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HYDROSTATIC PRESSURE EFFECTS UPON CELLULAR LEAKAGE AND ACTIVE TRANSPORT BY VIBRIO FISCHERI.

by

Kiyoshi .V. Masuda

B. Sc., The University of British Columbia, 1972

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

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ABSTRACT

The influence of hydrostatic pressure on active transport and efflux of metabolites was studied in a marine bacterium in order to explain observed effects of hydrostatic pressure on its survival and growth. Hydrostatic pressures exceeding ca. 400 atm cause the leakage of the macromolecules DNA, RNA, protein (including malate dehydrogenase) and amino acids into the menstruum from whole cells of Vibrio fischeri. Moderate pressures also inhibit the uptake rate of 1-aminocyclopentane-1-carboxylic acid (cycloleucine, analogue of L-valine and L-leucine) and increase the rate of its efflux. Increased values of K_m from Lineweaver-Burk plots of anaerobic cycloleucine uptake indicate a decrease in affinity of the amino acid transport system for substrate at increased pressures. Losses of intracellular metabolites and a decreased ability to accumulate substrate from the menstruum may contribute to inhibition of growth and survival of organisms in the ocean depths.

ACKNOWLEDGEMENT

I express my sincere thanks to Dr. Lawrence J. Albright for permitting me the opportunity to pursue graduate studies by sponsoring this research. I gratefully acknowledge the technical assistance of Dr. C. E. Milliken, Ms. T. M. Tuominen, and Mr. A. S. Dietz, and I thank the faculty and staff of the Department of Biological Sciences for cooperation and assistance during the course of this work.

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INTRODUCTION

Of the variables which govern oceanic life, hydrostatic pressure may be one of the most significant. This is particularly true in the ocean depths below the thermocline. In this environment, temperature and salinity are relatively constant around 2-3 C and 35 °/oo respectively, while pressure increases by approximately 1 atm with each 10 ~~m of de~~pth (56). Thus, the range in pressures in the oceans varies from 1 atm at the surface to approximately 1100 atm in the deepest known portion, the Marianas trench (61). Pressure increases coupled with decreasing penetration of light, a low temperature and low nutrient availability with depth may limit the number of microbes and their activities in the deeper regions of the oceans (81).

Until the late 19th century when bacterial cultures were first obtained from dredged samples, the deep waters of the oceans were considered sterile (41,93). Since then, various investigators have shown that many microbial species populate the ocean depths (12,23,41,45,61). However knowledge of their ecology and activities at these depths remains sparse.

Hydrostatic pressure affects reactions in which molecular volume changes are involved, favouring reactions or states in which the molecular volume decreases, and inhibiting those which involve or result in net increases (41). A reaction rate may be increased when an increase in the number of primary bonds results or when the activated complex is more ionized than the reactants (41,87).

The molecular volumes involved in the system depend upon the number and nature of interactions amoung the molecular and ionic constituents and water. Highly ionized hydrophilic groups occupy a minimal volume when the extent of ionic and hydrogen bonding with water is increased (41). Exposed hydrophobic groups cause an increase in volume due to localized ordering of the surrounding water molecules. When the hydrophobic groups of macromolecules are not exposed to water ('hydrophobic bonding'), the molecular volume is minimized due to fewer disruptions of the solution structure and an increased number of van der Waals' interactions.

Pressure may cause denaturation of macromolecules and enzymes due to conformational changes in proteins which affect the active site or result in dissociation of multimeric enzymes (69) depending on the degree of ionization and extent of hydrogen and hydrophobic bonding in the active and inactive states.

The mechanism of denaturation also may differ depending upon the range of pressure to which the system is exposed. Low and moderate pressure effects are often reversible when the system is returned to atmospheric pressure, while irreversible effects are exhibited in the high pressure range (41). Knowledge of how a process reacts to low (1-100 atm), moderate (100-300 atm), and high (exceeding 500 atm) pressures (32) may lead to an understanding of the reaction mechanism and how deep sea organisms adapt to their environment.

Moderate hydrostatic pressures (59) have been shown to slow or stop various catabolic and anabolic processes (3,6,33,71,72,79,80,87). Activities of enzymes from both terrestrial and marine species during and subsequent to exposure to hydrostatic pressures have been assayed. Both *in vivo* and *in vitro* comparisons of enzymes from bacterial strains isolated from terrestrial, marine, and deep sea sources, to determine whether there are functional or structural differences between them, have been reported. Recently, methods to make such comparisons *in situ* and in long term experiments have been reported (12,23,54). These types of experiments can clarify the role of high hydrostatic pressure as a selective agent. The presence of barosensitive enzymes in deep marine strains while their terrestrial counterparts show a remarkable degree of barotolerance continues to present a difficult problem. Extracellular catabolic enzymes are

important in the degradation of the organic detritus which forms part of the sediment and which might be attacked by the bacteria residing there. Chitinase, agarase, and amylase (44,98) in this group have been studied. Cytoplasmic and membrane-associated enzymes have also been studied (30,43,54,64,69,90).

Macromolecular syntheses, essential for cell growth and division, appear to be slowed or blocked by hydrostatic pressure (2,5,70). Protein synthesis appears to be the most sensitive (8,72,82,83) of these macromolecular syntheses. Pressure effects on the ribosome (32,35,36,73,75,85) and more specifically on the 30S particle of some species (72,74,75,85) appear to limit protein synthesis.

The action of pressure upon another important cellular function, active uptake, is not completely understood (32,47,68,84). Although Pope and Berger (11,72) have shown that increased pressures alter active uptake of metabolites by several microorganisms the question remains as to what is the limiting factor for activity and growth of marine bacterial cells under pressure. Since metabolism of these cells ultimately depends upon an exogenous source of metabolites, significant inhibition of active transport could account for some of the effects of pressure upon inhibition of cell activity and growth. If, in addition to active uptake

inhibition, metabolites present in intracellular pools are lost to the menstruum at moderate and high pressures, then lack of substrate for anabolic and catabolic processes, already occurring at lower rates, will further decrease the metabolic activity of the cells and subsequently lower growth and division rates. Accordingly, in this study uptake and efflux of various metabolites (DNA, RNA, protein, and amino acids) and cycloleucine, the non-metabolizable amino acid analogue of L-leucine and L-valine, by whole cells of V. fischeri were studied over the pressure range of 1 - 1000 atm.

MATERIALS AND METHODS

Cultures and Media

Vibrio fischeri (Beijerinck) Lehmann and Neumann ATCC 15381 (16,34) formerly referred to as Vibrio marinus (Russell) Ford strain MP-1 (15,21,22,61,64,65), a facultative marine (50) bacterium, was used throughout this investigation.

Stock cultures were grown (15 C) and maintained (5 C) on slants of 2216E agar (Difco, Detroit, Mich.) (49,94) in 15 x 75 mm screw-capped culture tubes.

Cells were grown aerobically on SDB medium (31) and anaerobically on the medium described by Albright and Morita (5). Transport studies were conducted using the medium of Drapeau and MacLeod (24). Leakage studies and centrifuge washes were performed in filtered Rila artificial seawater (F-RSW), which consisted of 27 °/oo Rila synthetic seawater salts (Rila Products, Teaneck, N.J.) dissolved in distilled water, pH adjusted to 7.4, and filtered through 0.45 µm HA membrane filters (Millipore Corporation, Montreal, Que.). All media and equipment were equilibrated to the working temperature (0, 5, 10, or 15 C) (14,40).

Cell Leakage Studies

Cells were grown to an absorbance of 0.535 @ 600 nm using 1 litre of SDB medium in a 2.8 litre Fernbach flask using an inoculum of 10 % from an overnight culture. The cultures were grown at 15 C at a shake rate of 120 strokes/minute and stroke length of 6.5 cm in a reciprocating incubator (Psychrotherm controlled environment chambre, New Brunswick Scientific, New Brunswick, N.J.). Harvesting was by centrifugation for 15 min at 0 C and 6000 x g using a GSA rotor in an RC2-B (Ivan Sorvall, Norwalk, Conn.) refrigerated centrifuge. The supernatant fluids were decanted, pellets combined, resuspended in and washed twice in F-RSW. After one additional wash the final resuspension was made in F-RSW to an absorbance of 0.500 @ 600 nm. This suspension was placed in an ice bath until used. Seven - 30 x 200 mm glass culture tubes were filled with the resuspended cells (approximately 100 ml) and stoppered (96). One of each of the tubes was held at 1, 50, 250, 500, 750 or 1000 atm for 1 h at 15 C using pressure cylinders similar to those described by ZoBell and Oppenheimer (60,99). The seventh tube was used as a 1 atm, t = 0 control. The tubes were depressurized and unstoppered following the 1 h incubation. For viability determinations, 1 ml aliquots were removed, and diluted samples plated onto 2216E agar (14,53,89). Colony forming units (cfu) were determined following 7 days incubation

at 15 C. The remainder of the cells were harvested as previously described. The supernatant fluids (51) were aspirated into screw-capped glass culture tubes and stored frozen at - 10 C until assayed.

In a second leakage study, 10 litres of SDB medium at 15 C were inoculated with 500 ml of overnight culture in a Microferm fermenter (New Brunswick Scientific, New Brunswick, N.J.), aerated at a 5000 ml/min flow rate, agitated at 250 rpm and held at 15 C. Antifoam A (Dow Corning Corporation, Midland, Mich.) was used to prevent foaming during growth (32). Cells were harvested at 4 C by centrifugation at a flow rate of 150-200 ml/min and 27000 x g utilizing the Szent-Gyorgi-Blum KSB-R continuous flow system (Ivan Sorvall, Norwalk, Conn.) with an SS-34 rotor in the RC2-B centrifuge. The pellets were combined, resuspended in F-RSW and washed 3 times as described previously. These washed cells were resuspended in F-RSW to an absorbance of 0.140 @ 600 nm following a 1:100 dilution in F-RSW. The cell suspension was then treated as previously described.

Supernatant Fluids Assay Methods

Supernatant fluids of the first study were assayed for protein by the Lowry method (48) utilizing the Folin phenol

reagent (27). RNA was determined by the orcinol pentose nucleic acid technique (77,78).

The supernatant fluids of the second study were assayed for DNA using the diphenylamine reaction (17), malic dehydrogenase (MDH) by the triphenyltetrazolium chloride (TPTZ) technique of Marsh (55) described by Morita and Haight (64), and total amino acids by the ninhydrin method (58,86) utilizing ninhydrin reagent prepared as per the Beckman AAAA manual (10).

Radioisotope Studies

Preparation of Cell Suspensions

Cells were grown at 15 C from an inoculum of 1 % to an absorbance of 0.410 @ 550 nm in 300 ml of SDB medium contained within a 500 ml Erlenmeyer flask using a reciprocating incubator. These cells were subsequently harvested and washed twice as previously described. The pellet from the second wash was resuspended in 10 ml F-RSW and placed in an ice-water bath. The cells were resuspended in Drapeau and MacLeod (24) transport medium at 15 C to an absorbance of 0.02 @ 550 nm (400 µg dry weight/ml).

Chase Experiment

Of the suspension prepared as described immediately above, 75 ml was transferred to a 200 ml Erlenmeyer flask; 3 ml of ^{14}C-cycloleucine (1,20) (1-aminocyclopentane-1-(carboxylic acid), Amersham/Searle, Chicago, Ill., diluted to specific activity 1 mCi/mmol) was added (final concentration 10^{-5} M) and mixed. Immediately, 25 ml portions of this suspension were added to each of three 50 ml Erlenmeyer flasks, designated ^{14}C, ^{12}C (to which non-radioactive cycloleucine (final concentration 0.01 M) was added at time = 4 h) and AK (acid-killed control, containing 0.35 ml conc. H_2SO_4 (37-39,68,91)). The flasks were incubated at 15 C with shaking. At appropriate time intervals (see fig. 4B) 1 - ml aliquots were removed from the flasks for filtration.

Kinetic Studies

Kinetic studies were performed in a similar manner (see figs. 5 - 9 for details). (Data for table II were obtained by incubation for 20 minutes at substrate concentrations of 3.57, 5.35, 8.83, and 17.86 μM.) Aliquots of cell suspension were added to varying quantities of ^{14}C-cycloleucine and mixed. The suspensions were then divided into two portions, ^{14}C and AK (as

defined above). Five ml volumes were taken up into 5 ml disposable plastic syringes (Becton-Dickinson, Baltimore, Md.), gases expelled (9,28,88,95,97), and the needle tips thrust into neoprene stoppers (32). The syringes were then pressurized in pressure cylinders as described previously.

At suitable time intervals, the appropriate syringes were depressurized and the contents immediately filtered through 25 mm 0.45 μm HA membrane filters (Millipore Corporation, Montreal, Que.) at 15 cm vacuum and washed twice with equal volumes of ice cold F-RSW (26,32). The moist filters were removed from the filtration apparatus and digested at 50 C for 1 h in sealed liquid scintillation vials containing 1.5 ml NCS (Amersham/Searle, Chicago, Ill.). The vial contents were cooled to room temperature, neutralized by addition of 50 μl glacial acetic acid, and brought to 20 ml with toluene based liquid scintillation cocktail (6 gm 2,5-diphenyloxazole and 75 mg p-bis[2(5-phenyloxazolyl)]-benzene per litre). The radioactivity of the vial contents were determined using a Beckman LS-250 liquid scintillation spectrometer (Beckman Instruments Inc., Fullerton, California) equipped with ^{3}H + ^{14}C, ^{14}C, and ^{3}H + ^{14}C + ^{32}P isosets and disintegrations per minute (dpm) calculation made by the channels ratio method.

RESULTS

Leakage Studies

The viability of V. fischeri in F-RSW following 1 h exposure to various hydrostatic pressures, is shown in figure 1. Within this time period all cells were killed at pressures greater than 750 atm. Between the pressures of approximately 200 and 750 atm, viability decreased. At 50 atm an apparent increase in cfu occurred; a similar phenomenon has been observed by other investigators (54,61,76,92).

Apparent leakage of orcinol reactive material (RNA) from the cells into the medium occurred over the pressure ranges of 1 to 200 atm and 700 to 1000 atm (fig. 1). However between the pressure intervals of 200 and 700 atm, cells excreted less RNA into the supernatant fluid than the controls.

Leakage of DNA into the menstruum did not occur to a great extent until the hydrostatic pressure exceeded 400 atm (fig. 3). The level of diphenylamine-reacting material (DNA) in the suspension medium remained almost constant from 1 to 250 atm. The rate of release of DNA increased linearly with pressures above 500 atm. This observation may be correlated to

cell viability (fig. 1) since the increase in DNA in the menstruum occurs near the lethal limit for the culture (4,67).

At all pressures greater than 1 atm, the protein in the supernatant fluid increased (fig. 1). Greatly increased levels of supernatant fluid protein were observed with pressures above 400 - 500 atm (the maximum pressure for growth of this organism) (4,67). There was also an apparent increase of protein in the supernatant between 1 and 50 atm (fig. 1).

The curves shown in figure 2 were obtained when the data for RNA and protein in the menstruum were related to the number of viable cells (cfu) and plotted as functions of pressure. There appears to be a slight decrease in RNA/cfu at 50 atm, compared to the control. However, the ratio increases markedly at pressures greater than 250 atm (fig. 2). The ratio of protein/cfu increased at a moderate rate to 250 atm, showing no apparent decline at 50 atm, followed by a very steep rise at higher pressures similar to that of RNA/cfu (fig.2).

MDH, a membrane-associated enzyme, (fig. 3) was released into the supernatant upon application of pressure. The release rates of MDH between 50 and 750 atm increased as a function of pressure. Changes in release rates were greater between 1 and 50 atm, and at pressures greater than 750 atm. The initial

rapid release of MDH may indicate losses of loosely bound molecules, while at the higher pressures losses may be due to major disruptions of the membrane. The rapid increase between 750 and 1000 atm is consistent with the extent of release of protein as shown in figure 1.

The concentration of total amino acids in the supernatant increased between 50 and 1000 atm (fig. 3). This curve exhibits a decrease in the amount of amino acids between 1 and 50 atm similar to the decrease of orcinol-reactive material shown in figure 2. Hydrostatic pressure between 500 and 750 atm resulted in the greatest increase of amino acids in the supernatant, with a less rapid increase between 750 and 1000 atm.

Radioisotope Studies

Aerobic Uptake

Aerobic uptake of ^{14}C-cycloleucine at 15 C over 4 h was determined (fig. 4B). The cells of this culture reached a saturation concentration of radioisotope label by 2 h with the period of linear uptake occurring during the first quarter hour. The amount of label in the cells decreased slightly in the 4 h following saturation, perhaps equilibrating the label initially transported into the cells. At 4 h, the concentration

of cycloleucine, in one flask, increased to 0.01 M with the addition of ^{12}C-cycloleucine. This increase of unlabelled substrate resulted in a significant loss of intracellular ^{14}C label, as shown in figure 4B. The rate of loss of label was similar to that of uptake of ^{14}C-cycloleucine by these cells, and demonstrates that the label was not bound into protein, but remained in an intracellular pool. The amount of label in these cells dropped to a level close to that of the acid-killed controls.

Further studies on the effect of low pH and low temperature on aerobic cycloleucine uptake (fig. 4A) were made. Uptake of ^{14}C-cycloleucine by both live and acid-killed cells at 5 and 15 C were studied. Uptake at 15 C by viable cells remained linear through 40 min incubation time. However, a decrease in temperature to 5 C markedly lowered the uptake to rates comparable to that of the acid-killed controls.

Uptake of label was studied at various concentrations and pressures. The values for slope, K_m, and V_m shown in table II were obtained from Lineweaver-Burk double reciprocal plots (not shown) of the resulting data (57). A pressure of 250 atm appears to inhibit cycloleucine uptake, as indicated by the increased slope of this curve. The negative values of K_m and V_m may indicate the influence of efflux, enzyme conformational changes, and high pressure oxygen effects upon uptake.

Anaerobic Uptake

Anaerobic accumulation of ^{14}C-cycloleucine by live cells increased at a constant rate to 20 minutes (fig. 5). A reduction in the amount of ^{14}C label associated with the acid-killed cells during incubation resulted in a curve for the net uptake of ^{14}C-cycloleucine.

Figure 6 (10 C) and table I (15 C) indicate that ^{14}C-cycloleucine uptake by cells is inhibited by hydrostatic pressures of 100, 250, and 500 atm. The curves (fig. 6) also demonstrate constant uptake rates up to 20 min.

The Lineweaver-Burk plots resulting from anaerobic uptake of ^{14}C-cycloleucine at 10 C at 1, 250, and 500 atm are shown in figure 7, and the K_m and V_m values shown in table III. As can be seen from this figure, hydrostatic pressure of 500 atm inhibits uptake to a greater extent than 250 atm. The type of inhibition is apparently competitive, however this effect may be due to conformational changes at the active site of the transport enzymes, causing an effect which mimics competitive inhibition.

A 2 h net uptake experiment is shown in figure 8. Uptake occurred at approximately a constant rate to 20 min and saturation occurred by 50 min. In the second hour, approximately 30 % of the accumulated label was released.

Figure 9 shows results from a similar experiment, where samples of the culture were pressurized at t = 60 min to 250 and 500 atm. While label was lost in the atmospheric pressure control, as expected from figure 8, both 250 and 500 atm resulted in a greater release of ^{14}C-cycloleucine.

Table I

^{14}C-cycloleucine uptake (DPM/ml) at 15 C and 1, 100, and 500 atm pressure sampled at 0, 5, 10, and 15 min. See Materials and Methods for details.

Time (min)	0	5	10	15
Pressure (atm)				
1	4	199	206	213
100		142	187	201
500		101	124	157

Table II

Values of K_m, V_m, and slope of Lineweaver-Burk plots for aerobic uptake of ^{14}C-cycloleucine by V. fischeri.

Pressure (Atm)	1	250
Slope (h)	67.54	131.5
K_m (nmol/ml)	20.56	-96.16
V_m (nmol/ml.h)	0.304	-0.731

Table III

K_m and V_m values for anaerobic uptake of ^{14}C-cycloleucine by V. fischeri. Data obtained from fig. 7.

Pressure (Atm)	1	250	500
K_m (nmol/ml)	42.32	67.02	109.1
V_m (nmol/ml.h)	3.76	3.507	3.319

Figure 1 Pressure effects (1 h, 15 C) upon culture viability (●), and leakage of protein (○) and RNA (□).

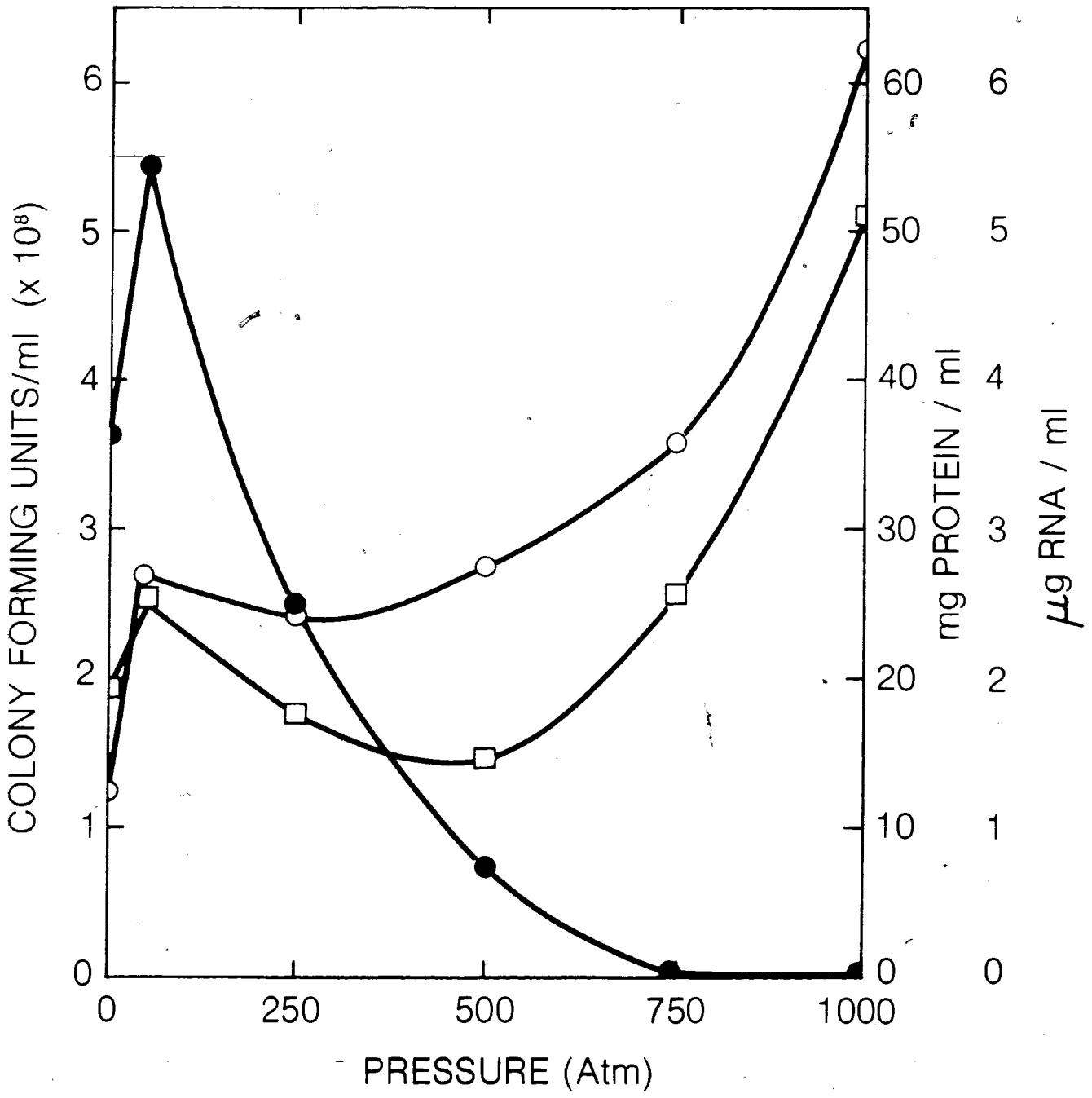

COLONY FORMING UNITS/ml (x 10⁸)
mg PROTEIN / ml
μg RNA / ml
PRESSURE (Atm)
0
250
500
750
1000
0
1
2
3
4
5
6
0
10
20
30
40
50
60
0
1
2
3
4
5
6

Figure 2 Pressure effects (1 h, 15 C) upon protein (●) and RNA (○) leakage per colony forming unit. Replot of data of fig. 1.

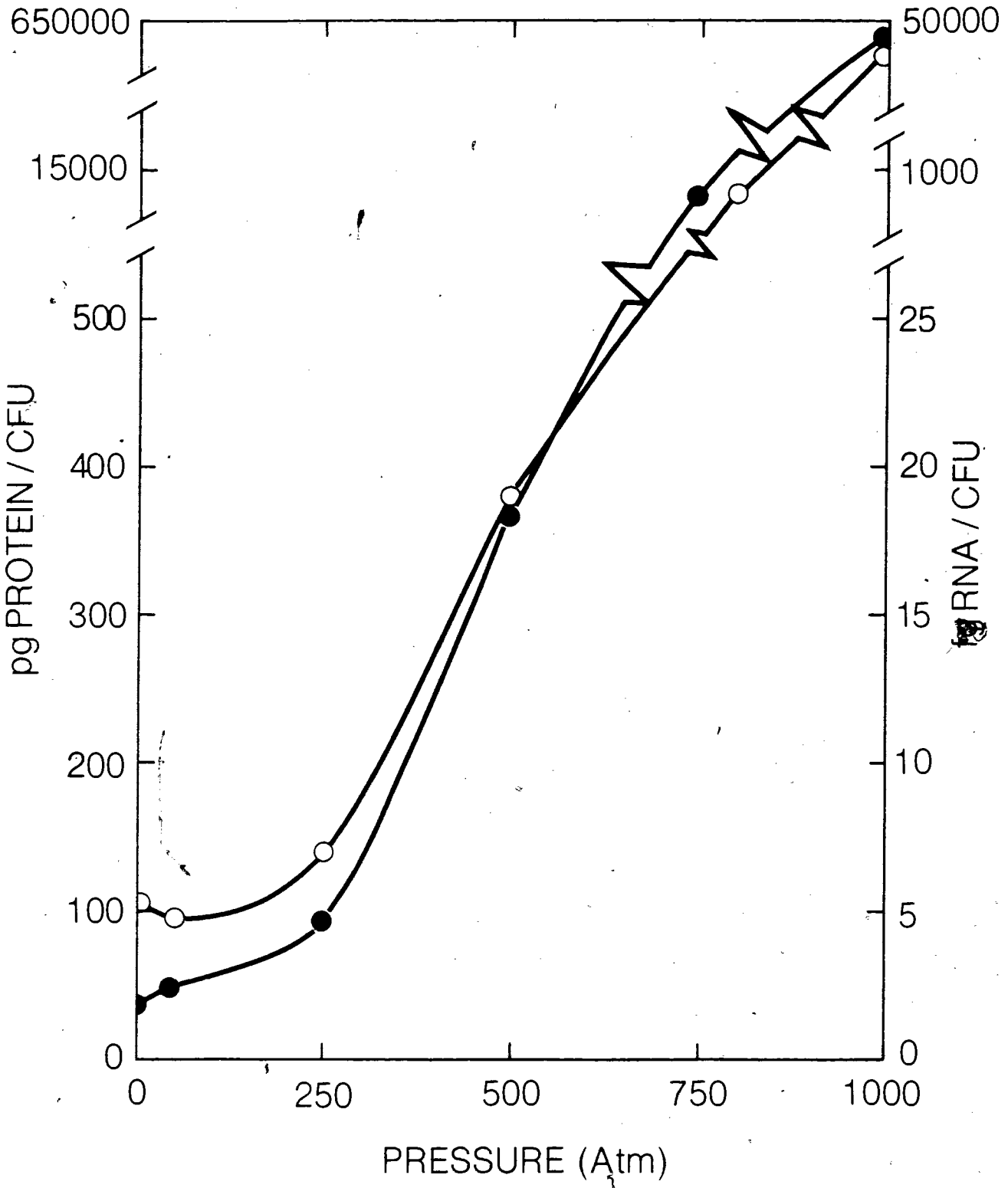

650000
15000
50000
1000
500
400
300
200
100
0
25
20
15
10
5
0
pg PROTEIN / CFU
pg RNA / CFU
0
250
500
750
1000
PRESSURE (Atm)

Figure 3 Pressure effects (1 h, 15 C) upon DNA (●), malate dehydrogenase (◯), and amino acid (□) leakage.

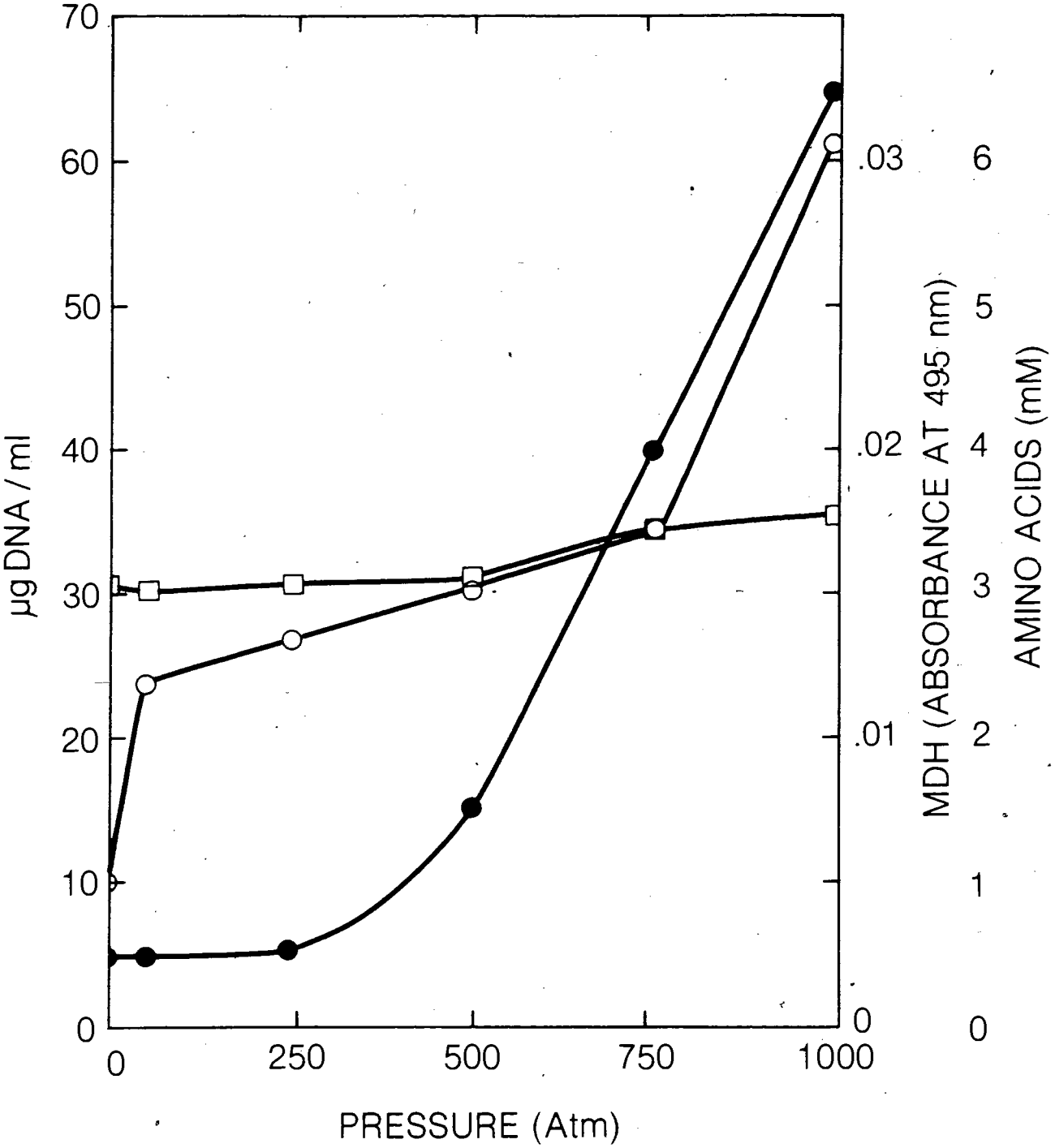

70
60
50
40
30
20
10
0
µg DNA / ml
.03
.02
.01
0
MDH (ABSORBANCE AT 495 nm)
6
5
4
3
2
1
0
AMINO ACIDS (mM)
0
250
500
750
1000
PRESSURE (Atm)

Figure 4 A. ^{14}C-cycloleucine content of viable (□,○) and acid-killed control (■,●) cells at 5 C and 15 C respectively.

B. ^{14}C-cycloleucine content of viable (○) and acid-killed control (●) cells at 15 C. At time = 4 h ^{12}C-cycloleucine was added to the viable cell culture (□). See Materials and Methods for details.

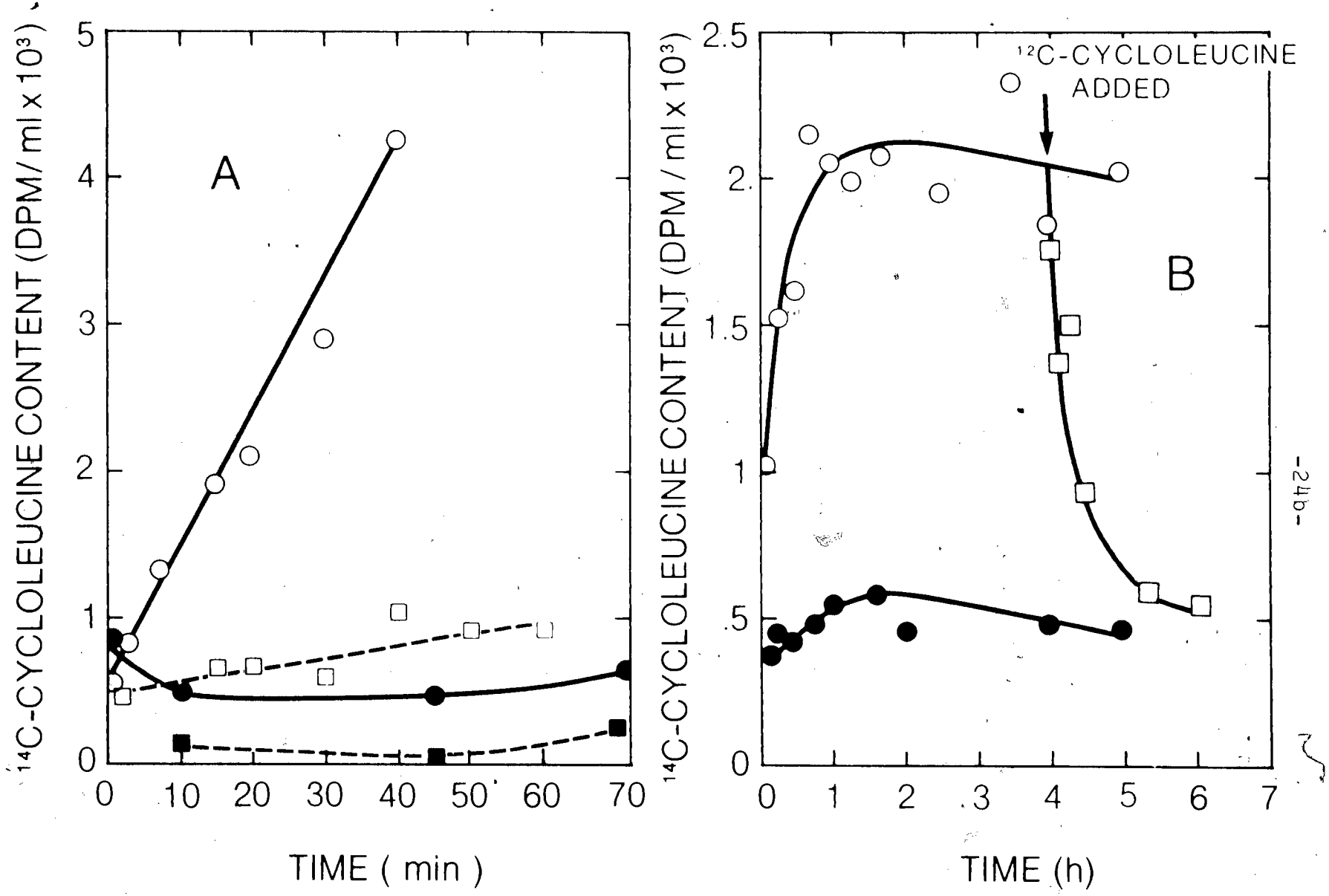

A
B
^{14}C-CYCLOLEUCINE CONTENT (DPM/ ml x 10^3)
TIME (min)
TIME (h)
^{12}C-CYCLOLEUCINE ADDED
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
.5
1.5
2.5

Figure 5 Anaerobic ^{14}C-cycloleucine content of viable (●) and acid-killed control (○) cells, and net uptake (□) at 10 C.

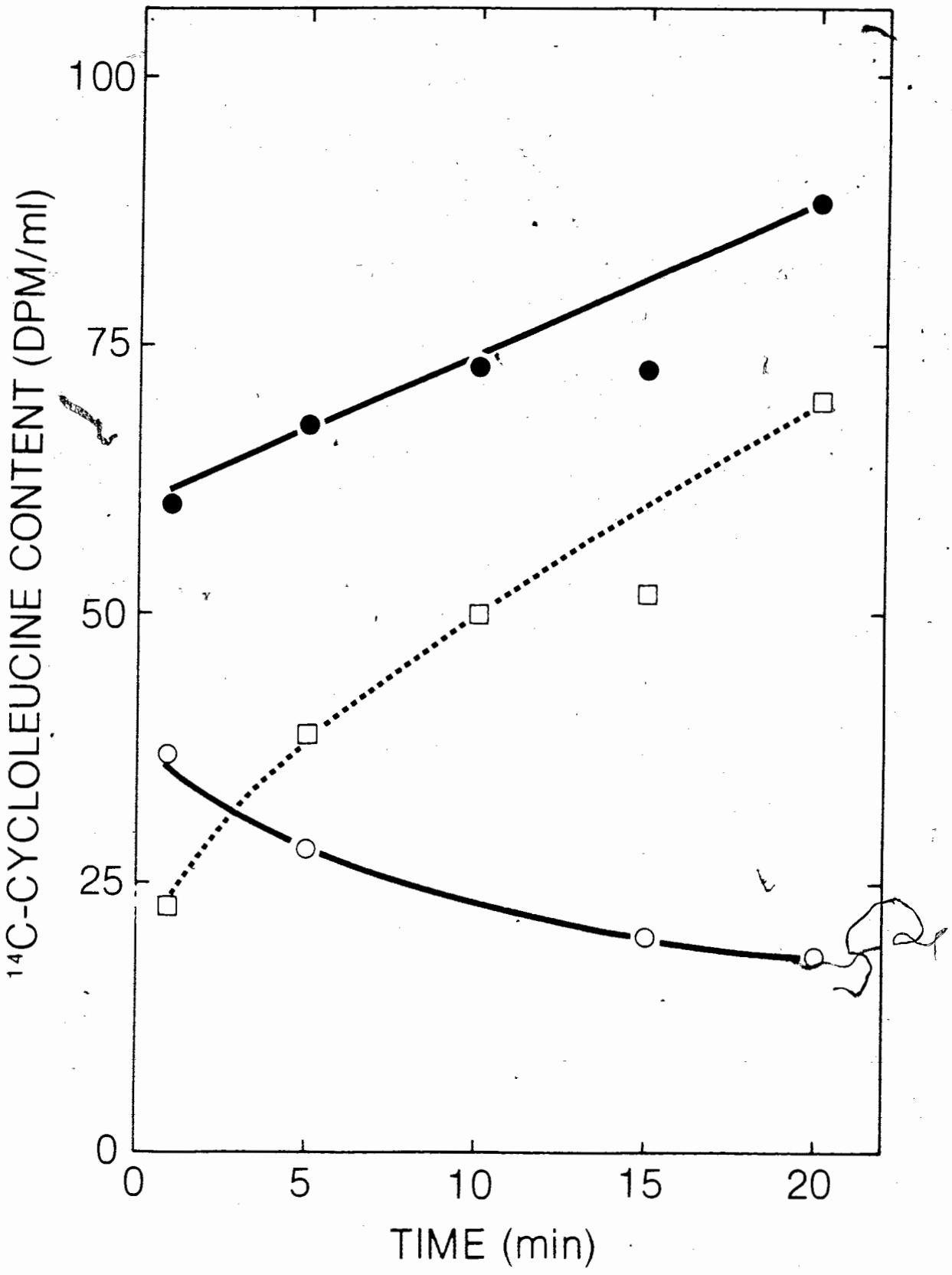

100
75
50
25
0
0
5
10
15
20
TIME (min)
^{14}C-CYCLOLEUCINE CONTENT (DPM/ml)

Figure 6 Anaerobic uptake of ^{14}C-cycloleucine by viable cells at 10 C and 1 (●), 250 (◯), and 500 (□) atm pressure. Data corrected for uptake by acid-killed control cells.

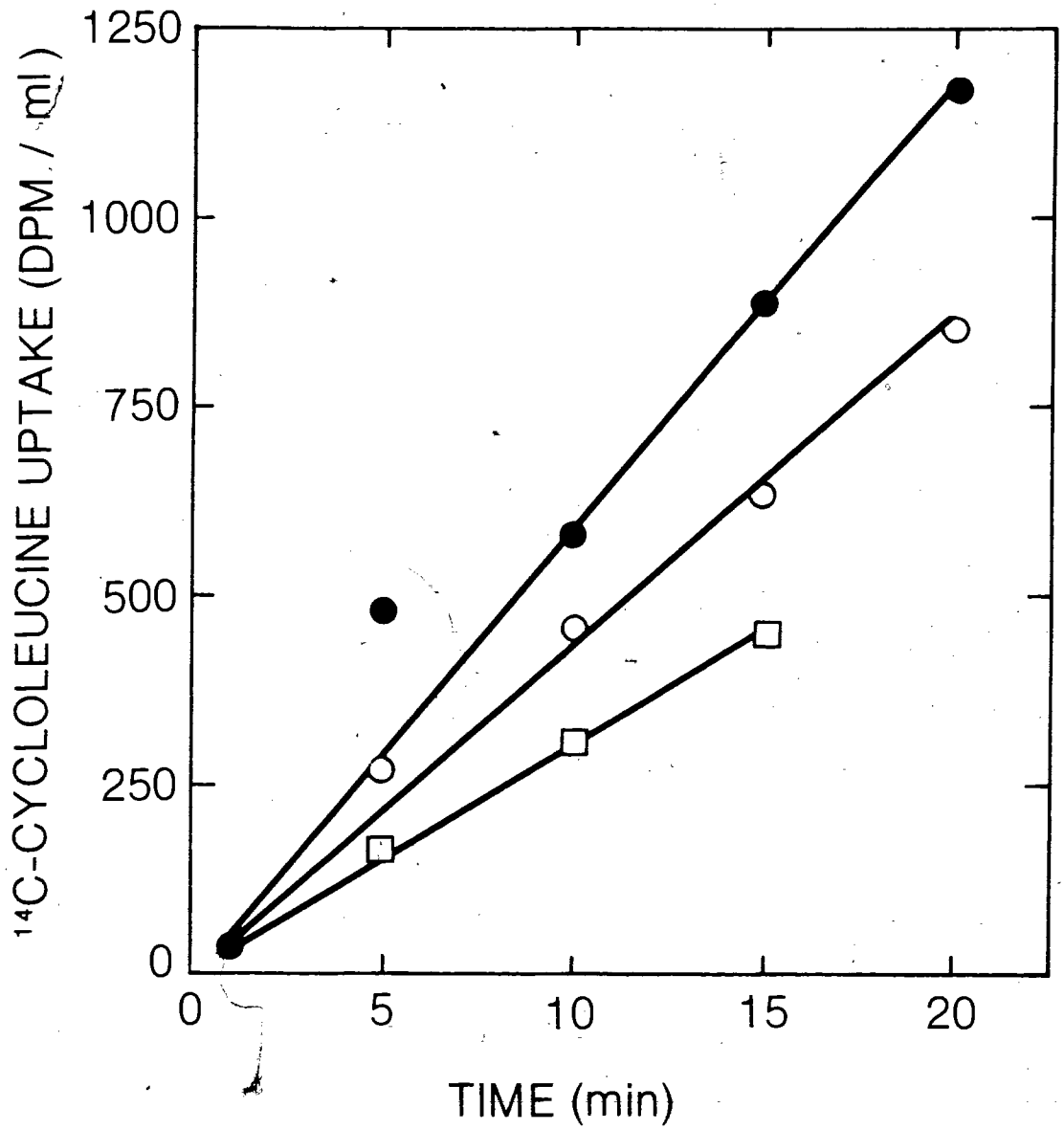

1250
1000
750
500
250
0
0
5
10
15
20
^{14}C-CYCLOLEUCINE UPTAKE (DPM / ml)
TIME (min)

Figure 7 Lineweaver-Burk plots of ^{14}C-cycloleucine uptake by viable cells under anaerobic conditions at 10 C and 1 (□), 250 (◯), and 500 (●) atm pressure. Data corrected for uptake by acid-killed control cells.

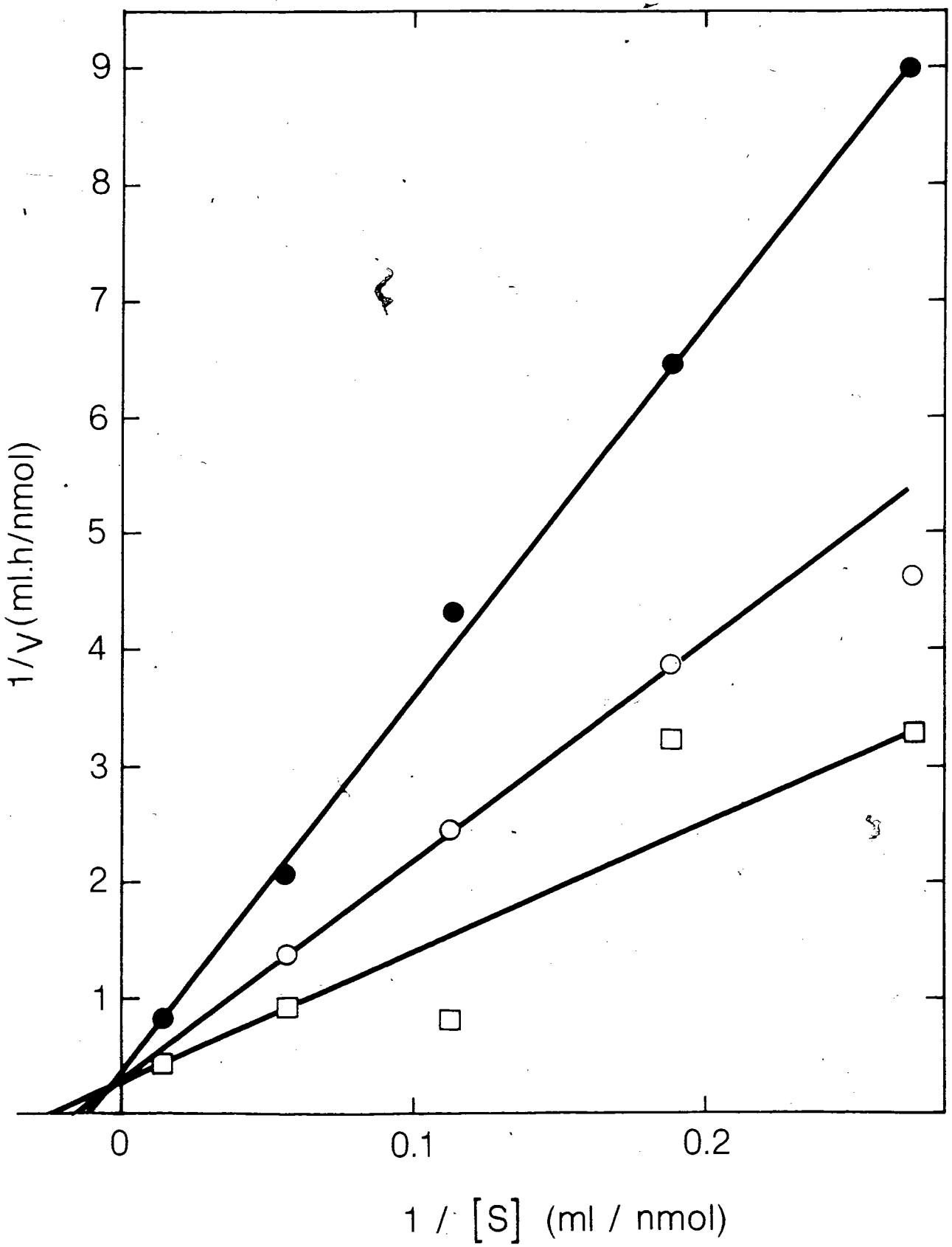

9
8
7
6
5
4
3
2
1
0
0.1
0.2
1/v (ml.h/nmol)
1 / [S] (ml / nmol)

Figure 8 Anaerobic uptake of ^{14}C-cycloleucine by viable cells at 10 C. Data corrected for uptake by acid-killed control cells.

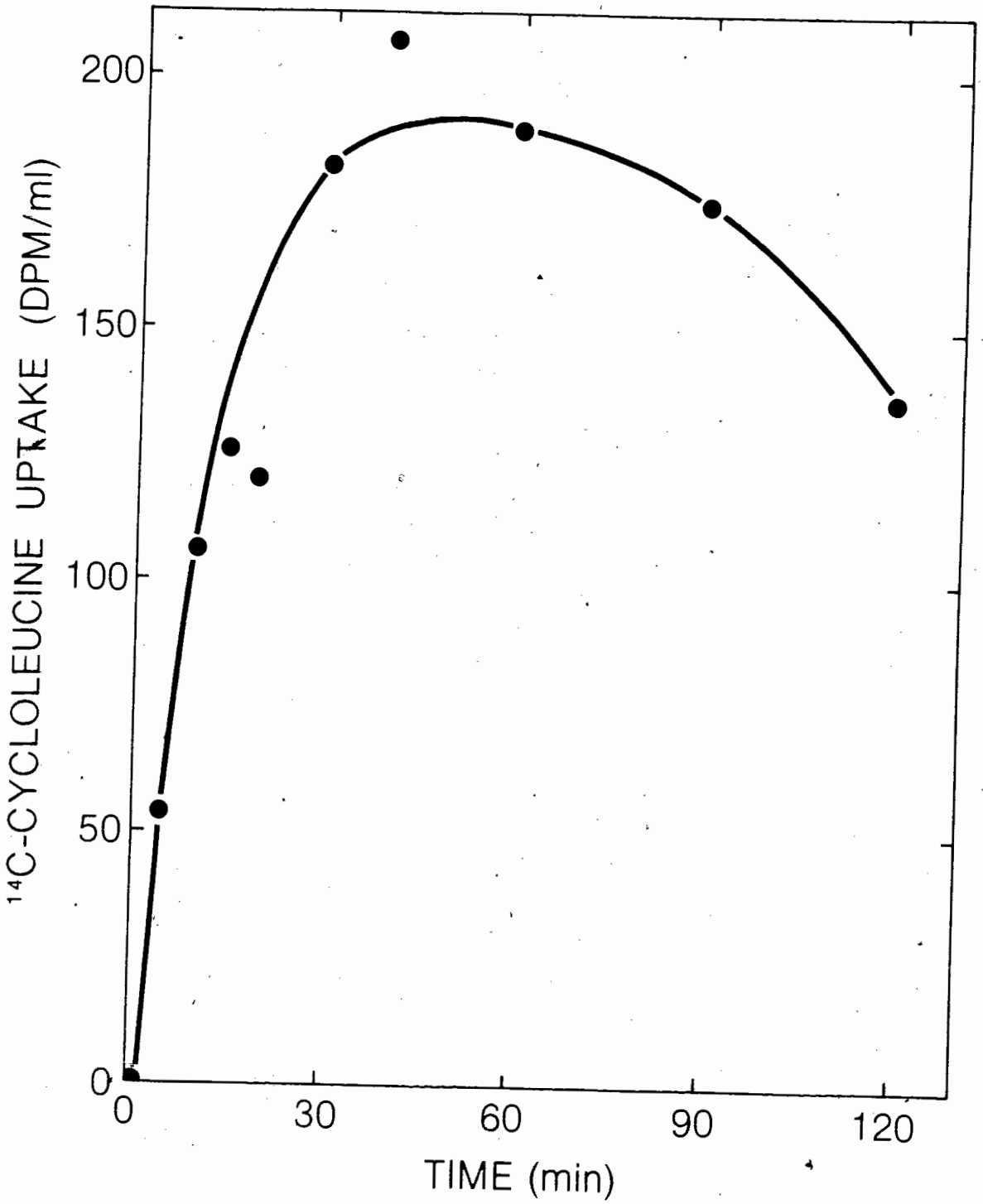
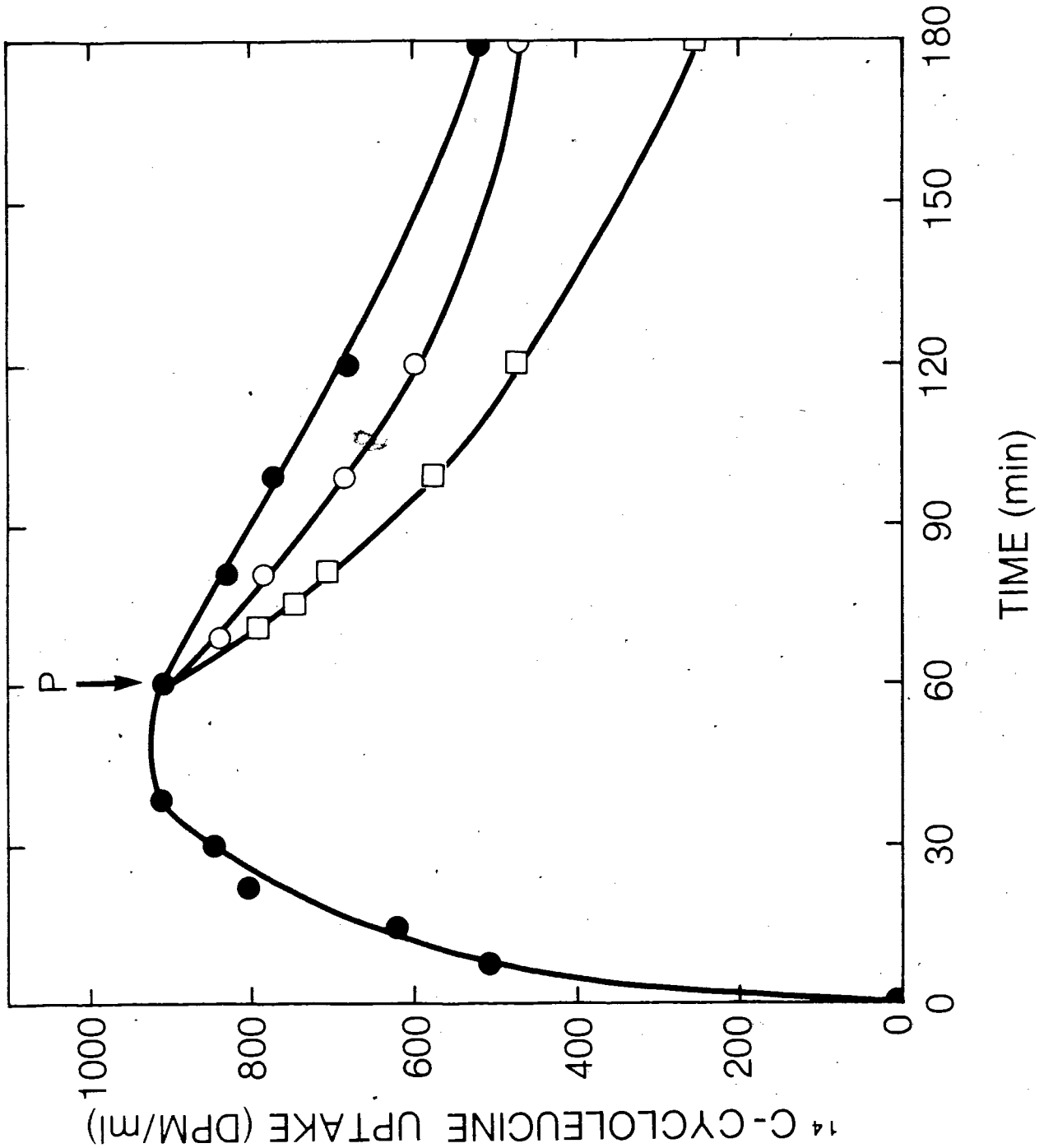

200
150
100
50
0
0
30
60
90
120
TIME (min)
^{14}C-CYCLOLEUCINE UPTAKE (DPM/ml)

Figure 9 Anaerobic loading of viable cells at 10 C and 1 atm (●) (0 to 60 min). At time = 60 min pressures of 1 (●), 250 (○), and 500 (□) atm were applied. Data corrected for uptake by acid-killed control cells.


^{14}C-CYCLOLEUCINE UPTAKE (DPM/ml)
1000
800
600
400
200
0
P
0
30
60
90
120
150
180
TIME (min)

DISCUSSION

Leakage Studies

The viability of V. fischeri, following pressurization, reported here (fig. 1) exhibits the characteristic phases reported in the literature (61,63). The peak in viability indicated at 50 atm is consistent with Morita's report of slightly better growth at 100 and 200 atm than at atmospheric pressure (61). Better growth at low pressures (ca. 100 atm) than at atmospheric pressure might be expected of a culture isolated from a water sample at 1200 m depth (65). Previous reports (4,62,63,67) indicating a decrease of viability at 300 atm (63) and with death occurring at pressures exceeding 800 atm (4,67) are in agreement with the viability vs. pressure curve (fig. 1). The results of these studies indicate that V. fischeri has adapted to the moderate pressures of the zone from which it was isolated (54), but cannot be considered a barophile (59) due to its sensitivity to high hydrostatic pressures.

In previous experiments testing the effect of temperature on V. fischeri, leakage of metabolites and macromolecules occurred along with loss of viability by the cells. Structures and/or reactions in marine bacteria which are sensitive to changes (usually increases) in temperature may also be

influenced by moderate and high hydrostatic pressures. An effect, demonstrable through the application of high temperatures may be counteracted or similarly affected by pressure, depending on the range of temperature and pressure experienced. In experiments conducted by Burton and Morita (18), Haight and Morita (31), and Kenis and Morita (42), temperatures exceeding the maximum growth temperature (20 C) resulted in losses of metabolites and macromolecules into the menstruum whereas no appreciable quantities of these materials were detected at 15 C. These materials, RNA, DNA, protein and amino acids, are biologically valuable molecules, and their loss to the menstruum would affect the ability of the cells to survive. The rates of leakage differed, where the rates of leakage were protein > RNA > DNA > amino acids. Haight and Morita (31) interpreted this result as an indication that the cell membrane was not rupturing, since this would result in the release of all of the components at similar rates, and should show high rates of release of small molecules such as amino acids.

Losses of intracellular molecules due to pressure were therefore tested (figs. 1-3). In all cases high hydrostatic pressures resulted in significant losses of macromolecules or metabolites. Leakage rates increased significantly at pressures exceeding 350 atm, however deviant rates of leakage were seen at lower pressures.

Release of RNA and protein show peaks at 50 atm (fig. 1) which appear to correlate with a similar viability peak (fig. 1) Increased amounts of RNA and protein may be a result of greater numbers of cells in the culture, due to the enhancing effect of low pressures (ca. 50 atm) on the growth rate of the culture (61). In the case of RNA, it is important to realize that the assay detected orcinol reactive material, which may consist of soluble or polymerized RNA-like material. In similar studies testing the effect of temperature on cellular leakage (31,42) it was found that the composition of this orcinol reactive material changed as the incubation temperature increased.

The ratios of released RNA or protein to viable cells at high pressures increase rapidly, as would be expected from the rapid decrease in viable cells at pressures greater than 250 atm (fig. 1). At high pressures, the number of viable cells become so low that the ratio of RNA or protein to viable cells increases markedly, regardless of any pressure induced increase in RNA or protein. Release of protein at low pressures (fig. 2) appears to increase with pressure when a correction is made for increased growth and cell division. Release of RNA into the menstruum at 50 atm when corrected for the number of viable cells appears to decrease slightly (fig. 2). A similar decrease in amino acids is seen in figure 3.

This effect may be due to slightly greater polymerization in the more rapidly dividing cells at pressures around 50 atm, resulting in fewer low molecular weight orcinol reactive materials and amino acids which may be released by the cells.

Accelerated losses of macromolecules and metabolites at pressures greater than 500 atm have been noted (figs. 1,3). If these results are compared to the viability curve (fig. 1), a correlation between cell death (loss of viability) and loss of RNA, DNA, protein, amino acids, and MDH is seen. The amount of MDH increases markedly between 750 and 1000 atm, the pressure range where death of the culture is most pronounced. Release of protein in this range also increases markedly. The amount of protein in the supernatant increases at a lesser rate between 250 and 500 atm. The pattern of protein release indicates that in the range of 250 to 750 atm during which the viability of the culture decreases, pressure causes the loss of loosely bound and membrane associated protein as well as other proteinaceous material, but probably not because of a lytic phenomenon. Release in the 750 to 1000 atm pressure range may indicate that membrane rupture had occurred. The increased concentration of amino acids in the supernatant, between 50 and 500 atm (fig. 3) may be due to inhibition of the cells' ability to concentrate amino acids from the

menstruum, as well as to some disruption of the membrane integrity. The much greater rate of loss shown between 500 and 750 atm may reflect the total inhibition of inward transport of amino acids coupled with severe disruption of the cell membrane allowing the amino acids to escape. The inability of the amino acid efflux to maintain the release rate, may reflect an exhaustion of the free amino acid pool limiting the effect of large scale lysis of the cells, indicated by the increased rate of release of RNA, protein (including MDH) and DNA. The release of DNA to the menstruum does not occur until the viability of the cells has decreased significantly. The pattern of release from 500 to 1000 atm may indicate that the losses are due to disruption of the membrane at lower pressures, and membrane rupture at higher pressures. If pressure dependent membrane rupture were occurring at pressures greater than 500 atm, losses of the smaller molecules should increase at a rate proportional to the rate of loss of DNA. The data support the observation of greater release at pressures greater than 750 atm, where the lethal effects of pressure are the most pronounced (fig. 1) and may indicate membrane rupture (fig. 1-3).

Thermally induced leakage (7,29) of MDH has been studied by Haight and Morita (31) and Kenis and Morita (42) as an indication of the proportion of the proteinaceous material

released which remains active. MDH in cell-free extracts of V. fischeri MP-1 was labile at 0 C, but the enzyme in whole cells remained active up to 18 C (46). The enzyme from a marine psychrophile PS207 was found to be heat-sensitive at 30 C, the maximal growth temperature for the organism (18), while the enzyme from the thermophile, Bacillus stearothermophilus NCA 2184, exhibited an optimum at 55 C and thermal inactivation at ca. 80 C (64). It was proposed (64) that the thermophile's enzyme was inactivated at higher temperatures due to an equilibrium shift favouring an inactive (higher molecular volume) conformation. When the thermophile's enzyme was subjected to high pressures (1000 atm) prior to heating, the increase in molecular volume caused by increased temperature (101 C) favouring denaturation was counteracted by a decrease in molecular volume due to high pressure (64). Low pressure (50 atm) release of MDH as shown in figure 3 is similar to the effect of incubation at 20 C in Kenis and Morita's study (42). Higher temperatures resulted in greater rates of release similar to those induced by moderate pressures (50-750 atm) in this study. MDH was released at temperatures higher than the maximum growth temperature, and its release was also affected by any pressure above atmospheric pressure.

Radioisotope Studies

The effect of pressure on amino acid uptake has been studied through the use of radioisotope labelled amino acids (11,32,52,68,84). This technique is valuable for studies involving uptake of amino acids, particularly when the respired label is also assayed, (32,52,68). In this study, where both uptake and efflux were studied, labelled non-metabolizable amino acid analogues such as cycloleucine (1,20) and α-aminoisobutyric acid (1,19,20,24-26,66) were preferred as substrates, since it was the free amino acid pool which was of interest (13). Respiration and assimilation of the radioisotope label into polypeptides, CO_2, carbohydrates, etc., results in accumulation of label into forms which would not be as readily influenced by pressure as would the intracellular free amino acid pool.

The uptake and chase experiment shown in figure 4B demonstrates that cycloleucine is transported aerobically into the cells and that the cycloleucine remains in an unbound mobile form. Accumulation of label by these cells at 10^{-5} M cycloleucine reached saturation by about 1 h in this experiment. The chase period after 4 h to a ^{14}C level comparable to that of the acid-killed cells shows that cycloleucine is not incorporated into immobile macromolecular

forms, such as polypeptides and proteins. The kinetics of label dilution are similar to those of uptake. The results of this experiment follow the classic appearance of active uptake into intracellular pools as reviewed by Britten and McLure (13).

The presence of molecular oxygen at high pressures has been previously shown to be detrimental to cellular processes (9,88,97). The aerobic ^{14}C-cycloleucine experiments were thus performed anaerobically to determine whether uptake inhibition was due to the increased pressure or because of the presence of molecular oxygen at high pressures.

Anaerobic uptake of cycloleucine over a 2 h period shows a similar uptake curve during the first hour (figs. 8,9), followed by loss of label during the second hour (fig. 8).

An experiment conducted to determine the duration of uptake at a linear rate, the effect of temperature, and the effectiveness of 0.01 M H_2SO_4 (fig. 4A), demonstrated that low pH and low tempeature limit the uptake of cycloleucine. The combination of low temperature and low pH results in less uptake than with either of these alone.

A short term anaerobic uptake experiment conducted over 20 min (fig. 5) establishes that uptake occurred at a constant rate

during this time. When the system was exposed to hydrostatic pressures of 250 and 500 atm, the uptake rate was depressed relative to the culture maintained at the ambient pressure (fig. 6, table I).

These results confirm that the gratuitous substrate cycloleucine was transported in a manner similar to that of amino acids, and that the transport mechanism may be similarly inhibited by hydrostatic pressure.

Further studies on the effect of hydrostatic pressures on the kinetics of cycloleucine transport into the cell were tested utilizing the Lineweaver-Burk double reciprocal plot (fig. 7, table II). The curves resulting from data plotted in this manner show inhibition of uptake both aerobically (table II) and anaerobically (fig. 7) by pressures of 250 and 500 atm.

In order to confirm the leakage studies performed with unlabelled cells, pressures of 250 and 500 atm were applied to an anaerobic culture which had been allowed to accumulate saturating concentrations of ^{14}C-cycloleucine, as shown in figure 9. The uptake curve follows the trend shown in previous uptake curves (figs. 6,8). Release rates of label at atmospheric pressure were increased at both 250 and 500 atm applied hydrostatic pressures. Moderate pressures (250 atm)

result in a loss of ca. 50 % of the accumulated label in 2 h., which is slightly greater than the loss shown at 1 atm, which indicates that although some of the label is lost, the depletion of the amino acid pool is not as marked as would be expected from the viability curve in figure 1 and from previous studies (61,62,65). The cells at 250 atm are inhibited by the loss of metabolites as shown by leakage of protein, MDH, RNA, DNA and ^{14}C-cycloleucine into the menstruum. Growth of the culture is inhibited by the loss of intracellular metabolite concentrations as well as by the inhibition of many enzyme mediated processes with moderate pressure. The losses may also include an uptake inhibition component due to pressure.

At higher pressures (500 atm), the ^{14}C-cycloleucine remaining intracellularly is reduced to ca. 25 % of the saturation level after 2 h. At this pressure, efflux of metabolites has become increasingly important as the concentration of intracellular components are markedly lowered, enzyme activities decrease and the membrane integrity is lost. Growth and cell division at this stage has been inhibited to the extent that the production of new cells cannot counteract the death rate.

CONCLUSIONS

The results of these studies indicate that uptake and efflux of amino acids by V. fischeri are affected by moderate and high hydrostatic pressures. Uptake of the non-metabolizable amino acid analogue cycloleucine was inhibited, both aerobically and anaerobically, in the sublethal range (below 800 atm) of pressures at its environmental and optimal growth temperatures. The increased K_m values may indicate a decreased affinity for this substrate by the amino acid transport system at high pressures. The data presented here support previous work indicating significant inhibition of active uptake of amino acids by pressure (11,32,68,84).

Efflux of accumulated cycloleucine from the intracellular amino acid pool, upon application of pressure, indicates an additional effect which may affect protein synthesis. Depletion of intracellular resources by release of amino acids, protein, including active enzymes such as malate dehydrogenase, RNA, and DNA would further decrease the ability of this organism to survive in the depths of the ocean. Under the conditions of low temperature and low nutrient availability prevailing in the deepest regions, and with significant inhibition of anabolic and catabolic enzyme activities, efflux of these metabolites may result in decreased numbers of microbes able to survive, grow, and divide.

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