was monitored by using a Beckman DB spectrophotometer at a wavelength of 600 nm and a light path of one centimeter. Unless otherwise

noted, cells were harvested in mid-log phase between an optical density of 0.08 and 0.135 (ca. $1-2 \times 10^7$ cell/ml) by centrifugation at 4080 x g and 5°C for 20 min. The resulting pellet was resuspended in approximately 200 ml cold sterile 4% SM and centrifuged again at 4080 x g for 20 min. This wash step was repeated once. The resulting washed cells were resuspended in one liter volumes of buffered 4% SM in Fernbach flasks at $1-2 \times 10^6$ cells/ml or, when noted, resuspended at the original cell concentration, $1-2 \times 10^7$ cells/ml. These suspensions were then starved at 5°C on a reciprocating shaker. Cells suspended at $1-2 \times 10^6$ cells/ml were used for experiments unless otherwise noted. When cells suspended at $1-2 \times 10^7$ cells/ml were used, they were diluted 1:9 in buffered SM just prior to commencing the experiment.

Viability

The spread plate technique was used to determine viability. Dilutions were made in sterile 4% SM and 0.1 ml of the appropriate cell dilution was spread on 0.1 X strength Lib-X agar plates in duplicate or triplicates. Plates were incubated at 5°C for one week and the colonies counted.

Uptake Experiments

Three techniques for terminating uptake were used, each providing observation of different fractions of substrate taken up by the cells. Non-acid samples were terminated by filtration only and accounted for all substrate taken up by cells, excluding that respired to $^{14}\text{CO}_2$. Samples acidified with 4 N H_2SO_4 to end uptake, accounted for substrate mineralized to $^{14}\text{CO}_2$ and that incorporated into cellular macromolecules.

Trichloroacetic acid (TCA) treatment of cells resulted in only substrate which was incorporated into cellular protein remaining associated with the cells when filtered.

After filtration and rinsing, all cell samples were treated the same. Samples were placed in scintillation vials, dried at 70-80°C for at least one hour and five milliliters of OMNIFLUOR added to each vial. A Beckman LS-100C liquid scintillation counter was used to count the samples, and DPM's were calculated by the external standard ratio method.

Uptake data was obtained with non-acid treatment unless indicated otherwise.

Non-Acid Treatment

Uptake samples were initiated by adding cells to an appropriate amount of ^{14}C -substrate. Samples were prepared either in (1) 20 ml serum bottles or (2) 125 or 250 ml Erlenmeyer flasks which were then capped and incubated at 5°C with intermittent shaking or in (3) 20 ml vials which were then capped, mixed ten seconds, and 3 or 5 ml taken up into disposable plastic syringes. Each syringe was immediately capped with a sealed needle and incubated at 5°C. At the end of the designated incubation time, samples were filtered through 0.45 μM Type HA Millipore filters and each filter then rinsed with two 7 ml volumes of cold 4% SM. All filters used for non-acid samples were pre-steamed for 15 minutes to remove electrostatic charges. During sample filtration the filter was not allowed to run dry until the rinsing was complete. When samples were run in serum bottles, the two 4% SM rinses were also used to rinse the bottles. For those samples run in Erlenmeyer flasks (more than one sample volume), duplicate three or five milliliter samples were

withdrawn and filtered at designated times. Non-acid sample blanks were run when uptake values were low enough to be significantly changed by blank subtraction. Non-acid blanks were estimated by filtering the designated amount of ^{14}C -substrate, followed by one 4% SM rinse, three milliliters of cells, and a second 4% SM rinse.

Sulfuric Acid Treatment

All acid samples were run in 20 or 50 ml serum bottles, depending on sample size. Cells were added to ^{14}C -substrate in the bottles and the bottles were immediately capped with the appropriate bucket and filter paper assemblies (Harrison, Wright, and Morita, 1971). Each bottle was gently swirled, then incubated at 5°C with intermittent shaking. Uptake was terminated by injecting 0.02 ml 4 N H_2SO_4 per ml sample into the sample through the rubber serum bottle cap, followed by 0.15 ml β -phenyl-ethylamine (PEA) which was carefully injected into the filter paper in the hanging plastic bucket assembly. Since the PEA captures the evolved $^{14}\text{CO}_2$, it was necessary to make sure all the PEA was absorbed by the filter paper and none was spilled. The caps were taken off after one hour of incubation with shaking, and each folded filter paper quickly removed, placed in respective scintillation vials, and the vials tightly capped. Each remaining acidified sample was then filtered through a $0.45\ \mu\text{m}$ Type HA Millipore filter and rinsed as described for non-acid samples. Ten milliliters of OMNIFLUOR were added directly to each vial containing a filter paper with the absorbed $^{14}\text{CO}_2$ and the samples counted. All filtered samples were treated as described. Acid blanks were estimated by acidifying cells just prior to adding ^{14}C -substrate. After the substrate was added the serum bottles were capped, PEA added and all

samples incubated for one hour with shaking. The blanks were then treated as all other samples.

Trichloroacetic Acid Treatment

These samples were initiated and incubated as described for acid samples, but were capped only with normal serum bottle caps. Cellular incorporation of the substrate was stopped by adding an equal volume of 20% TCA to each sample. One hour after addition of the TCA, each sample was filtered through a glass prefilter and rinsed three times with approximately 15 ml of 10% TCA (Kennell, 1967).

Kinetic Studies

Substrate (^{14}C) was mixed and incubated with cells in various concentrations ranging from 3.5×10^{-3} to $2.2 \times 10^1 \mu\text{M}$. Incubation times of 5, 10, and 30 min and 6 hr were used. The Lineweaver-Burk equation,

$$1/v_o = K_m/V_{\max} \cdot 1/[S] + 1/V_{\max}$$

was used to plot ($1/[S]$ vs $1/v$) the uptake data. Linear regression analysis was applied to calculate the best fit line and the resulting kinetic parameters, V_{\max} and K_m .

Competition Studies

Competition experiments were run primarily using AIB as the substrate and alanine as the competitor, while a few experiments reversing these roles were also conducted. Glutamate was also tested as a competitor to AIB uptake. Substrate and competitor concentrations ranged from 0.10 to 1.40 and 0.010 to 8.0 μM , respectively. Cells used for

these studies were harvested at densities of 1×10^7 up to 2×10^8 cells/ml and were starved at these cell concentrations. The ^{14}C -substrate and the competitor were always mixed before the cells were added unless otherwise noted. Incubation times ranged from five minutes to four hours.

Incubation at Increased Pressure

All samples incubated at increased pressure were run in three or five milliliter disposable plastic syringes. For uptake experiments, cells and substrate were prepared as described for non-acid samples with AIB at a final concentration of $1 \mu\text{M}$ unless otherwise noted. Duplicate samples were placed in each pressure cylinder and pressurized to the appropriate pressure (ZoBell and Oppenheimer, 1950). All cylinders were pre-equilibrated and incubated at 5°C . Samples were pressurized within two to five minutes after mixing cells and substrate. Within each experiment, the lag time for pressurization at each pressure was kept constant (i.e., three minutes for 200 atm samples, five minutes for 400 atm samples). Care was taken to exclude air bubbles from the syringes, to avoid possible toxic effects of oxygen at increased hydrostatic pressure (ZoBell and Hittle, 1967). At the end of each desired incubation period, the pressure was released instantaneously, unless indicated otherwise. Spot checks for pressure loss were made just prior to pressure release. All uptake samples were terminated as described for non-acid samples, requiring about 40 seconds from pressure release to filtration.

To test survival at increased pressure, 1.5 ml samples of starved cells were pressurized and released as described above. Samples were diluted and plated within 15 minutes of pressure release.

Pressure Release Effect on Uptake and Viability

Samples (for uptake and survival) were prepared and pressurized as described above and were incubated under one of the following sets of conditions: (1) at 1 atm; (2) at 1 atm until 0.5 to 5 minutes before stopping uptake, then pressurized to 400 or 800 atm, followed by instantaneous release of pressure at the end of the incubation time; (3) at 400 or 800 atm with instantaneous release of pressure at the end of incubation; (4) at 400 or 800 atm with a gradual release of pressure at a rate of about 100 atm per 15 s at the end of incubation.

Osmotic Shock Release of Substrate

Samples incubated at 1 and 400 atm for four or six hours were filtered and then rinsed with 10 ml 0.15 M NaCl or 0.50 M NaCl (exposure time approximately 1s/ml). Multiple samples from one flask were filtered after six hours incubation at 5°C and then either rinsed with salt solutions of various osmotic strengths, or rinsed with increasing volumes of 1% SM.

Oxygen Demand

Cells mixed with "cold" AIB (1 μ M) were used to fill BOD bottles and 10 and 30 ml syringes. All syringes and bottles were immediately sealed. Some of the syringes were immediately pressurized to 400 atm while the rest of the samples were incubated at 1 atm. All were incubated at 5°C. Fixation and titration of all samples were done according to a modified Winkler technique as described by Strickland and Parsons (1972). Controls were fixed within two minutes of the addition of substrate to

the cells. Samples were also fixed at 12 and 24 hr. Triplicate 50 ml volumes from the BOD bottles were titrated. Each ten milliliter syringe sample was titrated with 0.1 N sodium thiosulfate instead of the normal 0.5 N reagent. Titration results could therefore be directly compared to the 50 ml samples. Syringe samples of 25 ml were titrated with normal strength sodium thiosulfate and the results were doubled for comparison with the other data. Since this procedure was necessary only to check for significant oxygen depletion, the volumes of sodium thiosulfate titrated could be directly compared as relative measures of dissolved oxygen without conversion to actual oxygen concentrations.

The change in dissolved oxygen concentration from 0 to 10 hr at 1 atm and 5°C was also tested using a YSI Model #54 Oxygen Meter.

Data Analysis

Linear regression analysis was used to determine the best fit line for all linear graphical data. Slopes, x- and y-intercepts, and correlation coefficients were all calculated from these analyses.

Uptake data at increased pressures was expressed in numerous ways. "Relative uptake rate" consisted of the rate of uptake at an indicated pressure divided by the rate of uptake at 1 atm. If uptake was only determined at one point in time, and hence no rate could be calculated, pressure effect was expressed as "relative uptake," which is simply the total uptake at the indicated pressure divided by the total uptake at 1 atm for the same incubation time. "Actual uptake rates" are the rates of AIB uptake observed per milliliter of a starvation suspension (nmol/ml/hr). AIB uptake rates per viable cell were calculated by

dividing the "actual uptake rates" by the number of viable cells per milliliter in the starvation suspension.

RESULTS

Characterization of Starving Ant-300 -
AIB Uptake and Viability

Ant-300 cells ($1-2 \times 10^6$ cell/ml) starved two days and incubated with 1 μ M AIB exhibited a linear rate of AIB uptake between two and ten hours at one atm and 5°C (Fig. 1). Uptake at pressures up to 500 atm was also linear within a ten-hour period. In an initial experiment using 3-day starved cells to test uptake at 1 and 400 atm for 6 to 240 minutes, the one atm rate increased between one and two hours, while the 400 atm rate remained constant. Therefore, incubation times of two hours and above were predominantly used.

No significant incorporation of AIB into protein occurred, even after 41 hr, as evidenced by sulfuric acid and TCA treatment of cells incubated with AIB (see Appendix, Table 3). Mineralization of AIB was also non-existent (<1%). Total uptake of AIB (non-acidified cells) and alanine ($^{14}\text{CO}_2$ + non-acidified cells) by cells incubated for four hours with each substrate separately, showed AIB uptake to be about 5% of total alanine uptake.

Uptake of AIB by Ant-300 cells decreased during the first 3 to 5 days of starvation, then leveled off, remaining constant to at least 43 days (Fig. 2). By 90 days uptake decreased significantly. Cells starved at a concentration of $1-2 \times 10^7$ cells/ml and tested for uptake at 1 and 400 atm followed a similar pattern, with uptake at 400 atm remaining at a constant reduced level after 3 days.

Viable counts increased approximately 100% within the first two days of starvation, then remained fairly constant until about 12 days.

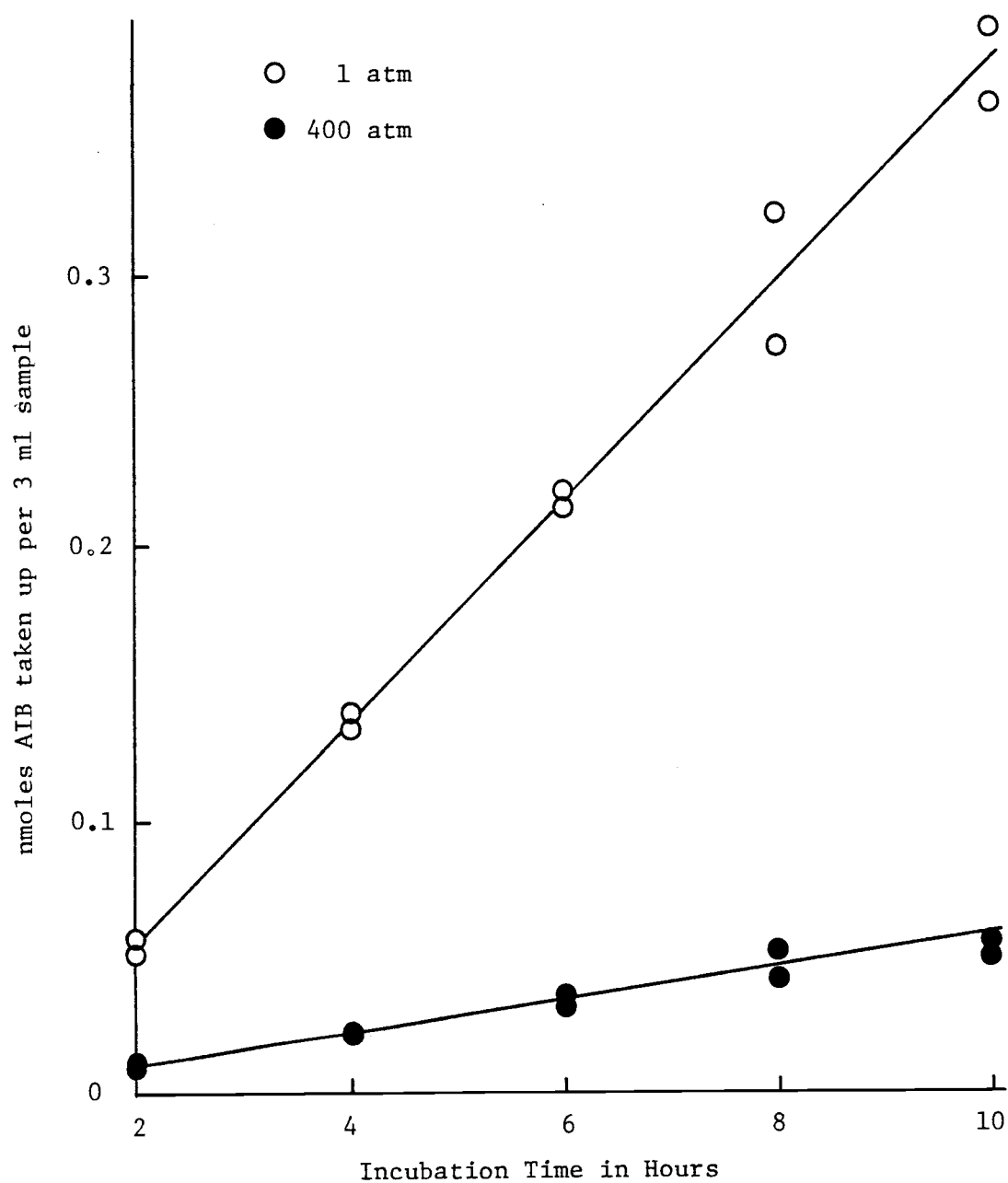


Fig. 1. Uptake of AIB ($1 \mu\text{M}$) with time by 2 day starved Ant-300 cells at 1 and 400 atm. Uptake was terminated with non-acid treatment.

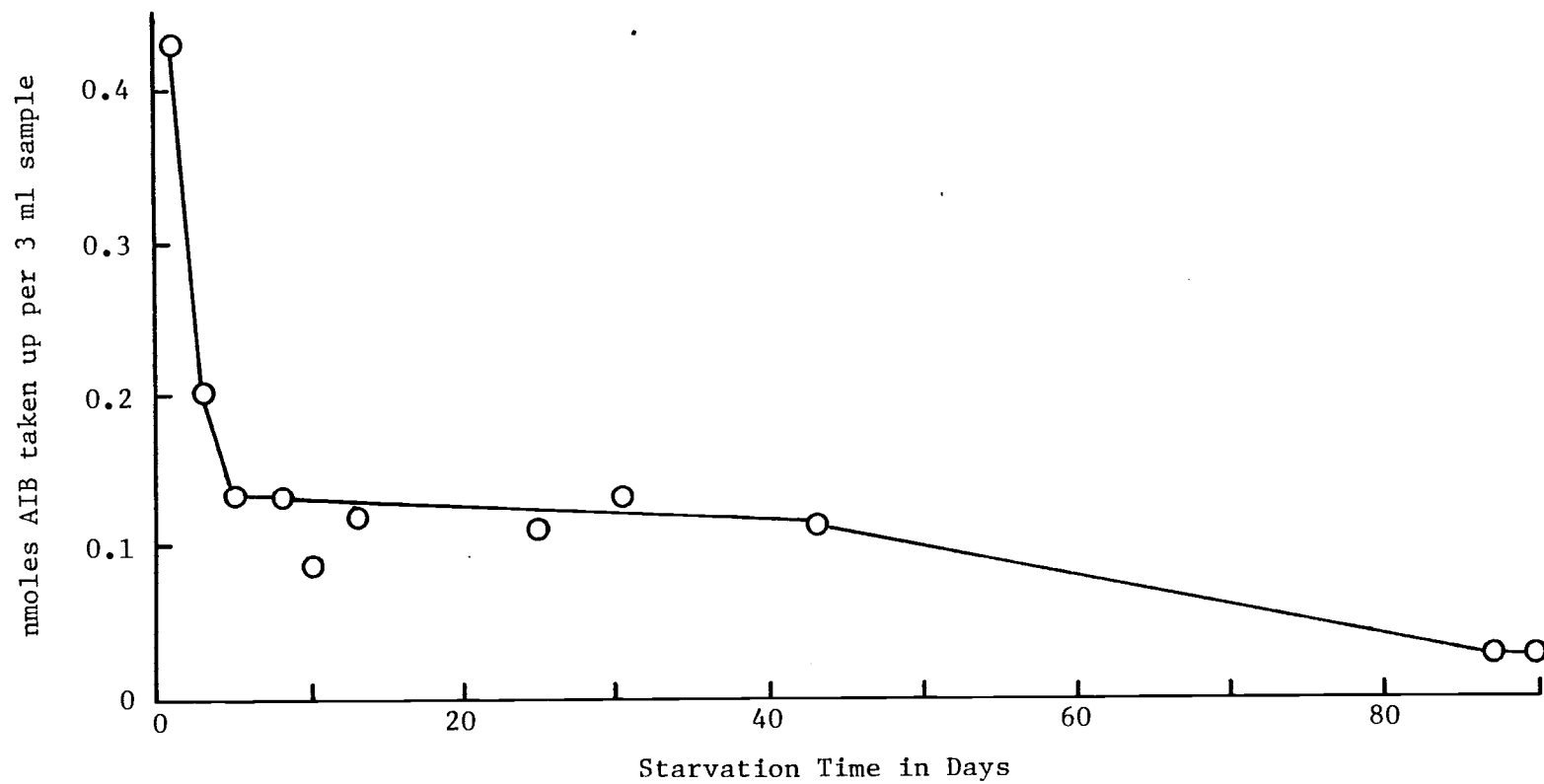


Fig. 2. Change in AIB uptake ability in starving Ant-300 cells. Cells starved at $1-2 \times 10^6$ cells/ml. Uptake incubation time was 4 hr and was terminated with non-acid treatment. Each point is an average of 2 to 4 samples.

After 12 to 14 days cells began to die at a logarithmic rate, with a half-life of about 20 days (Fig. 3).

Uptake of AIB conformed to typical saturation kinetics (see Appendix, Table 4). For cells starved 2 to 8 days and incubated with AIB for 10 or 30 min or 6 hr, V_{max} values ranged from 0.30 to 1.0×10^{-8} nmol/cell/hr with an average of 0.60×10^{-8} nmol/cell/hr. The corresponding K_m values ranged from 0.51 to $1.4 \mu M$ with an average of $0.9 \mu M$. No significant differences in V_{max} or K_m values were observed for the three different incubation times used. For alanine uptake (5 min) by 4 days starved cells, V_{max} and K_m values of 0.8 (range, 0.7 to 2.0×10^{-8} nmol/cell/hr and $0.44 \mu M$ were calculated, respectively. Comparable values for AIB uptake (10 min incubation time, 3-day starved cells) were 0.8×10^{-8} nmol/cell/hr and $0.8 \mu M$, respectively. A single experiment using cells starved less than 3.5 hr gave V_{max} and K_m values of 7.0×10^{-8} nmol/cell/hr and $5.0 \mu M$, respectively. No changes in V_{max} and K_m values were noted from 2 to 8 days of starvation. Six-day starved cells incubated with a range of AIB concentrations from 3.5×10^{-3} to $2.16 \times 10^1 \mu M$ for four hours at $5^\circ C$ resulted in no discernable differences in the V_{max} and K_m values.

In competitive inhibition studies, AIB uptake was inhibited about 70% at equal AIB and alanine concentrations, while a 98% inhibition was recorded when the alanine concentration was 1,000 times the AIB concentration. In a single experiment using alanine as a competitor at equal concentrations with AIB, the rate of AIB uptake was inhibited 74% during the first 15 minutes, after which the rate returned to the uninhibited level or higher (Fig. 4). With ^{14}C -alanine ($0.8 \mu M$) as the substrate

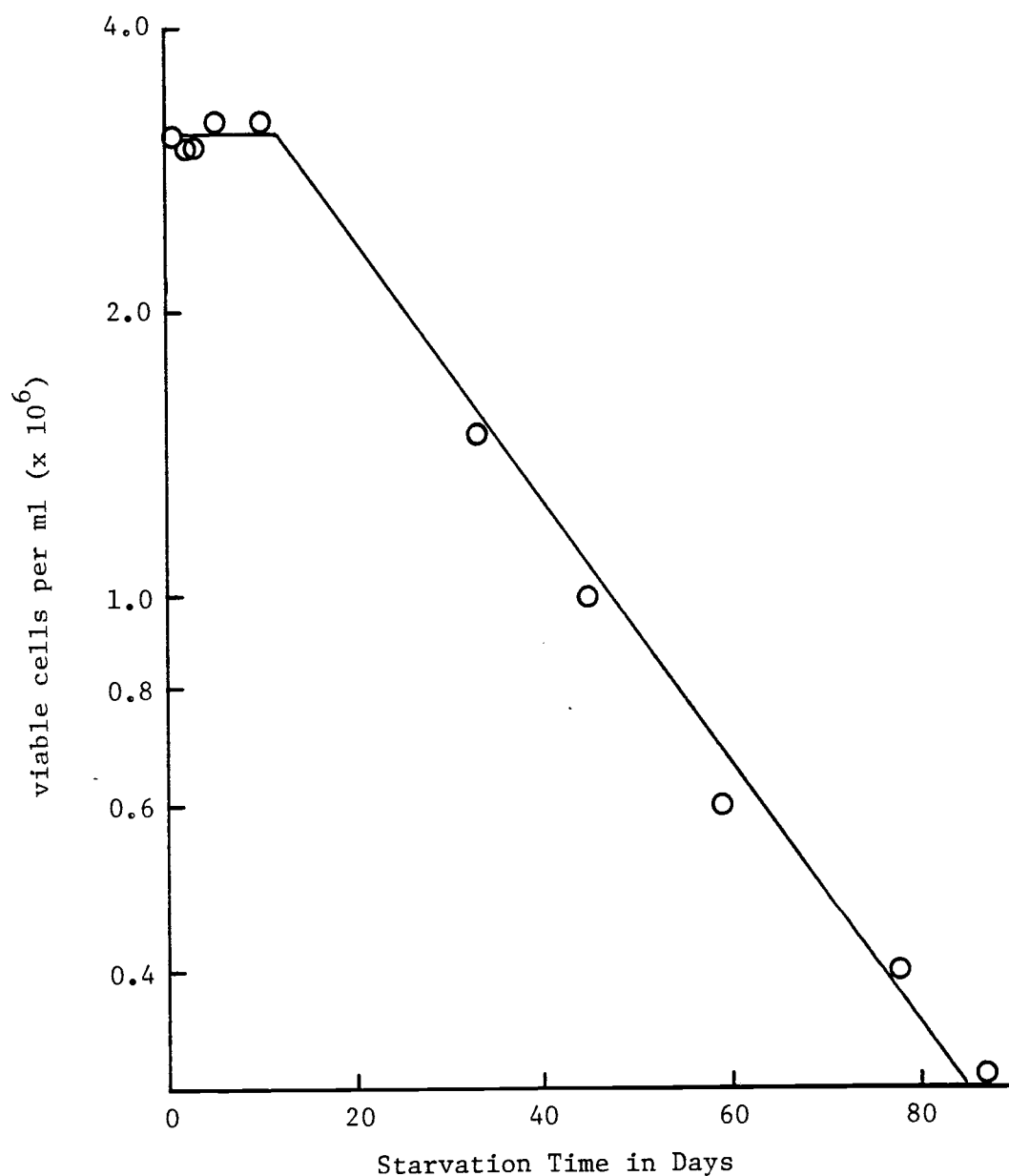


Fig. 3. Change in viable counts in a starving Ant-300 culture. Viability determined by plate counts. Viable counts at 0 time were not determined for this starvation suspension but would have been about 1.5×10^6 cells/ml.

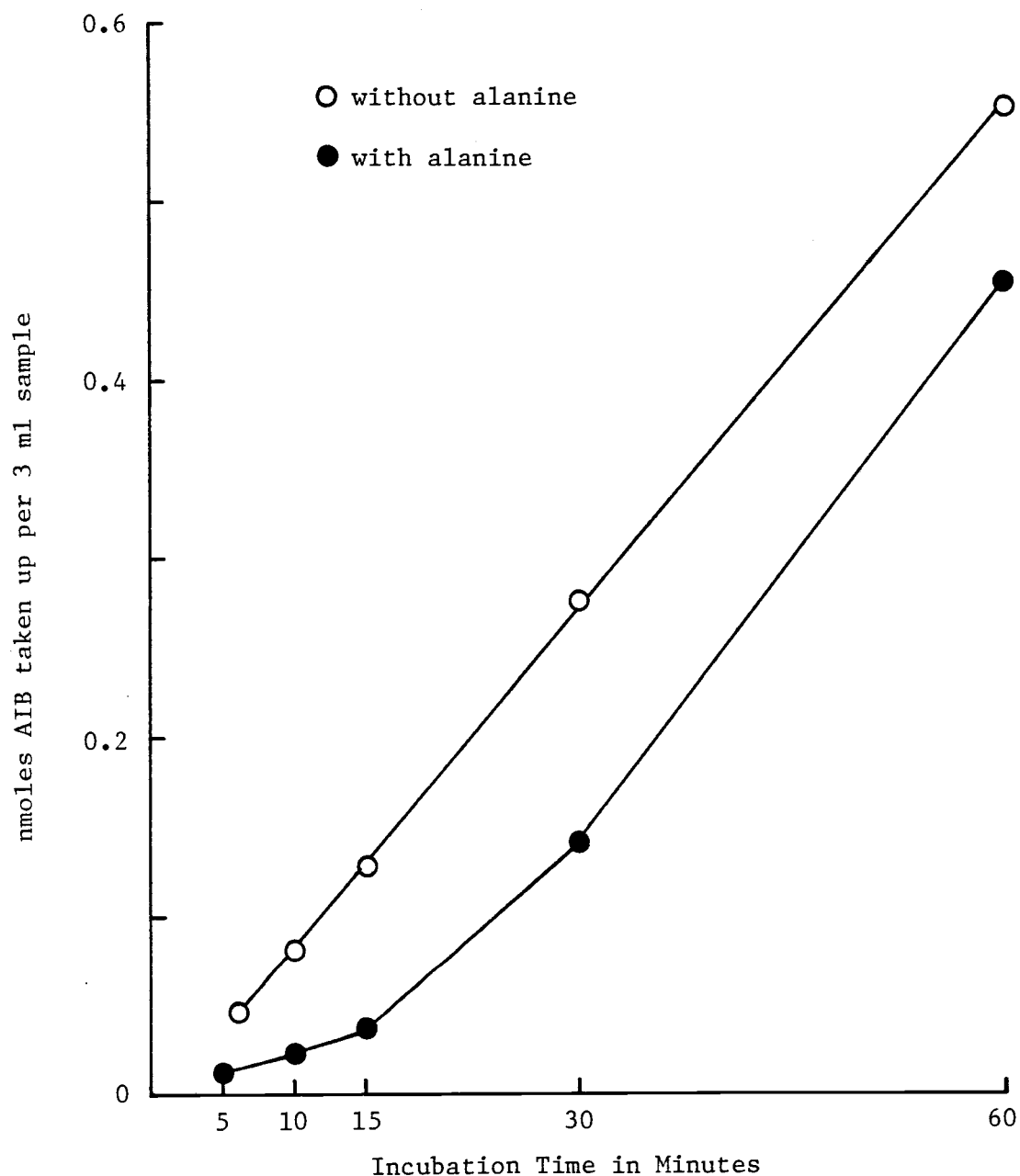


Fig. 4. Effect of alanine on uptake of AIB by Ant-300 cells. Both ^{14}C -AIB and alanine were added to final concentrations of $0.4 \mu\text{M}$. Cells were starved for 6 days at approximately 10^8 cells/ml and diluted 1:6 in buffered 4% SM just prior to beginning the experiment. Duplicate samples were averaged.

and AIB (8 μ M) as the competitor, the rate of total 14 C-alanine uptake was reduced 17% during the first two hours; but after two hours the rate returned to the uninhibited level. The rates of 14 C-alanine respiration, incorporation, and accumulation into the loosely bound pool are shown in Figures 5, 6, and 7. Due to the changing uptake rates observed, incubation times for kinetic competition experiments were kept between 5 and 30 minutes. K_m values usually increased when competitor was added, while V_{max} values generally decreased slightly (Table 1).

Glutamate was also observed to compete with AIB uptake. Addition of glutamate (2 μ M) to ten-week starved cells 30 min prior to AIB addition (1 μ M) resulted in a 60% reduction of AIB uptake when incubated for 30 min. Ten-week starved cells were also pre-incubated with 2 μ M glucose for 60 min, then AIB was added and the mixture incubated for another 30 min. Cells with glucose added showed a 30% increase in AIB uptake over cells not pre-incubated with glucose.

Cells incubated with AIB, filtered and then rinsed with 0.15 M NaCl for approximately ten seconds, showed 60 to 80% loss of substrate compared with cells rinsed with 0.5 M NaCl. Cells incubated at 400 atm lost nearly the same percent or slightly less than the 1 atm samples, when rinsed with 0.15 M NaCl. Cells rinsed with increasingly hypotonic solutions, 4% to 1% SM and 0.5 to 0.1 M NaCl, showed increasing losses of substrate (Fig. 8). Rinsing with 0.1 M NaCl caused the greatest substrate loss observed, 96%, while 1% SM caused losses of 80 to 87%. Exposure to 1% SM for increasing lengths of time between 0 and 40 s showed no more substrate was released after 10 s.

Using the Winkler technique, only slight utilization of available

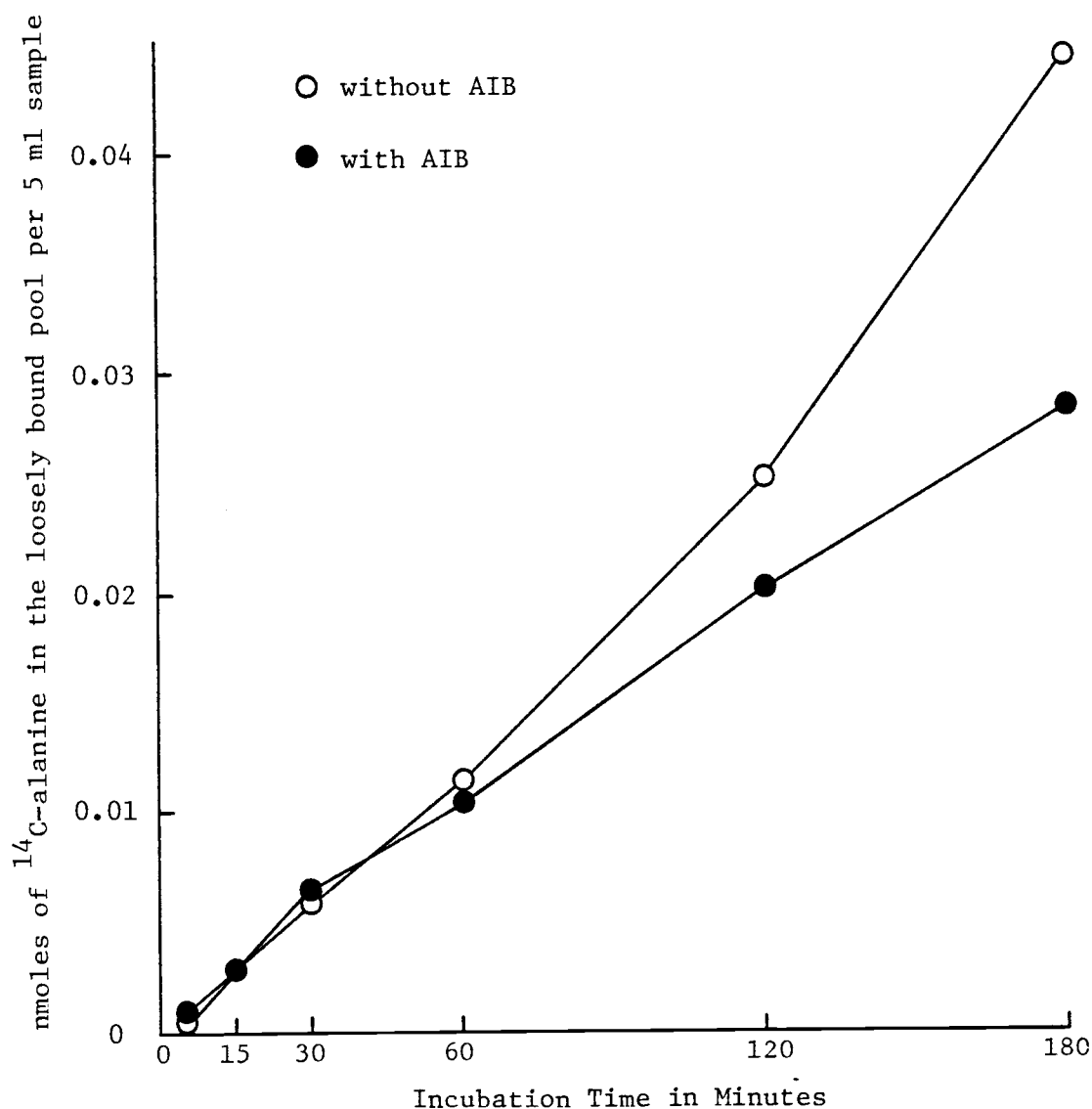


Fig. 5. Effect of "cold" AIB on accumulation of the loosely bound pool of ^{14}C -alanine uptake by Ant-300 cells. ^{14}C -alanine concentration was $0.7\ \mu\text{M}$. AIB, when added, was at $8.0\ \mu\text{M}$. For uptakes without AIB 4 day starved cells were used. Uptakes with AIB added were conducted the following day with cells from the same starvation suspension (starved 5 days). Cells were starved at 4×10^7 cells/ml and were diluted to 4×10^5 cells/ml just prior to beginning the experiment. Data points are an average of two samples.

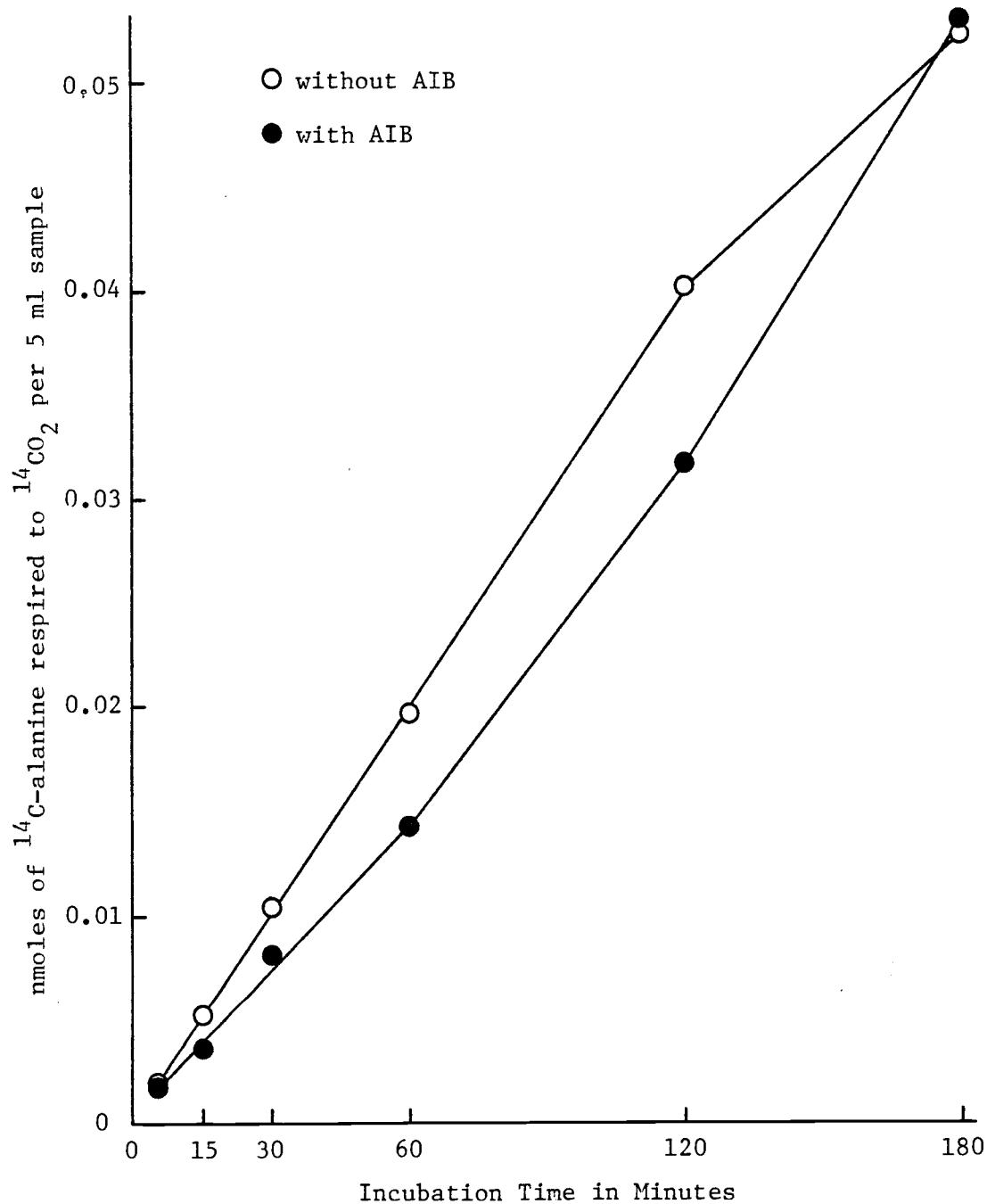


Fig. 6. Effect of "cold" AIB on respiration of ^{14}C -alanine to $^{14}\text{CO}_2$ by Ant-300 cells. All other experimental details as described in Fig. 5.

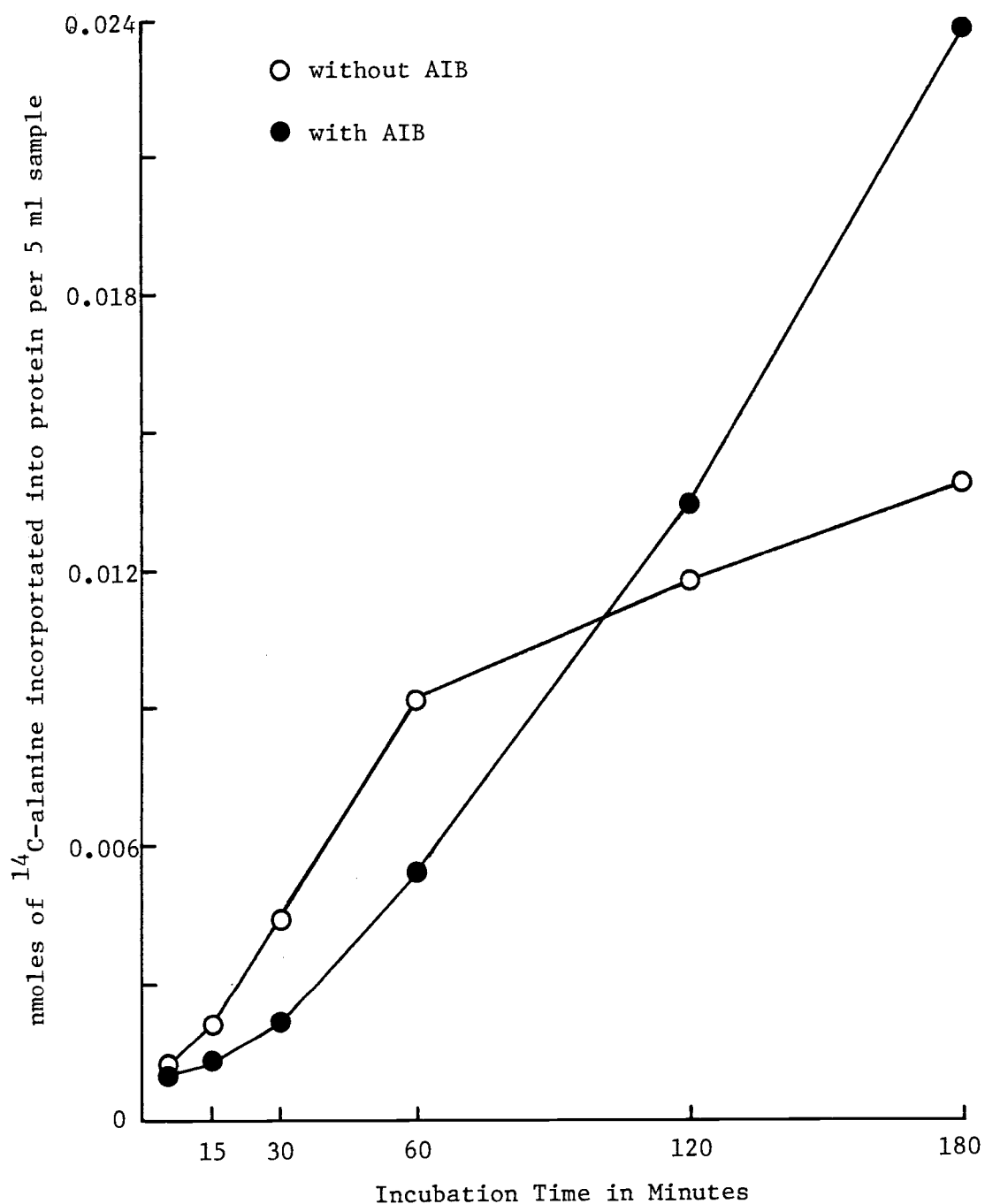


Fig. 7. Effect of "cold" AIB on ^{14}C -alanine incorporation into protein by Ant-300 cells. All other experimental details as described in Fig. 5.

Table 1. Changes in Vmax and Km values with competitive uptake between AIB and alanine.

Incubation time (min)	Starvation time (days)	Concentration range of ¹⁴ C-substrate (μM)	Competitor concentration (μM)	Vmax (x 10 ⁻⁸ nmol/cell/hr)	Km (μM)	Percent change with competitor added	
						Vmax	Km
		<u>AIB</u>	<u>alanine</u>				
10	2	0.10 - 0.80	0	1.0	0.9		
			0.010	1.1	1.0	+10	+11
10	3	0.35 - 1.40	0	0.78	0.77		
			0.14	0.65	2.4	-17	+210
30	2	0.15 - 1.0	0	0.41	0.68		
			0.060	0.30	0.81	-27	+19
30	2	0.35 - 1.40	0	0.70	0.79		
			0.070	0.66	1.18	-6	+49
30	2	0.70 - 1.40	0	0.67	0.90		
			0.070	0.50	0.85	-25	-6
		<u>alanine</u>	<u>AIB</u>				
5a	4	0.10 - 0.80	0	1.0	0.44		
			8.0	1.2	0.56	+20	+27

Correlation coefficients for Vmax and Km determinations were > 0.96.

^a Total alanine uptake measured (non-acid plus ¹⁴CO₂).

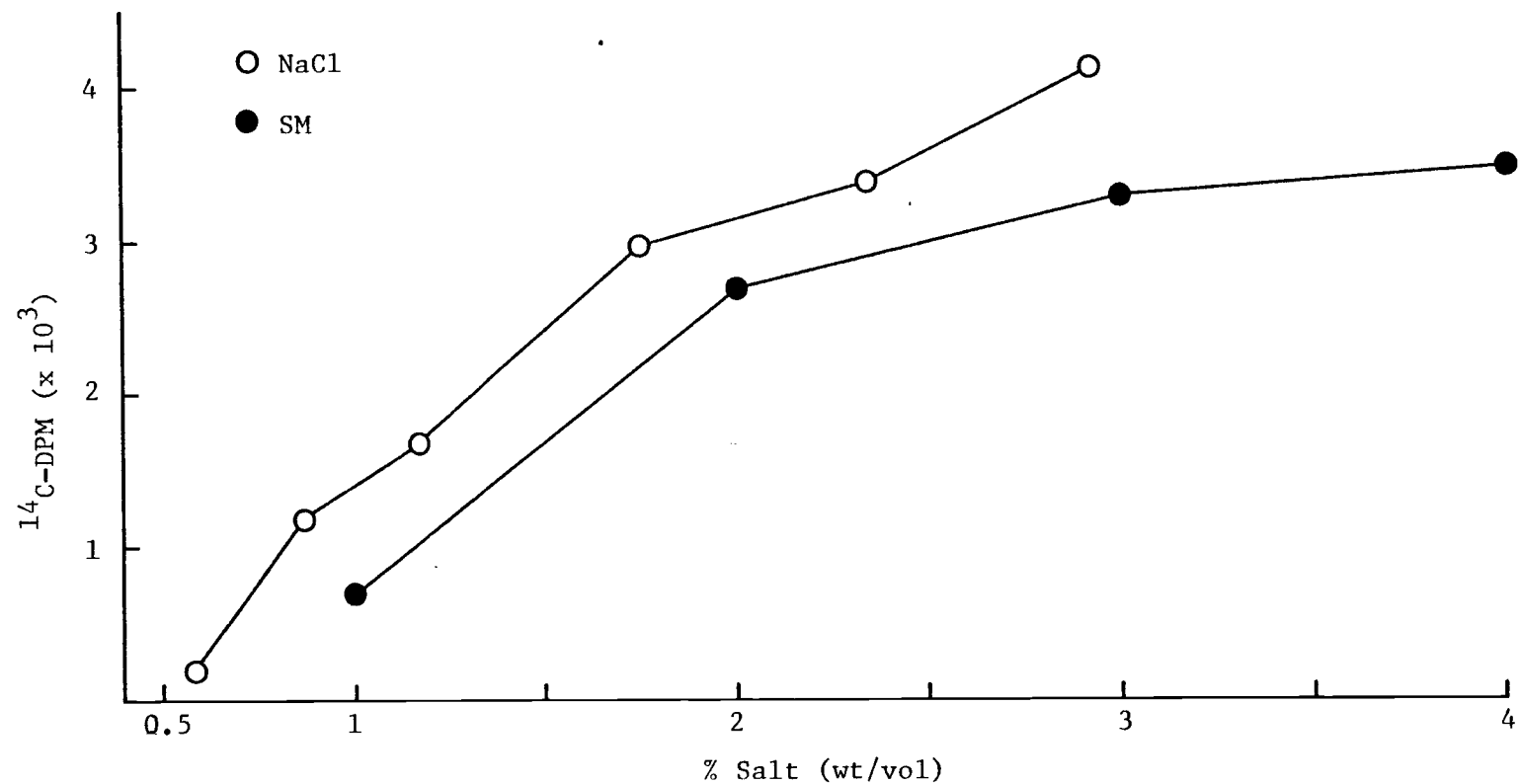


Fig. 8. Effect of hypotonic rinse solutions on AIB retention by Ant-300 cells. Two day starved cells were incubated 6 hr with AIB, then 3 ml samples were filtered and each rinsed with one of various salt solutions. Each data point is an average of two samples.

oxygen was detected in uptake samples at 1 atm for up to 24 hr (see Appendix, Table 5). Since it was only necessary to determine relative amounts of oxygen consumed, actual dissolved gas concentrations were not calculated. Those samples run and fixed in syringes showed consistently higher values, indicating that oxygen leaked into the syringes. Dissolved oxygen concentrations of 10.2 ppm at zero time and 10.0 to 10.1 ppm at ten hours were recorded using an oxygen meter, when AIB and 1.5 day starved cells were mixed and incubated in a BOD bottle at 5°C.

Pressure Release Effect on Viability and Substrate Retention

Five-day starved cells tested at 400 and 500 atm, with incubations of 5 to 50 hr, showed no difference in survival between cells released instantaneously from pressure to 1 atm and those released slowly.

Samples incubated at 1 atm with AIB, then briefly pressurized to 800 atm just prior to filtration, retained an average of 90% (range, 73 to 106%) of the substrate as compared to the control which was not pressurized. When pressurized to 400 atm, retention averaged 100%. The percentage of substrate retained by quick release samples as compared to slow release samples from 800 and 400 atm averaged 87% (73 to 102%) and 92%, respectively. Quick and slow release samples showed no difference in AIB uptake when incubated at 400 atm for 5, 10, 20, and 30 hr. Since pressures of 500 atm or less were predominantly used in this study and the retention of substrate with instantaneous release of pressure from 400 atm was 92 to 100%, all pressurized samples were released instantaneously.

Pressure Effects on AIB Uptake by Ant-300
Cells Starved 1.5 to 14 Days

For cells starved 1.5 to 14 days increased hydrostatic pressures resulted in decreased rates of AIB uptake (Fig. 9). At 400 atm, uptake by two and four-day starved cells was linear with time for at least 30 hr. Uptake was halted at 500 atm by 14 hr and no AIB was lost thereafter even up to 39 hr. Uptake was severely inhibited at 600 and 800 atm and incubation at pressure beyond 12 hr resulted in loss of accumulated substrate (Fig. 10).

The average and range of relative uptake rates at 1 to 500 atm for cells starved 1.5 to 14 days are given in Table 2. Experiments in which samples were run at all indicated pressures showed the largest difference in rate between 200 and 300 atm samples (Fig. 11). The differences in the average relative uptake rates (Table 2) cannot be compared as the data in Figure 11, since rates did vary between experiments and the pattern in Figure 11 was only consistently observed within any single experiment in which all pressures were tested.

Incubation of two-day starved cells at various pressures from 1 to 500 atm for 11 hr resulted in the following percent survival values: 100% at 1 atm; 88% at 100 atm; 94% at 200 atm; 82% at 300 atm; 88% at 400 atm; and 82% at 500 atm.

Calculation of ΔV^* (apparent activation volume) for AIB uptake by cells starved 1.5 to 14 days gave the following averages and ranges: at 100 atm, 32 ml/mol (17 - 51); at 200 atm, 64 ml/mol (23 - 89); at 300 atm, 102 ml/mol (80 - 119); at 400 atm, 127 ml/mol (101 - 149); and at 500 atm, 129 ml/mol (111 - 156). Experiments in which samples were run at all pressures gave the ΔV^* values shown in Figure 12 (see Fig. 11 for

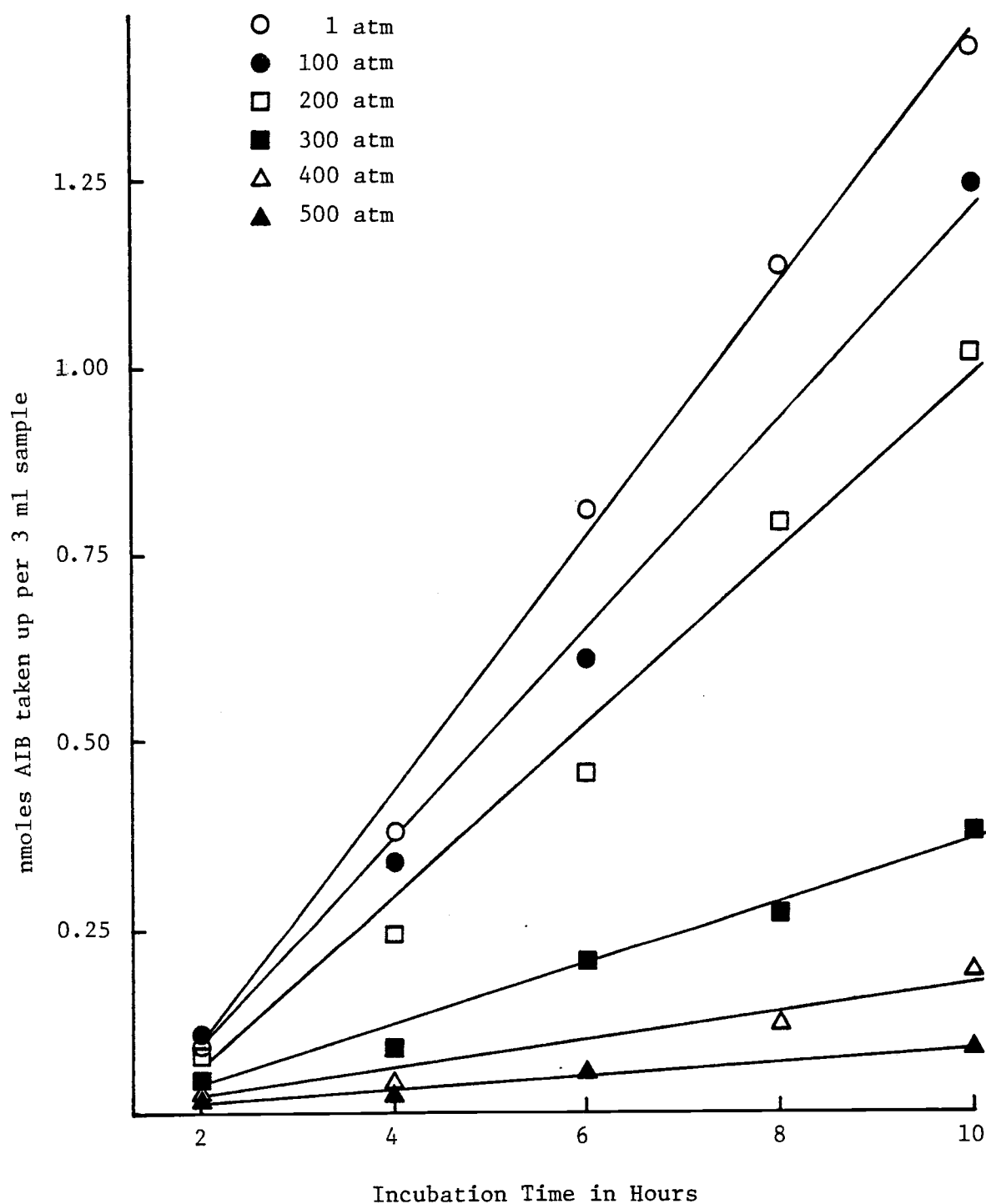


Fig. 9. Hydrostatic pressure effects on the rate of AIB uptake by Ant-300 cells. AIB concentration was $1.0 \mu\text{M}$. Cells were starved 1.5 days. Uptake was terminated with non-acid treatment.

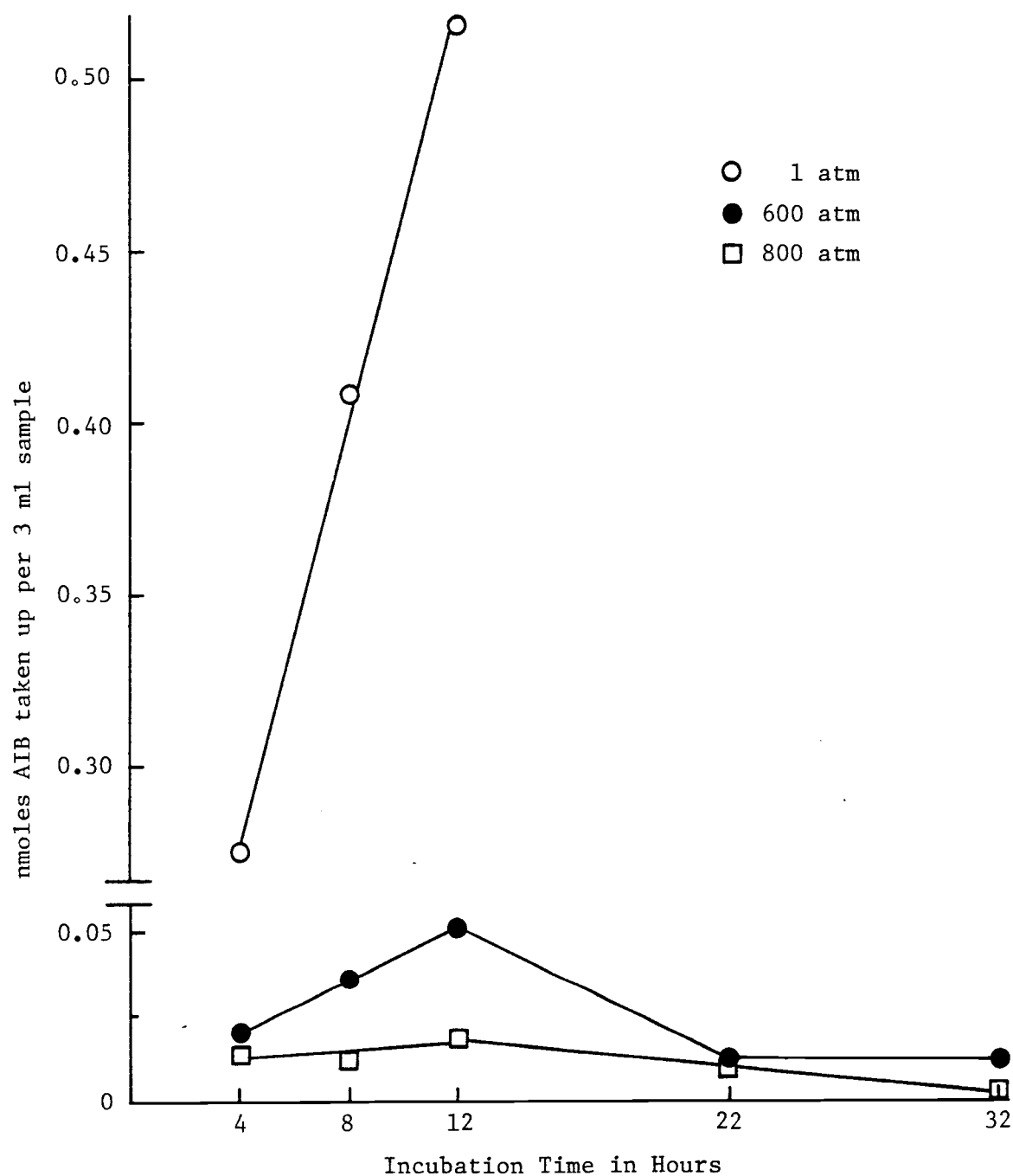


Fig. 10. Uptake of AIB by Ant-300 cells with time at 1, 600, and 800 atm. Cells were starved 4 days at $1-2 \times 10^7$ cells/ml and diluted 1:2 in buffered 4% SM just prior to beginning the experiment. AIB concentration was $1.0 \mu\text{M}$.

Table 2. Relative rates of AIB uptake by starved Ant-300 cells at increased hydrostatic pressures.

Hydrostatic pressure (atm)	Relative uptake rates		
	Average	Range	# of determinations
1	1.0	-	-
100	0.89	0.87 - 0.93	3
200	0.58	0.46 - 0.82	10
300	0.27	0.21 - 0.35	5
400	0.11	0.074 - 0.17	7
500	0.070	0.033 - 0.089	3

Cells were starved between 1.5 and 12 days. Uptake at 1 atm was used as the internal standard for each experiment. Rates were determined from 2 to 10 hr.

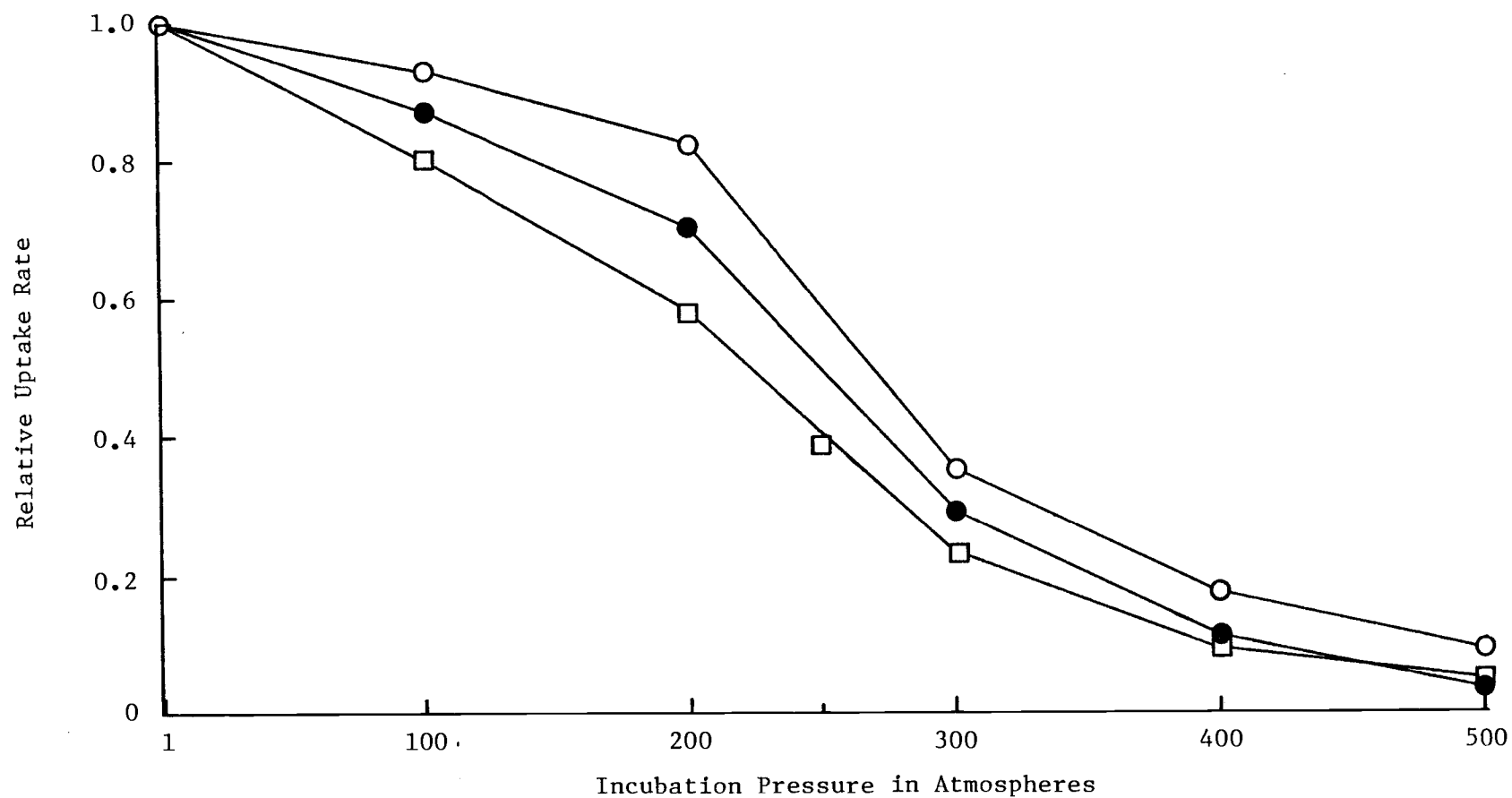


Fig. 11. Change in relative rates of AIB uptake by Ant-300 cells at increased hydrostatic pressures. Each different set of symbols represents one experiment in which uptake was tested at all the indicated pressures. The "open square" (□) data are relative uptakes (10 hr), except at 1 and 200 atm where rates were determined. The relative uptake at 200 atm was the same as its calculated relative rate. Cells were starved 1.5 to 10 days.

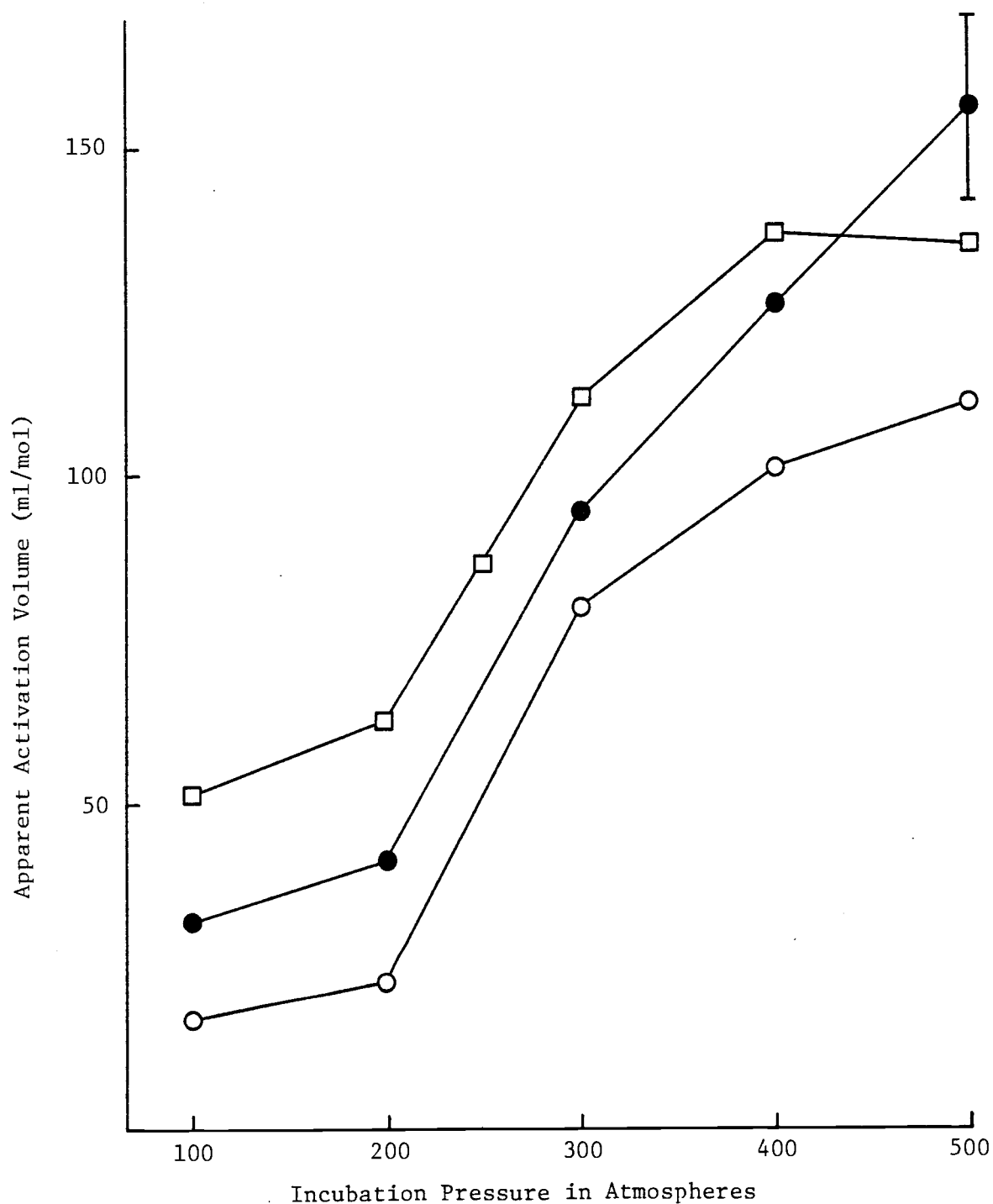


Fig. 12. Calculated apparent activation volumes (ΔV^*) at different pressures. The ΔV^* values were calculated from the relative uptake rates shown in Fig. 11. Each set of symbols represents one experiment in which all indicated pressures were tested. Cells were starved 1.5 to 10 days. Bracket denotes range of observation. For explanation of ΔV^* see literature review.

the corresponding relative uptake rates).

For AIB uptake by 6 to 8-day starved cells at 200 atm, V_{max} decreased an average of 40% (26 to 61% range) and K_m increased an average of 60% (-30 to 100%) compared to 1 atm samples (Fig. 13).

Pressure Effects on AIB Uptake by Ant-300 Cells
Starved 3.5 Hours to 90 Days

An initial drop in the relative rates at 200 and 300 atm was observed during the first 1.5 days of starvation (Fig. 14). Both starvation suspensions tested at 12 hr and 7 days showed a decrease in relative uptake rates at 200 atm, from 0.83 to 0.57 and 1.0 to 0.49 (Fig. 15). The one suspension tested also at 300 atm showed a decrease from 0.43 at 12 hr to 0.29 at 7 days. Preliminary experiments showed that relative uptake of AIB by Ant-300 at 400 atm also decreased during the first 2 to 3 days of starvation, then remained constant from 3 to at least 14 days.

Cell suspensions that were tested at 1.5 days of starvation up to 12 days showed no further decrease in relative uptake rates during that time period. Cells starved longer than 20 days, exhibited increasing relative uptake rates at 200 and 300 atm (Fig. 14). Uptake rates were not determined at 100, 400, and 500 atm for cells starved longer than 20 days.

Actual uptake rates by Ant-300 starving populations generally decreased at all pressures as starvation time increased (Fig. 16). Uptake rates of AIB per viable cell at 1, 200, and 300 atm decreased during the first 10 days of starvation, then recovered slightly by 30 to 40 days (Fig. 17). From 40 to 90 days the rates remained fairly constant.

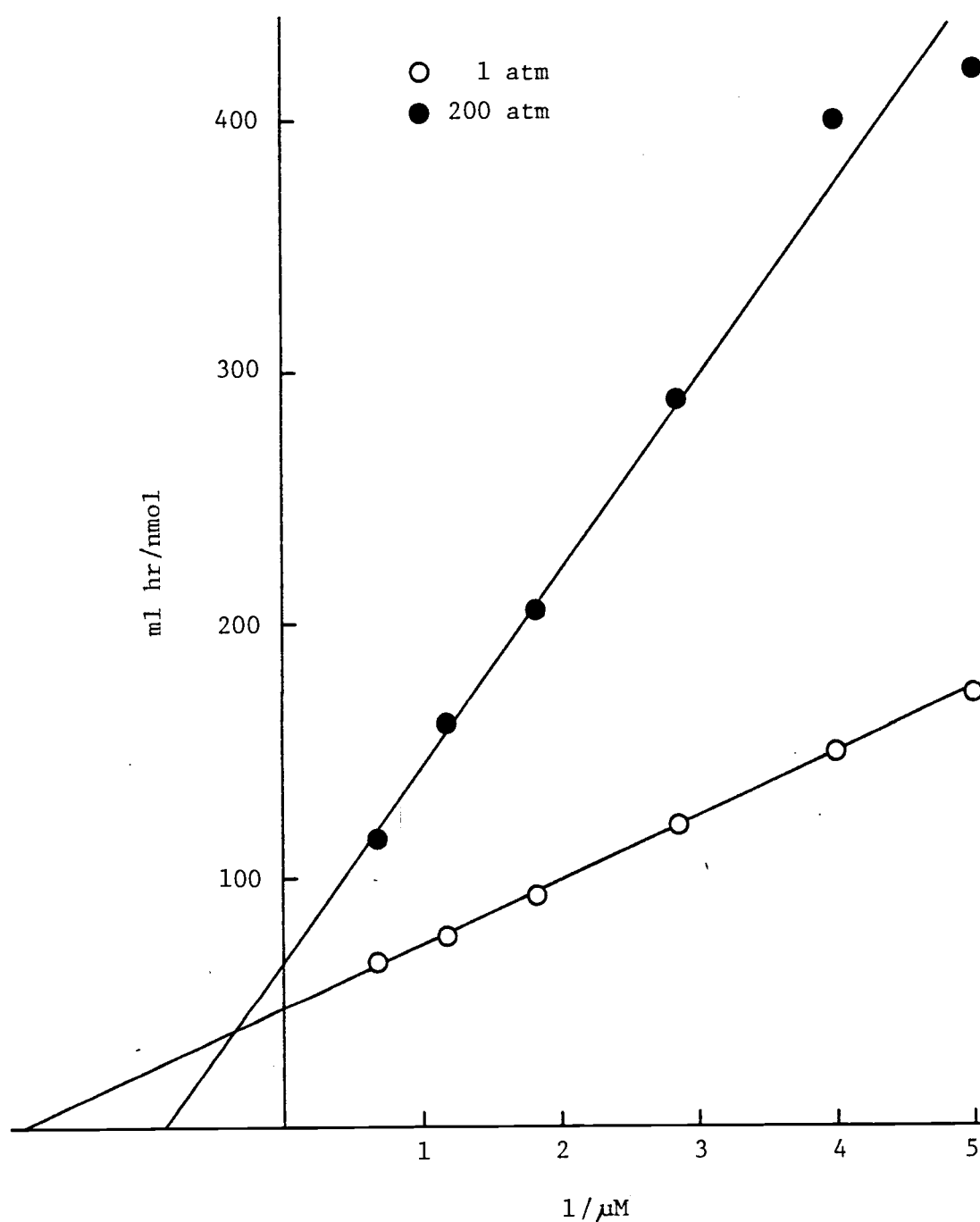


Fig. 13. Lineweaver-Burk plot of AIB uptake at six different substrate concentrations at 1 and 200 atm. Cells were starved 8 days at $1-2 \times 10^6$ cells/ml. Incubation time with AIB was 6 hr. Each data point is an average of two samples.

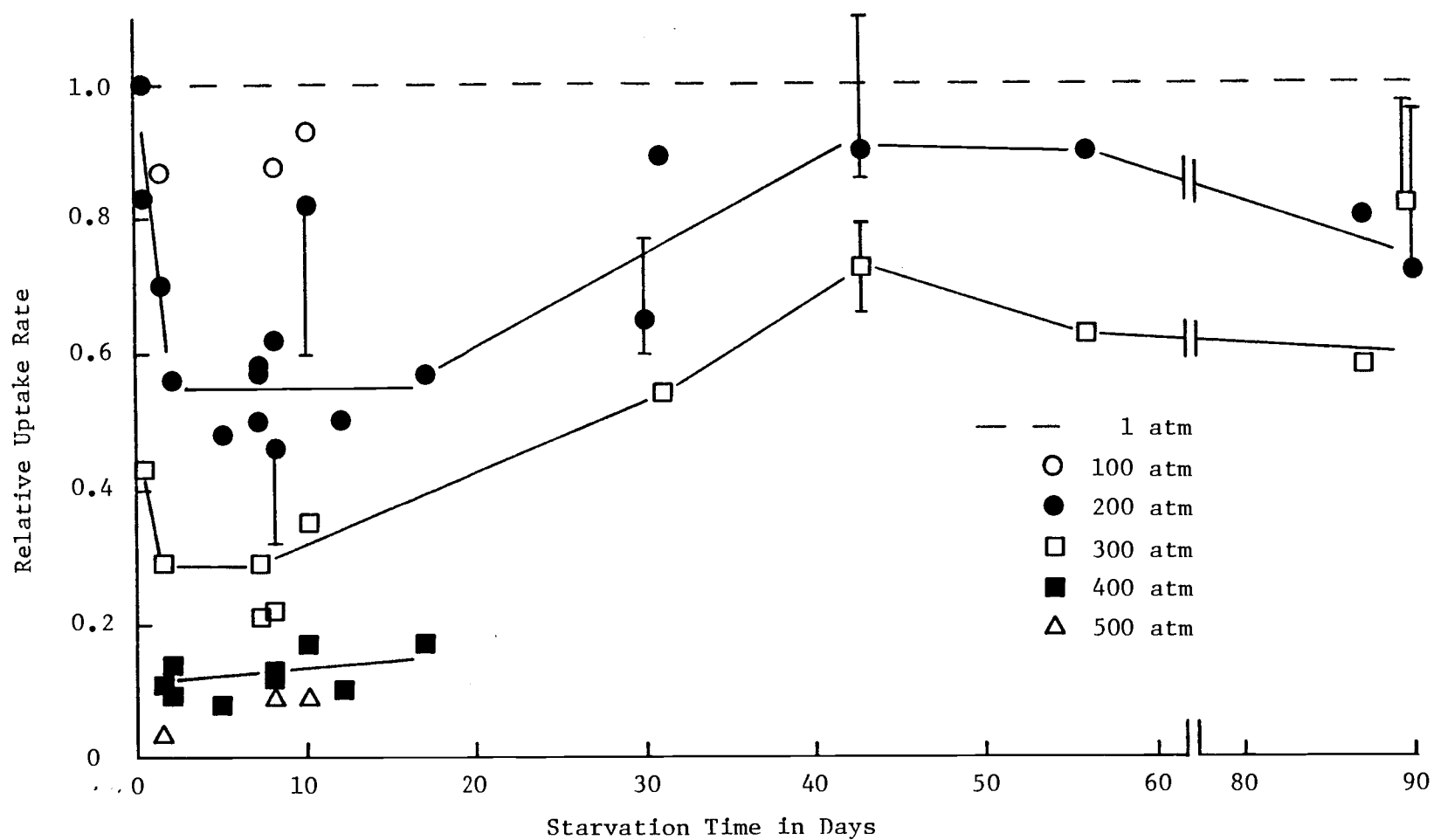


Fig. 14. Changes in relative uptake rates at 100 to 500 atm of starving Ant-300 cells. Brackets denote range of observation.

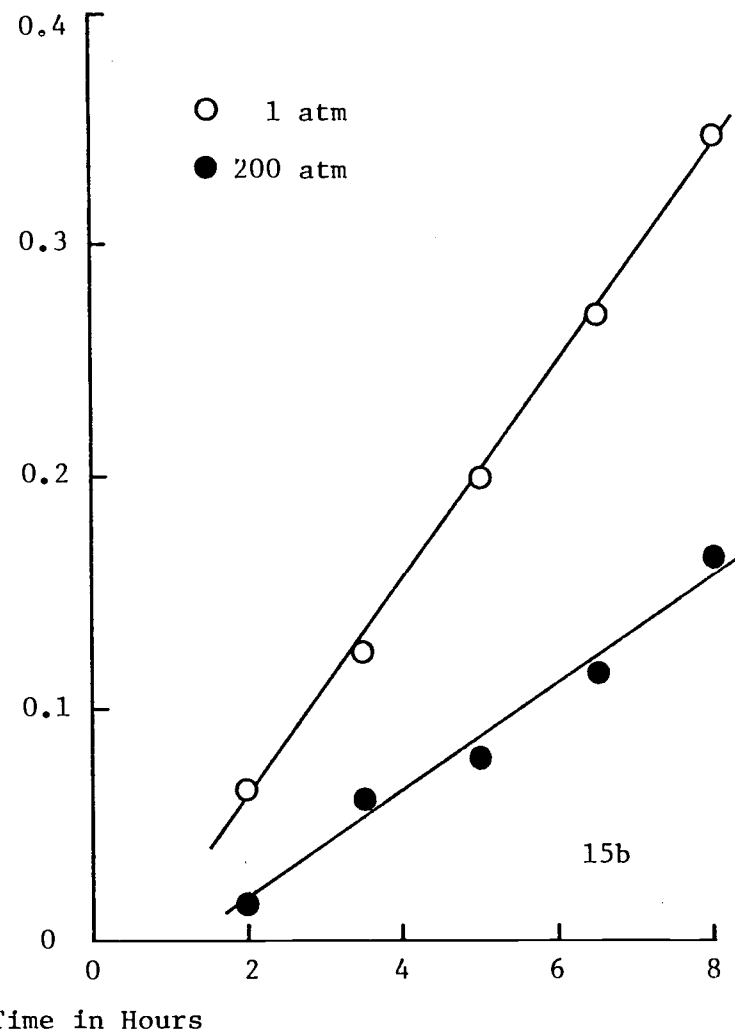
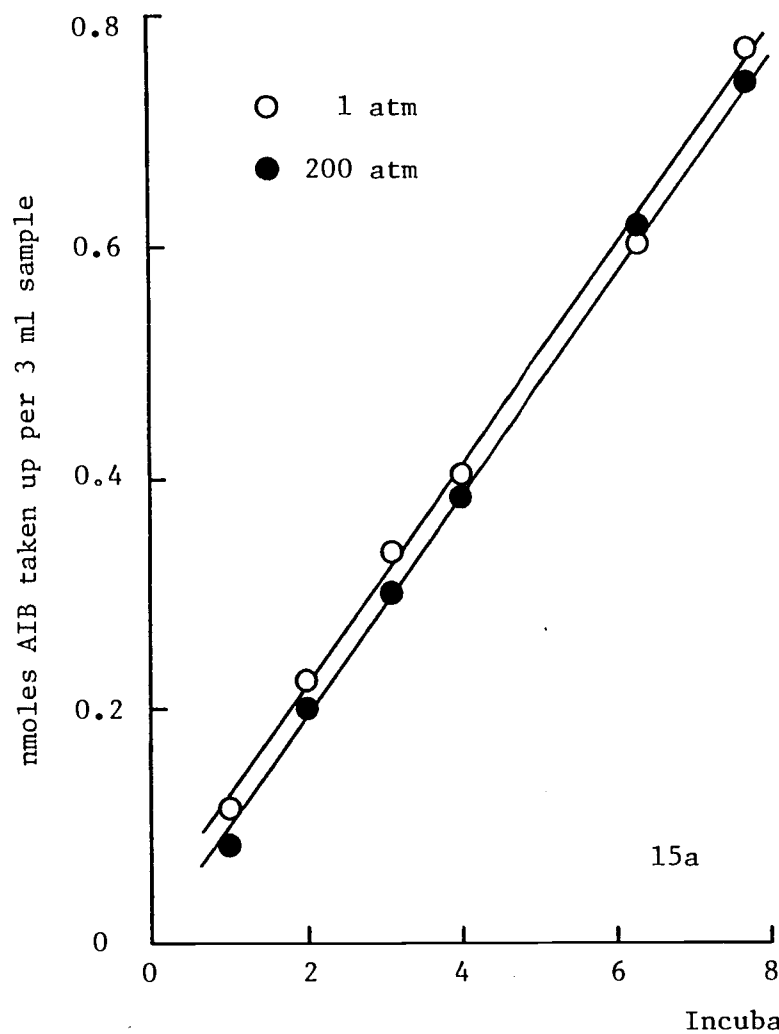


Fig. 15. Change in the barotolerance of AIB uptake upon initial starvation of Ant-300. Fig. 15a, cells starved 12 hr. Fig. 15b, cells starved 7 days. AIB concentration, 1.0 μ M. $1-2 \times 10^6$ cells/ml.

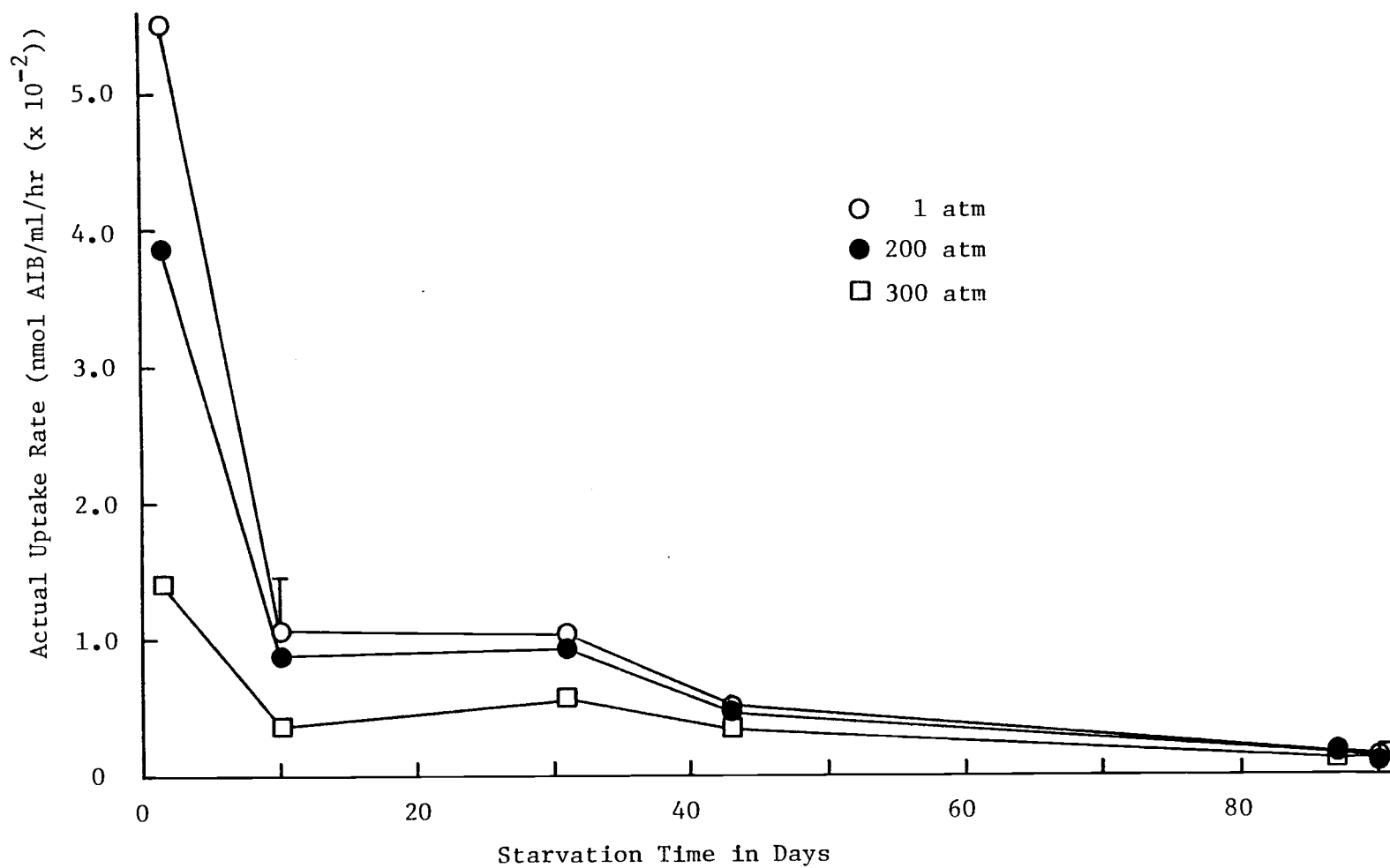


Fig. 16. Changes in actual AIB uptake rates of starving Ant-300 populations at 1, 200, and 300 atm. Bracket denotes range of observation.

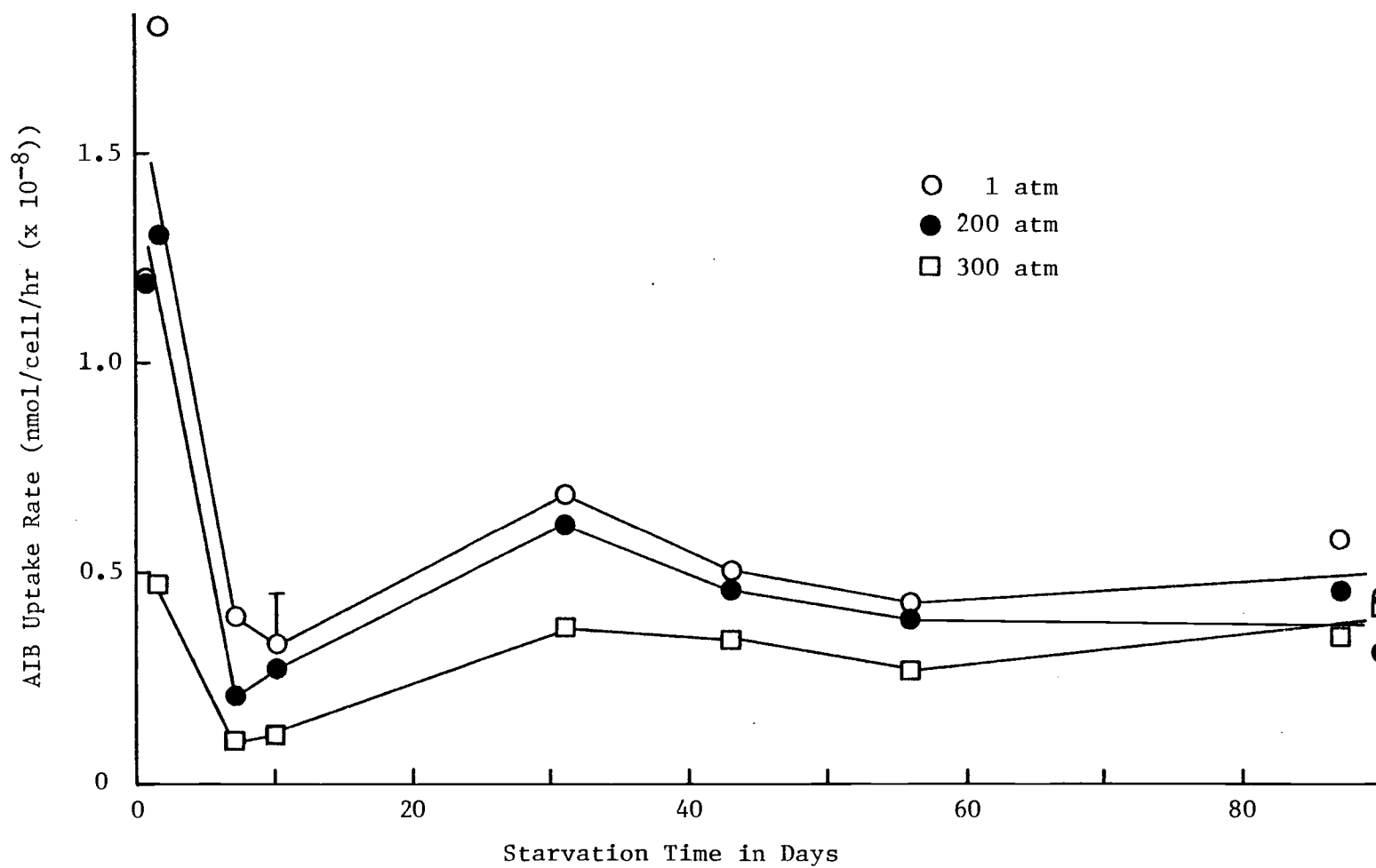


Fig. 17. Changes in rates of AIB uptake per viable cell at 1, 200, and 300 atm with starvation. Bracket denotes range of observation.

Starvation Survival of Ant-300 at Increased Pressures

Cells starved 11 days at 1 atm, then incubated at various pressures for up to 8 wk, survived as well at 1, 100, 200, and 300 atm (Fig. 18).

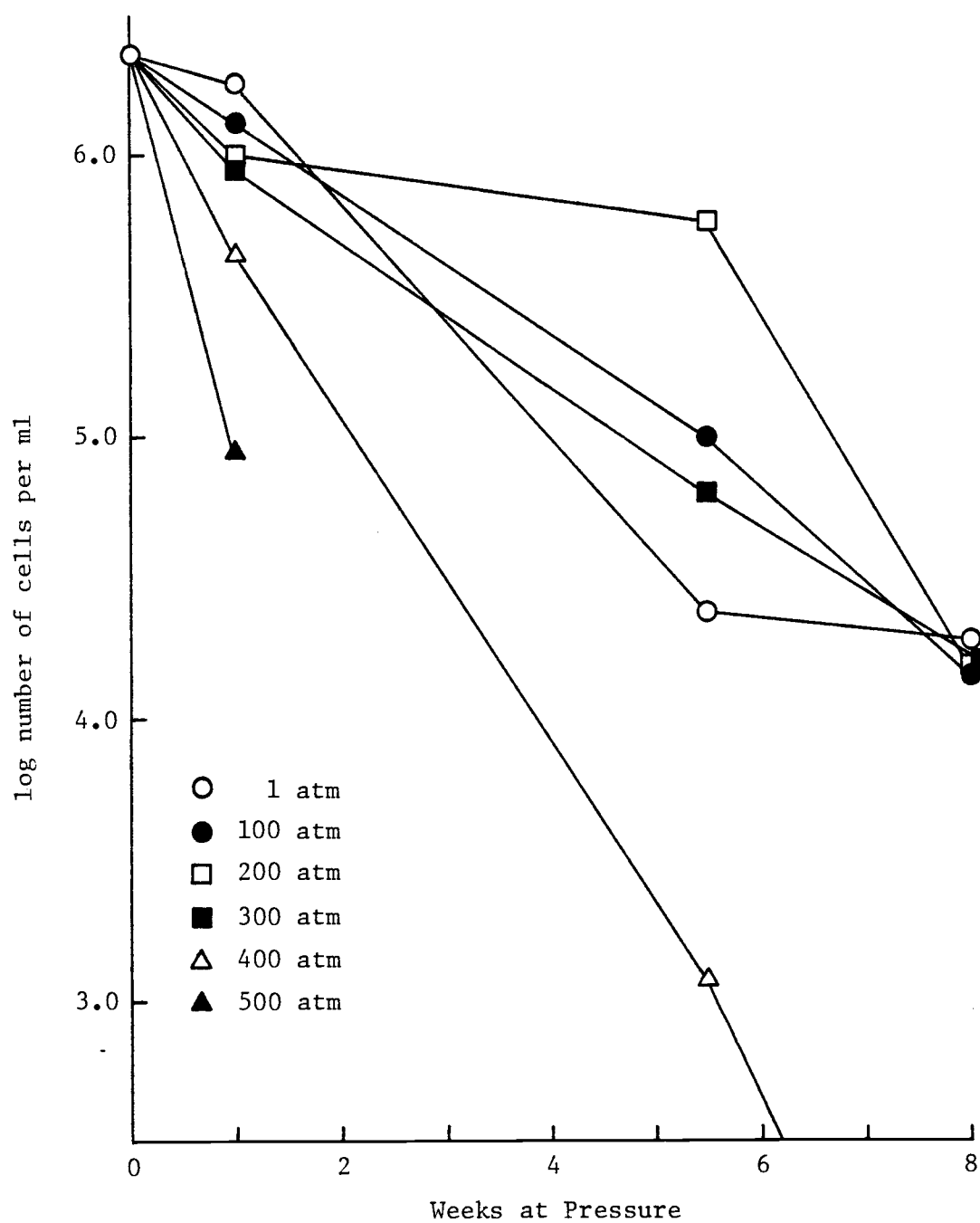


Fig. 18. Starvation survival of Ant-300 at moderate hydrostatic pressures. Cells were starved 11 days prior to pressurization.

DISCUSSION

Characterization of AIB Uptake by Starving
Ant-300 Cells at 1 Atm

No significant amount of AIB was respired or incorporated by Ant-300. Bacillus megaterium and a marine pseudomonad have also been reported to readily take up AIB but not metabolize it (Marquis and Gerhardt, 1964, and Drapeau and MacLeod, 1963). Horan, Midgley, and Dawes (1978) reported that about 96% of the AIB taken up by Staphylococcus epidermidis behaved as unchanged AIB when extracted and then examined by electrophoresis.

Ideally, this would permit testing of pressure effects on uptake without consideration of pressure effects on protein synthesis or respiration. However, in whole cells it is virtually impossible to isolate for observation any particular metabolic process or environmental effects on that process, due to the complex interdependence of whole cell systems. For example, though pressure effects on protein synthesis may not directly effect the intracellular concentration of AIB (since no AIB is incorporated into protein), it cannot be determined that pressure effects on protein synthesis or any other cellular process do not have some feedback control on transport, which could be altered by pressure.

Ant-300 cells were not capable of oxidizing AIB to CO_2 , indicating that no energy was gained by the cells from the analog. The first step leading to the oxidation of alanine or similar amino acids is a transamination reaction which entails removal of the $\alpha\text{-H}$ from the amino acid. The substitute $\alpha\text{-CH}_3$ group on AIB would be expected to block this reaction, preventing any metabolism or oxidation of the analog.

The fact that AIB uptake conforms to saturation kinetics strongly supports that it is mediated by a specific carrier as opposed to passive diffusion. Similar results were found for uptake of AIB by S. epidermidis (Horan, Midgley, and Dawes, 1978) and a marine pseudomonad (Fein and MacLeod, 1975).

The comparable V_{max} values for AIB and alanine uptake suggest that AIB was transported by a similar system as alanine. The lower K_m for alanine ($0.4 \mu M$) compared to the K_m for AIB ($0.8 \mu M$), would be expected for the natural amino acid substrate. Horan, Midgley, and Dawes (1978) observed similar results with S. epidermidis, recording K_m values of $0.12 mM$ and $0.38 mM$ for serine and AIB uptake, respectively. The lack of any discernable change in the V_{max} and K_m of AIB uptake by Ant-300 over a concentration range of 3.5×10^{-3} to $2.16 \times 10^{-1} \mu M$ indicates only one major transport system functions for AIB uptake over that range. Fein and MacLeod (1975) tested the kinetics of AIB uptake by a marine pseudomonad over a range of 2.5×10^{-5} to $8.0 \times 10^{-3} M$ and observed a shift in kinetics at $1.6 mM$, a concentration two orders of magnitude above the highest tested with Ant-300.

The concentration of AIB used for most experiments ($1 \mu M$), was very close to the average K_m determined ($0.9 \mu M$) for AIB uptake by two to eight day starved cells. This suggests that the substrate concentration used was within the normal operating range of the uptake system.

The AIB and alanine competition experiments showed that AIB and alanine share the same transport system(s) and that the system(s) possesses a higher affinity for alanine. This is consistent with the lower K_m observed for alanine uptake. The ability of alanine to almost

completely inhibit (98%) AIB uptake is evidence that AIB was taken up exclusively by transport system(s) which also transported alanine.

Glutamate, at a two-fold concentration, also inhibited AIB uptake (60%). Other researchers have noted inhibition of AIB uptake by various amino acids. Drapeau, Matula, and MacLeod (1966) noted that alanine, "cold" AIB, glycine, serine, cysteine, or threonine inhibited ^{14}C -AIB uptake by a marine pseudomonad by 97% or more when added at 100 times the ^{14}C -AIB concentration, while aspartic acid, phenylalanine, valine, proline, and leucine at 100 times the AIB concentration inhibited ^{14}C -AIB uptake 17 to 33%. Glycine, at 10 times the concentration of AIB, inhibited ^{14}C -AIB uptake by B. megaterium approximately 50% (Marquis and Gerhardt, 1964). Uptake of ^{14}C -glycine and ^{14}C -alanine by S. faecium was inhibited 23% and 17%, respectively, when "cold" AIB was added at 100 times the concentration of the substrate (Brock and Moo-Penn, 1962). Fein and MacLeod (1975) also showed that a mutant defective in AIB uptake (80% loss) had significantly reduced uptake capabilities for glycine, alanine, and serine, while the uptake of other amino acids was apparently unaffected. These results support the concepts that AIB shares a specific transport system(s) with a number of structurally related amino acids and that the affinity of the system(s) is usually higher for the natural amino acids.

The results of the kinetic competition experiments did not clearly fit competitive inhibition as expected. Pure competitive inhibition would cause an increase in K_m but no change in V_{max} , but the results of AIB uptake predominantly showed an increased K_m and also a decreased V_{max} when alanine was added as a competitor (Table 1). This type of

mixed inhibition is considered as basically noncompetitive by Westley (1969). Noncompetitive inhibition involves the competitor binding to the enzyme (or transport protein) at a site other than the active site and theoretically limits the maximum velocity of the reaction without affecting the affinity of the enzyme for its substrate. However, in reality often the bound competitor also lowers the affinity of the enzyme for the substrate, resulting in a mixed inhibition. For uptake, a mixed noncompetitive inhibition of alanine seems conceptually unlikely, though possible. The inhibition by alanine was probably basically competitive, with other unknown factors causing the decreased V_{max} . Fein and MacLeod (1975) reported that AIB competitively inhibited alanine uptake in a marine pseudomonad, while Horan, Midgley, and Dawes (1978) observed competitive inhibition of AIB uptake with serine using S. epidermidis.

The complexity of the competition studies was increased by the delayed stimulation of AIB or alanine uptake (as the substrate) by the corresponding competitor (Figs. 4 to 7). This observation is understandable for AIB uptake with alanine as the competitor, since alanine could be an energy source for starved cells. However, with ^{14}C -alanine as the substrate, the latent stimulation of uptake observed with "cold" AIB added as the competitor is not so clearly understood. The stimulated rate of ^{14}C -alanine incorporation (Fig. 7) indicates AIB was having some regulatory effect. Possibly AIB, being a non-metabolizable substrate, somehow affects a deregulation of protein synthesis and/or alanine uptake.

AIB uptake was also stimulated by glucose, probably again due to the energy available in glucose to the starved cells. This indicates that AIB uptake was an energy-requiring process. The ability of cells

at $1-2 \times 10^6$ cells/ml to take up 50% of the available substrate also indicates the uptake was not a passive process, but an active process against a concentration gradient. Horan, Midgley, and Dawes (1978) characterized the energetics of AIB uptake by S. epidermidis with a variety of experimental techniques and concluded that AIB uptake was an active process energized by a proton gradient across the cell membrane. They too observed a stimulation of AIB uptake when glucose was added. Proton conductors, lipid soluble ions, and inhibitors of the membrane-bound ATPase all inhibited AIB uptake (Horan, Midgley, and Dawes, 1978). A proton conductor was also reported to inhibit AIB uptake by a marine pseudomonad (Drapeau, Matula, and MacLeod, 1966). Overall the evidence suggests AIB was taken up by active transport.

The preliminary osmotic shock data indicated most of the AIB taken up was shock releasable and therefore probably bound extracellularly (Griffiths et al., 1974, and Schwarz and Colwell, 1975). It is likely that at least some of the AIB taken up by Ant-300 was transported intracellularly, considering the linear rates of AIB uptake over extended time periods, the saturation kinetics exhibited, the similar V_{max} and K_m values for AIB and alanine, and the apparent energy requirements for AIB uptake.

Since the cells apparently obtain no energy from AIB, uptake of this substrate would be an indicator of the endogenous energy available for uptake in starving cells. The limitation of using this type of non-metabolizable substrate is that it does not therefore completely reflect the natural response of an energy-limited starving cell when energy yielding substrates are taken up. Additionally, it is possible that the

unique nature of AIB could facilitate unnatural feedback controls on transport (Figs. 5, 6, and 7).

Overall, these data attest to the reliability of AIB uptake to reflect uptake of natural amino acids by Ant-300, but because of the reasons mentioned above, the emphasis of the ensuing results has been placed on the relative effects of starvation and pressure.

The oxygen utilization data showed that no significant amount of oxygen was consumed during the uptake incubation times used and hence oxygen limitation was of no concern in these experiments.

"Short Term" and "Long Term" Starvation

The goal of this research was to help elucidate the effects of pressure on uptake by cells under limited nutrient or starvation conditions. Study was primarily concentrated on the starvation period of 1.5 to 14 days due to the stability of viable counts and uptake ability during that time period. Further study of longer starvation times resulted in noticeable changes in the barotolerance characteristics beyond about 20 days of starvation (Fig. 14). This raises the question of whether the stable period from about 1.5 to 20 days of starvation was only a transition period of starving cells or was it indicative of limited nutrient or "partial" starvation conditions? In other words, is the shift to a "starvation metabolism" on all or nothing phenomenon?

The time period from 1.5 to 14 days will be described as short term starvation with the concept in mind that this may be reflective of limited nutrient (energy) conditions. Starvation beyond this time will be spoken of as long term starvation and be considered more representative of starvation with extended periods of essentially no available

nutrients. The use of short and long term in respect to starvation is primarily for clarity of discussion and is not meant to directly correlate to time periods of starvation in the natural environment, since exposure of microorganisms to starvation and increased pressure in nature could extend to hundreds of years or longer.

Pressure Effects on AIB Uptake by Short Term
Starved Ant-300 Cells

The observed inhibition of uptake in cells starved 1.5 to 14 days (Table 2) was consistent with the results of numerous other investigators. Inhibition of substrate uptake in this range of pressures using marine and terrestrial organisms and a wide variety of substrates has been reported. Since no significant number of cells died when subjected to pressures of 500 atm or less for up to 10 hr, the reduced uptake rates cannot be attributed to cell death. The results indicate that at 200 atm substrate uptake could be a limiting factor to the nutrient intake and hence the activity of short term starved Ant-300 cells. At 300 atm or higher pressures, the inhibition of uptake rates would probably drastically reduce the ability of the cells to accumulate nutrients. If 300 atm placed detrimental stress on Ant-300's metabolism, the inhibition of uptake would most likely further compound this stress due to the curtailed supply of external energy.

The large decrease in uptake rate between 200 and 300 atm suggested that at 300 atm the effect of pressure on transport has changed and possibly some critical alteration in the cells' metabolism may have occurred (Fig. 11). The upper limit of pressure at which Ant-300's uptake systems can function under partial starvation conditions apparently lies between

400 and 500 atm. The effects of 600 and 800 atm on AIB uptake, as would be expected, show these pressures to be above the functional level for continued uptake by Ant-300 cells (Fig. 10). The limitations of testing the effects of environmental conditions on microorganisms over short periods of time and then extrapolating the results to the natural environment are clearly shown in the results at 500 and 600 atm. The full effect of pressure was not observed until after 12 hr at pressure. These limitations are also recognized for the incubation times and conditions used in this study.

The calculated ΔV^* values were similar at 100 and 200 atm (Fig. 12). This suggests that the point or mechanism of pressure's effect was the same at 100 and 200 atm though more pronounced at 200 atm. The major shift in ΔV^* values between 200 and 300 atm again indicates that at 300 atm there was a change in the metabolic effect of pressure. This was probably due either to increased pressure causing a shift in the critical rate-limiting step in the process of uptake or to pressure inducing a significant change in the rate-limiting step affected (i.e., at a threshold pressure between 200 and 300 atm a conformational change in a transport protein occurred which altered substrate affinity). Theoretically, ΔV^* should be constant at all pressures. The fact that this was not observed is not surprising considering the basis for calculation of ΔV^* assumes an elementary process. Cellular uptake obviously is a rather complex system involving many steps, each with its own characteristic ΔV^* .

The observed 40% decrease in V_{max} and 60% increase in K_m at 200 atm as compared to 1 atm reflected the decreased potential rate of AIB

uptake and the decreased affinity of the system for the substrate.

Masuda and Albright (1978) reported essentially no change in the V_{max} of cycloleucine uptake by V. marinus MP-1 at 250 atm as compared to 1 atm, but the K_m increased about 71%. At 500 atm the V_{max} decreased 15% and the K_m increased about 140% relative to 1 atm. The increased K_m under pressure is of particular significance in light of the low concentration of nutrients in the ocean environment. Lee and Bada (1975) determined the concentrations of 11 different amino acids in Equatorial Pacific Ocean water at 2243 m (224 atm) and reported free serine and alanine were present at concentrations of approximately 15 and 6 nmol/liter, respectively, while aspartic acid, threonine, glutamic acid, proline, valine, isoleucine, leucine, tyrosine, and phenylalanine were each present at concentrations of 4 nmol/liter or less. Therefore, at comparable depths in the ocean, the extremely low concentrations of free amino acids and other nutrients would accentuate the detrimental effects of pressure on the ability of Ant-300 cells to scavenge energy-yielding substrates. For example, assuming alanine and AIB uptake kinetics for Ant-300 were altered the same at 200 atm, the estimated alanine uptake velocity at 200 atm would be about 2.7-fold less than at 1 atm, if the natural alanine concentration were 6 nmol/liter.

This would be consistent with the hypothesis of Laidler (1951), which predicts that if the apparent activation volume of substrate binding were positive, the pressure inhibition of binding and consequently of the entire process (i.e., uptake) would appear more pronounced at lower substrate concentrations. This is apparently borne out by calculations using the data of Wirsen and Jannasch (1975), which show that the

relative uptake of ^{14}C -glutamate at a concentration of 100 $\mu\text{g/ml}$ was less pressure sensitive than uptake of glutamate at 2 $\mu\text{g/ml}$. At 100 $\mu\text{g/ml}$, ^{14}C -glutamate relative uptake values were 1.0 at 1 atm, 0.47 at 50 atm, and 0.20 at 200 atm, while with ^{14}C -glutamate at 2 $\mu\text{g/ml}$ the respective values were 1.0, 0.34, and 0.15. These kinetic data in conjunction with the observed reduced uptake rates, further clarify how moderate pressures inhibit uptake and can reduce the potential metabolic activity of Ant-300 cells.

The implications of Laidler's theory on a cell in the ocean environment go beyond just uptake, since at increased pressure reduction of the internal substrate concentration could result in binding becoming a significant limiting factor to any cellular process requiring the substrate. The rate of such a cellular process would then be dependent on the pressure, the intracellular substrate concentration, and the ΔV^* and K_m of the substrate-binding step. If the ΔV^* values of binding are positive, the reduced intracellular substrate concentration could affect significant reductions in rates of such metabolic processes. It is even conceivable that the rate of an intracellular substrate-dependent process in whole cells could appear more pressure sensitive than substrate uptake, even though the reduced intracellular substrate concentration was primarily responsible for the increased pressure sensitivity of the process. The key to this is the variation in ΔV^* and K_m values between different metabolic processes.

An example of a substrate dependent process would be aminoacyl tRNA synthetase activity and the obvious consequences to protein synthesis. Hardon and Albright (1974) presented data indicating that

aminoacyl tRNA synthetase activity for phenylalanine in E. coli was inhibited at hydrostatic pressures of 100 to 300 atm. This inhibition was at least partially responsible for the observed reduced rates of protein synthesis at these moderate pressures. Decreased intracellular amino acid concentrations would most likely further limit the aminoacylation process at increased pressure, if the ΔV^* for binding were positive. An increase in the apparent pressure sensitivity of protein synthesis would result.

Schwarz and Colwell (1975) concluded uptake was not the critical limiting factor to microbial growth in their sediment samples under pressure, because a pool of amino acids believed to be bound intracellularly was the least pressure sensitive. Calculations using their data show relative rates of "intracellular" pool accumulation at in situ pressures ranged from 0.016 to 0.23, while relative rates of incorporation into protein ranged from 0.0093 to 0.031. The apparent rate of protein synthesis was more pressure sensitive than transport of substrate internally; however, the reduced levels of intracellular substrate cannot be ruled out as contributing to the decreased rates of protein synthesis.

These considerations emphasize (1) the potential effects of pressure inhibition of substrate uptake on cellular metabolism and (2) the complexity of pressure effects on the whole cell and the consequential difficulty of attempting to delineate a single metabolic process as the critical limiting factor to microbial growth under pressure.

Additionally, the ΔV^* calculations from AIB uptake by Ant-300 under pressure do fall in the range of values for other cellular processes of other organisms, indicating that the pressure sensitivity of uptake by

short term starved Ant-300 cells would probably be a contributing factor to the pressure sensitivity of the whole cell. Masuda and Albright (1978) also concluded that pressure effects on active uptake contributed to the limits placed on microbial growth by increased pressure.

Changes in Uptake Rates and Kinetics and Barotolerance of Uptake Upon Initial Starvation

The reduction in absolute rates of AIB uptake with starvation and the initial reduction in barotolerance of uptake were probably indicative of the curtailed energy supply in the starved cells (Figs. 14, 15, and 16). The immediate drop in uptake rates during the first ten days of starvation is consistent with the observations of Novitsky and Morita (1977). They demonstrated that the endogenous respiration of Ant-300 dropped over 80% during the first two days of starvation and by over 99% during the first seven days. Not only would cells most likely decrease their rate of energy usage upon starvation, but their total reserve of energy would be reduced. This would be caused by the immediate energy requirements for adjustments to starvation, such as cell fragmentation, as well as the longer term continuous requirements for cellular maintenance. Acid washing of Ant-300 cells from a ^{14}C -labelled culture starved 21 days or longer, resulted in no release of ^{14}C -material from the cells (Novitsky, 1977, Ph.D. thesis, Oregon State University). Novitsky concluded that the cells had completely utilized their "pool material and low molecular weight metabolites" by 21 days.

Other researchers have reported a drop in the energy charge or ATP concentration in cells at the onset of starvation. Chapman, Fall, and Atkinson (1971) found the energy charge of E. coli dropped slightly from

0.8 to between 0.6 to 0.7 within one hour after the glucose was completely used in a glucose-limited culture. Dawes and Large (1970) observed the intracellular ATP content of Zymomonas anaerobia decreased more than three-fold when glucose became limiting and then exhausted in a glucose-limited culture.

Therefore, less energy would probably be immediately available for metabolic processes such as transport. Horan, Midgley, and Dawes (1978) observed S. epidermidis cells began to lose AIB uptake ability after one hour of starvation and lost all uptake ability by four hours. Peptococcus prevotii showed a drop in its energy charge from about 0.65 to 0.30 by 20 hr of starvation and a concomitant loss of viability (Reece, Toth, and Dawes, 1976). After 15 hr of starvation the organism had lost its ability to utilize xanthine to produce ATP. From an unpublished data source the authors also reported that during starvation the organism lost its ability to transport serine. Since it was considered very probable that the membrane was energized for transport directly by ATP via an ATPase, the authors hypothesized that the depletion of the intracellular ATP supply would reduce the cells' ability to energize transport. As a result, organisms starved beyond a certain time period would be unable to recover even when nutrients were once again provided.

Assuming the supply of energy to uptake through ATP hydrolysis and substrate respiration was significantly reduced upon starvation, it would seem logical that as the magnitude of the uptake driving force (energy supply available to energize the membrane for uptake) decreased, the inhibition of uptake by pressure would be more pronounced.

Matsumura and Marquis (1977) observed that in S. faecalis the hydrolysis of ATP by the membrane ATPase, which can energize uptake in whole

cells, was stimulated by increased pressure (408 atm) at ATP concentrations above 0.5 mM. However, at ATP concentrations below 0.5 mM, the reaction was inhibited by pressure. The apparent K_m was increased approximately three-fold at 408 atm. This type of response could easily facilitate an increased inhibition of uptake upon starvation, since lower intracellular ATP concentrations would result in an increased pressure inhibition of ATP hydrolysis, which energizes uptake.

The relatively high barotolerance of uptake by cells starved only 6 to 12 hr is consistent with the data of Baross, Hanus, and Morita (1974), since Ant-300 cells growing in Lib-X would have no lack of energy. However, Novitsky and Morita (1978) found Ant-300 cells grown in a glucose medium then starved one week were more barotolerant at pressures up to 500 atm than growing cells or cells starved only a few hours. These differences are most likely due to the heterogeneous pressure sensitivities of the different pathways of substrate uptake and utilization. Marquis, Brown, and Fenn (1971) and Matsumura and Marquis (1977) have also reported that the barotolerance of S. faecalis growth was dependent on the substrate used.

Other factors, such as degradation of certain transport systems or related components, could also have played a part in the observed loss of uptake ability and barotolerance during the first few days of starvation. Novitsky (1977, Ph.D. thesis, Oregon State University), using Ant-300 cells grown in ^{14}C -(UL)-glucose, reported fluctuations in the ^{14}C -cellular protein during the first three weeks of starvation, resulting in a net loss of approximately 40% of the protein. This suggests a combination of degradation and synthesis was occurring with a general loss of cellular protein.

The only kinetic experiment conducted with cells starved less than 2 days showed that from 3.5 hr to 2 days the V_{max} and K_m values decreased. The V_{max} decrease could correlate with a reduction in the cellular energy supply upon starvation, but the decrease in both values indicates a change in the major uptake system. This could have been accomplished by the degradation of a high-velocity transport system and synthesis of a new system, which was more suited to low-velocity, high-affinity uptake. Or the "new" system may have already been present in the growing cells, but was simply not seen "kinetically" until the high-velocity system was removed. No changes in the kinetics of uptake were noted from two to eight days of starvation.

Recovery of Uptake Abilities After One Week of Starvation

The uptake ability (Figs. 2 and 16) and viable counts (Fig. 3) of a starving culture from 10 to 30 days, at first appear inconsistent. Uptake rates per viable cell (Fig. 17) increased by 30 days of starvation after a low around 7 to 10 days and relative uptake rates at 200 and 300 atm increased beyond 20 days.

Two significant changes in the starving cell suspension are pertinent to these observed results. First, by 30 days of starvation a significant number of "dead" (non-replicating) cells are present in the starvation suspension. Novitsky and Morita (1977) noted no significant decrease in the total number of cells observed by direct counts at least through six weeks, even though viable counts were decreasing. If the total number of cells did not decrease in this present study, there were approximately an equal number of dead cells and viable cells in the

starvation suspension after 30 days of starvation. Ant-300 cells killed at 600 atm (48 hr) then incubated with ^{14}C -glutamate for 30 min, took up about 1% of the total substrate taken up by viable cell controls (Personal communication, M. Glick, Dept. of Oceanography, Oregon State University, Corvallis, Oregon). This indicates that dead Ant-300 cells would have been incapable of contributing significantly to the uptake observed in this present study. However, due to different experimental conditions, uptake by dead cells can not be completely ruled out.

Secondly, Ant-300 cells starved 30 days are much smaller than non-starved cells. During the first week of starvation a rapid reduction in Ant-300 cell size occurred, followed by continued decrease in size for three weeks (Novitsky and Morita, 1976). Concomitant with the decrease in size, the cells changed from large non-starved rods ($1\text{ }\mu\text{m} \times 4\text{ }\mu\text{m}$) to small rods at one week of starvation then to small cocci by four weeks ($0.4\text{ }\mu\text{m}$ diameter). The changes in cell size and shape between non-starved cells and cells starved four to six weeks would result in a decreased surface area per cell, but an increased surface to cell volume ratio. If the number of uptake systems remained constant or was limited per unit membrane surface area, individual cells would have lost total uptake capability, but perhaps, as previously suggested by Novitsky (1977, Ph.D. thesis, Oregon State University), uptake capability per unit volume was increased.

As a result of the observations described above, three possible explanations of the AIB uptake data from 10 to 30 days are proposed. First, the cells that are apparently dying after 14 days of starvation, could actually still be functioning metabolically and be capable of

uptake, but simply be incapable of dividing. These "dead" cells could be beyond recovery for replication, or the procedure used to determine the viable counts (i.e., the media) might not have been the best to recover those stressed organisms. Secondly, it is also possible that the AIB was bound to cells which were even metabolically dead (by definition - no active energy-requiring processes). Either of these occurrences or both would have resulted in an apparent increase in the AIB uptake ability per viable cell.

If dead cells were capable of taking up or adsorbing a significant amount of AIB, their contribution to the total observed uptake would become continually larger as more cells died. It would seem likely that any kind of simple substrate adsorption, specific or non-specific, by dead cells would be much less pressure sensitive than an active biological system.

The last possibility, is that the remaining viable cells made some physiological adjustments from 10 to 30 days which increased their uptake capabilities and barotolerance. The low point of AIB uptake rate per viable cell at 7 to 10 days may be due to the combination of the (1) the reduced energy supply, and (2) the great loss of surface area and associated transport systems per viable cell, which occurred during the first week of starvation. Beyond one week the change in shape from a rod to a cocci may have facilitated the additional decrease in size (determined by differential filtration) by three weeks without much more loss of surface area. However, even if no more surface area was lost from one to three weeks, additional uptake systems or other pertinent cellular adjustments would have had to be made by the starving cells in

order to actually increase their uptake capabilities per cell and facilitate more barotolerant transport.

As previously mentioned, Novitsky (1977, Ph.D. thesis, Oregon State University) reported a fluctuation in ^{14}C -cellular protein during the first three weeks of starvation; and it is, therefore, feasible that additional transport systems were synthesized at the expense of other cellular components. Calculations using Novitsky's data show cells starved 34 days had a respiration rate of ^{14}C -glutamate per unit cell surface area that ranged from 1.5 to 16 times (depending on whether dead cells were capable of respiring or not) the rate for non-starved cells. This indicates a possible increased potential in starved cells for energy coupling to transport. If the cells could supply the energy to utilize an increased coupling potential, this type of adjustment might itself help alleviate the inhibitory effects of pressure or it could be indicative of other pertinent changes in the energy coupling system.

Another possible cellular adjustment would be a shift toward a substrate binding system that initially required less energy, for example, a hypothetical non-energy requiring binding protein. This would help if the energy coupling to uptake were the most pressure sensitive step. However, at low substrate concentrations binding can be the rate determining step in a pressure inhibited process.

Synthesis of additional uptake system components or cellular adjustments to facilitate increased uptake capabilities, at the cost of other cellular systems and energy would be understandable in light of the dilute nutrient concentrations in the ocean and the primary requirement of any cell for energy to sustain viability. Synthesis of different

transport systems (i.e., carriers) which were specifically more baro-tolerant would be very unlikely.

Beyond 30 to 40 days of starvation, though the AIB uptake rates for the starvation culture continue to decrease (Fig. 16), the concurrent decrease in viable counts (Fig. 3) results in a fairly constant uptake rate per viable cell at one atm up to 90 days (Fig. 17). This is indirect evidence that the observed uptake was primarily attributable to viable cells.

Pressure Effects on Ant-300 Starvation Survival

Survival at 300 atm compared to the cell death rates at 400 and 500 atm (Fig. 18) indicated that the critical pressure above which the death of starving Ant-300 cells was accelerated, was between 300 and 400 atm. Due to the nature of starvation, pressure inhibition of uptake can be ruled out as the cause of the observed rapid death rates at 400 and 500 atm. This critical pressure was also observed by Baross, Hanus, and Morita (1974) for growth of Ant-300 in Lib-X at 5°C. The starvation survival of Ant-300 cells grown in a glucose medium was unaffected or enhanced at 250 atm, but at 300 atm cell death rates were increased compared to the 1 atm control (Novitsky and Morita, 1978b). In both this study and that of Novitsky and Morita (1978b) cells were starved about one week prior to pressurization, therefore indicating that not only was the barotolerance of growth dependent on the carbon and energy source, but the barotolerance of starving cells was also dependent on the nutrient source during pre-starvation growth. This could be due to differences in the catabolic systems, the cellular energy coupling systems, and the total cellular energy reserve present upon starvation.

The survival data for 1 to 300 atm indicate that cells starved at 100, 200, and 300 atm survived at least as well as cells at 1 atm for up to eight weeks.

The trend in survival at 300 atm did not at first appear consistent with the major reduction in uptake rate observed at 300 atm in cells starved 1.5 to 14 days. The large difference between uptake rates at 200 and 300 atm suggested that at 300 atm major detrimental metabolic alterations were occurring which over time would accelerate cell death. However, the increased barotolerance of AIB uptake beyond 20 days of starvation at 300 atm (Fig. 14), does agree with the general barotolerance of the organism. It is important to recognize the limitations in comparing the uptake data and survival data, since all the uptake data was acquired using cells starved at one atmosphere then pressurized for the ten hour uptake period, while the survival data was acquired by incubating the cells at pressure during starvation.

The hypotheses of Baross, Hanus, and Morita (1975) and Novitsky and Morita (1978b), linking metabolic inactivity and barotolerance, correlate with the observed survival of starving Ant-300 under pressure. The inactivity of the cells in this situation was induced by starvation. As previously mentioned, for Ant-300 cells growing under optimal conditions, moderate pressures had no effect or an inhibitory effect depending on the medium, however survival of starving cells was either unaffected or enhanced by pressures up to 250 to 300 atm for six to eight weeks.

The metabolic state of an organism which would favor its survival or propagation could be dependent primarily on availability of energy.

At one extreme a cell with sufficient available nutrients would benefit most from a high level of metabolic activity, to grow or maintain its own systems, while a cell with no available nutrients would benefit most from the lowest possible level of metabolic activity necessary to maintain its own viability. This has been generally shown with simple temperature variations as well as the pressure effects at low temperatures previously mentioned. Postgate and Hunter (1962) observed that Aerobacter aerogenes grown at 40°C, then starved at various temperatures from 10°C to 50°C, survived best at 20°C. Streptococcus lactis grown at 30°C and starved at temperatures from 3°C to 45°C, exhibited the best survival at the lowest temperature (Thomas and Batt, 1968).

In accordance with the ideal gas law ($PV = nRT$) increasing pressure may have a similar effect as decreasing temperature, and moderate pressure may as a result act to further "freeze" a cell's systems in a less active state, which would require less maintenance energy. So while certain metabolic activities might require more energy under pressure (Matsumura and Marquis, 1977), when minimal metabolic activity would be advantageous, moderate hydrostatic pressure could enhance survival.

CONCLUSION

If the short-term starved cells are indicative of cells under limited nutrient conditions, pressure's observed inhibition of uptake rates and reduction of the uptake system's affinity for its substrate would very likely be a limiting factor to the cells' activity within the range of moderate pressures at which the cells survive. The evidence that a curtailed energy supply reduces the barotolerance of uptake and other metabolic processes, reinforces the critical nature of the pressure sensitivity of uptake to a pressure stressed organism.

Under extended starvation Ant-300 cells are apparently capable of making adjustments in their uptake systems that would be advantageous to starvation survival at one atmosphere and at moderate pressures. Though the reduced uptake rates (5 to 40%) observed at 200 and 300 atm and 90 days can not be ruled out as a limiting factor to the survival potential of Ant-300 under pressure, the recovered uptake capabilities of a starved cell may in fact be sufficient to meet the minimal cellular energy requirements for maintaining viability under starvation conditions and increased pressure. Or perhaps the primary importance of maintaining an efficient uptake system under long-term starvation in the natural environment would be to provide the initial input of energy necessary for a cell to resume growth when nutrients once again became available. The significance of starvation associated barotolerance was discussed by Novitsky and Morita (1978b). They considered it "reasonable to assume that cells in the natural environment would encounter starvation or low nutrient conditions before a large increase in pressure as they sediment or are down-welled from a productive surface area."

These observations and the survival data indicate that Ant-300 may be well adapted to survival at increased pressure under extended starvation, but may not be capable of growth under pressure and limited nutrient conditions.

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APPENDICES

Table 3. Ability of starved Ant-300 to respire and incorporate AIB, as determined using H_2SO_4 and TCA treatments to terminate uptake.

Incubation time (hr)	Non-acid treatment (DPM)	H_2SO_4 treatment	
		Respiration (DPM)	Incorporation (DPM)
0.25	9000	13	0
22.5	15000 ^a	120	0-200
<hr style="border-top: 1px dashed black;"/>			
Incubation time (hr)	TCA treatment - Incorporation		
	AIB (DPM)	Alanine (DPM)	
0.50	25	10000 ^b	
41.5	150	--	

Cells starved 3 to 14 days. All values corrected for controls.

^aThis non-acid sample was incubated only 4 hr, but used the same cells as the 22.5 hr H_2SO_4 samples.

^bThe alanine sample was corrected for the difference in specific activity between alanine and AIB.

Table 4. Vmax and Km values for AIB and alanine uptake by Ant-300 starved various lengths of time.

Substrate	Incubation time	Starvation time	V_{\max} ($\times 10^{-8}$ nmol/cell/hr)	Km (μ M)	r
ala	5 min	4 days	0.8 (0.7-2) ^a	0.4	0.998
AIB	10 min	3.5 hr	7	5.0	0.993
AIB	10 min	2 days	1.0	0.9	0.995
AIB	10 min	3 days	0.8	0.8	0.986
AIB	30 min	2 days	0.41	0.68	0.996
AIB	30 min	2 days	0.6	0.8	0.991
AIB	30 min	2 days	0.5 - 0.8	0.9 - 1.7	0.98
AIB	30 min	3 days	0.30	1.4	0.997
AIB	30 min	4 days	0.30	1.4	0.993
AIB	4 hr	3 days	0.11	13	0.995
AIB	6 hr	6 days	0.4 - 0.8	1.5 - 4	0.98
AIB	6 hr	7 days	0.2 - 0.5	0.6 - 2	0.93
AIB	6 hr	8 days	0.70	0.81	0.998
AIB	6 hr	8 days	0.65	0.51	0.993

All 5, 10, and 30 min and 4 hr experiments were run with cells starved at an original density of $1-2 \times 10^7$ cells/ml. The 6 hr uptakes used cells starved at $1-2 \times 10^6$ cells/ml.

^a This range is due to uncertainty in the viable counts. The value not in parentheses is the most likely within the range.

Table 5. Dissolved oxygen utilization concomitant with AIB uptake by starved Ant-300 cells.

Hydrostatic pressure (atm)	Incubation time (hr)	Starvation time (days)	Sodium thiosulfate titrated per 50 ml sample ^a (ml)	
			BOD bottles	Syringes
1	0	2	4.82	-
1	11	2	4.72	5.99
1	23	2	4.80	-
400	23	2	-	4.8 - 6.0
1	0	12	5.16	-
1	24	12	5.05	-
400	24	12	-	5.82

^a Each value is an average of duplicate or triplicate determinations.