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Title: EFFECTS OF LONG-TERM NUTRIENT STARVATION ON A MARINE PSYCHROPHILIC VIBRIO

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Ant-300, a psychrophilic marine vibrio, was starved for periods in excess of one year. Cells starved at a high initial cell density increased in numbers from 100 to 800% of the initial number of cells during the first week. Fifty percent of the population remained viable for six weeks while a portion of the population remained viable for more than one year. Cells starved at a low initial cell density increased in numbers over 400 fold during the first week and after 70 weeks of starvation over 15 times the original number of cells were still viable.

Observation of cellular DNA with Feulgen staining prior to starvation showed that the average number of nuclear bodies per cell varied from 1.44 to 4.02 depending on the age of the culture. A linear relationship was found between the average number of nuclear bodies per cell and the increase in numbers upon starvation. The data indicate that DNA replication is not necessary for the increase

in cell numbers and suggest that the cells divide by fragmentation under starvation conditions.

Concomitant with the increase in cell numbers was a decrease in cell size and a change in shape from a rod to a coccus. After three weeks of starvation, 50% of the viable cells were able to pass through a 0.4 μm Nuclepore filter. Electron microscopy of thin sections of the small cells revealed normal cell structure except for an enlarged periplasmic space. When inoculated into a fresh medium, small starved cells grew without a significant lag and regained "normal" size and shape within 48 h.

During the first two days of starvation, the endogenous respiration of the cells decreased over 80%; after six weeks of starvation cellular DNA and protein had been reduced 46 and 43%, respectively.

Additions of nutrients to starving cell populations showed that phosphate or nitrate had little effect while glucose caused a highly accelerated cell death. The addition of glucose and nitrate or glutamate to starving cell suspensions resulted in recovery and growth of the cells with no detectable loss in viability.

Cells starved in seawater or a starvation menstruum amended with small amounts of amino acids showed a slight increase in cell numbers but was otherwise characteristic of complete starvation.

Effects of Long-Term Nutrient Starvation on a Marine Psychrophilic Vibrio

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INTRODUCTION

Compared to other natural environments, the nutrient content of the ocean is very low. Although estimates vary, the amount of dissolved organic matter in the sea is usually less than 1 mg C/liter for surface water and 0.5 mg C/liter for deep water (56). In addition to temperature and hydrostatic pressure, nutrient availability has been suggested as an important factor in the distribution of bacteria in the oceanic environment (97). Thus, more bacteria are generally found near river outfalls, in sediments, and in surface waters. Due to the constant circulation and mixing of the oceans, a high concentration of cells in a productive area such as an estuary will eventually be diluted and dispersed. Moreover, it has been estimated that converging surface waters that mix and subsequently sink will not appear at the surface again for 1100 years. How then can the bacteria in these waters survive great periods of time with little or no nutrients between the nutritive areas of the ocean? The present study has attempted to answer this question by investigating the survival of a marine Vibrio sp. under starvation and dilute nutrient conditions.

In comparison to other facets of microbiology, very little is known about the starvation and death of microbes since most microbiological studies have been conducted using rich media containing

several grams of nutrients per liter. Even for the isolation of marine bacteria high nutrient media has been employed. ZoBell (97) has suggested a medium containing 5 g of peptone and 1 g of yeast extract per liter of seawater for the isolation and enumeration of most marine bacteria. More recently other rich media have been suggested (75) for the cultivation of marine bacteria. These media contain laboratory reagents whose presence is doubtful in large quantities in the open ocean. When the starvation survival of bacteria has been investigated it has been found that most bacterial strains are not well suited for survival. For example: after 36 hours of starvation the viability of a population of Escherichia coli will be reduced by one-half (30). For most all other strains studied, greater than 50% of the initial number of cells are dead after only five days of starvation. To date, the best survival has been demonstrated by Arthrobacter crystallopoietes with a starvation half-life approaching 100 days (8). Morita (59) first questioned whether some marine bacteria are transients or not actually metabolizing in the oceanic environment. This point was reiterated by Sieburth et al. (73) when they suggested that marine bacteria "free" in the water column may be nothing more than transients starving to death, even though there have been no reports of starvation survival for any marine bacteria. Strange (79) has stated that "Bacterial populations in saline buffer may survive completely for hours or days... but prolonged starvation ultimately leads to death"

even though there have been no reports of starvation experiments lasting longer than 80 days. The purpose of the present study therefore was to determine the survival characteristics of a marine bacterium subjected to long-term nutrient starvation. The goal of this study was three-fold: to determine any similarities between cells in the natural environment which may be under starvation and laboratory starved cells; to determine the length of starvation needed to completely eliminate a population; and to study and characterize the physiological effects of nutrient deprivation and starvation recovery of a marine bacterium.

LITERATURE REVIEW

Prior to 1960 reports on the effects of starvation on bacteria were few in number. In 1918 Winslow and Cohen (94) determined that populations of Bacterium coli[1] and Bacterium aerogenes[2] would be reduced by one-half after three days in tap water. Other early reports dealt with the effects of temperature and pH (24) and salt concentration (71, 95) upon the viability of various bacteria. Over 30 years passed before Dagley and Sykes (25) reported differences in macromolecular sedimentation rates in crude cell extracts prepared from starved and nonstarved cells.

After 1960 a limited number of reports began to appear. In general, it was found that bacteria are not well adapted for survival under nonnutrient conditions. The starvation half-life or 50% survival time[3] for most bacterial species studied varies from a few hours to

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[1] Presently classified as Escherichia coli (13).

[2] Bacterium aerogenes, first described by Escherich in 1885, is no longer recognized as a valid taxon (13). From the description of this organism (12), it would most likely be presently classified as Klebsiella pneumoniae.

[3] The half-life or 50% survival time for starving bacterial suspensions is defined as the length of time needed to reduce the viable population by one half. It can be calculated from standard plate counts, from a combination of direct counts and plate counts, or from slide culture techniques (61, 63). Since no standard method has been accepted and differences have been observed among the techniques (82), results reported by various investigators may not be strictly comparable.

a few days (Table 1); however, the 50% survival time for Arthrobacter crystallopoietes approaches 100 days (8). Survival, therefore, appears to be species specific and much of the literature on starvation pertains to determining what factors determine the survival characteristics of certain organisms. Harrison (42) has stated, "log phase cells in the absence of food die as the result of loss of cell substance." More specifically, Boylen and Ensign (9) found that 40% of the cellular protein of A. crystallopoietes was degraded after 30 days of starvation. Protein degradation during starvation has also been reported by others (17, 34, 39, 52, 78, 82). Robertson and Batt (69) found that Nocardia corallina degraded cellular protein but only after complete degradation of its polysaccharide. Montague and Dawes (58) reported extensive (80%) loss of cellular RNA in Petpococcus prevotii after 15 h of starvation. Boylen and Ensign (9) reported an 85% loss of RNA from A. crystallopoietes after 30 days of starvation. Degradation of RNA seems nearly universal in starving bacteria (14, 17, 22, 23, 27, 39, 47, 50, 52, 69, 82, 86); however, DNA is not usually degraded (9, 14, 27, 29, 47, 86, 92) or it increases slightly (9, 11, 17, 39, 52, 84). There has been only one report of DNA degradation during starvation. Harrison and Lawrence (43) reported that Aerobacter aerogenes[4] degraded 48% of its DNA in 19 hours; however, at

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[4]The generic name Aerobacter has been rejected as a taxon in the 8th edition of Bergey's Manual of Determinative Bacteriology (13)

Table 1. Half-life starvation times for various bacteria.

Organism	Half-life[a]	Reference
Aerobacter aerogenes	5	64
Streptococcus lactis	7	85
Klebsiella aerogenes	10	16
Peptococcus prevotii	12	58
Sphaerotilus discophorus	12	77
Streptococcus lactis	24	87
Escherichia coli	36	30
Azotobacter agilis	48	76
Sarcina lutea	60	14
Bacterium coli	72	94
Pseudomonas aeruginosa	96	23
Micrococcus halodenitrificans	110	74
Salmonella enteritidis	140	31
Nocardia corallina	480	69
Arthrobacter globiformis	1344	51
Arthrobacter crystallopoietes	2400	8

[a]When half-lives were not reported as such by the investigators they were calculated from their data. Half-lives can vary greatly depending on experimental conditions, in each case only the longest time is presented. Values presented are in hours.

this time less than 1% of the population was viable. This contradicts the data of Postgate and Hunter (64) who reported no significant reduction in DNA for the same organism. Ribosomes (10, 40, 54), carbohydrates (9, 10, 69, 80, 82), free amino acids (14), and lipids (69, 86) have also been reported as being degraded during starvation. Robertson and Batt (69) found no relationship between protein, RNA, or DNA and loss of viability. This is consistent with the other literature to date with the exception of Lovett (50) who found that deuterium oxide retarded the rate of hydrolysis of RNA and prolonged the survival of A. aerogenes.[5]

Certain compounds such as polysaccharides, glycogen and poly-β-hydroxybutyric acid (PHB) are found in bacteria and are considered as storage or reserve materials. One would expect cells that contain a large amount of these materials to survive longer than cells devoid of such compounds. Sierra and Gibbons (74) found that PHB rich Micrococcus halodenitrificans cells survived ten times as long as did

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due to misuse and confusion. The editors suggest that bacteria labeled Aerobacter aerogenes that are non-motile should be placed in the genus Klebsiella while those motile by peritrichous flagella should be included in Enterobacter. The authors describe their strain as being non-motile; it would therefore be presently classified as Klebsiella pneumoniae.

[5]Strain NCTC 418, the same organism used by Harrison and Lawrence (43) and Postgate and Hunter (64, 65).

PHB poor cells. Stokes and Parson (77), Sobek et al. (76), and Strange et al. (82) also reported increased survival times for cells containing PHB. Chen and Alexander (21) found that the ability of soil isolates to survive carbon starvation was correlated to PHB content but not to the glycogen content of the cells. Burleigh and Dawes (14) reported that glycogen rich Sarcina lutea did not survive as well as glycogen poor cells. Dawes and Ribbons (28) found that glycogen does not favor the survival of E. coli and that cells containing glycogen (up to 23% of the dry weight of the cell) oxidized it within one to three hours. This is consistent with the data of Strange (80) but not with that of van Houte and Jansen (91) who found that glycogen favored the survival of Streptococcus mitis. Wilkinson (93) hypothesized the production of storage compounds in bacteria as a result of "hot house" conditions (growth on rich laboratory media) and therefore questioned the existence of such compounds in nature. He also points out that these compounds are never more than 50% of the dry weight of the cell and therefore cannot serve as a carbon and/or energy source for extensive multiplication.

Dawes and Large (27) found that the intracellular concentration of adenosine triphosphate correlated with the starvation viability of Zymomonas sp. Chapman et al. (20) found that a rapid fall in viability

coincided with a steep drop in the adenylate energy charge[6] of E. coli while Montague and Dawes (58) found that the loss of viability of P. prevotii became more rapid when the energy charge fell below 0.4 to 0.5.

Robertson and Batt (69) noted a 90% decrease in endogenous respiration after 48 h of starvation for N. corallina (half-life 480 h) and suggested that a bacterium would have to step-down its endogenous respiration in order to survive for long periods of time. This is consistent with the data of Luscombe and Gray (51) which shows a 94% decrease in respiration for Arthrobacter globiformis (half-life 56 days). Also in agreement is the data of Boylen and Ensign (8) which show an 80-fold decrease in endogenous respiration for A. crystallopoietes which has the longest 50% survival time yet reported. On the other hand, Dawes and Holmes (26) and Burleigh and Dawes (14) reported a 95% reduction in endogenous respiration for starving S. lutea which has a half-life of only 60 h. In addition, Postgate and Hunter (64) reported an 80% decrease in endogenous

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[6] The adenylate energy charge is defined by the following equation:

$$\frac{[ATP] + 0.5\,[ADP]}{[ATP] + [ADP] + [AMP]}$$

respiration for Aerobacter aerogenes which has one of the shortest half-lives yet reported. Finally, a study of soil isolates by Chen and Alexander (21) showed that starvation survival was not related to endogenous respiration.

Of obvious importance to a cell under starvation is the retention of enzymes and enzyme activity. Boylen and Ensign (9) found that glucose oxidation in A. crystallopoietes was stable for 14 days of starvation while Meganathan and Ensign (55) found a 90% decrease in catalase but no decrease in succinic dehydrogenase, fumarase, or aconitase in the same organism. In contrast, Fan and Rodwell (34) reported a 5-30% decrease per hour in activity for the enzymes studied[7] in Pseudomonas putida, while Bentley and Dawes (7) reported a 35% decrease in threonine dehydratase activity in 33 h for P. prevotii.

Postgate and Hunter (64, 65) reported on the protective effect of Mg^{2+} ions on starving suspensions. This observation has been confirmed by others (9, 67, 85, 88) and is believed to be the result of the protective effect of Mg^{2+} ions on ribosomes.

Cryptic growth or the growth of bacteria on nutrients released from dead cells was first reported by Ryan (70). The effect of cryptic growth on starving bacterial suspensions was described by

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[7] L-arginine oxidase; α-ketoarginine decarboxylase; and γ-guanidinobutyrate amidinohydrolase.

Postgate and Hunter (64, 65) who calculated that a population of A. aerogenes could produce one new organism at the expense of 47 dead ones. Druilhet and Sobek (31) determined that 6.7 heat killed Salmonella enteritidis cells were needed for the doubling of one cell.

Other factors influencing the starvation survival of bacteria are the initial concentration of the cell suspension (population effect) and the biological history of the culture. Harrison (42) first described the population effect and showed that starved populations died at a slower rate as the density of the population increased, thus implying an interaction between the individuals of the population. At very dense population levels (above 1×10^9 cells/ml) he noted a reverse population effect which he attributed to anoxia. Postgate and Hunter (66) and Thomas and Batt (85) confirmed Harrison's finding although they did not observe the reverse effect. Postgate and Hunter (64, 65) observed that the faster the cells grew, the slower they died under starvation conditions. Strange et al. (82) found better survival for bacteria grown in a rich medium than for those grown in a poor one. Stokes and Parson (77) found that exponentially growing Sphaerotilus discophorus cells were more resistant to death than were cells from stationary phase while Harrison et al. (43) found the opposite to be true for A. aerogenes. Thomas and Batt (85) found that the phase of growth of Streptococcus lactis prior to starvation was not important for survival.

Postgate and Hunter (66, 67) first observed that the addition of the growth-limiting substrate[8] to a starving suspension of cells caused an increase in the death rate which they termed substrate accelerated death. They were able to show substrate accelerated death for cultures limited by carbon, nitrogen or phosphorus. Strange and Hunter (83) also noted substrate accelerated death by ammonium ions while Strange and Dark (81) confirmed Postgate and Hunter's data but observed that specificity among carbon substrates didn't occur, i.e., glucose or ribose was effective in accelerating the death of glycerol-limited cultures. This is in agreement with the data of Thomas and Batt (85) who found that carbohydrates accelerated the death of S. lutea irregardless of the limiting nutrient and that of Boylen and Ensign (8) who found that glucose accelerated the death of A. crystallopoietes grown in batch culture. Calcott and Postgate (15, 16) found that glycerol accelerated the death of Klebsiella aerogenes[9] only if the recovery medium contained glycerol and that 3'-5'-cyclic adenosine monophosphate prevented substrate accelerated death.

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[8] Prior to starvation the cells were grown in a continuous culture. The growth-limiting substrate is the compound which is added in limited quantity so as to keep the cells growing at a specific density of growth rate.

[9] Klebsiella aerogenes is not recognized as a valid taxon (13). The authors indicate that this strain was formerly known as Aerobacter aerogenes NCTC 418; i.e. Klebsiella pneumoniae.

Boylen and Pate (10) reported that starvation did not change the size or shape of rod or spherical cells of A. crystallopoietes but Luscombe and Gray (51) reported that 70 days of starvation would cause rod shape A. globiformis cells to revert to their spherical shape. MacKelvie et al. (52) reported that Pseudomonas aeruginosa cells grown on a chemically defined medium were small and stayed the same under starvation while cells grown on a rich medium decreased in size upon starvation but they failed to report the magnitude of this decrease. Ultrastructural changes of bacteria under starvation conditions were reported by Thomas et al. (89) who found unfolding of the mesomes and dislocation of the nuclear material after starvation of S. lactis. Boylen and Pate (10) reported an increase in vesicular membranes and nucleoplasm in addition to a decrease in ribosomes and glycogen deposits during the starvation of A. crystallopoietes.

MATERIALS AND METHODS

Organism, Media and Reagents

Ant-300, a psychrophilic marine vibrio isolated from surface water of the Antarctic Convergence during cruise #46 of the R/V ELTANIN, was used for this study. Due to the psychrophilic nature of this organism all equipment, media, and reagents were cooled to 4 C prior to use except where noted.

4% SALT MIXTURE (SM)

NaCl	26.0 g
KCl	0.8
$MgCl_2 \cdot 6H_2O$	5.6
$MgSO_4 \cdot 7H_2O$	7.6
Distilled water	1.0 liter

GLUCOSE MEDIUM (GM)

Glucose	2.5 g
Yeast extract (Difco)	0.2
$NaNO_3$	0.5
Na_2HPO_4	0.5
$FeSO_4$	0.0005
SM	1.0 liter
pH	7.5

BORATE SOLUTION

Boric acid	12.4 g
NaOH (1.0 N)	100.0 ml
Distilled water qs	1.0 liter

BORATE BUFFERED SM (BSM)

NaCl	26.0 g
KCl	0.8
$MgCl_2 \cdot 6H_2O$	5.6
$MgSO_4 \cdot 7H_2O$	7.6
HCl (0.1 N)	43.5 ml
Borate solution	52.5
Distilled water	904.0
pH	7.5

Glucose and dibasic sodium phosphate were autoclaved separately as 25 and 5% solutions, respectively. The solid medium contained 1.2% agar as a solidifying agent.

Ant-300 was maintained on GM slants covered with sterile mineral oil stored at 1 C. The cells were starved in SM buffered with sterile Na_2HPO_4 (final concentration 0.05%) unless otherwise noted. For phosphate-free starvation BSM was used. Starvation at various salt concentrations was conducted using buffered SM with the salt concentrations adjusted proportionately.

LIB-X MEDIUM

Trypticase (BBL)	2.3 g
Yeast extract (Difco)	1.2
Sodium citrate	0.3
L-glutamic acid	0.3
$NaNO_3$	0.05
$FeSO_4$	0.005
Rila salts	38.0
Distilled water	1.0 liter
pH	7.5

SDB MEDIUM

Glucose	0.5 g
Succinic acid	0.2
Polypeptone (BBL)	5.0
Yeast extract (Difco)	3.0
NaCl	15.0
Rila salts	5.0
$FeSO_4$	0.005
Distilled water	1.0 liter
pH	7.5

MEDIUM 2216E

Neopeptone (Difco)	5.0 g
Yeast extract (Difco)	1.0
$FePO_4$	0.1
Rila salts	26.25
Distilled water	1.0
pH	7.5

The pH adjustments were made with 2.0 N NaOH or HCl. Solid media contained 1.2% agar. GM, Lib-X, SDB, and 2216E were prepared full strength as presented and 0.1X strength by adding one-tenth the amount of nutrients with the exception of SM, NaCl, rila salts, and agar which were added in the normal amount.

Buffered SM, as free from organic material as possible, was prepared by placing the salts in a furnace at 500 C for 2 h followed by dissolution in freshly prepared double distilled water. The pH of the solution was adjusted to 7.5 with HCl. The solution was sterilized by filtration through a sterile distilled water washed Nuclepore filter. All glassware used in this preparation was soaked in a saturated solution of $Na_2Cr_2O_7 \cdot 2H_2O$ in concentrated sulfuric acid for 24 h and washed with double distilled water prior to use.

An amino acid mixture was prepared to simulate the natural concentrations of amino acids found in seawater as determined by Hobbie et al. (46). The composition of this mixture is shown in Table 2. A 100X strength stock solution was prepared, filter sterilized, and frozen until used.

Natural seawater was obtained from a surface water sample collected approximately 20 miles off the coast of Newport, Oregon. The water was filter sterilized after collection and stored at 1 C until used.

A stock solution of nalidixic acid (Calbiochem) containing 10 mg/ml in 0.1 N NaOH was prepared and filter sterilized immediately before use.

Chitin, purified from the cuttlebones of commercially-available squid (Loligo opalescens), was kindly provided by Dr. Thomas Goodrich. The chitin was suspended in distilled water to a concentration of 0.2 g/ml and sterilized by autoclaving for 30 min at 121 C and 15 psi.

Omnifluor (4 g Omnifluor (New England Nuclear) per liter toluene) and Triton X-100 fluor (8 g Omnifluor per 500 ml Triton X-100 (Rohm and Haas) plus 1 liter toluene) were used as liquid

Table 2. Composition of amino acid mixture as found in seawater.[a]

Amino acid	Concentration (μg/liter)
L-Glycine	16.85
L-Serine	4.91
L-Ornithine	3.17
L-Aspartic Acid	1.92
L-Threonine	1.50
L-Alanine	1.49
L-Methionine	1.31
L-Glutamic Acid	1.00
L-Histidine	0.95
L-Lysine	0.95
L-Valine	0.82
L-Tyrosine	0.75
L-Leucine	0.63
L-Proline	0.58
L-Isoleucine	0.57
L-Arginine	0.55
L-Phenylalanine	0.18

[a] After Hobbie et al. (46).

scintillation cocktails.

Growth and Starvation Conditions

Ant-300 was grown in 100 ml of medium in a 250 ml Erlenmeyer flask at 5 C on a reciprocating shaker (New Brunswick Psychrotherm), harvested during the exponential phase of growth (80 h after inoculation unless otherwise noted) by centrifugation for 15 min at 4,100 x g and 4 C, and washed twice with cold SM. Cell concentrations of 10^2 to 10^7 cells/ml were suspended in 200 ml of buffered SM (5 C) in a 1 liter Erlenmeyer flask which was shaken at 5 C except as noted. Cells were grown in GM unless otherwise noted. Growth and starvation were monitored spectrophotometrically using a Beckman DB spectrophotometer operating at 600 nm with a light path of 1 cm.

Viability Assessment

Viability was determined by the spread plate technique. After the appropriate dilution of the starving suspension in SM, 0.1 ml was spread on the surface of an agar plate. Duplicate determinations were made in all cases and cells were considered viable if they could produce a visible colony after 1 wk incubation at 5 C. GM agar was used except where noted. Direct counts were obtained using epifluorescence microscopy. A portion of the starving cell suspension was fixed with glutaraldehyde (final concentration 1.0%) at 5 C for

1 h. The cells were then filtered onto a 0.2 μm Nuclepore filter, stained with acridine orange according to the method of Zimmerman and Meyer-Reil (96), and observed with a Zeiss epifluorescent microscope. The cells in at least 50 fields from the center to the edge of the filter were counted and the number of cells/ml of the original suspension was calculated using an area conversion factor between the microscopic field and the working surface of the filter.

Starvation under Hydrostatic Pressure

Duplicate washed cell suspensions suspended in SM were loaded into 3-ml disposable syringes (Becton, Dickinson and Co.) which were capped with sealed hypodermic needles and placed into precooled pressure cylinders. Pressure was immediately applied and the cylinders were placed in a water bath at 5 C. Samples were removed at various times from separate cylinders and assayed for viability.

Light Microscopy

Heat fixed smears were stained with crystal violet for 1 min and observed under oil immersion. Photomicrographs were recorded on Ilford FP4 film using a Leitz Orthomat camera and a Leitz Ortholux microscope.

Electron Microscopy

Cells starved for 5 wk and exponentially growing cells were harvested by centrifugation for 30 min at 7,700 x g and 4 C. Both pellets were then fixed with 3% (v/v) glutaraldehyde in SM buffered with Sorenson phosphate buffer (37) (pH 7.4, 0.125 M) for 3 h at 0 C, embedded in 2% agar and postfixed in 1% (w/v) OsO_4 for 3 h at 4 C. The fixed preparations were stained with uranyl acetate (saturated solution in 70% ethanol) for 3 h during dehydration in a graded ethanol series and embedded in a modified Mollen-Hauer (57) resin containing 53% dodecenyl succinic anhydride, 35% Araldite 6005, and 12% Epon 812. Sections were cut with a diamond knife, stained with lead citrate (68), and observed using a Philips EM300 electron microscope operating at 60 kv.

Preparation of ^{14}C labeled Cells

Prior to starvation cells were grown for at least two generations in GM containing ^{14}C-(UL)-glucose (specific activity 4.06 mCi/mmole; final concentration 0.2 μCi/ml). Unlabeled glucose was omitted from the medium.

Determination of Endogenous Metabolism

At various times during starvation 5 ml portions of a ^{14}C labeled

starving cell suspension were placed into four 50 ml serum bottles and capped with a rubber stopper fitted with a cup and filter paper assembly. Two of the bottles were placed in an incubator at 5 C. After an incubation period sufficient to produce a significant quantity of $^{14}CO_2$ (3-24 h), 1 ml of 3.0 N HCl was injected through the cap into the sample and 0.2 ml of β-phenethylamine was injected onto the filter paper to collect the evolved $^{14}CO_2$. After shaking for 20 min at room temperature the filter paper was removed and placed into a scintillation vial. The contents of the serum bottles were then filtered through 0.45 μm Millipore Type HA filters and washed twice with 5 ml portions of cold SM. The filters were dried and placed into scintillation vials. The filter paper and membrane filters were then covered with 5 ml of Omnifluor and the radioactivity was determined using a Nuclear Chicago Mark I liquid scintillation counter. The remaining two bottles which served as blanks were injected with acid and treated as described above immediately after collection of the sample. The amount of $^{14}CO_2$ contained in the starvation menstruum at time 0 and was subtracted from the value obtained from the incubated samples. Also at the time of collection the total cellular carbon was determined as follows. Two 5 ml samples of the starving suspension were filtered through 0.45 μm filters and washed twice with 5 ml portions of cold SM. The filters were dried and assayed for radioactivity as described. Endogenous respiration was calculated

as the amount of $^{14}CO_2$ evolved per h expressed as a percentage of the total cellular carbon. The amount of carbon present in the cells in substrate pools and low molecular weight metabolites was calculated from the difference in counts between the salt washed and acid treated cells (5).

Protein and DNA Determination

Protein and DNA content of both the starving cells and the starvation menstruum was determined. At various times during starvation 5 ml portions of a ^{14}C labeled starving cell suspension were removed and centrifuged at 4,100 x g for 15 min at 4 C. The supernatant was decanted and the pellet was resuspended in 1 ml of 1.0 N NaOH. Both the supernatant and the resuspended pellet were then frozen until assayed. Prior to the assay all samples were thawed at room temperature and the tubes containing the cellular fractions were placed in a boiling water bath for 15 min to assure cellular lysis. All samples were then cooled on ice and protein and DNA were determined according to the procedure of Kennell (48). The isolated DNA fragments were assayed for radioactivity as described previously after mixing 1 ml of the preparation with 10 ml of Triton X-100 fluor. The dried filter containing the protein was placed in a scintillation vial, covered with 5 ml of Omnifluor and counted as described previously.

Observation of Cellular DNA

Cellular DNA was observed after Feulgen staining of the cells. The technique of Feulgen and Rossenbeck (36) was modified to produce the best microscopic image for Ant-300 as follows. Ten ml of a culture was fixed with glutaraldehyde (final concentration 5%) for 2 h at 5 C, centrifuged at 4,100 x g for 15 min at 4 C, washed twice, and resuspended in sodium phosphate buffer (pH 7.1, 0.1 M). A drop of the suspension was then spread on a microscope slide, air dried, fixed in 70% ethanol for 5 min, and washed with distilled water. The slide was then immersed in 1.0 N HCl for 15 min at 60 C. After a distilled water wash the smear was covered with a basic fuchsin dye solution for 1 h. The slide was then washed with sulfite solution three times (5 min each) followed by distilled water. After air drying the cells were viewed with a phase contrast microscope. The dye solution was prepared by dissolving 5 g basic fuchsin (BBL) in 1 liter boiling water. After cooling to 50 C, 10 g Na_2SO_3 and 100 ml 1.0 N HCl were added and the solution was stored overnight in the dark. Five g of Norite was then added and the solution was filtered until clear. The resulting filtrate was stored at 1 C in the dark until used. The sulfite solution was prepared by combining 10 ml of 1.0 N HCl, 10 ml of a 5% solution of Na_2SO_3 and 180 ml of distilled water. When viewed under phase contrast dark bands were observed within the cells which

were designated as nuclear bodies. The nuclear bodies in at least 100 cells were counted and the average number of nuclear bodies per cell was calculated.

Viable Cell Size Determination

Viable cell size during starvation was determined by passing portions of the starving cell suspension through Nuclepore polycarbonate filters with different pore sizes. Plate counts of the filtrate were expressed as a percentage of the counts obtained from the non-filtered sample.

Respiration of Exogenous L-glutamic Acid during Starvation

The ability of small starving cells to respire glutamate after 34 days of starvation was determined by diluting a portion of the starving suspension that had been filtered through a 0.4 μm Nuclepore filter 1:100, 1:200 and 1:400 with buffered SM and placing 5 ml aliquots into 50 ml serum bottles. One-tenth ml of ^{14}C-(UL)-L-glutamic acid (20 μCi/ml; 237 mCi/mmole) was added to each bottle which was then capped with a rubber stopper fitted with a cup and filter paper assembly. The bottles were then incubated at 5 C with shaking and at various times duplicate samples were removed and assayed for $^{14}CO_2$ evolution as previously described. The rate of respiration

was expressed as the rate of $^{14}CO_2$ evolution after conversion to pmole glutamate equivalents. The respiration rate of 10^6 viable cells was calculated by extrapolating the graph of viable cells vs. the rate of respiration for the three dilutions used. Respiration of L-glutamic acid by nonstarved cells was determined in the same manner after the appropriate dilutions of a washed exponentially growing culture.

Cryptic Respiration

The ability of starving cells to take up and respire materials released from other starving cells was determined by starving two cell populations, one uniformly labeled with ^{14}C and one unlabeled. At various times 15 ml of the labeled suspension was filtered through a 0.2 μm Nuclepore filter. At the same time 10 ml of the unlabeled suspension was centrifuged at 4,100 x g for 15 min at 4 C. The supernatant from the unlabeled culture was discarded and the pellet was resuspended in 10 ml of the labeled filtrate. The suspension was then divided into two 5 ml aliquots each placed into a serum bottle which was capped with a rubber stopper and filter paper assembly. The bottles were incubated at 5 C with shaking for 24 h. The remaining 5 ml of the filtrate served as a blank and was placed into a capped serum bottle and incubated with the others. After incubation, the amount of material associated with the cells and the $^{14}CO_2$ evolved was determined as described previously.

RESULTS

Ant-300, a typical psychrophile (60), has a growth range in GM with 4% SM from less than -1 to 13.5 C and grows optimally from 4.5 to 7.5 C. At 5 C Ant-300 will grow over a salt concentration range from 2 to 7.5% SM with an optimum near 4%. Ant-300 cannot divide at hydrostatic pressures greater than 400 atm (6).

When placed into a starvation menstruum under optimum conditions of 5 C and 4% SM, Ant-300 increased in numbers during the first wk of starvation (Fig. 1). The total number of cells continued to increase slightly after the first wk of starvation while the viable cells decreased in number. After 2 wk 50% of the total number of cells were still viable while 50% of the initial number of cells remained viable for 5 to 7 wk. After one year of starvation 10^3 cells/ml were still viable. Extensive cellular lysis was not noted during starvation as judged by the absence of cellular debris during microscopic observation of the starving suspension and by the absence of a significant decrease in the total number of cells.

Also during the first wk of starvation the optical density of the starving cell suspension decreased 62% (Fig. 2). After 7 wk of starvation the optical density decreased an additional 21%.

Starvation in 4% SM at 5 C or less was similar to starvation at 5 C, however at 10 C the initial increase in cell numbers was only

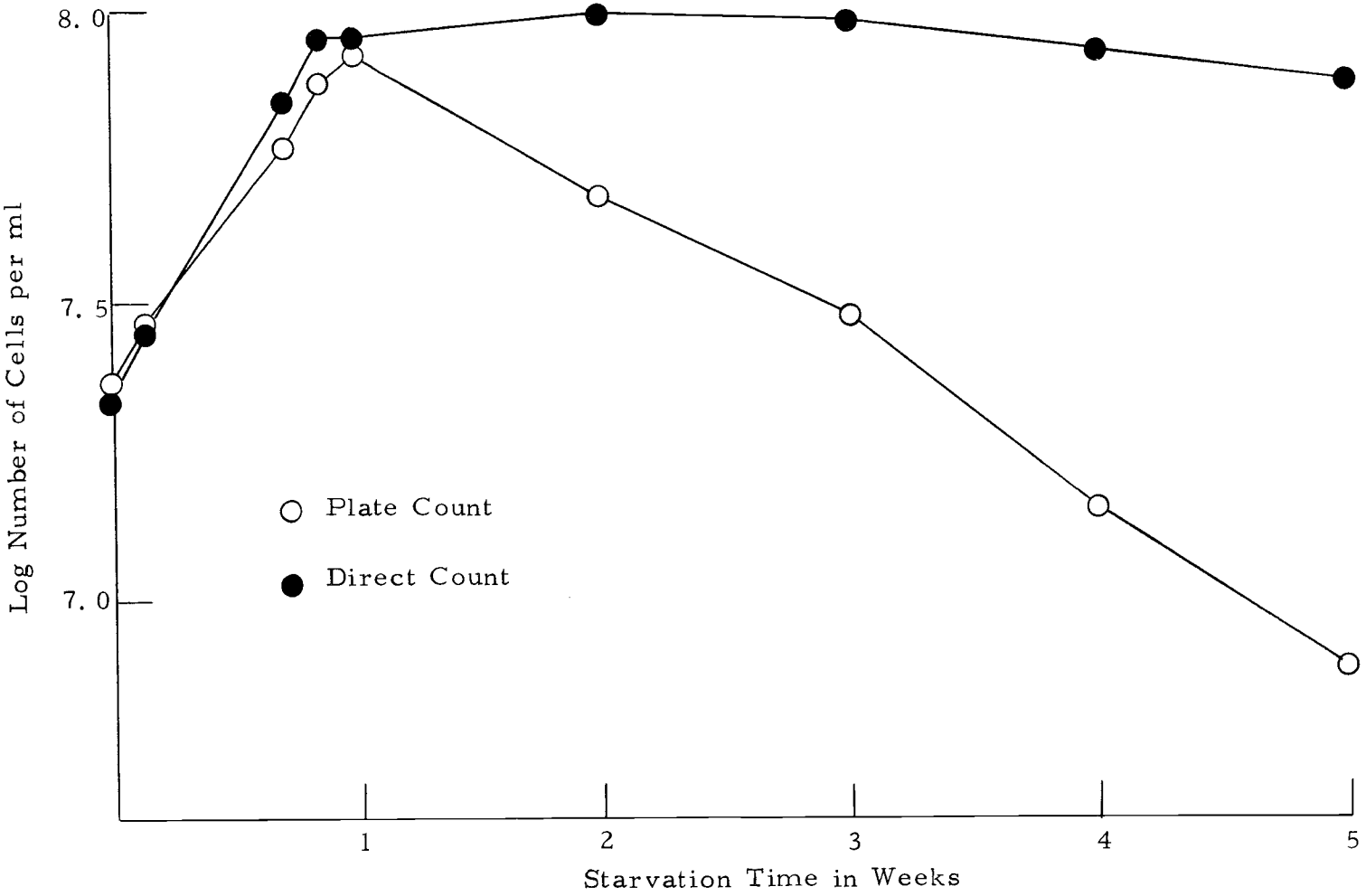

Fig. 1. Direct and viable counts of a starving population of Ant-300. Cells were harvested from exponential growth, washed, and starved in a buffered salt mixture.

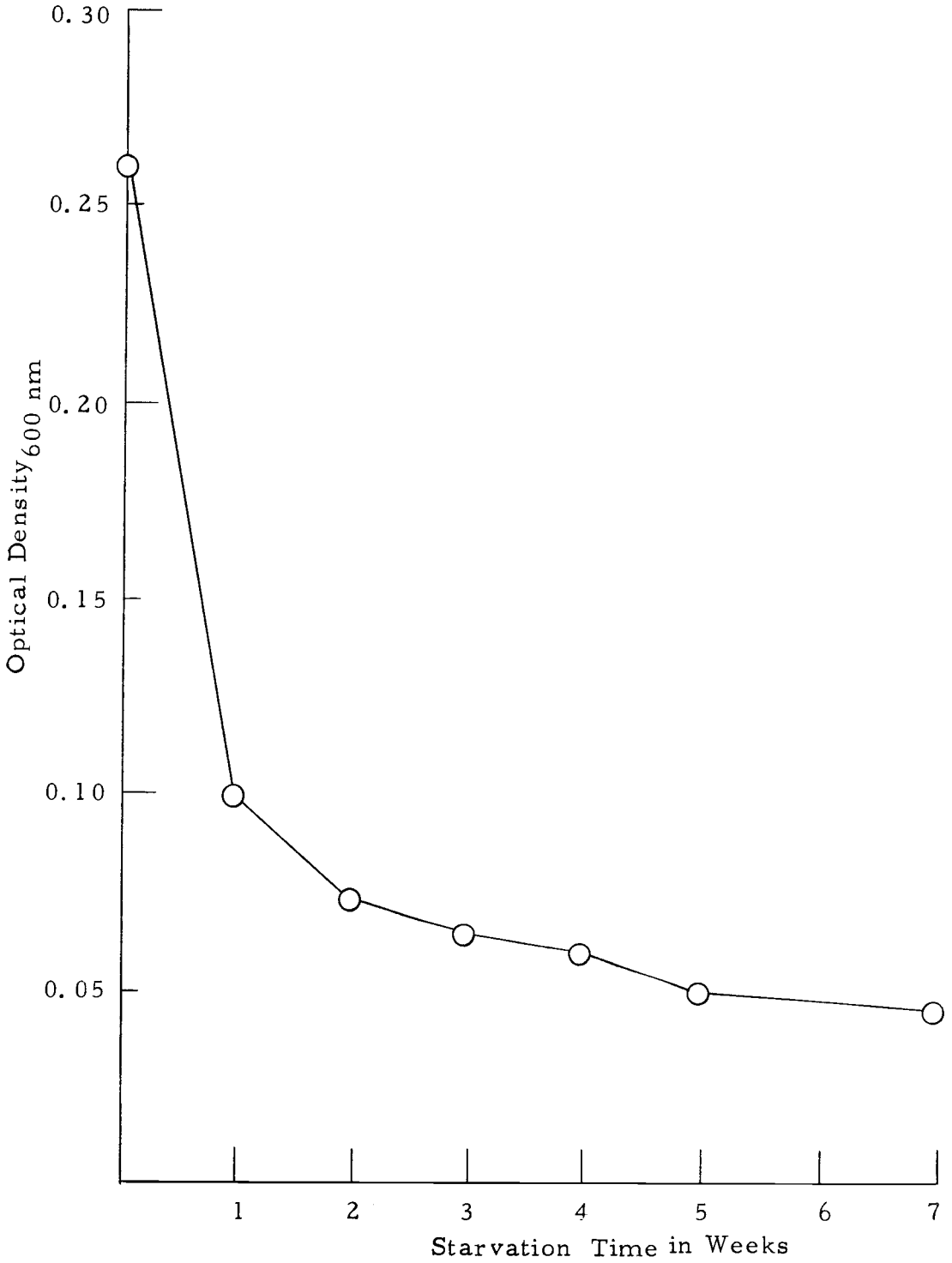

Fig. 2. Optical density of a starving population of Ant-300.

50% and 50% of the initial number of cells remained viable for only 2 wk. Starvation at 5 C at various salt concentrations showed the optimum for survival was 4%. In 2 or 6% SM, 50% of the initial number of cells remained viable for only 3 wk. In 2% SM, no increase in cell numbers was noted. The viability of Ant-300 under starvation conditions at 5 C, 4% SM, and under hydrostatic pressure is presented in Table 3. No increase in cell numbers was noted and even under moderate pressure the viability was drastically reduced within a few days. However, if the cells were starved for 1 wk prior to pressurization of the starving suspension, 100% viability (compared to a cell suspension starving at 1 atm) was maintained for over 6 wk at 250 atm.

Cells grown in Lib-X medium and subsequently starved did not survive as well as cells grown in GM (Fig. 3). When starved in phosphate-free BSM, Ant 300 showed the same survival pattern as when starved in phosphate buffered SM.

The increase in cell numbers upon starvation was found to be dependent on the age of the culture prior to starvation as shown in Fig. 4. Generally the older the culture, the greater the increase. Increases from 100 to 800% of the initial number of cells were noted from logarithmically growing cultures (65 to 115 h after inoculation). Irregardless of the initial increase in cell numbers, 100 to 200% of the initial number of cells were still viable after 6 wk of starvation.

The effect of various media on the recovery of Ant-300 after

Table 3. Starvation of Ant-300 under hydrostatic pressure.

Time (days)	Percent of the initial number of cells				
	1 atm	100 atm	200 atm	300 atm	400 atm
0	100	100	100	100	100
0.5	126	-	-	71	46
1	159	72	46	30	33
2	246	74	69	37	-
3	251	56	50	-	-
5	312	62	-	-	-

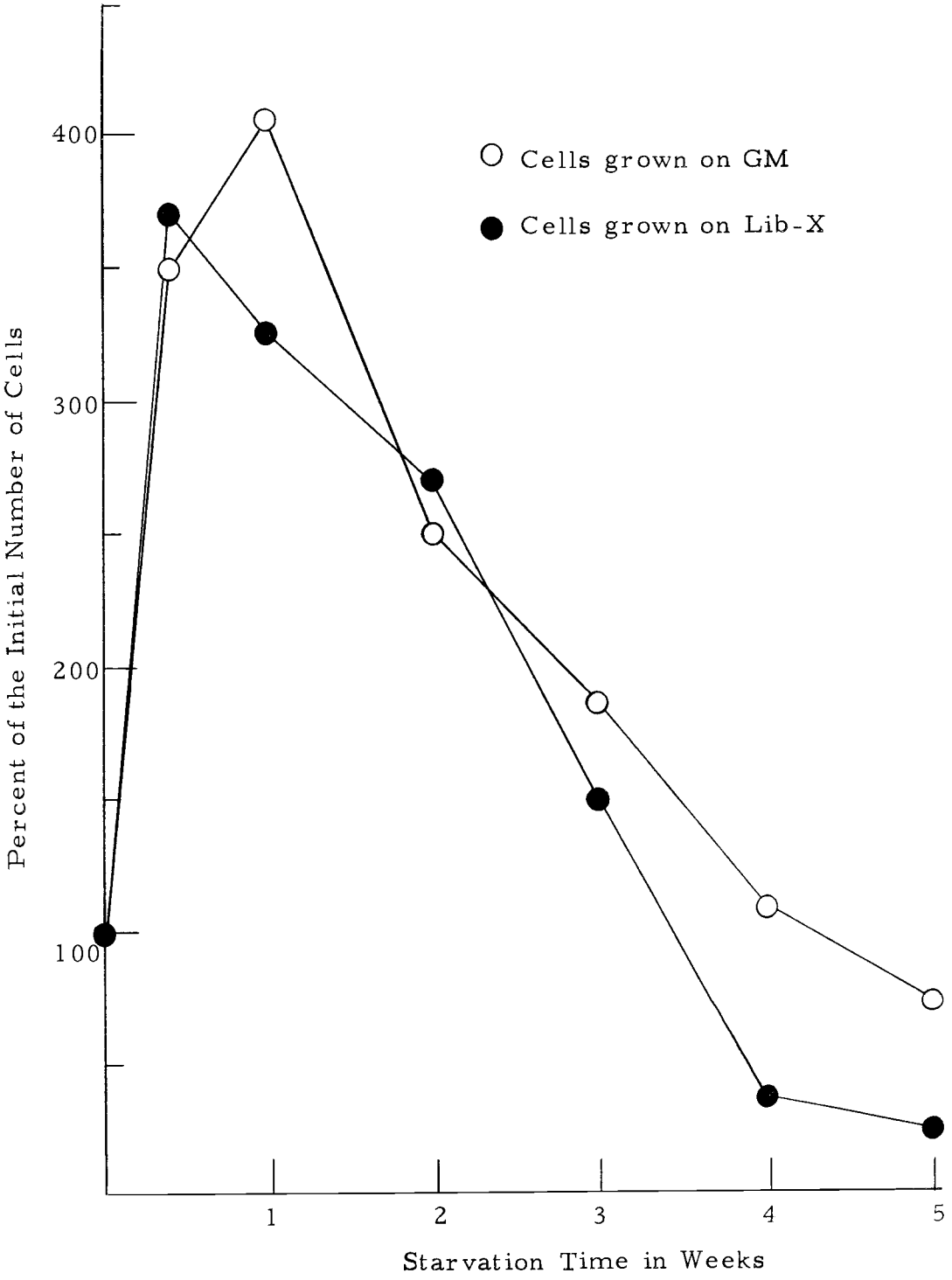

Fig. 3. Viability of a starving population of Ant-300 grown on Lib-X. Previous to starvation cells were grown in either GM or Lib-X. Viability was determined by plate counts on the respective medium.

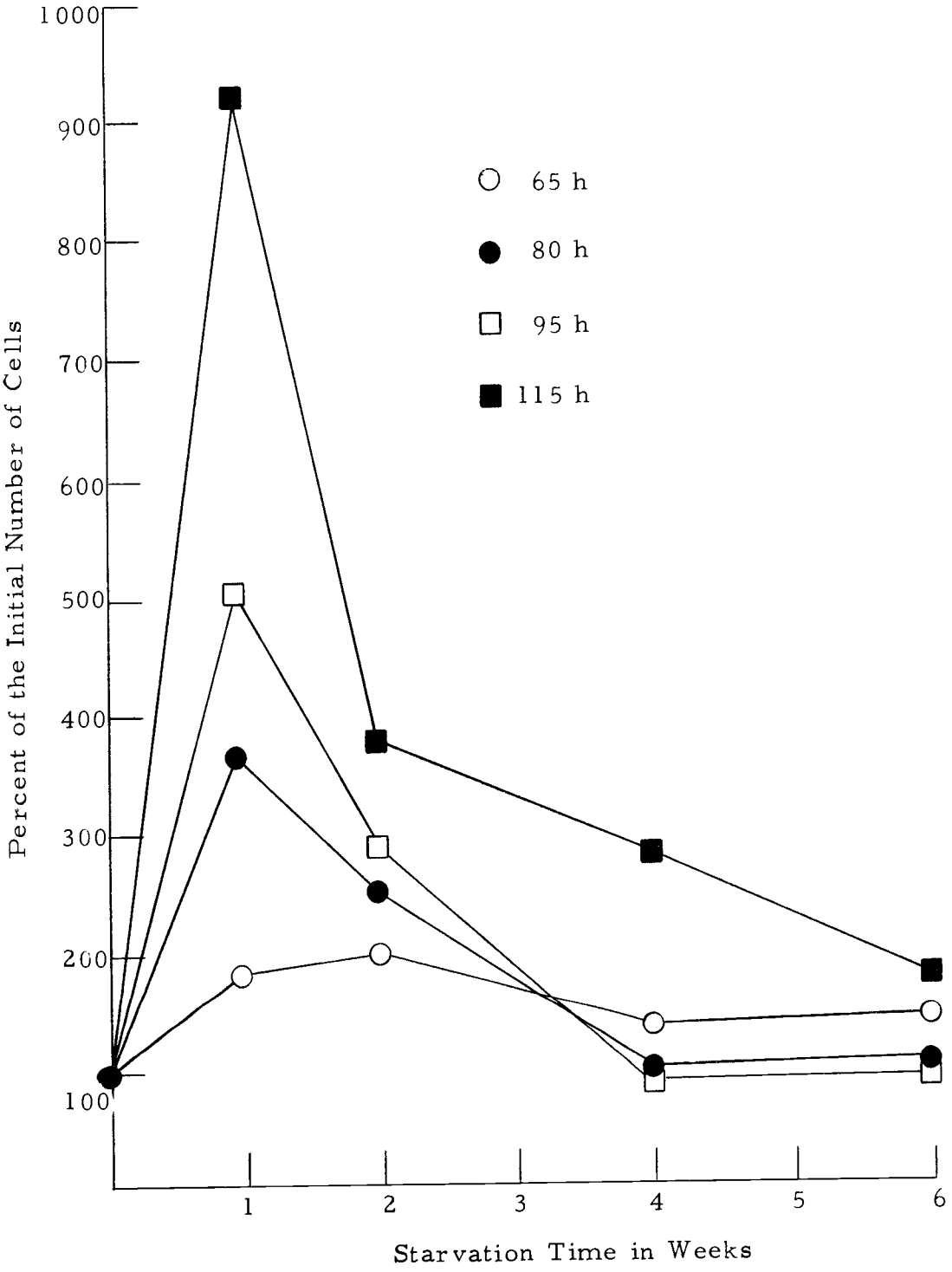

Fig. 4. Viability of Ant-300 starved from various periods of growth. Ant-300 was harvested after various periods of growth, washed and starved in a buffered salt mixture. Viability was determined by plate counts.

four and 26 days of starvation is shown in Table 4. After 4 days of starvation greater than 90% of the cells able to form colonies on GM agar also grew on the other media tested with the exception of 0.1X strength GM. Similar results were obtained after 26 days of starvation with the exception of 2216E agar which only supported the growth of 69% of the cells able to grow on GM agar. In all cases media prepared at 0.1X strength gave higher counts than did the full strength media with the exception of GM agar.

The endogenous respiration of the starving cells is shown in Fig. 5. During the first two days of starvation, endogenous respiration decreased over 80%. After 7 days, respiration had been reduced to 0.0071% per h and remained constant thereafter.

Total cellular carbon measured as ^{14}C DPM decreased rapidly during the first three days of starvation after which time it continued to decrease but at a slower rate as shown in Fig. 6. The amount of radioactivity associated with the cells after acid washing (Fig. 6) decreased then increased sharply during the first 6 h of starvation. After 24 h it decreased at a steady rate. The difference between the salt washed and acid treated cells decreased during the first 21 days of starvation after which time no significant difference was observed.

The respiration of L-glutamic acid by starved and nonstarved cells is shown in Fig. 7. After a 15 min lag period, nonstarved cells respired labeled glutamate at a rate of 0.49 pmoles/min/10^6 viable

Table 4. Starvation recovery of Ant-300 on various media.

Medium	Percent viable cells	
	After 4 days of starvation	After 26 days of starvation
GM[a]	100	100
0.1X GM	7	11
Lib-X	93	94
0.1X Lib-X	108	105
SDB	98	96
0.1X SDB	116	99
2216E	95	68
0.1X 2216E	101	93

[a]Arbitrarily set at 100%

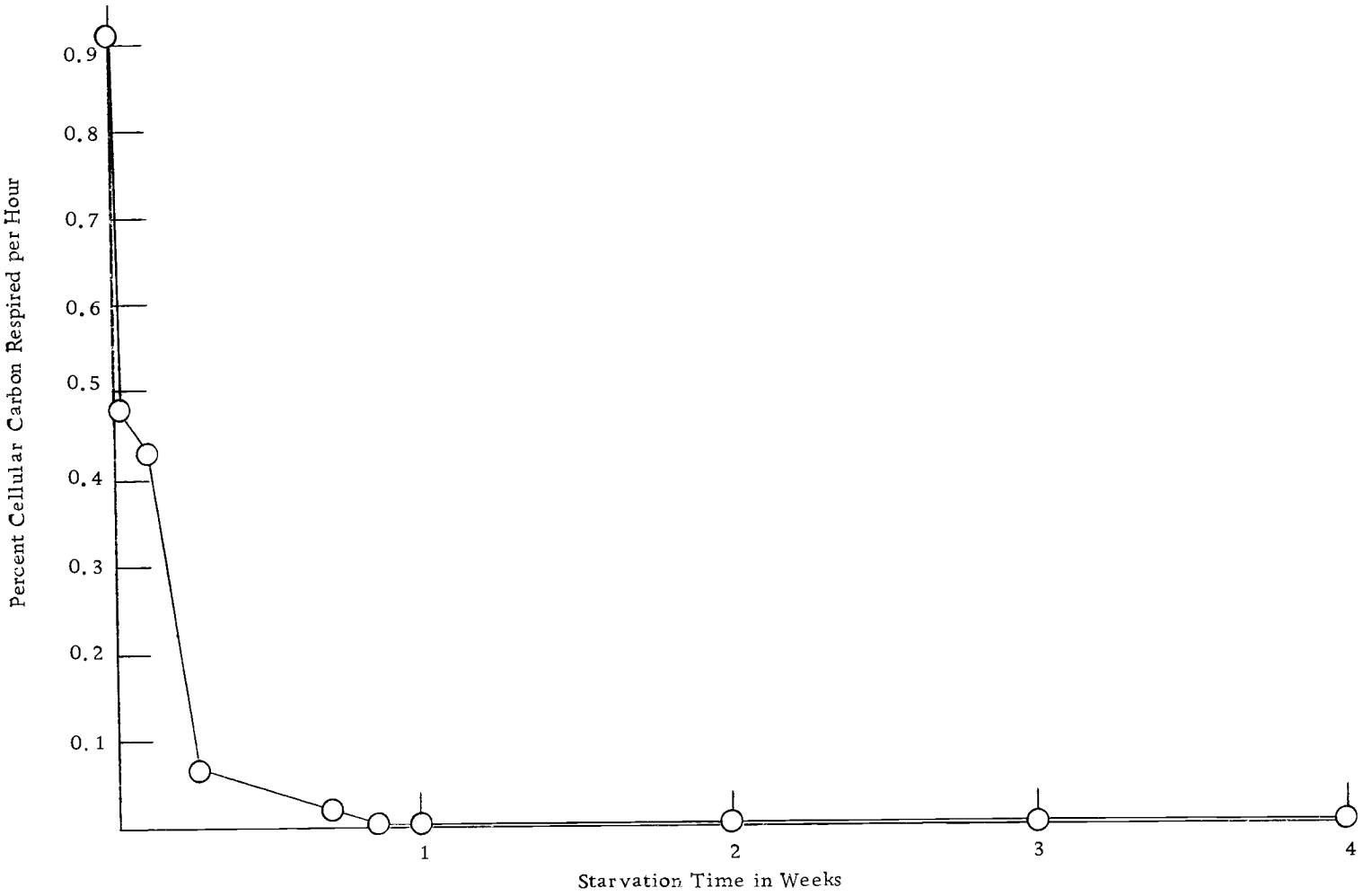

Fig. 5. Endogenous respiration of starving Ant-300 cells. Previous to starvation Ant-300 was grown in ^{14}C-glucose. At various times during starvation a portion of the suspension was removed and the amount of $^{14}CO_2$ evolved was measured as a function of time and the total amount of ^{14}C associated with the cells.

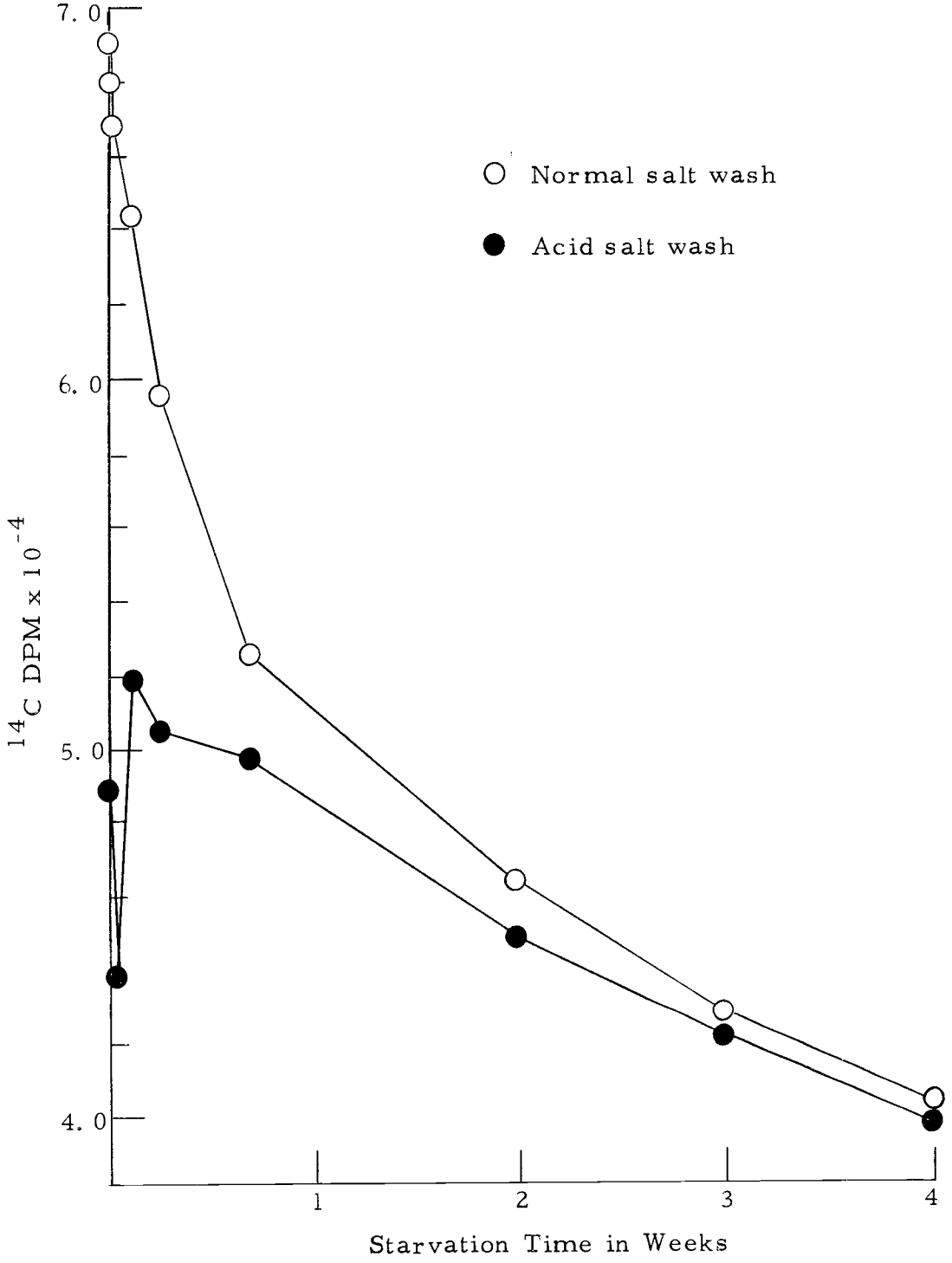

Fig. 6. Cellular carbon of Ant-300 during starvation. A ^{14}C labeled culture of Ant-300 was starved and at various times 5 ml portions were removed, filtered, and washed with SM or SM containing HCl.

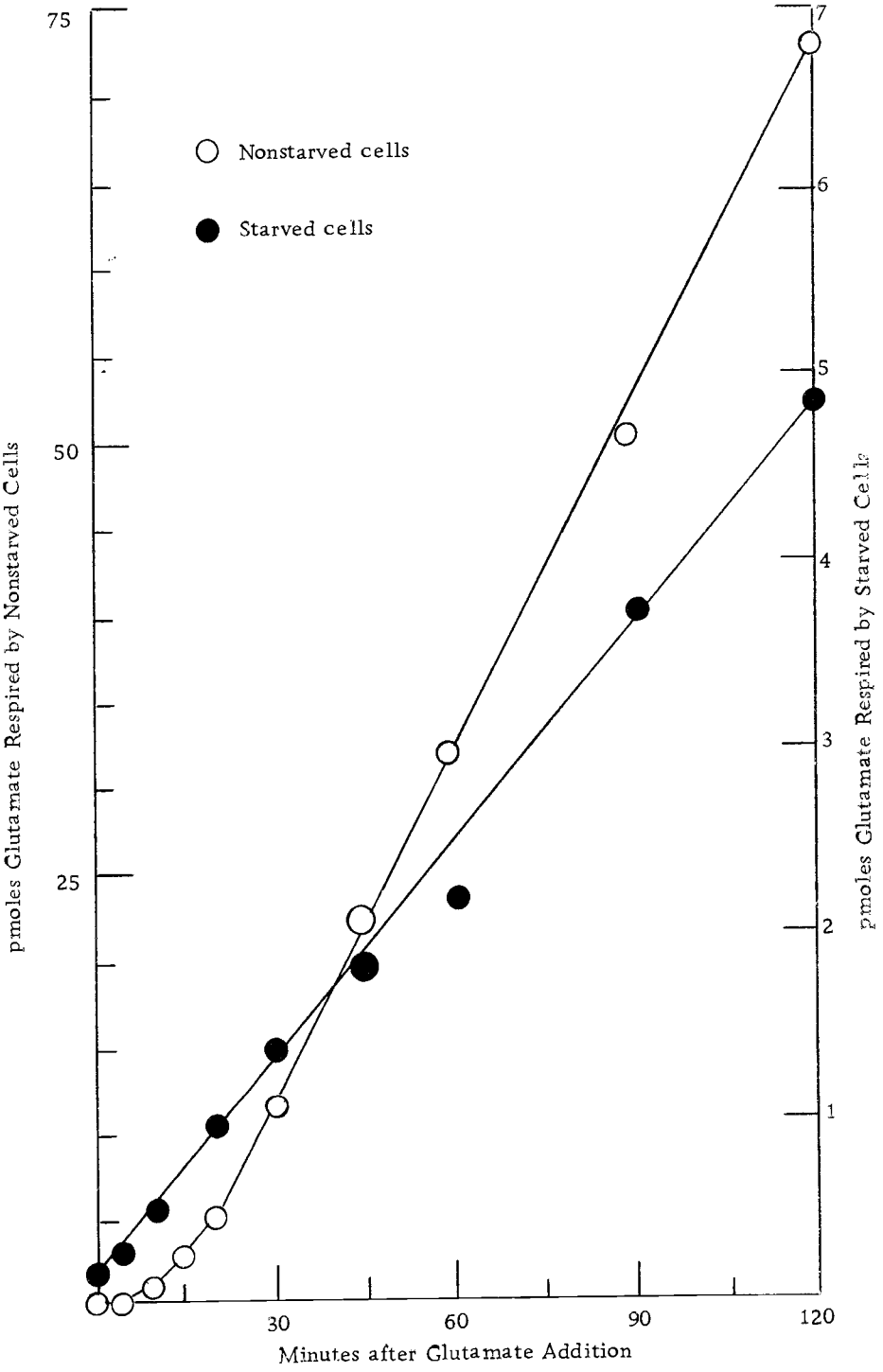

Fig. 7. Respiration of L-glutamic acid by starved and nonstarved Ant-300 cells. Nonstarved cells and cells starved for 34 days and passed through a 0.4 um filter were assayed for glutamate respiration. ^{14}C glutamic acid was added to 5 ml portions of the cell suspensions and the amount of $^{14}CO_2$ evolved was measured at various times. The nonstarved suspension contained 4.6×10^5 viable cells/ml and the starved suspension contained 5.6×10^4 viable cells/ml.

cells. Cells starved for 34 days and passed through a 0.4 μm filter respired labeled glutamate without a lag period at a rate of 0.31 pmoles/min/10^6 viable cells, i.e., a 36% reduction from the rate for nonstarved cells on a per viable cell basis. A linear relationship was found between the rate of $^{14}CO_2$ evolution and the number of viable cells for both the starved and nonstarved cells, however the slopes of the lines differed: 5.6×10^{-7} pmoles/min/viable cell for nonstarved cells and 2.7×10^{-7} pmoles/min/viable cell for starved cells.

The uptake and respiration of materials released from starving cells is shown in Table 5. During the 6 wk of starvation the cells were able to take up only 4.8% of the released materials during the first 24 h of incubation and only 0.6% during the last incubation period. The starving cells were able to respire 10.6% of the carbon released from the starving cells during the first 24 h incubation period. After this time no further respiration of the leakage materials was noted. After 6 wk of starvation, the labeled filtrate was added to a suspension of washed, exponentially growing cells. These nonstarved cells were only able to take up and respire 6.65 and 0.52%, respectively, of the labeled materials during a 24 h incubation.

The DNA and protein content of a starving cell population and the starvation menstruum is shown in Fig. 8. Cellular DNA decreased rapidly during the first 14 days of starvation. Thereafter

Table 5. Uptake and respiration of starvation leakage material by a starving population of Ant-300.

Starvation time (days)	Percent leakage material taken up[a]	Percent material taken up converted to CO_2[a]
0	4.8	10.6
1	2.8	0
2	1.3	0
3	1.7	0
4	2.5	0
5	1.1	0
6	1.1	0
7	1.1	0
14	1.1	0
21	0.9	0
28	0.7	0
35	1.0	0
42	0.6	0

[a]During a 24 h incubation period.

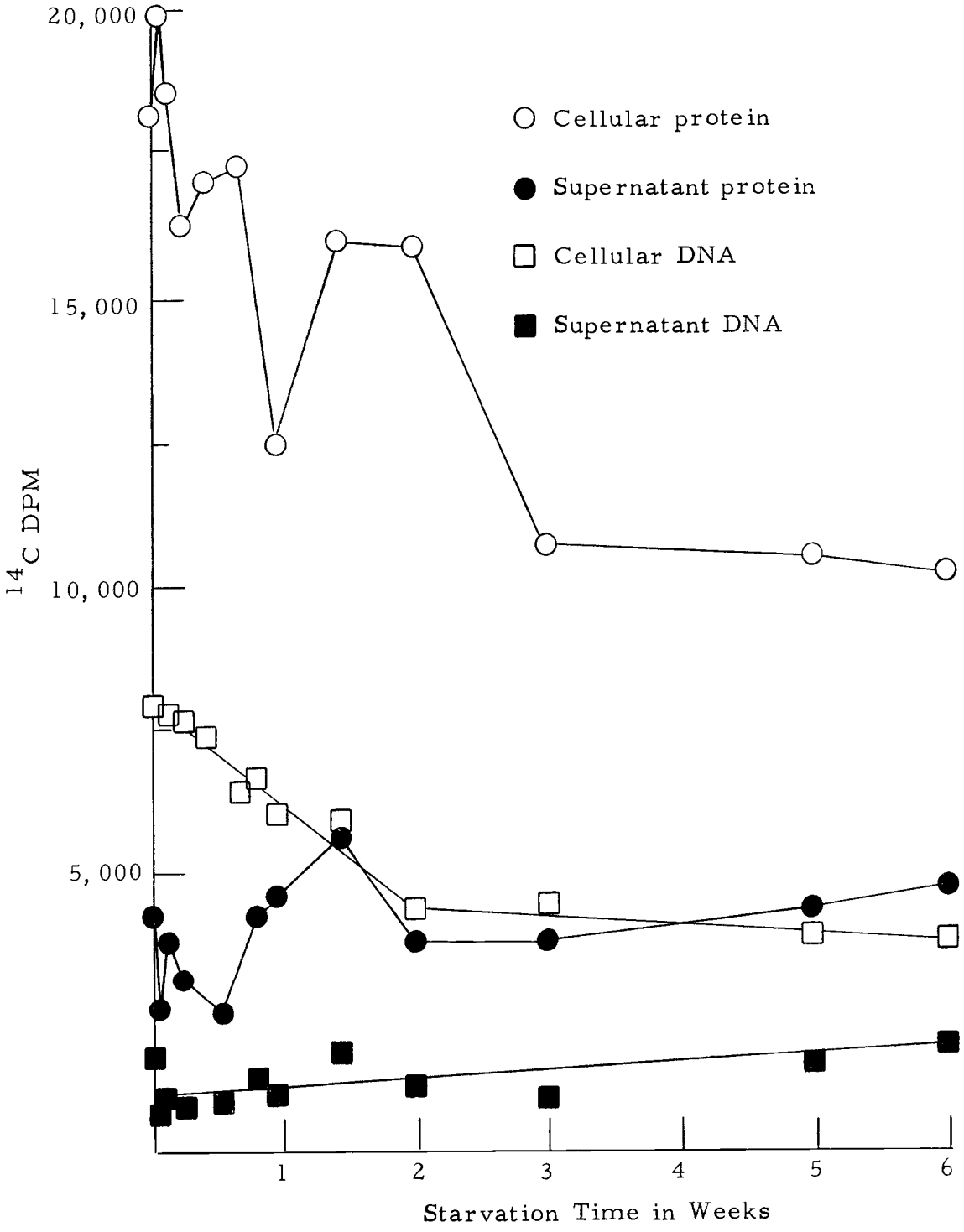

Fig. 8. DNA and protein content of starving Ant-300 cells. At various times during starvation 5 ml portions of the cell suspension were removed and centrifuged to pellet the cells. DNA and protein were determined for both the cells and the menstruum. Prior to starvation the cells were uniformly labeled with ^{14}C.

it continued to decrease slightly. During 6 wk of starvation the cellular DNA decreased 46%. The DNA released into the starvation menstruum could account for only 5% of this decrease. After fluctuating for 21 days the cellular protein remained constant for the remaining 3 wk of starvation. During the first 7 days of starvation the protein content of the cells and the menstruum were correlated: as the content of the cells increased, the content of the menstruum decreased. After day 7, the menstruum protein concentration slightly decreased then increased while the cellular protein concentration increased then decreased sharply before reaching a constant level after 21 days of starvation. After 6 wk of starvation, the protein associated with the cells showed a net decrease of 43%. The net increase of protein detected in the menstruum could account for only 6% of this amount.

The average number of nuclear bodies per cell of an exponentially growing culture varied from 1.44 to 4.02 depending on the age of the culture as shown in Table 6. To determine the significance of these multiple nuclear bodies during starvation a culture of Ant-300 was grown and at various times portions were removed and prepared for starvation. At the same time the average number of nuclear bodies per cell of each culture portion was determined. Plate counts were made during starvation and, as can be seen in Fig. 9, a linear relation was found between the average number of nuclear bodies

Table 6. Average number of nuclear bodies per cell in an exponentially growing Ant-300 culture.

Time after inoculation (h)	Average number of nuclear bodies per cell
63	1.44
71	3.17
78	2.86
82	3.13
95	4.02

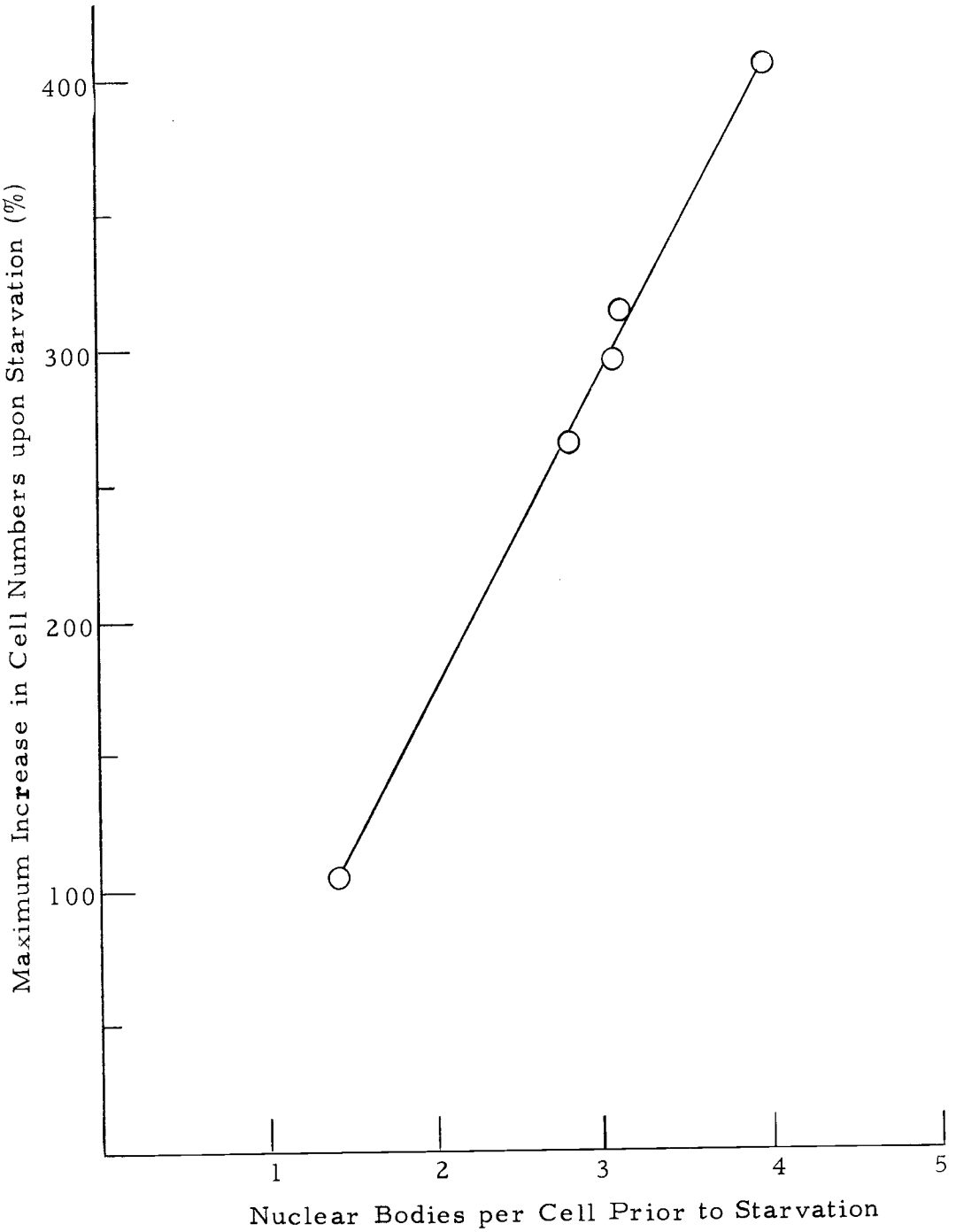

Fig. 9. Nuclear bodies vs. maximum increase in cell numbers. At various times during the growth of Ant-300 portions were removed and prepared for starvation. At the same time the average number of nuclear bodies per cell of each portion was determined by Feulgen staining. The maximum increase in cell numbers during starvation was determined by plate counts.

per cell of each culture portion was determined. Plate counts were made during starvation and, as can be seen in Fig. 9, a linear relation was found between the average number of nuclear bodies per cell and the percent increase in cell numbers. No relationship was found between the average number of nuclear bodies per cell and the age of the culture.

Nalidixic acid added to an exponentially growing population of Ant-300 (final concentration 20 μg/ml) caused growth to cease after 1 h followed by a decrease in viable cells (Fig. 10). However, nalidixic acid added to a population of cells beginning starvation had no effect on the initial increase in cell numbers.

Ant-300 cells decreased in size and changed in shape during starvation (Fig. 11). After 1 wk and 6 wk of starvation the cells appeared as small rods and small cocci, respectively. Filtration of the population through filters of various pore sizes showed the cell size distribution during starvation as presented in Fig. 12. Size reduction was most rapid during the first two days of starvation and continued for 3 wk. After 2 wk of starvation, 100% of the viable cells were able to pass through 3.0 and 1.0 μm filters. After 3 wk of starvation 100% of the viable cells were able to pass through 0.8 and 0.6 μm filters. Initially no cells were capable of passing through a 0.4 μm filter but 3 wk of starvation resulted in filterable viable cells (50%). After 4 wk of starvation, 100% of the initial number of cells were still viable. At no time during starvation (up to and including 65 wk) were any cells able to pass through a 0.2 μm Nuclepore filter

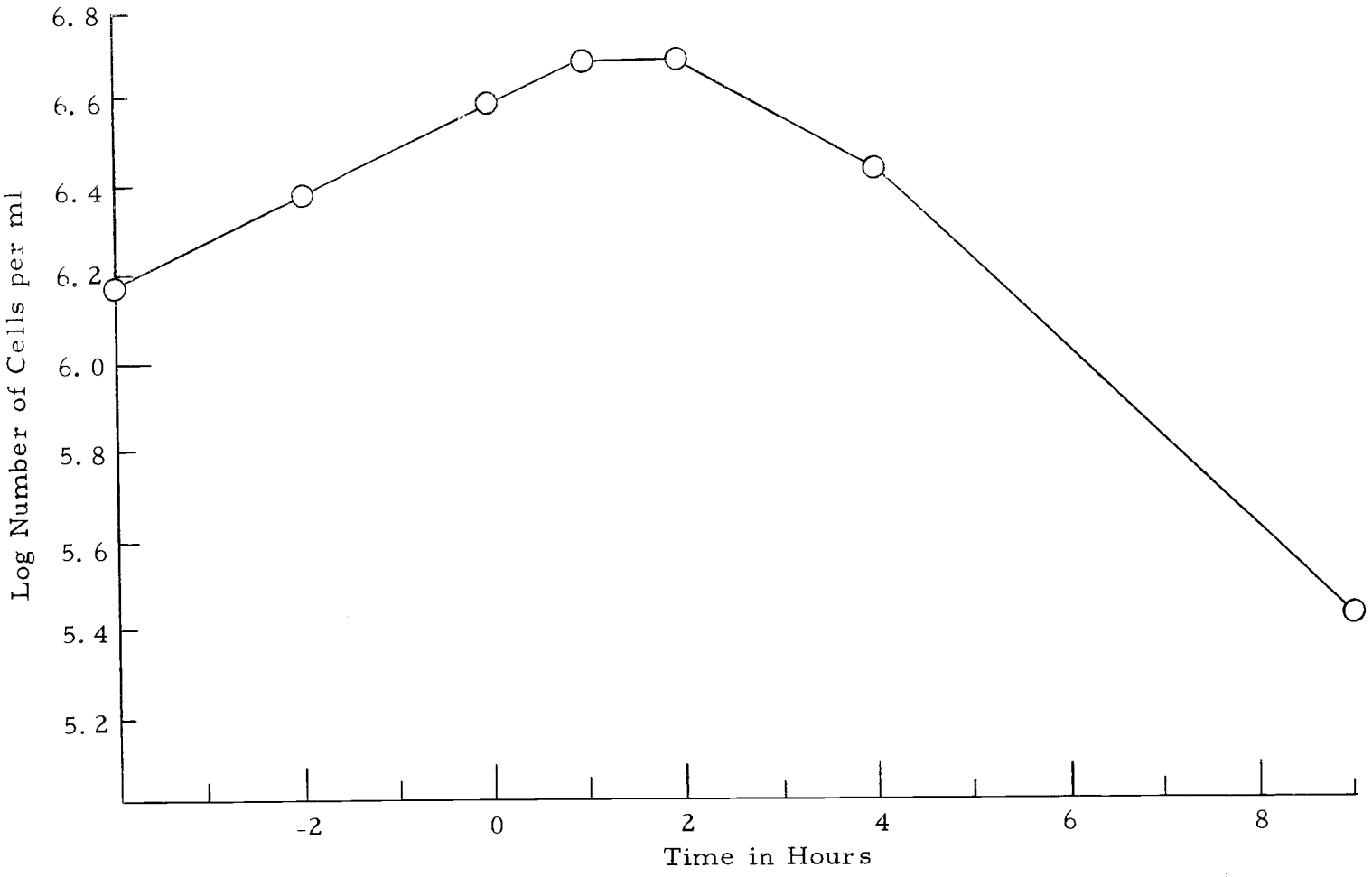

Fig. 10. Effect of nalidixic acid on the growth of Ant -300. Ant-300 was grown in GM and nalidixic acid (final concentration 20 ug/ml) was added at zero time.

Fig. 11. Photomicrographs of starved and nonstarved Ant -300 cells. (a) Zero time; (b) one week after starvation; (c) six weeks after starvation. Bars represent 5 μm.

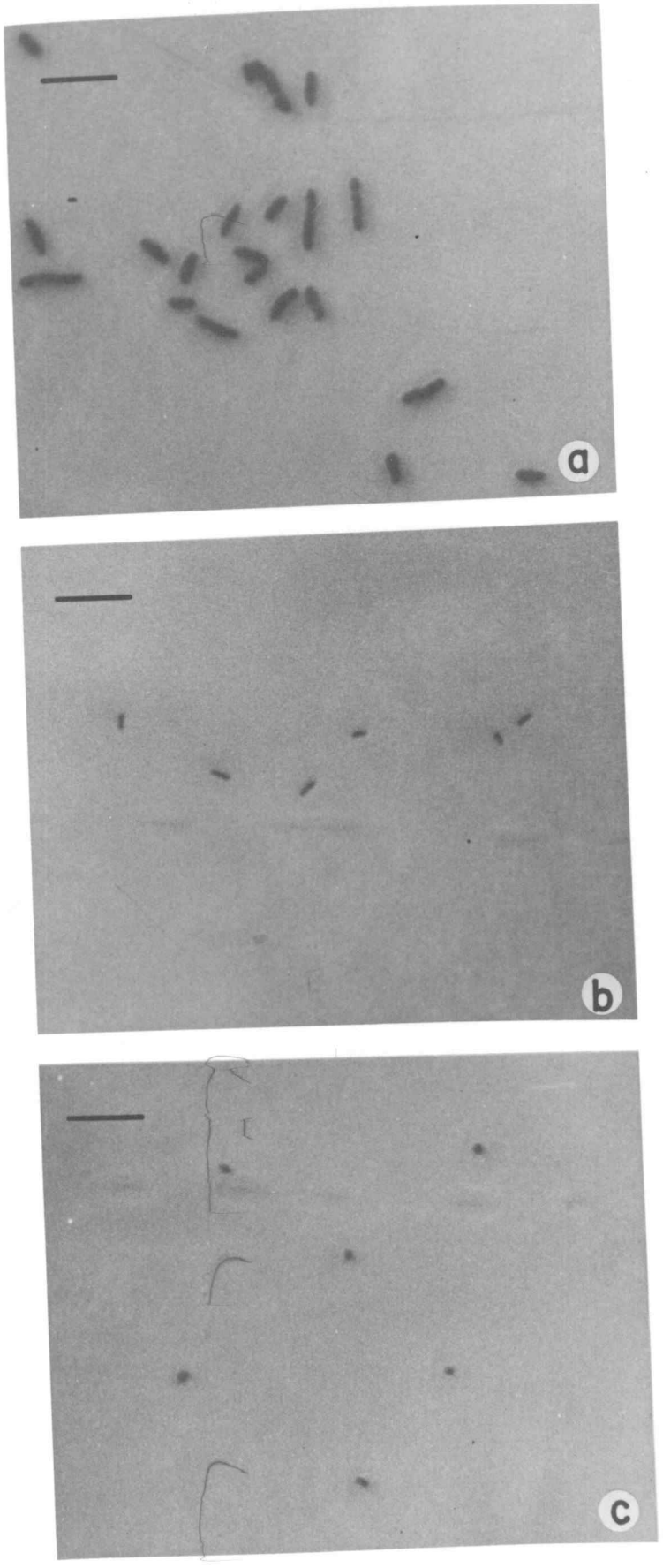

Fig. 11.

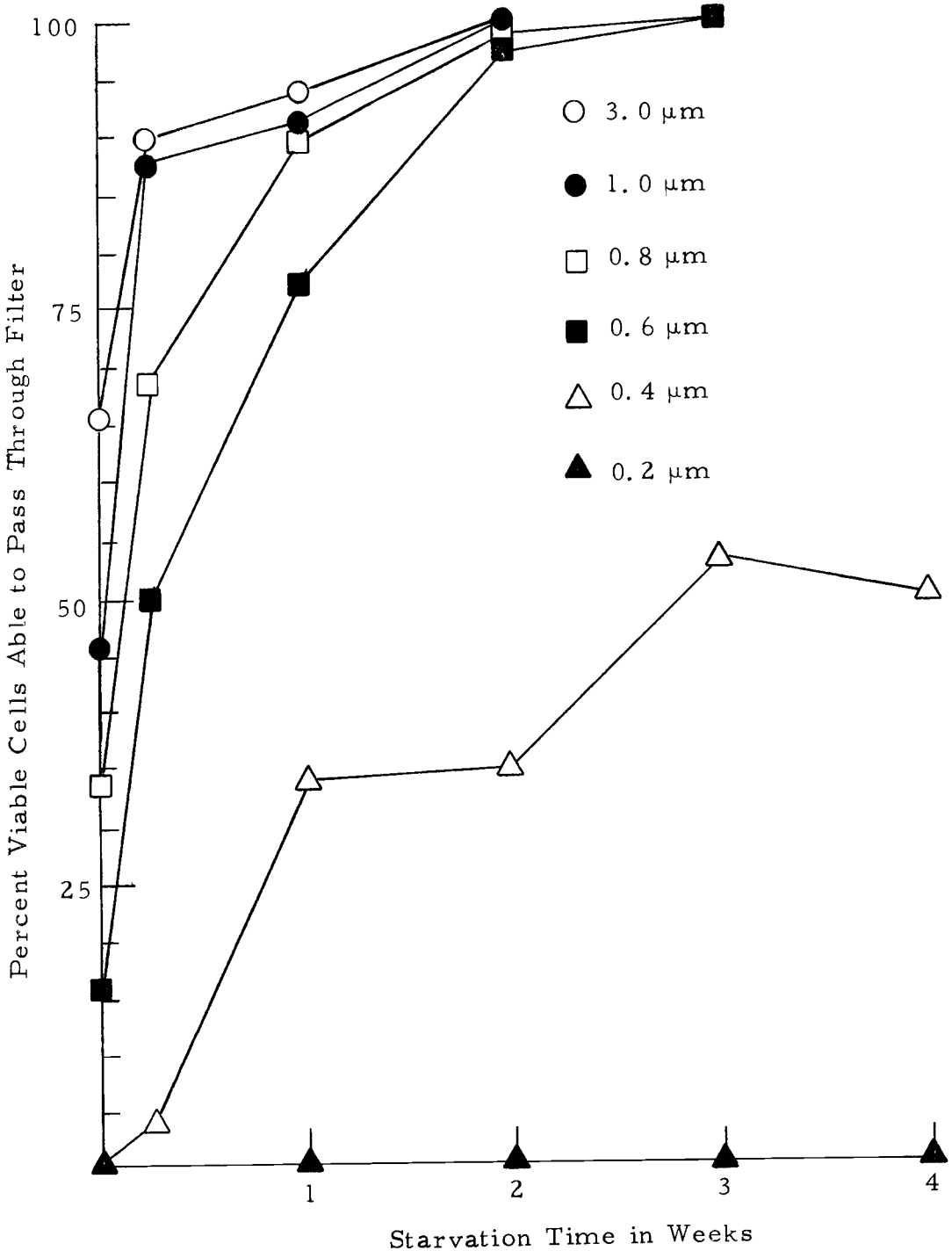

Fig. 12. Size distribution of starving Ant-300 cells. Ant-300 was starved and at various times portions of the cell suspension were passed through Nuclepore filters with various pore sizes. Viability of each filtrate was determined by plate counts and expressed as a percentage of the counts obtained from the nonfiltered sample.

or a 0.45 μm cellulose membrane filter (Millipore, Type HA).

Electron microscopy of a thin section of nonstarved Ant-300 cells is shown in Fig. 13a. The cells are typical for Gram negative cells and show no unusual structures. The small starved cells (Fig. 13b and c) appear smaller and roughly spherical. The ultrastructure is generally the same as nonstarved cells except for an enlarged periplasmic space containing stainable material observed in all the starved cells examined. No unusual membrane structures were observed.

After 5 wk of starvation cells were passed through a 0.4 μm filter and inoculated into fresh GM. The cells grew without a significant lag period and had a generation time of 6 h, equal to that for nonstarved cells. When inoculated into a fresh medium, the small cells immediately began to increase in size (Table 7) and regain their rod shape. After 48 h (8 generations) the cells were indistinguishable in size from nonstarved cells. The increase in size was almost synchronous for all the cells in the population; however, even after 60 h of growth, a few small cells were still observed.

To determine the effect of starvation on a population's ability to survive subsequent starvation, cells were starved, recovered from starvation in GM, and starved again in buffered SM. Cells recovered from 7 wk of starvation, grown and re-starved responded to starvation similar to cells not previously starved. However, cells starved for nine months, grown, and re-starved, died faster than normal

Fig. 13. Electron micrographs of starved and nonstarved Ant-300 cells. (a) Nonstarved cells; bar represents 1.0 μm. (b) and (c) Cells starved for five weeks; bar represents 0.2 μm.

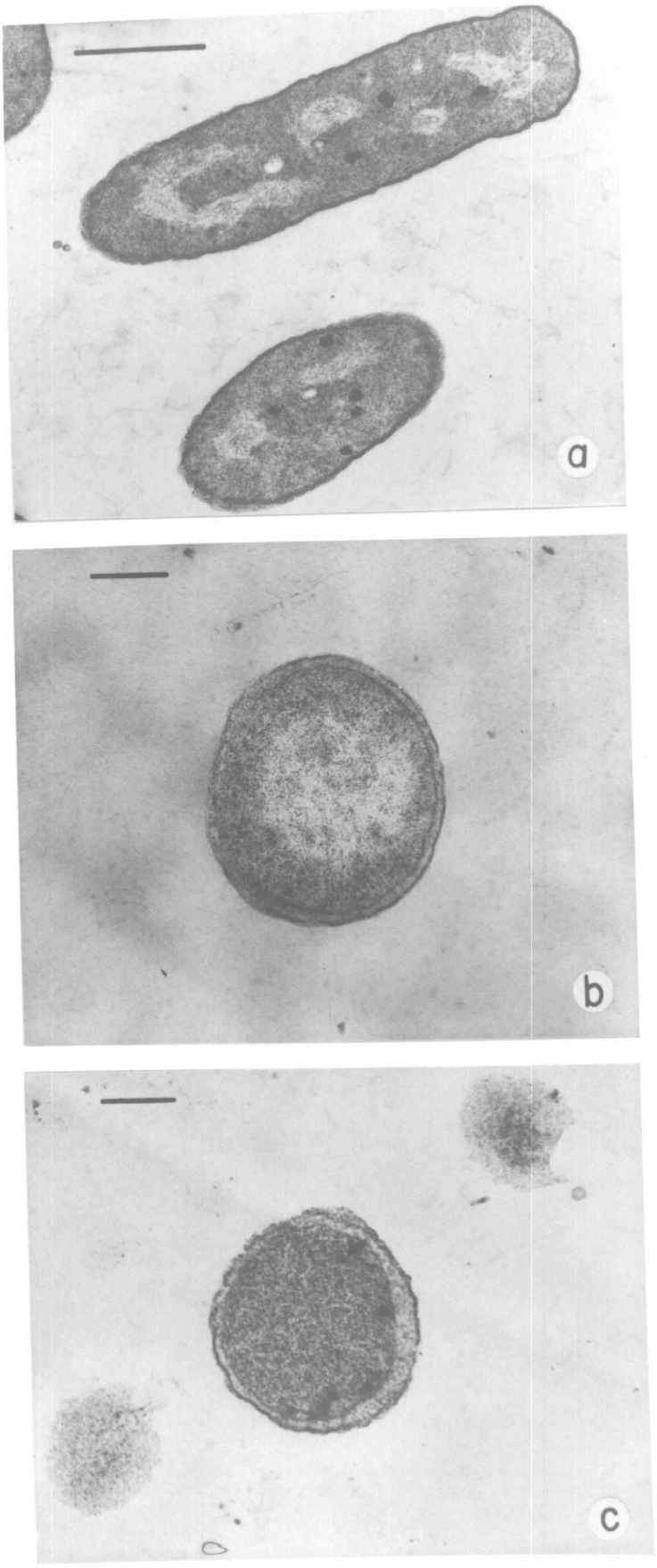

Fig. 13.

Table 7. Size increase of filterable[a] starving cells after inoculation into fresh media.

Time (h)	Percent cells able to pass through a 1.0 μm filter
0	100
6	93
12	71
24	36
30	32
36	20
48	18

[a]Passed through a 0.4 μm filter.

cells (50% of the initial number of cells remained viable for only 3 wk) as shown in Fig. 14. Both cultures increased in numbers, decreased in size, and formed coccoidal cells during starvation, typical of normal cells.

The starvation of Ant-300 in GM after depletion of glucose is shown in Fig. 15. Glucose was added in an amount (1.84 mg) sufficient to support the growth of Ant-300 to an optical density of approximately 0.2. After 29 h of growth the optical density reached a maximum of 0.2 and then declined. Growing exponentially to this point, the cells continued to divide and increased in numbers (375%) for an additional 22 h after which time the number of viable cells decreased similar to cells under complete starvation conditions. Fifty percent of the initial number of cells (cell count at 29 h) were still viable after 5.5 wk.

The effect of the addition of glucose or nitrate (final concentrations 0.1%) to a starving cell suspension is shown in Fig. 16. While nitrate had little effect on the viability of the starving suspension, glucose caused an immediate loss of viability (81%) within 24 h. The addition of a dilute amino acid mixture (Table 2) after seven days of starvation caused a slight increase (6%) in viable cells but had no effect on the long-term survival of the population.

The starvation of Ant-300 in the presence of chitin (final concentration 0.1%) is shown in Fig. 17. Chitin was observed to increase

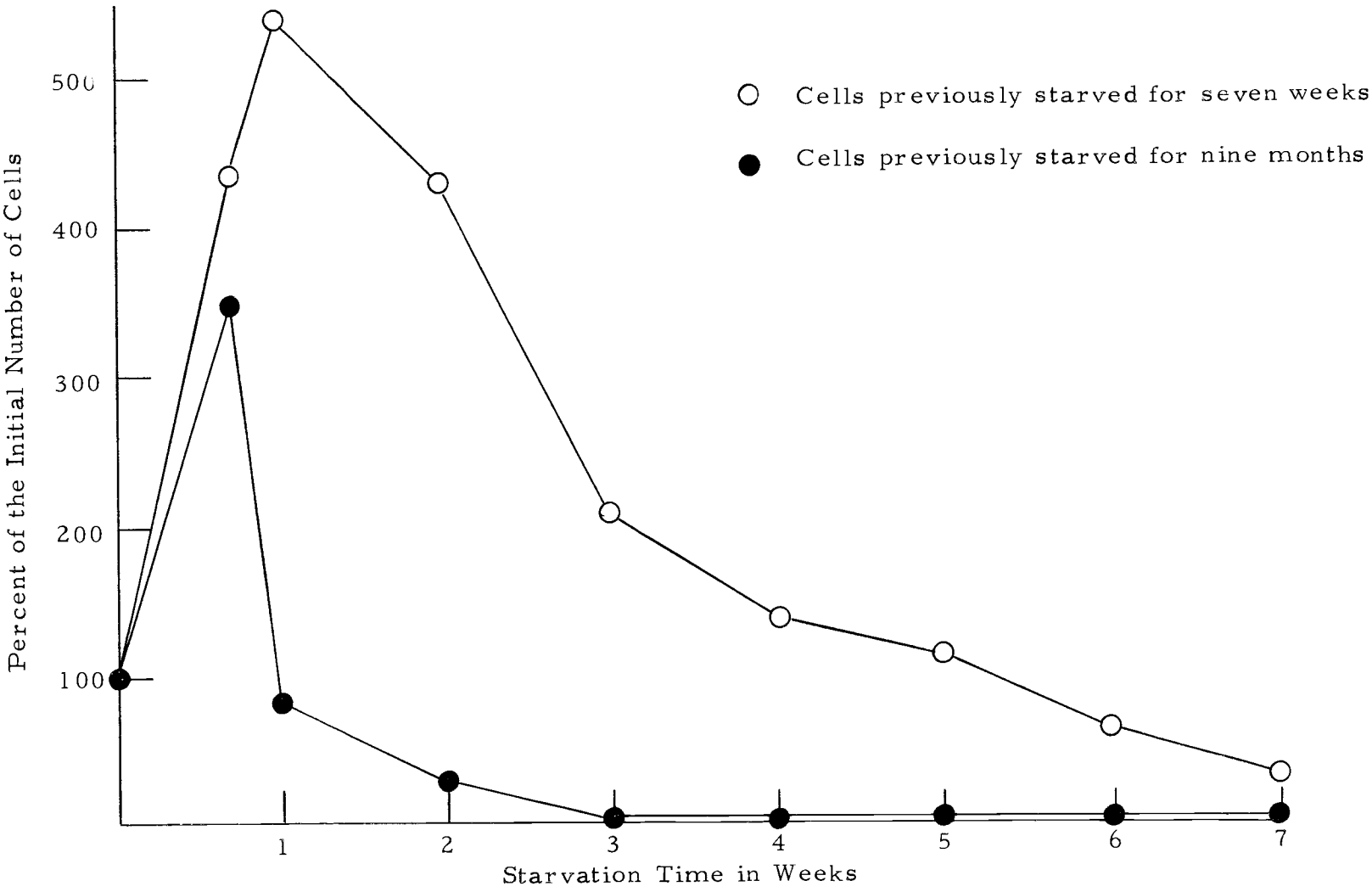

Fig. 14. Starvation of Ant-300 cells previously starved for seven weeks and for nine months. After starvation for seven weeks and for nine months Ant-300 was inoculated into GM and starved from the logarithmic growth phase. Viability was determined by plate counts.

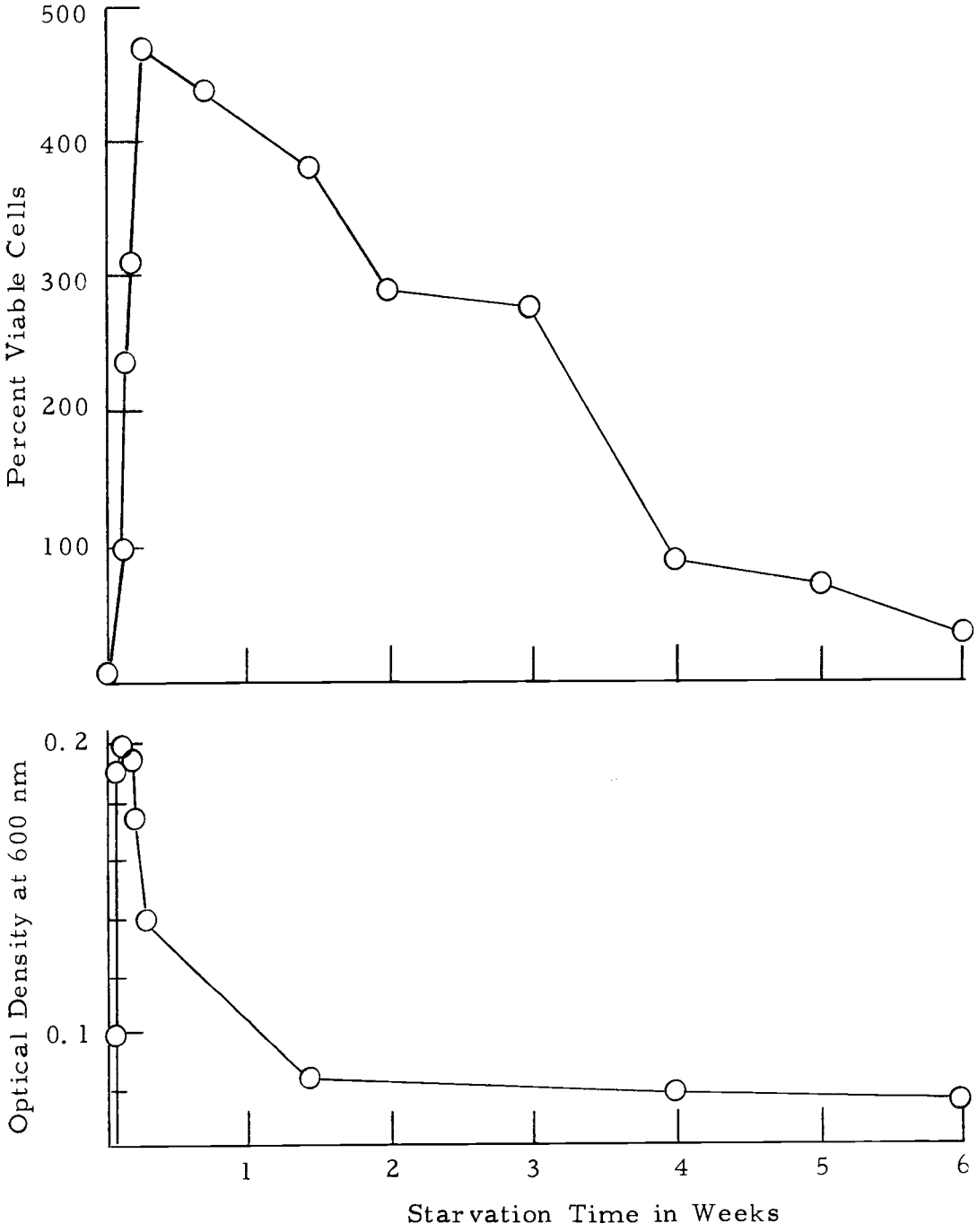

Fig. 15. Starvation of Ant-300 in GM after depletion of glucose. Ant 300 was grown in GM with a limited amount of glucose. Viability (top) was determined by plate counts. The time (29 hours) of maximum OD (bottom) was arbitrarily set as 100%.

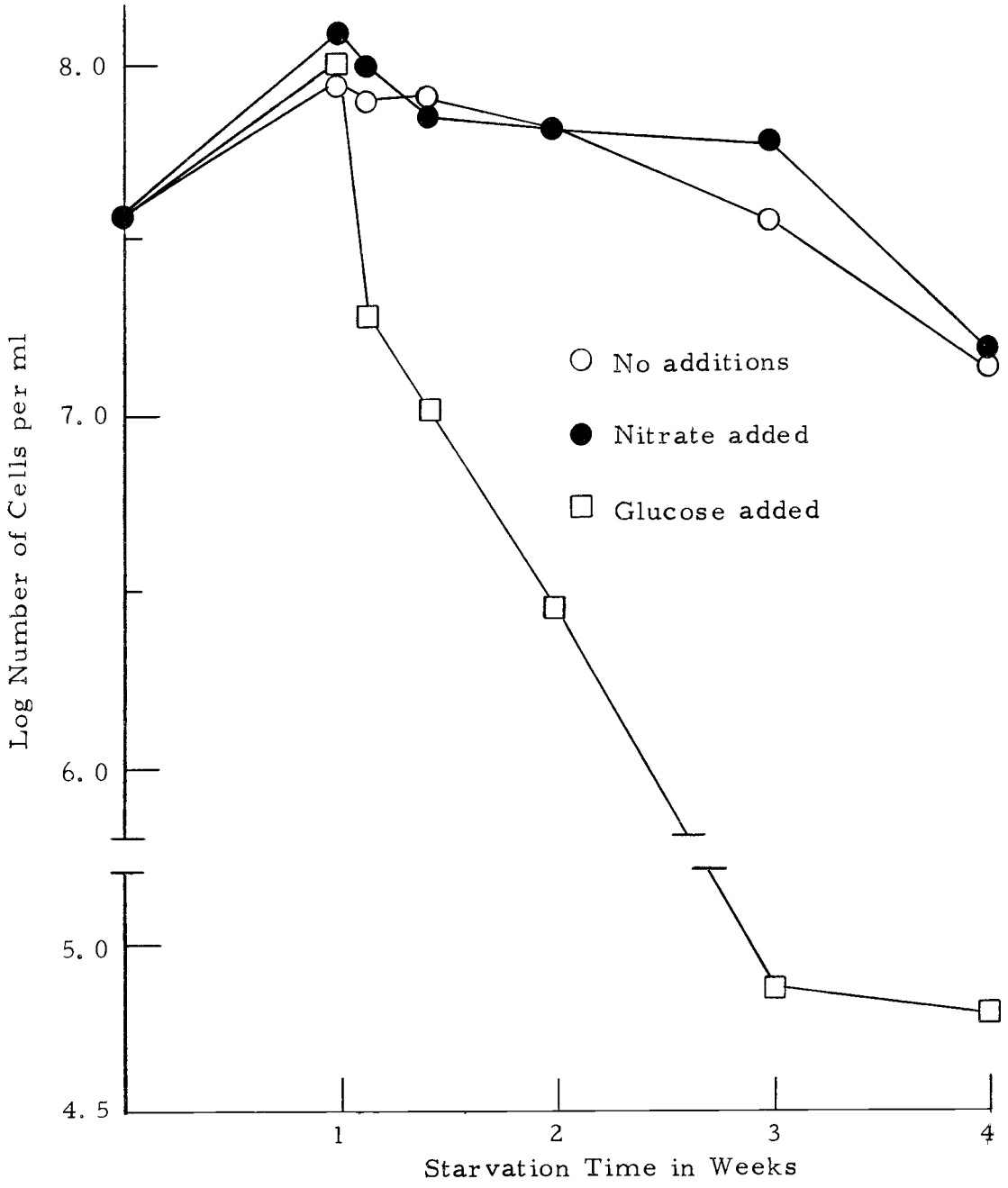

Fig. 16. Effect of glucose or nitrate on starving populations of Ant-300. Glucose and $NaNO_3$ were added separately (final concentration 0.1%) to populations of Ant-300 after seven days of starvation. Viability was determined by plate counts.

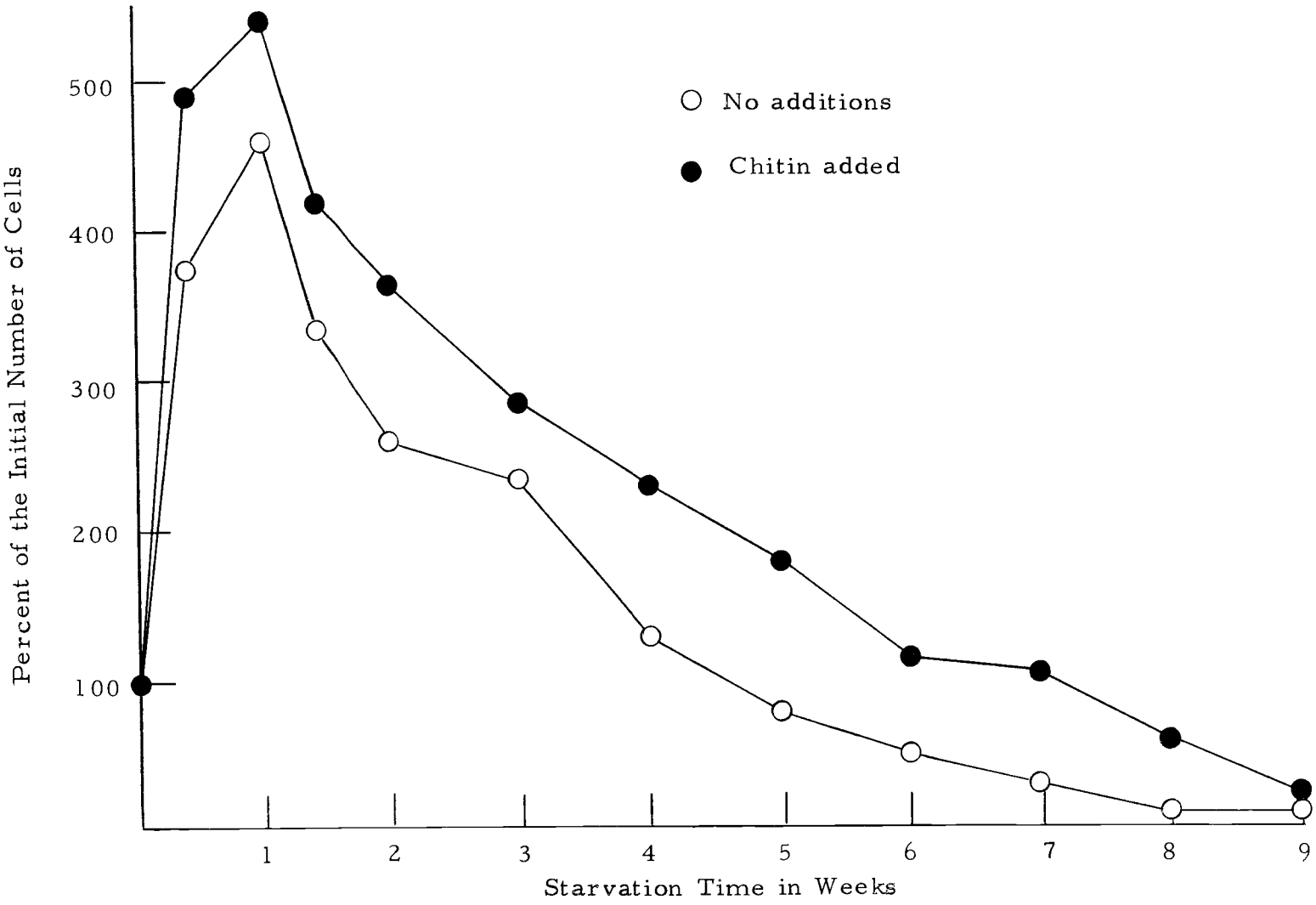

Fig. 17. Starvation of Ant-300 in the presence of chitin. Ant-300 was starved in the absence and presence (final concentration 0.1%) of chitin. Viability was determined by plate counts.

the magnitude of the initial increase in cell numbers and prolong the 50% survival time by approximately 2 wk. However, after 9 wk the viable counts in the chitin and control cultures were nearly identical. The survival of Ant-300 starved in the presence of glucose or sodium nitrate (final concentrations 0.1%) is presented in Fig. 18. The effect of nitrate was similar to the effect of chitin but glucose caused an increase followed by a sharp decrease in viable cells such that after 24 h over 97% of the original number of cells were no longer viable.

The viability of Ant-300 starved at various initial cell concentrations from 2.7×10^2 to 1.8×10^7 cells/ml is shown in Fig. 19. Ant-300 was prepared for starvation as described then serially diluted to produce the various initial cell concentrations. The magnitude of the initial increase in cell numbers was inversely dependent upon the initial cell concentration and varied from 216 to 92,122% (Table 8). Direct counts of populations starving at low initial cell densities confirmed that this increase was indeed an increase in cell numbers. Even though initially the cell densities varied 100,000-fold, after 2 wk of starvation the viable cell concentrations in all the suspensions did not vary more than 10-fold with the exception of the suspension with the highest concentration of cells which remained significantly higher than the other cultures throughout the starvation period (Fig. 19). Cells diluted after seven days of starvation, such that the cell concentration of the final suspension was equal to that of the initial

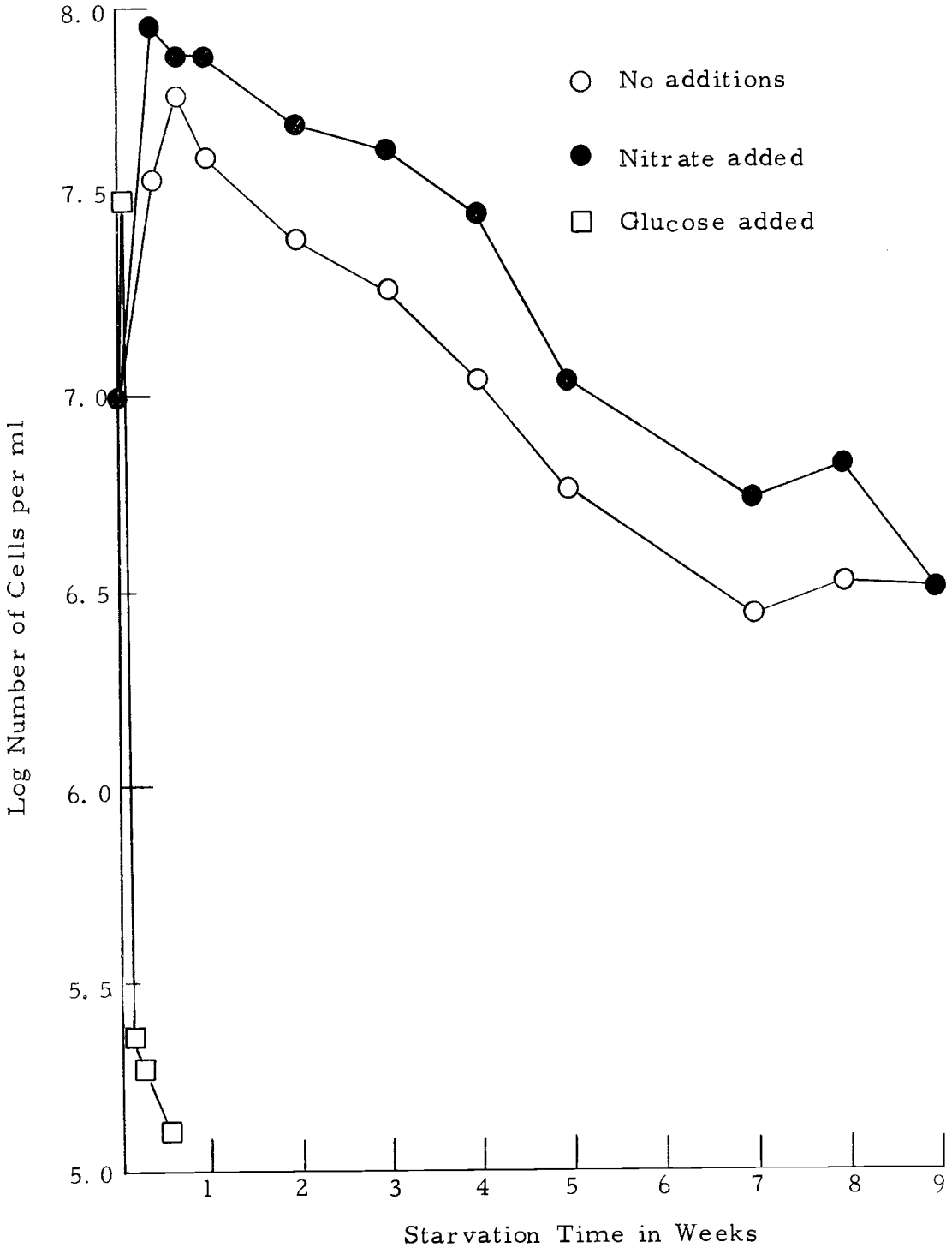

Fig. 18. Starvation of Ant-300 in the presence of glucose or nitrate, Ant-300 was starved in the presence of glucose or $NaNO_3$ (final concentration 0.1%). Viability was determined by plate counts.

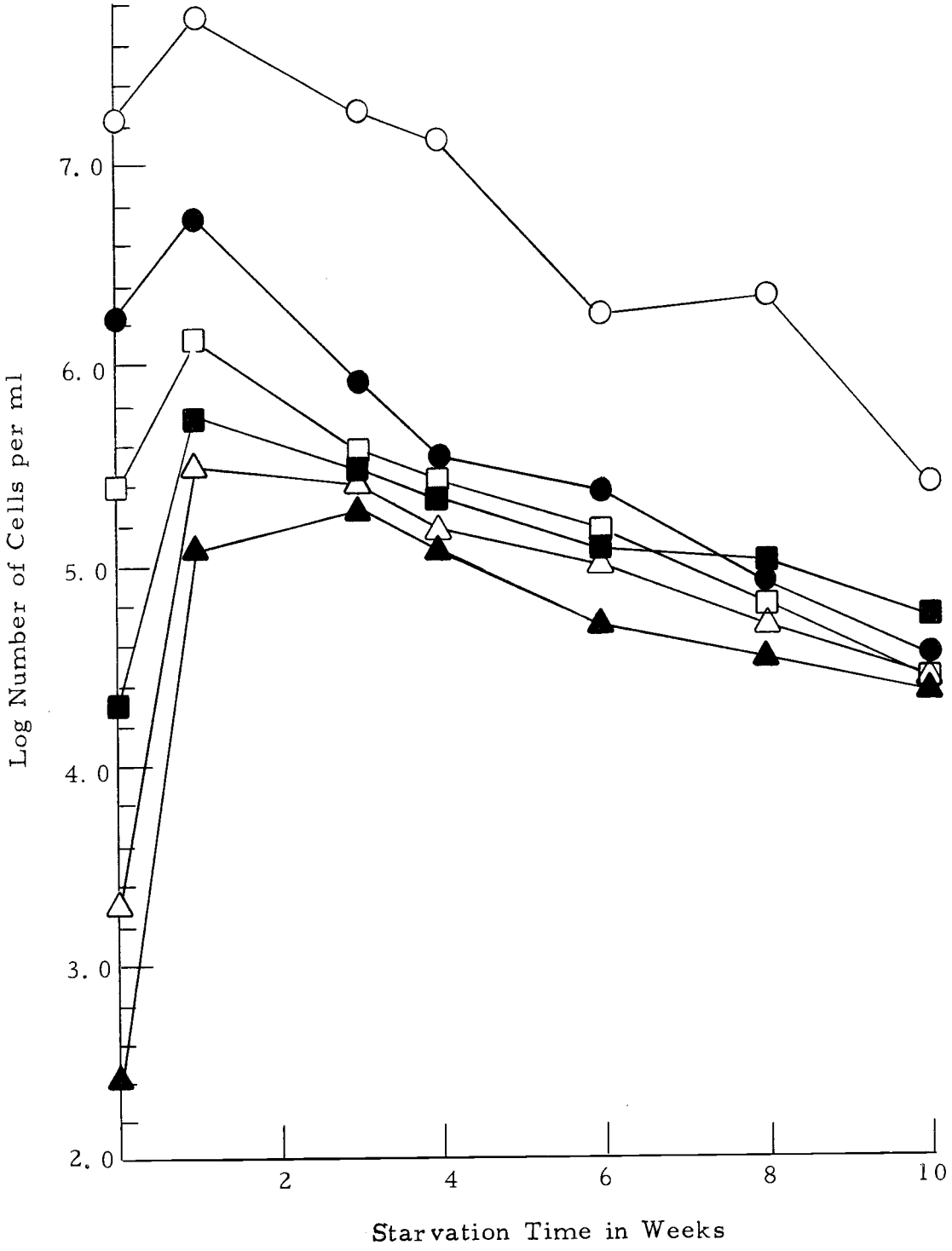

Fig. 19. Effect of initial cell density on the viability of Ant-300 during starvation. Ant-300 was prepared for starvation then serially diluted to produce the various initial cell concentrations. Viability was determined by plate counts.

Table 8. Maximum increase in cell numbers upon starvation of Ant-300 at various initial cell concentrations.

Initial cell concentration (cells/ml)	Percent increase in cell numbers
1.77×10^7	216
1.77×10^6	216
2.45×10^5	435
2.08×10^4	2,592
2.01×10^3	15,422
2.70×10^2	92,122

suspension, died at a rate equal to that for the undiluted sample (Fig. 20).

Long-term starvation of Ant-300 at low initial cell densities showed that after 70 wk of starvation, over 15 times the original number of cells were still viable (Fig. 21). To determine the extent of any possible contamination in reagents and glassware a specially prepared SM, as free from organic material as possible, was used as a starvation menstruum. In addition, all glassware was acid washed and freshly prepared double distilled water was used throughout the procedure. The results of this experiment are shown in Fig. 22. As can be seen by this graph, only a small difference was noted between the normally starved cells and cells starved in specially prepared glassware and SM.

The starvation of Ant-300 in natural seawater or in SM with added amino acids is shown in Fig. 23. Starved in seawater, Ant-300 showed a similar initial increase in cell numbers as when starved in SM but not a greater viability after 7 wk of starvation. Cells starved in buffered SM amended with amino acids at a concentration similar to that found in seawater, showed a starvation viability similar to that for seawater. Increasing the concentrations of the amino acids ten-fold resulted in a delayed but not greater maximum increase in cell numbers compared to cells starved in SM.

Cells starved in a SM menstruum, which had previously been

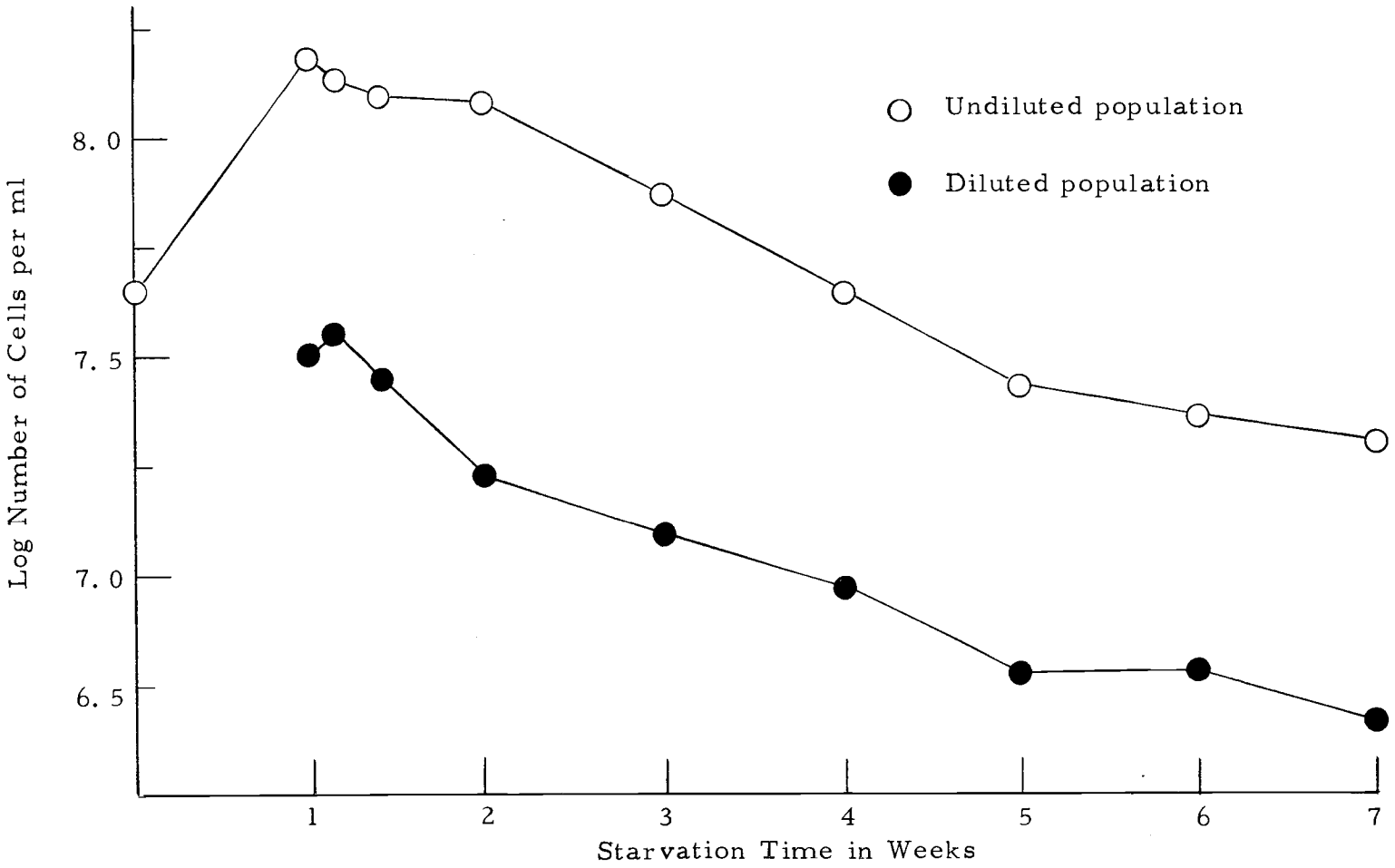

Fig. 20. Starvation of Ant-300 upon dilution of the population. After seven days of starvation a portion of the starving population was diluted to a cell concentration approximately equal to that of the initial population. Viability was determined by plate counts.

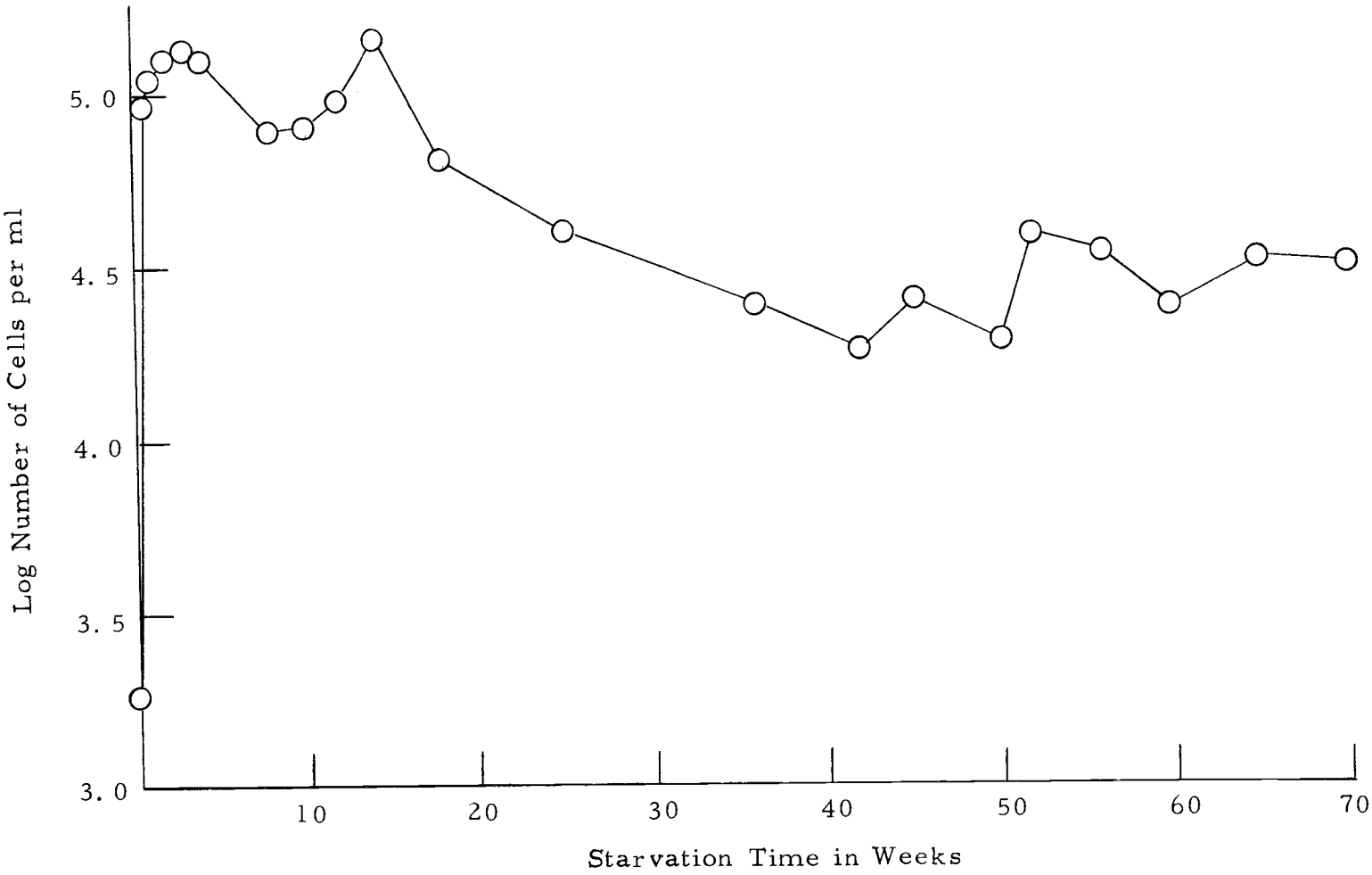

Fig. 21. Long-term survival of Ant-300 starved at a low cell density. Ant-300 was starved at an initial cell density of 1.88×10^3 cells/ml. Viability was determined by plate counts.

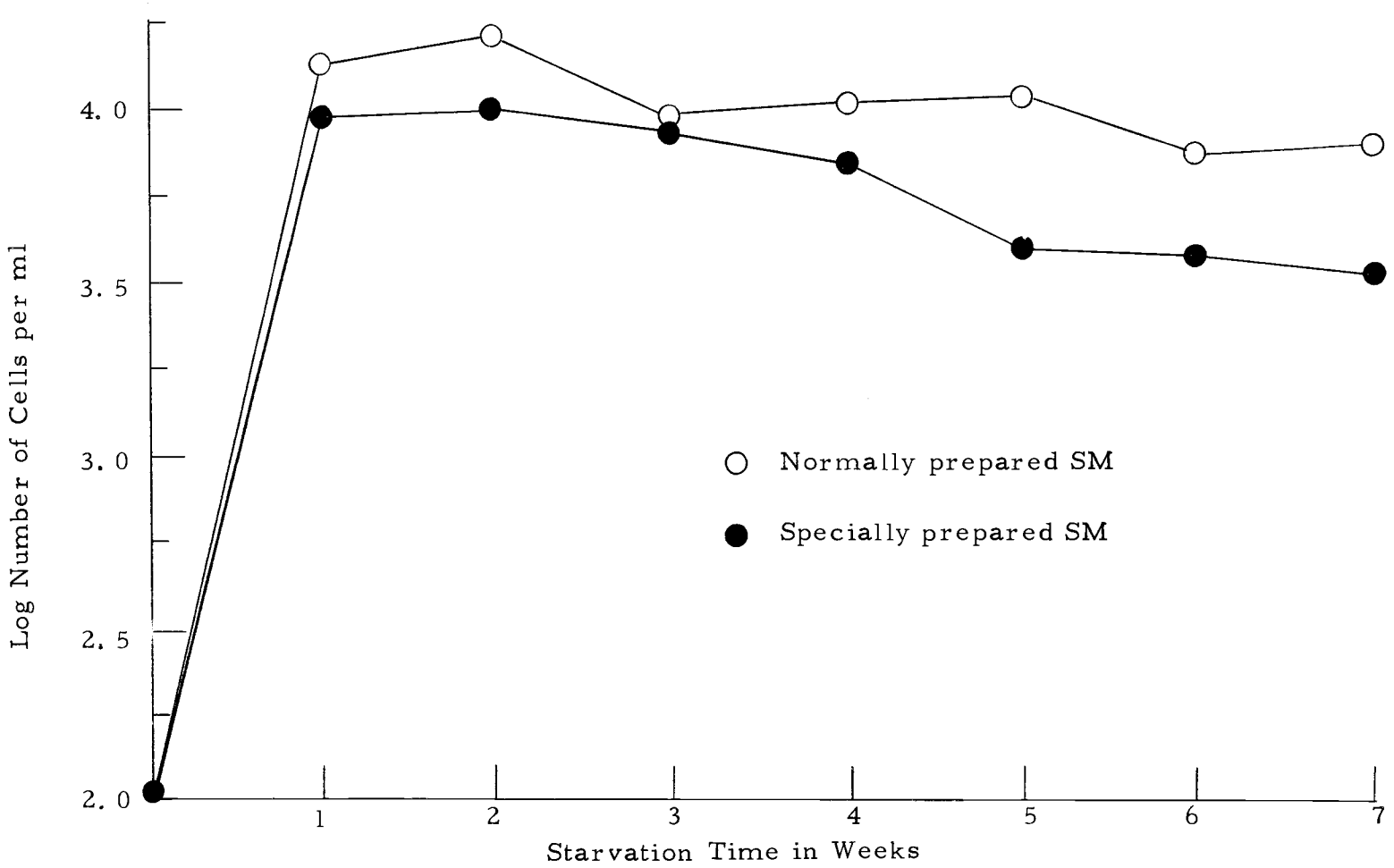

Fig. 22. Starvation of Ant-300 at a low cell density in a specially prepared starvation menstruum. Ant-300 was starved in normally prepared SM and in SM prepared to be as free from organic material as possible. Viability was determined by plate counts.

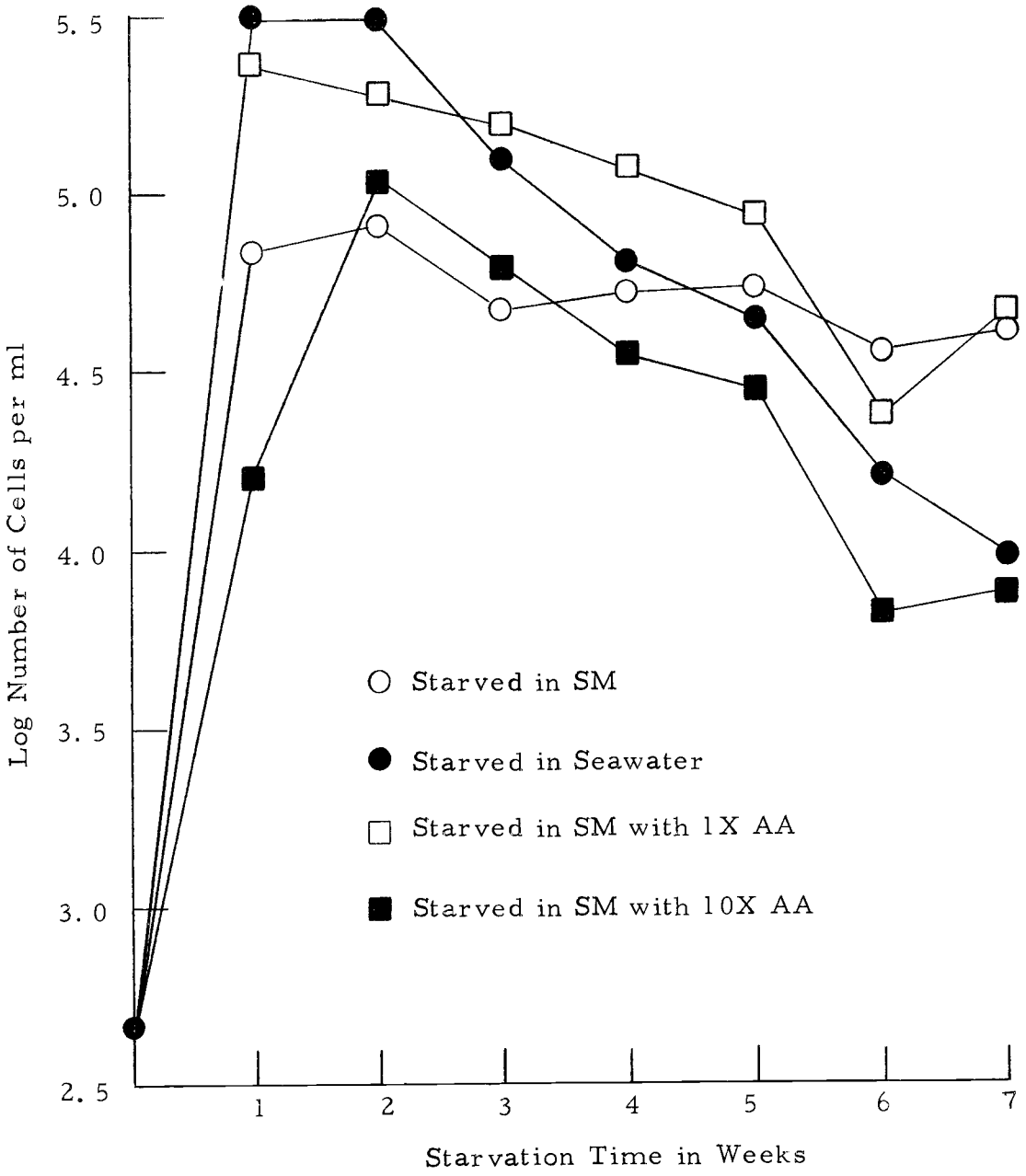

Fig. 23. Starvation of Ant-300 in seawater and in menstra amended with amino acids. Ant-300 was starved at a low cell density in seawater and in menstra amended with an amino acid mixture at single strength (1X AA) and 10X strength (10X AA). Viability was determined by plate counts.

used as a starvation menstruum for 1 wk followed by filter sterilization, showed a greater increase in cell numbers (137%) than cells starved in SM for the first time. However, cells starved in the recycled menstruum died at a faster rate than did the control suspension as shown in Fig. 24.

The addition of low level nutrients (GM; final concentration 1.0%) to a cell population starving for 7 wk produced an immediate increase in cell numbers (100-fold) followed by a decline in cell numbers as shown in Fig. 25. Fifteen wk after the nutrient addition (wk 22 of starvation) viable cell numbers were reduced to slightly below pre-addition levels and remained constant for an additional 3 wk.

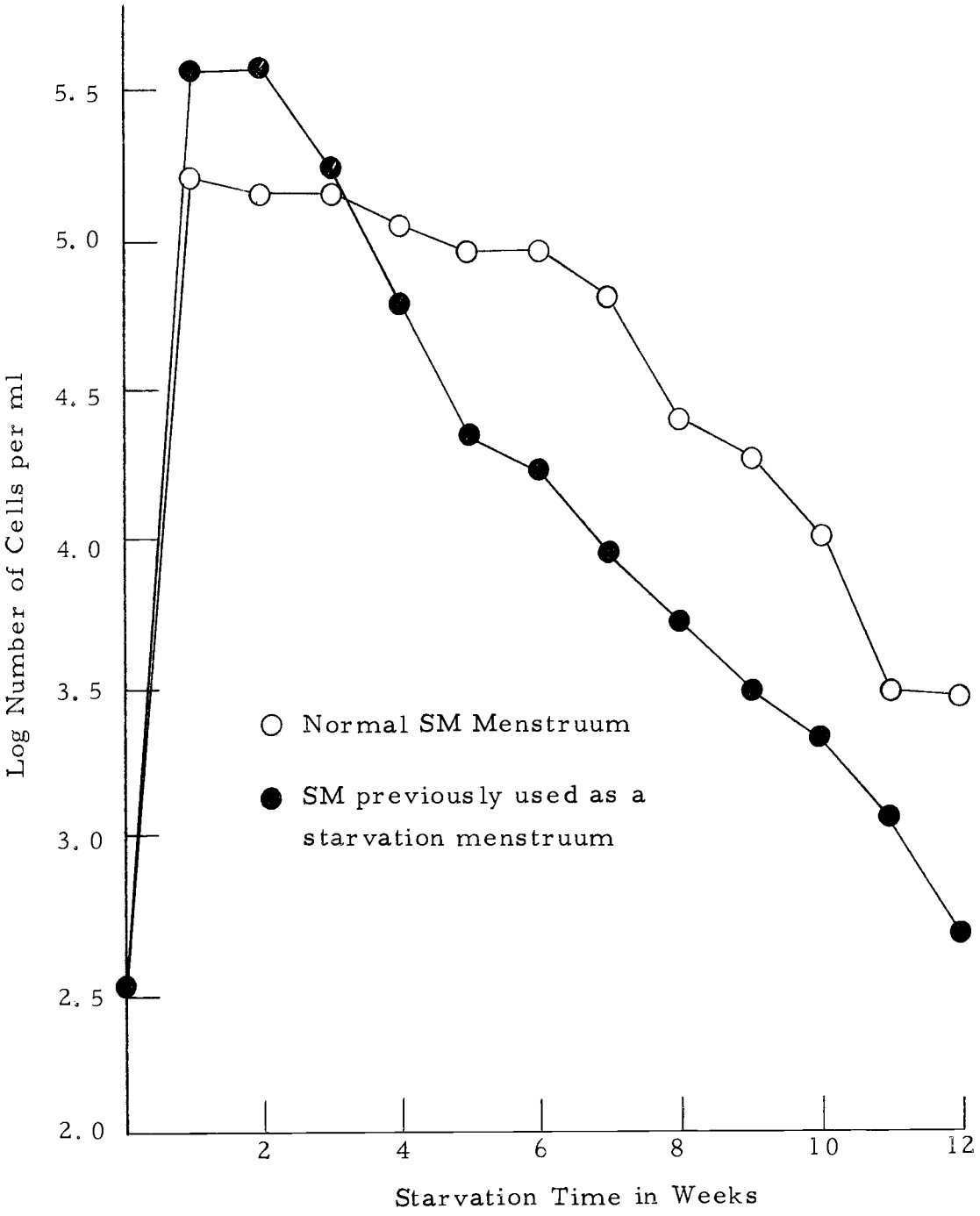

Fig. 24. Starvation of Ant-300 in a menstruum previously used as a starvation menstruum. Ant-300 was starved for one week in buffered SM which was then filter sterilized and used again as a starvation menstruum for a population of Ant-300. Viability was determined by plate counts.

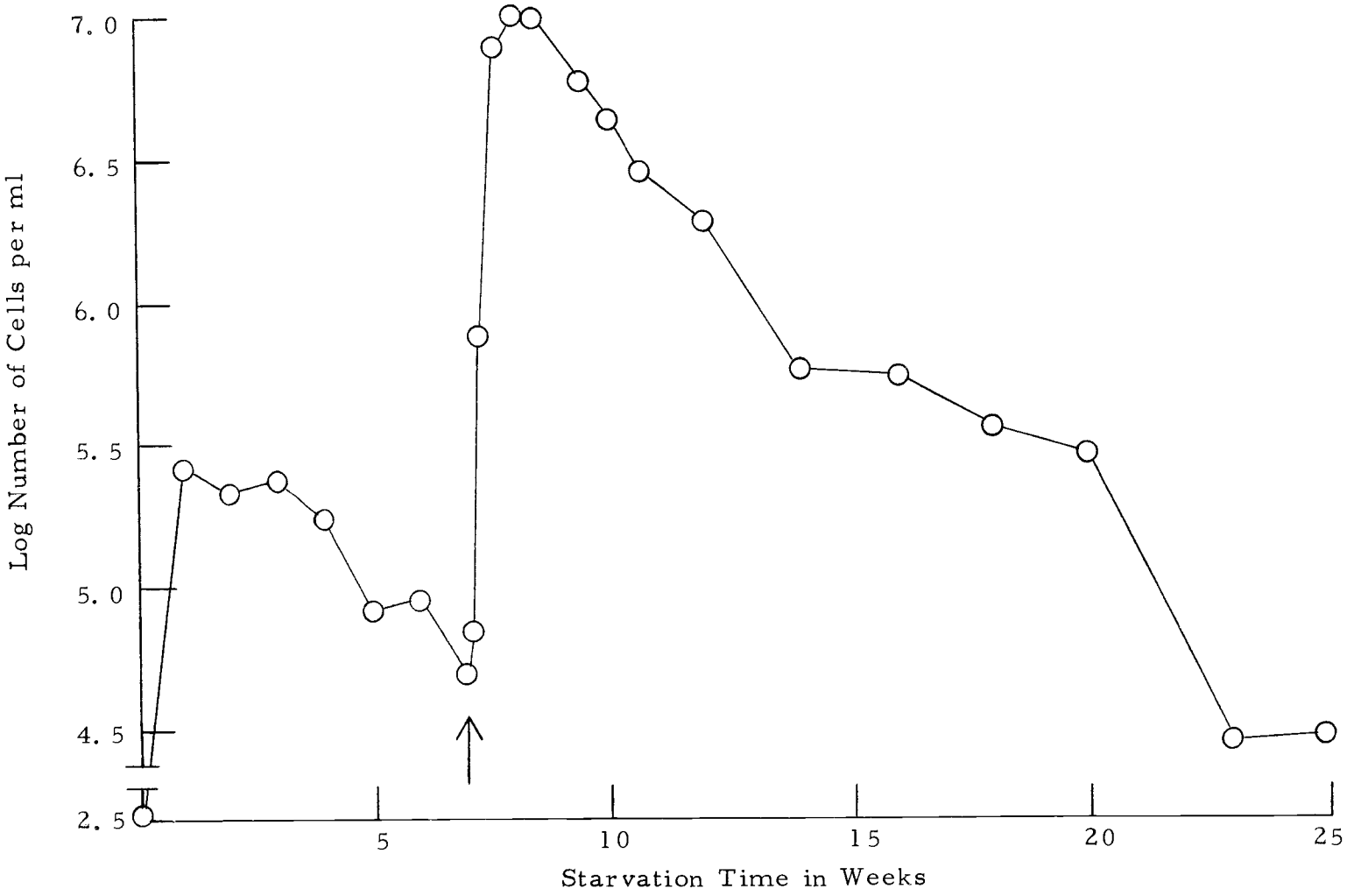

Fig. 25. Addition of low level nutrients to a starving suspension of Ant-300. After seven weeks of starvation GM (final concentration 1.0%) was added to a suspension of Ant-300. Viability was determined by plate counts. Arrow marks the time of nutrient addition.

DISCUSSION

The concept of death, be it for multicellular or unicellular organisms, has been well studied but still remains poorly defined. Bacterial death can be considered as the inability to replicate, the irreversible loss of some or all metabolic activity, and/or the loss of structural integrity. Obviously any definition of death has its limitations and no single explanation has been universally accepted. Probably for this reason, most investigators have avoided the question of death and have defined what they considered viability. Likewise in this study, cells were defined as viable if they could produce a visible colony on a suitable medium; conversely, cells were considered dead if they could not replicate. It is important to recognize throughout this discussion that these "dead" cells may be viable in other respects and may therefore contribute to the observed results. It must also be realized that the definition used for viable cells is rather narrow. It is possible that cells considered dead under these experimental conditions may be viable under more favorable conditions.

Just as there are many definitions for viability, there are many ways to determine it. Total direct counts of cells, plate counts of viable cells, slide culture techniques to determine total and viable cells, and combinations of these have all been used. Total counts

are affected by cell lysis and give no information about viability; plate counts give viable numbers but no information on the percentage of viable cells unless compared to an arbitrary point; and slide cultures give an estimate of the percentage of viable cells but are also affected by cellular lysis. The slide culture techniques, made popular for viability determination of starving bacterial populations by Postgate and Hunter (64, 65), have an additional disadvantage heretofore unrecognized: if a starving population such as Ant-300 increases in cell numbers it cannot be monitored by slide culture since the viability so determined cannot exceed 100 percent. The present study, therefore, has relied upon plate counts for the determination of viability which were expressed in reference to the original number of cells rather than to the total number of cells. Reporting data in this manner allows the determination of the survival capabilities of the cells in relation to the initial population. This relationship must be considered when hypothesizing the survival potential of any organism in the natural environment. Since the gene pool of a species can be conserved by the survival of only one organism, the concern in the literature over half-life survival times and the ratios of living to dead cells is therefore a moot point in considering the ultimate survival of a species.

The starvation viability of Ant-300 (Fig. 1) is characterized by an increase in cell numbers during the first week of starvation

followed by a decrease such that after 6-7 wk of starvation, 50% of the initial number of cells are still viable. Compared to other bacteria cited in the literature (Table 1) this starvation viability is exceeded only by Arthrobacter globiformis and A. crystallopoietes, on a half-life basis. The initial increase in numbers appears to be unique since no such increase in cell numbers has ever been reported for any other starving bacteria. The increase in cell numbers is related to the age of the culture prior to starvation (Fig. 4). Generally the older the culture the greater the increase. Irregardless of the increase however, the time needed to reduce the initial viable population by one-half is approximately the same for all cultures provided that the cells are starved at approximately the same initial cell concentration. It appears that the age of the culture is not important in determining its survival potential as was reported by Thomas and Batt (85) for Streptococcus lactis. However, the initial increase in cell numbers is dependent upon the age of the culture and the magnitude of this increase can be an important factor in population survival if the culture were to be dispersed after the increase. In order for the cultures to have similar numbers of viable cells after 6 wk of starvation, the cultures with the greatest increase in numbers must also have the greatest death rate afterwards (Fig. 4). The reason for this increased death rate is unclear. At high population densities, Harrison (42) noted an increased death rate for Klebsiella pneumoniae

which he attributed to anoxia. To determine if this was also true for Ant-300, a culture was diluted after the initial increase but the death rates for both cultures were identical. It appears that this phenomenon therefore is characteristic of the cells rather than of the population. If this is the case then older cultures die more rapidly than younger ones (also observed by Stokes and Parson (77) for Sphaerotilus discophorus) and their survival depends solely on the large initial increase in cell numbers.

When grown in a rich medium (Lib-X) Ant-300 did not survive as well as when grown in a relatively simple medium (GM). This contradicts the data of Strange et al. (82) on K. pneumoniae starvation. Although the differences are not great, they may be due to differences in the cellular metabolism under the two nutrient conditions. Although this is an interesting point it probably has no significance in the natural environment. Of more importance is the role of phosphate during starvation. Cells starved for phosphate in addition to carbon and nitrogen survive as well as cells starved in the presence of phosphate. This indicates that the cells are able to store enough phosphate during growth or are able to recycle the existing pool to an extent necessary for survival.

The media used to recover cells from starvation has a two-fold significance: it is directly related to the assessment of viability; and if cells in the natural environment are in a starvation state then the

media used of their isolation is in fact starvation recovery media. It was found (Table 4) that 0.1X strength media supported the recovery of more cells from starvation than did the full strength media under identical conditions. The only exception was 0.1X GM which gave significantly lower counts even with nonstarved cells. Although the differences between the concentrated and dilute media for most cases are small, a 37% increase in viable cells was noted when 0.1X 2216E was substituted for full strength 2216E, a medium suggested and used (97) for the isolation of marine bacteria.

Under starvation conditions, Ant-300 reduces its endogenous respiration over 99% within the first wk of starvation. At this rate, if respiration was the only loss of carbon, it would take approximately 58 wk to reduce the cellular carbon by one-half. Actual measurements indicate that one-half of the cellular carbon is lost after only 39 days, a value close to the time when the viability of Ant-300 has been reduced to 50% of the initial number of cells. This apparent relationship may only be coincidental however, since total cellular carbon was measured for both viable and dead cells. A sharp decrease in endogenous respiration may be a factor in long-term survival since the bacterial strains with the three longest survival times, Nocardia corallina (69), Arthrobacter globiformis (51), and A. crystallopoietes (8), all step-down their endogenous respiration over 90%. Evidence that a decrease in endogenous respiration is not coupled to a long

survival time has been reported (14, 26, 64), but with rapidly dying populations it is difficult to determine with certainty whether the decrease in respiration is actually a survival mechanism or as a result of, or previous to, cell death. Halvorson (41) showed that agents which inhibit respiration such as 2, 4, -dinitrophenol, sodium azide, and arsentate also suppress protein and nucleic acid degradation in yeast cells. Similar results were reported by Mandelstam (53) for protein degradation in Escherichia coli. It is possible that starvation induced respiration reduction acts in the same manner to prevent macromolecular degradation leading to increased survival.

The decrease in total cellular carbon (Fig. 6) represents carbon loss due to respiration, leakage, and degradation of cellular components. The radioactivity associated with the acid washed cells represents cellular macromolecular carbon. The sharp decrease then increase during the first day of starvation may represent an excretion and reabsorption of enzymes or other macromolecules when the cells are placed into a new menstruum. The difference between the two values, representing pool material and low molecular weight metabolites, decreases to extinction in 21 days. The continued decrease of cellular carbon indicates a steady degradation and/or excretion of cellular components. Extrapolating this graph, all of the cellular carbon would be depleted after 15 wk of starvation. Obviously this extrapolation cannot be valid.

Cryptic growth, or growth on materials released from dead cells is a factor in starvation studies and regrowth may balance or exceed death. Extensive cryptic growth in the early stages of starvation was not noted during the starvation of Ant-300 as judged by the absence of an increase in viable numbers after the first wk of starvation. In addition, no large amounts of cellular debris were noted under microscopic examination of starving populations. In the latter stages of starvation (after 10 wk) some small increases in viable counts were noted which may have been due to cryptic growth. Another possibility of starving cells is the uptake of released materials subsequently used for metabolic processes other than reproduction. The possibility of cryptic uptake or respiration has been ruled out in the starvation of Ant-300 as no significant uptake or respiration of released materials was noted for starving cells except for the initial incubation period (Table 5). At this time leakage would be minimal but any nutrients carried over from the growth medium would be expected to be utilized. Repeated washing of the cells before starvation could not reduce this amount any more than the normal two washings. This indicates either a constant leakage of materials from the cells or removal of loosely bound materials from the cell surface. The carry over of nutrients from the growth medium is always a consideration in starvation survival but, as was shown, the uptake and respiration of such materials was minimal and of short

duration. Nonstarved cells were able to take up and respire only 6.7 and 0.5%, respectively, of the materials released by cells starving for 6 wk. This indicates that even nonstarved cells are unable to effectively utilize this leakage material. It can be assumed that this leakage material represents metabolic end products or macromolecules not normally utilized by Ant-300, however, it is not possible from these data to rule out the release of ectocrine compounds, nitrogenous compounds, or phosphate which may be utilized by the remaining viable cells.

The ability of small starving cells to take up and respire L-glutamic acid was shown (Fig. 7). Small starved cells take up and respire glutamate without a noticeable lag indicating the necessary enzymes are still present even after 34 days of starvation. A 15 min lag period, characteristic of nonstarved cells, may not necessarily indicate a lag in glutamate respiration but rather a lag in the observation of labeled CO_2 evolution. Nonstarved cells would be expected to contain a pool of unlabeled gluatamate thus producing a quantity of unlabeled CO_2 before the $^{14}CO_2$ begins to appear. Starved cells, on the other hand, have been shown to contain no pool material past 21 days of starvation and therefore $^{14}CO_2$ appears immediately. Glutamate respiration can proceed in a number of different ways including transamination or deamination to α-ketoglutarate, an intermediate of the tricarboxylic acid cycle. Radioactivity associated with

the acid washed cells indicate incorporation of the glutamate into macromolecules also. Although the exact pathway of glutamate utilization in Ant-300 is not known, the addition of 0.1% glutamate to a suspension of cells starved for 34 days resulted in recovery and growth of the cells. It is apparent, therefore, that the enzymes of glutamate respiration and assimilation are not eliminated during starvation; however, a 36% reduction in respiration rate on a per viable cell basis was noted for starved cells. The validity of comparing glutamate uptake on a per viable cell basis is uncertain since the starved cells are considerably smaller and have a greater surface to volume ratio than the nonstarved cells. In addition, the starved cell suspension contained a considerable number of dead[10] cells. For these reasons the significance of a reduction in respiration rate due to starvation is unclear. It may well be possible that starved cells would actually take up and respire more glutamate if compared on some other basis such as dry weight or cell volume.

The fluctuating protein content of the starving cells and starvation menstruum (Fig. 8) appears to indicate protein synthesis and degradation in addition to some leakage and reabsorption during the

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[10] As stated before, "dead" has been defined as unable to reproduce, whether or not these dead cells can take up and respire exogenous substrates has not been determined.

first 21 days. The net degradation of cellular protein indicates the utilization of protein during starvation and is consistent with the results of others (9, 17, 34, 39, 82). The degradation of DNA, generally considered a stable cellular component, by starving cells without a loss in viability is unprecedented. The reason for DNA degradation is not clear at this time. The DNA loss, however, is biphasic: during the first 14 days degradation is rapid followed by continued degradation but at a much slower rate. This may indicate that during the first 14 days the cells are degrading extraneous DNA such as extrachromosomal DNA or partially replicated chromosome copies. The effects of DNA degradation on the cell can be severe; however, attempts to determine detrimental effects on the cells from DNA loss such as the loss of genetic traits during starvation have been unsuccessful.

On first observation, the increase in viable cells during the first wk of starvation with a concomitant decrease in DNA appears paradoxical: cells increasing in number would be expected to increase in DNA as well. One possible explanation of the observed results is a fragmentation process similar to that observed for Arthorbacter sp. which undergo an increase in viable cells without an increase in cell mass (32). Feulgen stained Ant-300 cells show distinct dark bands which were designated as nuclear bodies. The number of nuclear bodies per cell so observed does not remain constant but generally

increases with the age of the culture (Table 6). Cells starved from various times during the exponential growth phase increase in numbers in proportion to the average number of nuclear bodies per cell observed prior to starvation (Fig. 9). The nuclear bodies therefore appear to be complete nuclei, several of which are produced by a cell during growth conditions and subsequently packaged into functional cells upon starvation. Functioning in this manner, the additional nuclei produced serve as reserve material and permit a population to increase in numbers upon starvation with a minimum expenditure of energy. If this hypothesis is true, once a cell enters starvation conditions, no synthesis of DNA need occur. Nalidixic acid, an inhibitor of DNA synthesis (38, 44), when added to a young culture of Ant-300 caused growth to stop after 1 h (Fig. 10), permitting only a 20% increase in cell numbers. However, when Ant-300 is starved in the presence of nalidixic acid it has no effect and the cells increase in numbers identical to control cultures, indicating that DNA synthesis is not required. The mechanism for the production of multiple nuclei and this altered type of cell division is not known but may be similar to the recovery of Escherichia coli from the effects of penicillin. Hirokawa (45) observed that spheroplasts induced by penicillin treatment increased four-fold in DNA before giving rise to four rod-shaped cells. He also observed three to four ultraviolet light absorbing areas within each spheroplast.

Harrison and Lawrence (43) reported that starvation selected for or produced starvation resistant cells and that in a population of starved cells up to 50% of the cells showed better survival on subsequent starvation. Restarvation of Ant-300 (Fig. 14) showed that after 7 wk of starvation, the survivors were no more or less resistant to starvation than were cells not previously starved. Moreover, cells surviving nine months of starvation, grown and starved again, did not survive as well as cells not previously starved. Clearly, the survival capability of Ant-300 is not due to a segment of the population normally resistant to starvation or to a mutation of the culture during starvation. The reduced starvation capacity of long-term starved cells is unclear but has serious implications if cells in the natural environment are exposed to repeated cycles of growth and long-term starvation. The diminished capacity for repeated starvation may be due to cell damage during starvation which is not repaired in the relatively short growth period.

The growth of bacteria can be restricted by limiting the amount of any one of several nutrients that may be present in the growth medium. Likewise, it may be reasoned that cells may be under starvation conditions not only in the complete absence of nutrients but also when only one or a few essential nutrients are lacking. Moreover, cells in the natural environment would most likely encounter starvation conditions gradually as a particular nutrient

is depleted rather than abruptly as in the laboratory. With this in mind, Ant-300 was grown with a limited amount of glucose (Fig. 15). After cessation of growth (as determined by optical density) the cells increased in number and then died as did cells completely starved. This being the case, it appears that cells starved for a particular nutrient (in this experiment, carbon) survive only as well as cells completely starved. The starvation of Ant-300 in the presence of glucose or nitrate (Fig. 18) showed that cells survive slightly better in the presence of nitrate but die rapidly in the presence of glucose. Apparently starving cells can utilize nitrate to an advantage but glucose causes a rapid decline in viable cells. Although the pH of the suspension remained the same, ruling out the possibility of death due to acid production, it is possible that the unbalanced metabolism of glucose causes cell death. Ant-300 starved in the presence of chitin (Fig. 17) survives slightly better than cells without chitin but the long-term effects are minimal. Chitin, the major skeletal component of most marine invertebrate species, is produced annually in great quantities and would therefore be expected to be a major source of nutrients for marine bacteria. Although Ant-300 produces a chitinase (T. Goodrich, personal communication) attempts to grow it on chitin as a sole source of carbon and/or nitrogen were unsuccessful. Apparently Ant-300 is also unable to utilize chitin under starvation conditions to any great advantage. The slight effect

on starvation may be due to contaminants in the chitin preparation or the attachment surface that the particulate chitin offers.

Substrate accelerated death was noted for glucose but not nitrate on Ant-300 (Figs. 16 and 18). Nitrate added on day 7 had little effect, less than when nitrate was present from the onset of starvation indicating that the effect of nitrate is expressed during the first wk of starvation quite possibly in conjunction with a stored carbon compound. The effect of a glucose addition on day 7 was similar to the addition of glucose on day 0 indicating that previous starvation is not required for accelerated death following certain nutrient additions.

The reduction in size of Ant-300 cells is most pronounced during the first wk of starvation (Fig. 12), the same period when the population increases in numbers. This observation is consistent with the hypothesis of a fragmentation of large multinucleated cells into smaller uninucleated ones. However, size reduction continues after the first wk indicating that size reduction is not solely due to fragmentation. In contrast, the morphology change is a gradual process commencing after the first wk and continuing until the fourth wk of starvation at which time no further apparent changes occur. This may indicate that fragmentation and a reduction in size is necessary before a morphological change can take place.

Nuclepore filters were chosen for this study because the average minimum sizes of the particles they retain are similar to the

stated pore size of the filter whereas cellulose membrane filters are able to retain particles much smaller than their stated pore size (72). This is probably the reason why Ant-300 cells that were able to pass through a 0.4 μm polycarbonate filter were retained by a 0.45 μm cellulose filter. Using polycarbonate filters, if the sample volume is large or contains a dense suspension of particles the filter will retain particles smaller than the stated pore size (72). To avoid this problem samples passed through filters were of minimum volume (3 ml). Taking this into account, the data indicate that after 3 wk of starvation at least 50% of the population is between 0.2 and 0.4 μm in diameter and no more than 50% of the population is between 0.4 and 0.6 μm in diameter. Direct counts and plate counts of the nonfiltered suspension and the suspension of cells passed through the 0.4 μm filter showed that approximately the same percentage of cells were viable in both cases. The small cells, therefore, are not more resistant to starvation, but reflect the viability of the entire population.

The occurrence of small bacterial cells in the natural environment has been reported by Casida (19) who observed cells in soil that were approximately 0.5 to 0.8 μm in diameter. Casida (18) also isolated soil organisms 0.5 μm and less in diameter. Bae et al. (3) reported that 72% of the soil organisms examined were less than 0.3 μm in diameter. Oppenheimer (62) reported the occurrence of up to 12 viable cells per ml of seawater filtered through a membrane

with an approximate pore size of 0.4 μm, while Anderson and Heffernan (2) found from 12 to greater than 1000 filterable organisms per 40 ml of seawater. Observation of bacteria in seawater using the technique of Zimmermann and Meyer-Reil (96) indicates that there are many small cells in the marine environment (Meyer-Reil, Geesey, and Morita; unpublished data). These small spherical cells may represent as yet unisolated species of bacteria or isolated species that have increased in size and changed morphology upon laboratory cultivation. Small cells in the natural environment may have arisen through a natural starvation process similar to that used to induce small cell formation in Ant-300.

The fact that the small cells are able to grow indicates that they are complete cells unlike the minicells of Escherichia coli which contain no DNA and are unable to divide (1). The formation of E. coli minicells appears to be an alteration of normal cell division with a population producing approximately one minicell for every two normal cells in the culture (1). In a starving culture of Ant-300, all the cells undergo size reduction indicating a generalized physiological mechanism.

Specialized groups of bacteria are known to change morphology and this subject has been reviewed by Ensign (32). Changes in morphology of Vibrio sp. have been reported by Felter et al. (35) and Baker and Park (4). In all cases the changes in morphology

appear to be part of a cellular life cycle. Although the sphere-rod-sphere cycle of Arthrobacter crystallopoietes has been nutritionally controlled (33), there have been no reports of changes in cell size or morphology due to nutrient starvation.

Ant-300 is normally a straight or curved rod (Fig. 11a). Growing in liquid or on solid media no morphological changes are observed except for an increase in cell length in stationary phase cultures. The change in morphology upon starvation appears to be a direct effect of nutrient deprivation rather than part of a life cycle. When inoculated into a fresh medium, starving cells increase in size and regain their "normal" shape. Cells recovered from starvation, grown on GM, and starved again show the same size reduction and morphology change. Of the 25 filterable strains isolated by Anderson and Heffernan (2) only eight retained their filterable property after cultivation. They hypothesize the existence of a life cycle or growth on rich media to explain this finding. The existence of a life cycle cannot be ruled out as Tuckett and Moore (90) reported filterable particles produced during the life cycle of Cellvibrio gilvus that gave rise to normal size cells. However, the present study showed that growth on rich media after starvation does produce an increase in cell size.

Electron microscopy of the small starving cells shows no structures resembling those of cysts or spores. It is apparent that

the prolonged survival characteristics of Ant-300 are not due to the formation of specialized cells. Electron microscopy did reveal an enlarged periplasmic space similar to that of natural soil "dwarf" organisms examined by Bae et al. (3). Together with the size similarity, this may indicate that cells under starvation conditions more closely resemble cells in the natural environment than do laboratory strains grown on rich media. This may also indicate that relatively large cells are the result of laboratory cultural conditions and are not indicative of cells in the natural environment. Since most microbiological studies are carried out using rich media unlike most natural habitats, the data obtained must be interpreted carefully. Studying bacteria using dilute media or under starvation conditions may more closely simulate normal natural conditions for bacterial populations as well as allow the expression of the organism's natural morphology.

To date, the study of bacterial survival under starvation conditions has been conducted using cell suspensions with a relatively high cell density (10^6-10^8 cells/ml). In fact, increased survival has been observed with increasing cell concentrations (42, 66, 85). The advantage of using high cell densities in any bacteriological study are many. High cell densities allow multiple small volume samples to be taken with enough material to permit standard biochemical tests, microscopic examination, and subsamples for additional experiments. Indeed, many of the results reported in this study would have

been impossible to obtain with a less dense suspension. However, the disadvantages of high cell densities are equally numerous. With a few exceptions, cells are never found in the natural environment in such great concentrations especially in pure culture. When cells are found in high numbers, it is usually in a nutritive environment. The study of bacteria at low population densities, therefore, may provide a more realistic approach to the study of starvation.

Ant-300 starved at various initial cell densities (Fig. 19) showed that starvation at high cell densities is not indicative of starvation at all densities. In addition to an increased maximum increase in cell numbers (Table 8), low initial cell densities cause an apparent tremendous increase in survival. This observation is a direct contradiction of the data of Harrison (42), Postgate and Hunter (66), and Thomas and Batt (85) who found greater survival for denser populations (population effect). This reverse population effect in Ant-300 may reflect a buildup of toxic materials or anoxia in the dense cultures. The determination of the time needed to completely eliminate a population of Ant-300 starved at low initial densities has proven elusive. After 70 wk of starvation over 15 times the original number of cells were still viable. In addition, the shape of the survival curve excludes the possibility of determining the starvation survival of Ant-300 by extrapolation. Limited by "the patience and perseverance of the investigators," Boylen and Ensign (8) were only able to

determine the half life of Arthrobacter crystallopoietes (100 days) by extrapolation. Continuing this extrapolation, a population of A. crystallopoietes would be completely eliminated in 190 days. The starvation survival of Ant-300, therefore, easily exceeds the longest heretofore reported starvation survival by at least 2.5 times.

The reason for the magnified increase in cell numbers and the increased longevity is unclear. Nalidixic acid added to a low density suspension resulted in an increase similar to cells starved at a high cell density indicating that DNA replication and growth are occurring. However, cell size reduction and morphology change still occurred indicating starvation conditions. Possible explanations for this growth include: growth on nutrients carried over from the growth medium; autotrophic growth; growth on contaminants in the menstruum; growth on volatile compounds from the air; and growth on stored reserve material. Repeated washings of the cells prior to starvation (up to five times) did not affect the survival characteristics of the cells. Furthermore, carry over of nutrients would be expected to be minute after the repeated washings and the $1:10^5$ or greater dilution necessary to reduce the cell density. Ant-300 is not known to be autotrophic. Attempts to show utilization of CO_2 by Ant-300 as judged by the uptake of $NaH^{14}CO_3$ proved unsuccessful. The fact that the starvation menstruum is able to support roughly the same number of viable cells after 2 wk of starvation irregardless of the

initial number of cells (Fig. 19) tends to support the possibility of growth on contaminants in the starvation menstruum. However, starvation of Ant 300 in a specially prepared menstruum showed little difference from cells starved in normally prepared menstra (Fig. 22). After dissolution of the specially prepared salts, a white precipitate (probably carbonate) formed and was accompanied by a high pH (9. 0). The precipitate was dissolved and the pH was reduced by the addition of HCl. The precipitate reappeared after 3 wk of starvation at which time the pH had risen to 8. 5. This precipitate and high pH may have been the cause of the differences observed. Furthermore, cells starved in a menstruum which had previously been used as a starvation menstruum for 1 wk also showed an initial increase in cell numbers (Fig. 24). If the original menstruum did contain contaminants sufficient for the initial increase in numbers, its second use would be expected to show no such increase. However, the increased death rate of the cells starving in the recycled menstruum indicates that the initially starving cells did change the menstruum so that even at low cell densities there are interactions between the cells of the population. It therefore appears that the extensive growth is not due to impurities in the menstruum. It also seems unlikely that trace nutrients either from the growth medium or as impurities in the menstruum could support the cells for extended periods of time (over 1 yr) since it would be expected that the cells would utilize these

nutrients in the first wk of starvation. The possibility of growth or maintenance on volatile compounds has not been investigated. At best, the amount of nutrients obtained in this manner would be expected to be slight.

On first inspection, it appears impossible for cells to have enough reserve material to permit an over 400-fold increase in cell numbers. However, the efficient utilization of reserve materials and cellular constituents may permit the cells to increase in numbers without a significant increase in biomass. The evidence for this type of replication includes the following. Ant-300 has been shown to contain several nuclei per cell prior to starvation and to decrease its cellular DNA and protein by 40% upon starvation. The DNA and protein detected in the menstruum can only account for 5% of this decrease. Also, the material released by starving cells cannot be taken up or utilized extensively even by nonstarved cells. Ant-300 has also been shown to change in size and shape from a rod (approximately 1 μm X 4 μm) into spheres (approximately 0.4 μm in diameter). Using these figures, Ant-300 reduces its cellular volume 11-fold. In addition, prior to starvation, 30% of the cellular carbon is present in low molecular weight components that disappear during starvation. Lastly, Ant-300 reduces its endogenous metabolism over 99% during the first week of starvation thus conserving carbon that would otherwise be lost through respiration. The production of numerous small

cells by Ant-300, therefore, indicates an efficient species survival mechanism instituted by starvation conditions.

The "starvation" of Ant-300 in seawater (Fig. 23) produced an initial increase in cell numbers similar to cells starved in buffered SM. This response was similar to starvation in the presence of a mixture of amino acids at a concentration similar to that found in natural seawater (1X AA). When the concentration of amino acids was increased ten-fold, the initial increase was not greater than either seawater or SM amended with 1X AA, and the survival was less than with no additions. More growth would be expected in seawater and SM amended with amino acids due to the increased nutrient content but the results obtained do not confirm this idea. In addition, these results tend to rule out the possibility that the starvation survival of Ant-300 at low population densities is affected by contaminants in the menstruum or carry over nutrients. The results do indicate that the response of the cells in starvation menstra is close to their response in seawater, and, under certain conditions, starvation survival may be better under more dilute nutrient concentrations. The addition of amino acids may be causing a form of substrate accelerated death albeit of much less intensity than glucose accelerated death.

The ability of starving cells to utilize added nutrients is of significance in the natural environment in the mineralization of compounds introduced into the environment. If the addition of

nutrients causes accelerated death, a large percentage of the microbial population would die. On the other hand, if substrate accelerated death is an artifact of the laboratory or only due to the unbalanced utilization of certain compounds, added nutrients will be mineralized and utilized for the production of bacterial biomass. The addition of low level nutrients to a starving population of Ant-300 produced the response shown in Fig. 25: an increase in viable numbers (biomass) with no detectable death. Undoubtedly, some of this material was also mineralized. The 200-fold increase in cell numbers was followed by a decline in numbers typical of starvation at high population densities.

Lamanna and Mallette (49) have suggested that evolution should have selected for starvation resistant microbes, but this may only be true for microbes normally subjected to starvation stress. Ant-300 appears to be well adapted for survival under the sparce nutrient conditions of the open ocean. It is not surprising that the optimum temperature and salinity for survival are close to the optima for growth, and fall within the ranges expected in situ for Ant-300. Ant-300 also appears well adapted for starvation survival under hydrostatic pressure if allowed to starve for a short period of time before pressurization. This may indicate that a reduction in metabolic activity or other starvation induced changes are necessary for barotolerance. It seems reasonable to assume that cells in the natural environment would encounter starvation conditions before a large

increase in pressure as they sediment or are downwelled from a productive surface region. In addition, the starvation induced increase in viable cells together with the decrease in size of the cells increases the bacterial surface area as well as the surface to volume ratio of the individual cells. A population with increased surface area or a cell with an increased surface to volume ratio would be expected to capture substrates more efficiently and therefore have a selective advantage especially in an environment where the nutrient content is very low. Ant-300 has been starved for periods exceeding 1 yr, a relatively long period of time, but the length of time needed to completely eliminate a population is still not certain. Perhaps the answer can only be expressed in terms of geological time.

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