

AN ABSTRACT OF THE THESIS OF

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Title: THE ACTIVITY OF EXTRACELLULAR ENZYMES IN STARVED AND  
UNSTARVED CELLS OF A PSYCHROPHILIC MARINE VIBRIO

Abstract Approved: ~~\_\_\_\_\_~~  
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The psychrophilic marine Vibrio sp., ANT-300, was able to utilize a variety of carbon sources that can be considered typical for the marine environment. The organism grew abundantly on glucose and serine. Chitin was hydrolyzed even when it was offered as the only carbon source, while no growth occurred on laminarin which is a common exudate of macroalgae. ANT-300 was not able to hydrolyze either DNA or RNA. Fatty acid ester hydrolysis was shown for Tween 20, 40, 60, 80, and 85.

The presence of a fatty acid esterase with biphasic substrate kinetics was demonstrated ( $K_m = 22 \mu\text{M}$  and  $K_m = 420 \mu\text{M}$ ). This esterase has a temperature optimum of  $35^\circ\text{C}$ , a pH optimum of pH 9, and displayed maximal activity for fatty acid esters of p-nitrophenyl with a chain length of 8 carbon atoms. Both enzyme systems were maintained on starvation, but the  $K_m$  of the low affinity system decreased greatly ( $K_m = 79 \mu\text{M}$ ). Calculated on the basis of viable cells and on the basis of direct cell counts there was an increase in activity of the enzyme during the starvation period of 57

days. Although about 30% of the observed activity was due to activity in the supernatant the results did not change when this was factored into the calculations.

ANT-300 also has an extracellular alkaline phosphatase with a temperature optimum of 13°C, monophasic substrate kinetics, and an optimal activity for AMP as a substrate in starved and unstarved cells. This phosphatase was maintained on starvation and increased its substrate affinity slightly as indicated by a drop in  $K_m$  from an initial 88  $\mu\text{M}$  to 60  $\mu\text{M}$ . Calculated on a per cell basis for viable and total cell counts the activity increased on starvation but about 30% of this activity was again due to the supernatant. If this was considered in the calculations, an increase in activity could only be assessed for viable cells while there was a decrease for direct counts.

It has been demonstrated that ANT-300 maintains one of the extracellular enzyme systems examined over a period of 57 days without an exogenous carbon source and even increases its activity during this time. Biphasic kinetics for an extracellular fatty acid esterase have been demonstrated which are maintained on starvation. The results presented in this thesis indicate that starved microorganisms are well prepared to utilize bioavailable dissolved organic carbon (DOC) sources and would therefore influence the composition of DOC even in such nutrient-poor environments as the deep sea.

THE ACTIVITY OF EXTRACELLULAR ENZYMES IN STARVED AND  
UNSTARVED CELLS OF A PSYCHROPHILIC MARINE VIBRIO

by

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# THE ACTIVITY OF EXTRACELLULAR ENZYMES IN STARVED AND UNSTARVED CELLS OF A PSYCHROPHILIC MARINE VIBRIO

## INTRODUCTION

The marine environment represents a variable habitat with fluctuating chemical, physical, and biological characteristics. Bacteria living in this environment have special adaptations to survive the external stresses imposed on them. Unlike laboratory cultures that grow under optimal conditions with respect to the supply of organic carbon, marine bacteria frequently encounter severe nutrient limitations (Morita, 1985). The importance of energy for growth and reproduction cannot be overemphasized as it determines the role that microorganisms play in the ocean (cf. Azam, 1982). The newly emerging "microbial loop" hypothesis may serve as an example. The concept is that bacteria salvage organic material released during phytoplankton productivity or as a result of zooplankton grazing that would otherwise be lost from the traditional food chain (Azam et al., 1983; Azam, 1986). Bacteria would then serve as prey for heterotrophic microflagellates and ciliates which can in return supplement the classical food chain by serving as prey for higher trophic levels (ZoBell, 1946). Although there is tentative evidence supporting this hypothesis, it still lacks proof and there are presently heated discussions about the existence of the "microbial

returned to the metazoan food web (Ducklow et al., 1986; Sherr and Sherr, 1987; Sherr et al., 1987; Roman et al., 1988; Sherr and Sherr, 1988; Bjørnsen et al., 1988; Nygaard et al., 1988). Despite the ongoing controversy in this field of research, the example demonstrates the metabolic and ecological potential of marine bacteria which is only limited by the availability of nutrients. The concept of "starvation-survival" has therefore become an area of extensive research in the attempt to determine the behavior of microorganisms under conditions of nutrient deprivation.

Nutrients required to support growth and reproduction are generally scarce in the marine environment. This holds especially true for the open ocean where DOC concentrations range from 0.3 to 1.2 mg/l (Riley and Chester, 1971). Particulate organic carbon (POC) is exceeded by the amount of DOC by a factor of 10. Bacteria utilize only DOC, a considerable fraction of which may be recalcitrant. It is, therefore, likely that microbial "famine" in the open ocean is the rule rather than the exception (Morita, 1982; Morita, 1988).

On the other hand, if the ocean is such an oligotrophic environment, how can deep sea isolates survive in an environment that is characterized by the absence of bioavailable carbon? Estimates of DOC residence times range from 1000 (Broecker, 1963) to 6600 years (Williams and Druffel, 1987) and indicate that there is indeed a

considerable fraction of refractory carbon. Survival of nutrient deprivation for extended periods of time would clearly make morphological and physiological adaptations necessary. Examination of these changes is the subject of current starvation-survival studies that were initiated by those of Novitsky and Morita (1976, 1977) on the marine psychrophilic Vibrio sp. ANT-300. They described a number of distinct morphological changes that occurred after the initiation of the starvation process. The starvation pattern of ANT-300 involved an initial increase in viable cells by fragmentation into ultramicrocells (0.2  $\mu\text{m}$  diameter, Novitsky and Morita, 1977), followed by a steep decline in the number of viable cells until finally a stable level was reached. How many cells were produced in the fragmentation process depended on the length of time the organisms had been in exponential growth phase prior to starvation (the longer the time, the larger the number of cells produced, Novitsky and Morita, 1977) as well as the initial cell concentration (Novitsky and Morita, 1978a). The nutritional history of the organism prior to starvation, may also play a role. Cells that were previously nitrogen-limited, for example, survive better than glucose-limited cells (Jones and Rhodes-Roberts, 1981). The ecological advantage of ultramicrocells may be avoidance of predation and increased ability to adhere to surfaces (Kefford et al., 1982; Kjelleberg et al., 1982, Marshall, 1988), which

together with an increased surface area/volume ratio, improves the scavenging capabilities of the organism (Morita, 1986). In order to guarantee the survival of one individual and thereby the species, it is also advantageous to increase cell numbers. It should, however, be noted that the starvation-survival pattern of ANT-300 is only one out of four known (Morita, 1985). The other patterns involve either a steep decline without the initial increase in the number of viable cells, or an increase followed by the maintenance of a constant level of viable cells, or maintenance of the initial cell numbers with neither the initial increase nor the decline (Amy and Morita, 1983a). Organisms that display either of these starvation patterns still await further study. It is unfortunate that ANT-300, although the model organism for starvation-survival studies, still lacks a valid classification since its isolation from the Antarctic convergence in 1972 (Gillespie and Jones, cf. Baross et al., 1974).

Physiological changes during starvation, that may enable microorganisms to survive times of nutrient limitation, have been reported by Amy and Morita (1983b). Within 30 days after initiation of their starvation experiment, changes in protein patterns were detected by two-dimensional gel electrophoresis. While new proteins appeared, previous ones disappeared which was further supported by the release of  $^{35}\text{S}$ -containing material,

implying protein degradation. These results match similar findings of Groat and Matin (1986) who resolved at least 30 new polypeptides in a carbon-starved Escherichia coli culture, employing the method introduced by Amy and Morita (1983). Novitsky and Morita (1977) and Mårdén et al. (1985) found that endogenous respiration in ANT-300 after an initial increase on starvation, decreased by 99%. The increase in respiratory activity coincided with the onset of increased protein biosynthesis (Nyström et al., 1986). Jouper-Jaan et al. (1986) starved three bacterial isolates from marine waters and found that two synthesized new proteins during starvation, while one decreased its protein content. Interestingly, the inhibition of protein synthesis in microorganisms that do show enhanced protein levels as a starvation-survival strategy, decreases their survival capabilities (Jouper-Jaan et al., 1986). Moyer (M.S. thesis, Oregon State University, 1988) examined total DNA, RNA, and protein levels during starvation of chemostat cultures of three different growth rates. He found that slow growth rate cells ( $D = 0.015 \text{ h}^{-1}$ ) displayed enhanced viability with increased protein levels of 130% of the initial concentrations. This coincided with low DNA and RNA content and the lowest biovolume of all growth rates examined. The combination of morphological and physiological changes indicates the complexity of the starvation-survival process which does not follow a set

pattern and differs among species.

Although the mode of substrate capture under low nutrient conditions and the characteristics of the enzymes involved must be an essential survival mechanism of nutrient limited microorganisms, the studies available for ANT-300 in this area of research were so far limited to the biochemical description of an alanine and an arginine uptake system. Novitsky and Morita (1979) found an uptake system for alanine in ANT-300 with optimal uptake between 10 to 20°C. Considerable amounts of  $\alpha$ -aminoisobutyrate taken up at 5° C were released when the temperature was raised to 25° C which indicates that the integrity of the cell is temperature dependent. Geesey and Morita (1979) examined two arginine uptake systems in the same organism that were specific for L-arginine. Arginine uptake proceeded through a high affinity ( $K_t = 4.5 \times 10^{-6}$  M) and a low affinity ( $K_t = 1.7 \times 10^{-8}$  M) system. Substrate capture at high arginine concentrations involved the low affinity uptake system and was connected with a chemotactic response of the organism towards its substrate (optimal chemotaxis occurred at an arginine concentration of  $10^{-4}$  M). Substrate retention capabilities depended on the external salt concentration with arginine being released under hypotonic conditions (Geesey and Morita, 1981).

Azam and Hodson (1981) reported the presence of multiphasic kinetics in natural microbial marine

assemblages. The multiphasic kinetics could not be explained by diffusion processes into algae or bacteria and were maintained when the majority of the algal cells was removed. Their findings were followed by the description of multiphasic uptake system for D-glucose in a marine bacterium (Nissen et al., 1984). For each of the four phases of the uptake system separate values for  $K_m$  and  $v_{max}$  could be determined. They concluded that multiphasic uptake kinetics were an adaptation to an oligotrophic environment with fluctuating glucose levels.

This thesis addresses the biochemical characteristics of two extracellular enzymes in starved and unstarved ANT-300 cells and thereby tries to determine the nature of the changing proteins by a different approach. It was assumed that extracellular enzymes could belong to the proteins that disappear on starvation as they may be superfluous in times of nutrient deprivation. Although the presence of hydrolytic extracellular enzymes has been previously reported for marine microorganisms (Corpe and Winters, 1972; Rosso and Azam, 1987), changes in enzymatic activity as a strategy for starvation survival have not been investigated.

## MATERIALS AND METHODS

### Organism, Media, and Reagents

The organism used for the following experiments was a psychrophilic marine Vibrio sp., designated ANT-300, which was isolated from a depth of 300 m at station 18 during cruise 46 of R/V Eltanin in 1972 to the Antarctic Convergence (Baross et al., 1974).

For the experiments described ANT-300 was grown on modified Novitsky's glucose medium (MNG; Novitsky, Ph.D. thesis, Oregon State University, 1977), unless noted otherwise.

Cells referred to as starved have been kept in a mineral salt solution (SM) without an exogenous carbon or nitrogen source. The composition and modifications of the media used are as follows:

#### Buffered 4% Salt Mixture (SM)

NaCl	26.0 g
KCl	0.8
MgCl <sub>2</sub> ·6H <sub>2</sub> O	5.6
MgSO <sub>4</sub> ·7H <sub>2</sub> O	7.6
Tris Buffer	3.2
Distilled water	1.0 l

Modified Novitsky's Glucose Medium (MNG)

Glucose	2.5 g
Yeast Extract (Difco)	0.005
NaNO <sub>3</sub>	0.5
K <sub>2</sub> HPO <sub>4</sub>	0.5
Fe(III)Na EDTA	0.007
NH <sub>4</sub> Cl	1.0
SM	1.0 l
pH	7.8

Glucose and dibasic potassium phosphate were autoclaved separately as 25% and 5% solutions, respectively. The solidifying agent for the solid medium was agar in 1.5% concentration. For the determination of the nutritional spectrum of ANT-300, glucose was replaced or supplemented with other carbon sources.

As a modification MNG contained TRIS buffer, replacing K<sub>2</sub>HPO<sub>4</sub> which then served solely as a phosphate source. In addition FeSO<sub>4</sub> was replaced by the more soluble Fe(III)Na EDTA. The medium was also supplemented with NH<sub>4</sub>Cl in order to offer nitrogen in the form of ammonia as well as nitrate (NaNO<sub>3</sub>). The concentration of yeast extract in the medium was adjusted to 0.005 g/l after preliminary experiments showed that this concentration ensured sufficient growth when the medium was supplemented with another carbon source

while growth on yeast extract alone was negligible.

1/10<sup>th</sup> Strength Lib-X (SLX)

Trypticase-peptone (BBL)	0.23 g
Yeast Extract (Difco)	0.12
L-glutamic acid	0.03
Sodium citrate	0.03
NaNO <sub>3</sub>	0.05
Fe(III)Na EDTA	0.007
Rila Salts	38.0
Tris Buffer	3.2
Distilled water	1.0 l
pH	7.8

The pH was adjusted with concentrated HCl and 10 N NaOH.

The following vitamin solution was employed in the attempt to replace the yeast extract completely.

Vitamin Stock Solution

p-Aminobenzoate	30.0 mg
Pyridoxine	60.0
Riboflavine	100.0
Choline	10.0
Myo-inositol	100.0
Ca-pantothenate	250.0
Thiamine	125.0
d-Biotin	5.0
Nicotinic acid	20.0
Distilled water	1.0 l

Of this stock solution 0.1% was added to MNG to replace the yeast extract. All media and solutions were autoclaved for 20 minutes at 121°C.

For the pH optimization of the esterase and the phosphatase assays a buffer with a pH range from 5.5 to 11 was used. The desired pH was adjusted with concentrated HCl or 10 N NaOH respectively.

Buffer (pH 5.5 to 11)

CHES (2-[N-Cyclohexylamino]ethane-sulfonic acid)	5.18 g
CAPS (3-[Cyclohexylamino]-1-propane-sulfonic acid)	5.53
BIS-TRIS ([bis(2-Hydroxyethyl)imino-tris(Hydroxymethyl)methane])	5.32
BIS-TRIS Propane (1,3-bis[tris-(Hydroxymethyl)-methylamino]propane)	7.06
Distilled water	1.0 l

**Growth and Starvation Conditions**

Due the psychrophilic nature of ANT-300 all equipment, media and reagents were kept at 5°C unless noted otherwise. ANT-300 was grown in 300 ml batches of MNG in 1 l Erlenmeyer flasks on a rotary shaker (110 rpm) at 5°C. With a 10% inoculum the organisms usually reached log phase within 72 hours. Growth was assessed with a spectrophotometer (Bausch and Lomb Spectronic 710) at 600 nm with a light path of 1 cm. ANT-300 was maintained on MNG agar plates, from which fresh cultures were grown up regularly in liquid medium.

For the starvation experiments two 1 l batches of ANT-300 were grown in 2.8 l Fernbach flasks (MNG at 5°C, 110 rpm on rotary shaker) for 72 hours. The organisms were then harvested by centrifugation (Sorvall RC5; 7970 g, 10 min, 4°C). The cells were pooled, washed twice with chilled SM

and finally resuspended in 1.5 l SM. After equilibration the batch was split up into three independently starving cultures. Samples of 10 ml were taken over a period of two months from each starvation menstruum. Prior to sampling cell suspensions were stirred with a magnetic stirrer for 5 minutes to resuspend cells that had settled out. The enzyme assays were carried out within an hour after sampling at room temperature (25°C) with cell suspensions of the same temperature.

#### **Viability Assessment**

The viability of cells during the starvation experiments was determined by the spread plate technique. After an appropriate dilution series had been made from the sampled cell suspension, 0.1 ml were spread on the surface of MNG agar plates. For all dilutions plates were spread in duplicates, except for the first sampling time ( $t_0$ ) when quadruplicate plate counts were used. Cells were considered viable, if visible growth occurred after incubation for one week at 5°C.

#### **Direct Counts with Epifluorescent Microscopy**

The cells were fixed after sampling with 30 % formaldehyde (pH 8, 0.5 ml/4.5 ml sample volume) and kept at 5°C until stained with acridine orange. Samples were usually processed within 3 weeks. The staining procedure

employed was the one described by Hobbie et al. (1977) in an improved version (Hoff, 1984, Ph.D. thesis, University of Bergen, Norway). The cells were filtered onto Nuclepore filters (0.2  $\mu\text{m}$  pore size) with silver membrane prefilters (Selas Flotronics, 0.8  $\mu\text{m}$  pore size) as supports to ensure uniform cell distribution. They were then stained with acridine orange for 10 minutes, rinsed, and air-dried. For counting the filters were mounted on a slide and the cells were counted under an Zeiss epifluorescent microscope. For the determination of the total number of cells per ml, 20 fields were counted in a crosslike fashion, the results averaged, and the number of cells/ml were calculated considering dilutions, area conversion and magnification of the microscope.

#### **Statistical Treatment of the Starvation Data**

The results from all three starvation menstruums for the  $\text{OD}_{600}$ , viable counts, acridine orange epifluorescent microscopic counts, as well as for the fatty acid esterase and the phosphatase activities were averaged and the standard error of the mean was determined. The biochemical characterizations of the fatty acid esterase and phosphatase were repeated at least twice. These data were not averaged as they were obtained from different batches of starved and unstarved cultures. This did not influence the precision of the results.

## Determination of the Nutritional Spectrum of ANT-300

### A. Utilization of Different Carbon Sources

ANT-300 cells were grown on different carbon sources replacing the glucose in MNG. The concentration of each substrate tested was 2.5 g/l. A 1% inoculum from a log phase ANT-300 culture was used in the experiment. The substances used can be divided into three categories:

1. CARBOHYDRATES: d-ribose  
d-trehalose  
d-cellobiose  
d-fructose  
d-mannitol  
d-galactose  
l-arabinose  
d-glucose  
d-maltose
2. ORGANIC ACIDS: sodium citrate  
sodium succinate  
sodium acetate  
sodium pyruvate
3. AMINO ACIDS: l-glutamate  
l-methionine  
l-serine

All substrates were made up with or without yeast extract (0.005 g/l). The organic acids were used as their

sodium salts and the pH of the medium was adjusted when necessary. Each carbon source had an uninoculated control for comparative purposes. Growth usually occurred within ten days.

#### **B. The Presence of Extracellular Hydrolytic Enzymes in ANT-300**

For the following assays the media employed were supplemented with 1.5% agar as solidifying agent. The procedures described were derived from the Manual of Methods in General Bacteriology (Smibert and Krieg, 1981) unless noted otherwise. All plates were inoculated with cells from an actively growing ANT-300 culture. The incubation time was usually two weeks. Incubation temperature was 5°C.

##### **Gelatin Hydrolysis**

For the determination of gelatin hydrolysis, MNG was supplemented with 0.4% gelatin. Plates were poured and inoculated with a single streak. After growth occurred, the plates were flooded with 15% HgCl<sub>2</sub> in 20% concentrated HCl. Clear zones around the colonies indicated gelatin hydrolysis.

##### **Starch Hydrolysis**

Starch hydrolysis was examined by supplementing SLX with 0.2% soluble starch. After the incubation period the inoculated plates were flooded with iodine solution. Clear zones around the colonies indicated a positive test result.

### **Nucleic Acid Hydrolysis**

MNG was supplemented with 0.2% RNA or DNA to replace  $K_2HPO_4$ , respectively. Toluidine blue O was added to the medium in a final concentration of 100 mg/l. A control containing  $K_2HPO_4$  and toluidine blue O but no nucleic acids served to exclude interactions of the dye with the medium. A positive test would show a bright pink zone around the colonies on the otherwise blue plate.

### **Lipid Hydrolysis**

Fatty acid esters of polyoxyethylene monosorbitan (TWEEN 20, 40, 60, 80, and 85) replaced glucose in MNG in this experiment. The substrates were added to medium with and without yeast extract, respectively. The medium always contained 0.01%  $CaCl_2$ . A halo around the colonies indicated positive results.

### **Lipase Assay**

The glucose in MNG was replaced by an oil emulsion (2.5% final concentration) and supplemented with a spirit blue solution following the procedures described by Starr (1941). The production of a lavender to purple color around the colonies was indicative for lipid hydrolysis.

### **Chitin Hydrolysis**

Colloidal chitin was added to MNG as the only carbon source and was then poured as an overlay on MNG lacking any carbon sources (Hsu and Lockwood, 1975). A clearing around the colonies indicated chitin utilization.

### **Laminarin Hydrolysis**

Laminarin was sterilized with acetone in an autoclaved Wheaton bottle. After the acetone evaporated, MNG (no carbon source) was added and the laminarin was suspended by stirring. A clearing of the medium around the colonies was indicative of laminarin hydrolysis.

### **Phospholipid Hydrolysis**

Phosphatidylcholine was prepared as a 1% solution in absolute alcohol. This stock solution was filtered through a 0.2  $\mu\text{m}$  Nuclepore filter and added to  $\text{K}_2\text{HPO}_4$ -free MNG to a concentration of 10 or 20%. A clearing around the colonies indicated hydrolysis of the phospholipid.

### **C. Assays of Extracellular Enzymes employing p-Nitrophenyl Substrates**

The following enzyme assays employed p-nitrophenyl-derivatives as substrates (Sigma). Enzyme assays were carried out at 25°C with log phase cell suspensions in SM. Reactions were stopped with 20% sodium dodecyl sulfate (SDS) after two hours. Where no enzyme activity could be determined, the assay was repeated with an extension of the incubation time to 24 hours. The release of p-nitrophenol, which is a direct measure of the activity of the enzyme examined, was then assessed at 410 nm.

### **Sulfatase Activity**

The production of aryl-sulfatase was examined by using

5 mM p-nitrophenyl sulfate as a substrate on cells that had been grown in MNG and in the same medium with 2 mM methionine replacing MgSO<sub>4</sub> for enzyme induction.

#### **$\alpha$ - and $\beta$ -Glucosidase Activity**

Cells used in this assay were grown on SLX to prevent repression of the glucosidases by glucose. The substrates employed were p-nitrophenyl- $\alpha$ - and  $\beta$ -glucoside in a concentration of 10 mM. An induction of the enzymes was attempted by using ANT-300 cells that had been grown on sterile-filtered maltose or cellobiose.

#### **Enzyme Assays for the Starvation Experiments**

The following enzyme assays were chosen for the starvation experiments under two criteria:

- As a decrease in activity on starvation was expected the enzyme activity in unstarved cells had to be high with a low reagent blank.
- At least one assay should be targeted towards monitoring the energy metabolism of the cell, while the other one should reflect changes in activity towards nutrients other than carbon (nitrogen, phosphorus) on starvation.

The release of p-nitrophenol was measured as described earlier. The polystyrene cuvettes used were replaced after each sampling time. Samples were processed in duplicates with cell, supernatant and reagent controls. To determine the amount of activity due to the supernatant the cells were

removed from 2 ml of the sample by centrifugation prior to the enzyme assays.

#### **A. Fatty Acid Esterase**

The esterase activity was assessed using an enzyme assay described by Kolattukudy et al. (1981) for a fungal cutinase. The marine nature of ANT-300 made some modifications necessary: the concentration of the substrate (p-nitrophenyl laurate, Sigma) was doubled to reduce the amount of added distilled water and to avoid osmotic stress on the organism. The enzyme reaction was stopped using 20% SDS as mentioned earlier. The cells were then removed from the assay by centrifugation and the absorbance of the supernatant was determined spectrophotometrically. Unless noted otherwise this assay was employed for all experiments. Alterations were made in order to adapt the assay to the special circumstances for the determination of the following parameters.

For the substrate kinetics experiments methyl-cellosolve was used as a solvent for p-nitrophenyl laurate. Final concentrations ranged from 0.0098 mM to 20 mM. Substrate kinetics were determined for starved (57 days) and unstarved cells respectively.

The pH optimum was determined by replacing the phosphate buffer of the original assay with an equal volume of the buffer covering the range of 5.5 to 11 (see p.12).

Unfortunately it was not possible to obtain a final pH above 9.8, as the buffering capacity of the buffer decreased considerably above pH 9.5. As the development of color in the released p-nitrophenol is pH dependent, 1 ml of a 4 M Tris buffer solution (pH 8) were added to 3 ml of the reaction mix.

The hydrolysis of even numbered fatty acid esters of varying chain lengths by starved and unstarved cells was examined using p-nitrophenyl esters of fatty acids with chain lengths between 2 and 18 carbon atoms.

#### **B. Phosphatase**

The biochemical properties of an alkaline phosphatase were examined by using a p-nitrophenyl phosphate as a substrate (Morita and Howe, 1957). The assay had a total volume of 2.5 ml, consisting of 500  $\mu$ l cell suspension, 500  $\mu$ l substrate solution, and 1.5 ml SM. The p-nitrophenyl phosphate was prepared with SM to make a final concentration of 5 mM. The enzyme reaction was stopped after 5 minutes incubation with 500  $\mu$ l of 20% SDS. As in the fatty acid esterase assay the cells were removed by centrifugation before the samples were measured in the spectrophotometer. All samples were run in duplicates with reagent controls to determine the extent of autohydrolysis of the substrate. This assay was altered where necessary to meet the objectives of the different phosphatase experiments.

For the enzyme kinetics experiment the substrate concentration varied from 0.024 mM to 50 mM. Kinetics were determined for both starved (two months) and unstarved cells.

The utilization of different phosphate substrates by starved and unstarved cells was examined. The substrates (ATP, AMP,  $\beta$ -glycerophosphate, glucose-1-phosphate, glucose-6-phosphate; Sigma) were made up as 5 mM solutions in SM with the exception of inositol hexaphosphate (Sigma) which precipitated in SM and was therefore made up as a 50 mM solution in distilled water. Of this solution 500  $\mu$ l were added to 4.5 ml SM to make a final concentration of 5 mM. All other substrates were used as the original 5 mM solutions. The substrate solutions were then inoculated with 10 % of a cell suspension of ANT-300 and supplemented with 500  $\mu$ l toluene as bacteriostatic agent. The toluene did not inhibit the enzyme activity but prevented the released orthophosphate from being taken up by the cells. Uninoculated controls were run together with each substrate. After 30 minutes incubation at 25°C the amount of liberated orthophosphate was determined spectrophotometrically at 800 nm following the procedures described in the Manual of Chemical and Biological Methods for Seawater Analysis (Parsons et al., 1984).

For the determination of the pH optimum, the SM of the assay as described above, was replaced with an equal amount

of the buffer with a pH range of 5.5 to 11 (see p.12). The reaction mix was then adjusted to the desired pH. After the reaction was stopped with SDS, 1 ml of 4 M TRIS (pH 8) was added which resulted in a uniform pH 8 in all cases.

## RESULTS

### Growth Characteristics of ANT-300 on Modified Novitsky's Glucose Medium

The attempt to replace the yeast extract in MNG with the vitamin solution or with either of its components was unsuccessful for organic acids as a carbon source. In this case ANT-300 obviously required one or more components of the yeast extract. The lower cell yield when ANT-300 was grown in the absence of yeast extract made a fully defined growth medium for the organism undesirable. For the observation of the enzyme assays during the starvation experiments initial high cell densities were important. Therefore, the amount of yeast extract in the medium was reduced 80-fold from originally 0.1 g/l to 0.00125 g/l. A yeast extract concentration of 0.005 g/l was considered optimal as this provided sufficient growth of the organisms in the presence of glucose, while growth on yeast extract alone was negligible. Figure 1 illustrates the growth of ANT-300 on 0.005 g/l yeast extract with and without 2.5 g/l glucose.

### Utilization of Different Carbon Sources

The ability of ANT-300 to grow on different carbon sources is summarized in Table 1. The organism was able to utilize a variety of mono- and disaccharides. For all sugars that were utilized, growth occurred in the presence and absence of yeast extract.

Figure 1: Growth of ANT-300 on modified Novitsky's glucose medium (MNG) with (YE= yeast extract and glucose) and without (GLC= glucose only) yeast extract.

Figure 1.

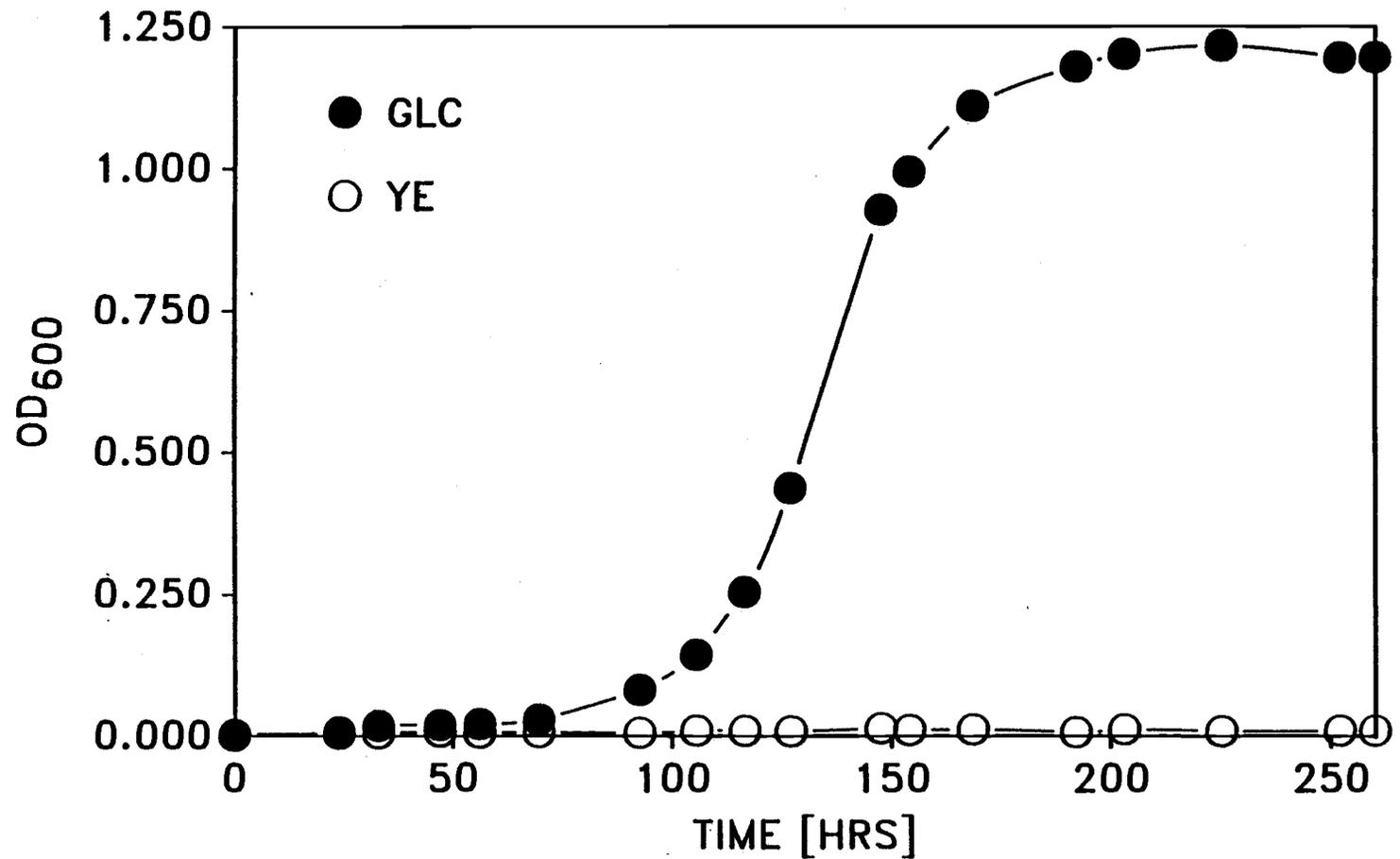


Table 1. Growth of ANT-300 on different carbon sources.

Carbon source	with yeast extract	without yeast extract
Citrate	-	-
Succinate	+	-
Acetate	-	-
Pyruvate	+	-
Glutamate	+	+
Serine	+	+
Methionine	-	-
Methionine/glucose	+	+
Ribose	-	-
Trehalose	-	-
Cellobiose	-	-
Fructose	+	+
Mannitol	-	-
Galactose	-	-
Arabinose	-	-
Glucose	+	+
Maltose	+	+

ANT-300 was able to grow on succinate and pyruvate in the presence of yeast extract. The organism did not grow on acetate or citrate.

Out of the three amino acids tested serine and glutamate resulted in abundant growth on medium with and without yeast extract. ANT-300 was not able to grow solely on methionine. Growth occurred only when methionine was supplemented with glucose which was a utilizable carbon source for the organism. In the case of the methionine-glucose combination growth could also be observed in the absence of yeast extract, which is similar to the pattern described earlier for growth on glucose alone.

#### **Extracellular Hydrolytic Enzymes**

ANT-300 was unable to hydrolyze gelatin or starch. Lipid hydrolysis took place for all substrates offered. Abundant growth occurred on fatty acid esters as the only carbon source with or without the yeast extract supplement. The lipase assay showed positive results accordingly. ANT-300 hydrolyzed chitin and was able to use it as the only carbon source. No growth occurred on the laminarin plates which indicated an inability of the organism to utilize the polysaccharide as the sole carbon source. The organism could not hydrolyze DNA or RNA. Phospholipid hydrolysis did not occur. As the p-nitrophenyl enzyme assays showed, ANT-300 lacks aryl-sulfatase and  $\alpha$ - and  $\beta$ -glucosidase. These results did not change when the incubation period was extended to 24 hours or when an induction of the enzyme was attempted. The presence of extracellular enzymes in ANT-300 is summarized in Table 2.

Table 2. The presence of extracellular hydrolytic enzymes in ANT-300.

---

Chitinase	+
Laminarinase	-
$\alpha$ -Glucosidase	-
$\beta$ -Glucosidase	-
Aryl-sulfatase	-
Phosphodiesterase	-
Gelatinase	-
DNA hydrolase	-
RNA hydrolase	-
Fatty acid esterase	+
Lipase	+
Phosphatase	+

---

### Description of the Enzymes for the Starvation Experiments

#### A. Fatty Acid Esterase

The extracellular esterase of ANT-300 has a temperature optimum of 35°C (Figure 2). Between 3°C and 10°C the amount of p-nitrophenol released was only 5% that of the optimal temperature. The activity of the enzyme in the natural environment of the organism with average temperatures 5°C would therefore be below optimum.

The pH dependence of the enzyme activity is illustrated in

Figure 2: Fatty acid esterase temperature profile in unstarved ANT-300 cells.

Figure 2.

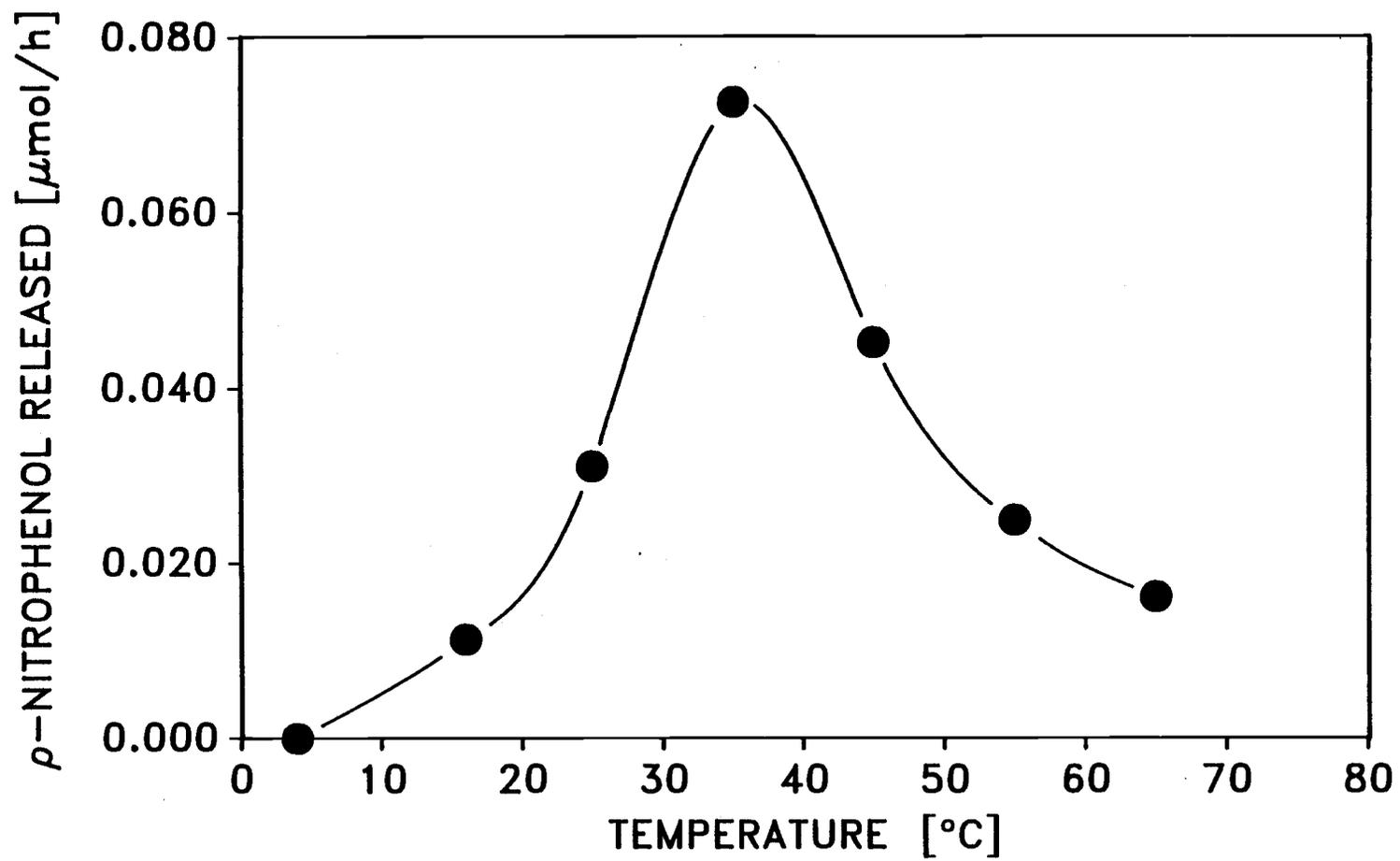


Figure 3: Fatty acid esterase pH profile for unstarved ANT-300 cells.

Figure 3.

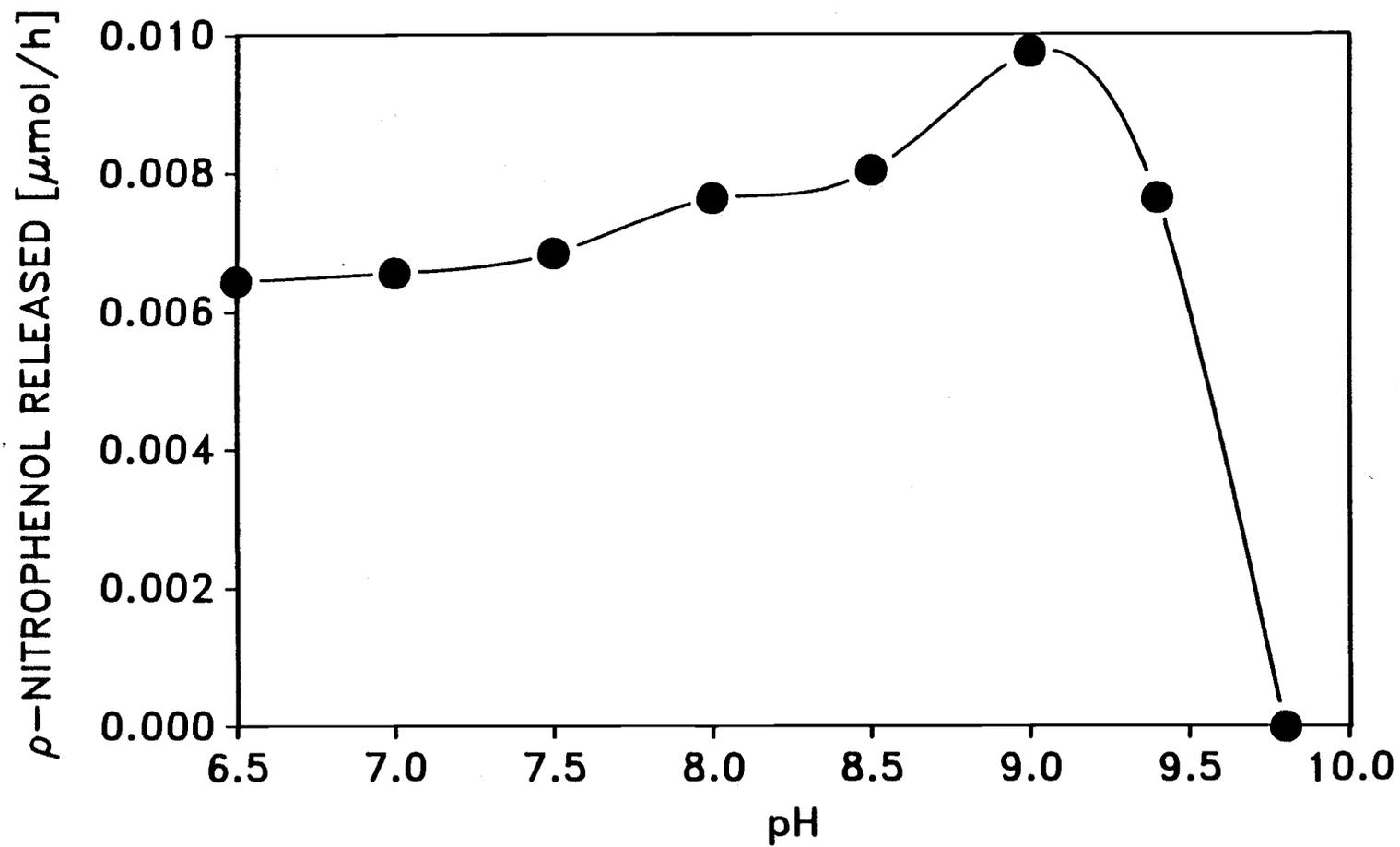


Figure 4: Hydrolysis of different chain length fatty acids esters by starved and unstarved cultures of ANT-300.

Figure 4.

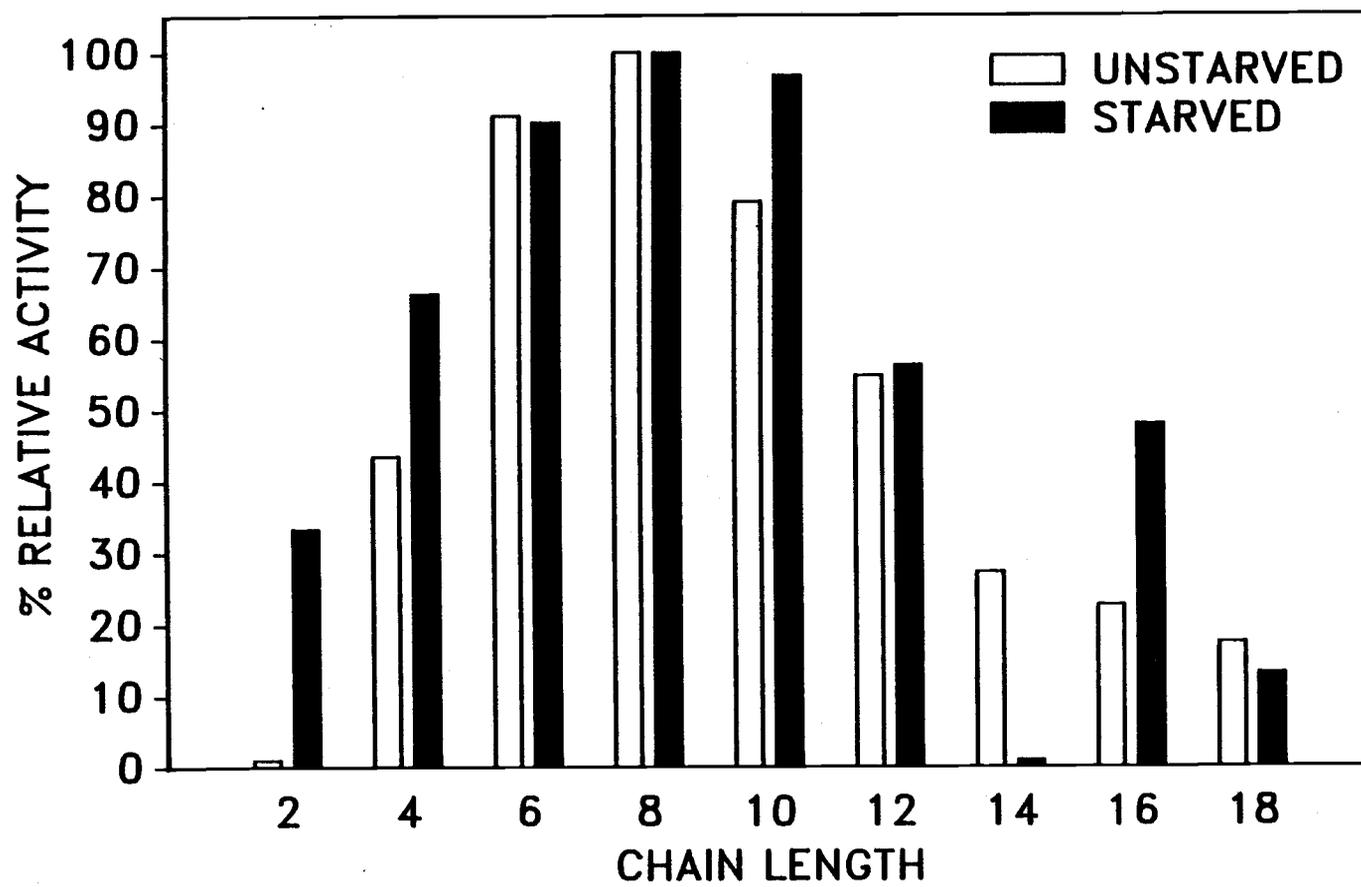


Figure 5: Michaelis-Menten plot of the fatty acid esterase substrate kinetics for unstarved ANT-300 cells. The insert is an enlargement of the substrate kinetics at substrate concentrations from 0 to 1mM p-nitrophenyl-laurate.

Figure 5.

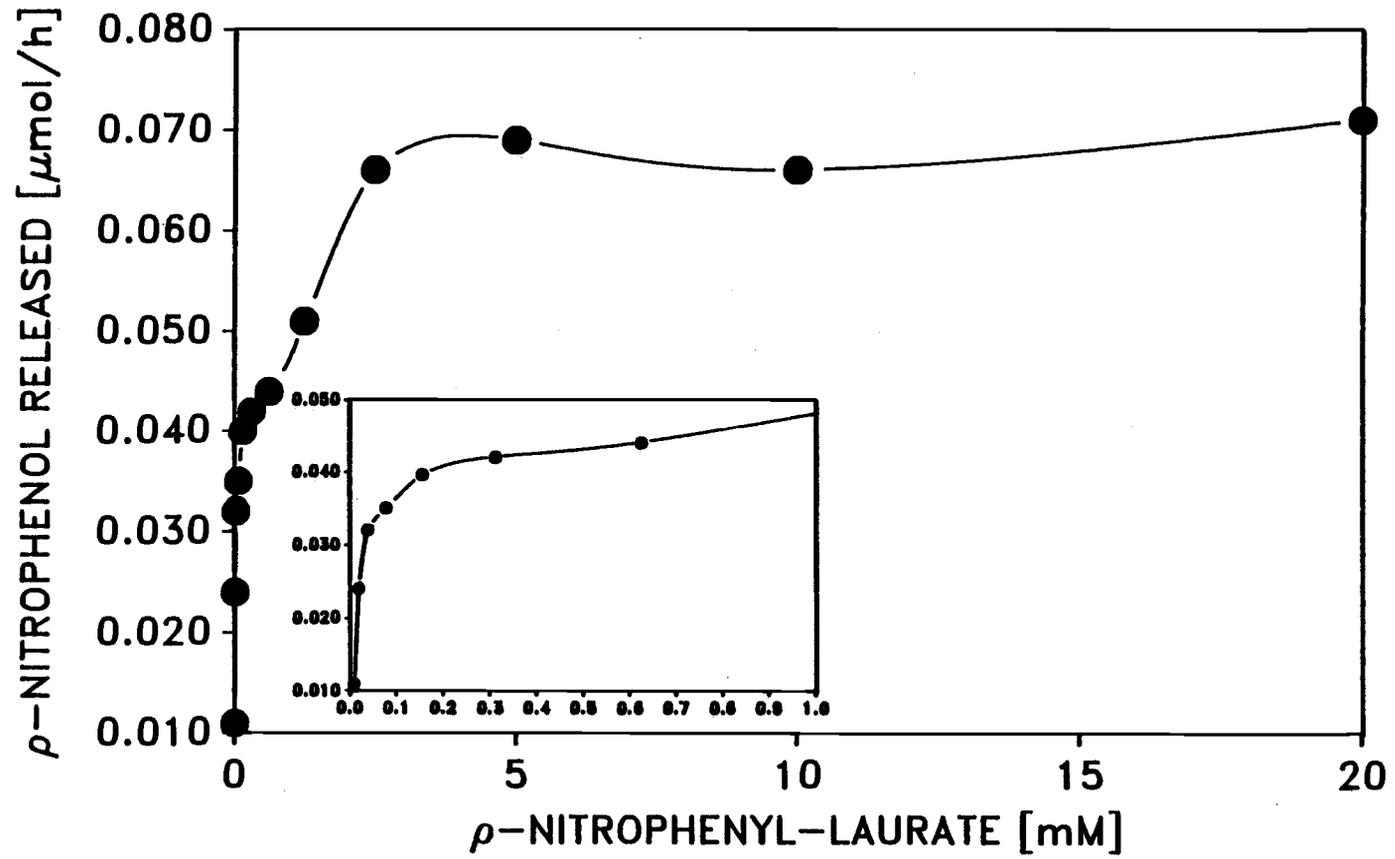


Figure 6: Hanes-Woolf transformation of the fatty acid esterase kinetics data for unstarved cells. Low affinity system ( $K_m = 420 \mu\text{M}$ ) at the top, high affinity system ( $K_m = 22 \mu\text{M}$ ) at the bottom. [ $K = 10^3$ ]

Figure 6.

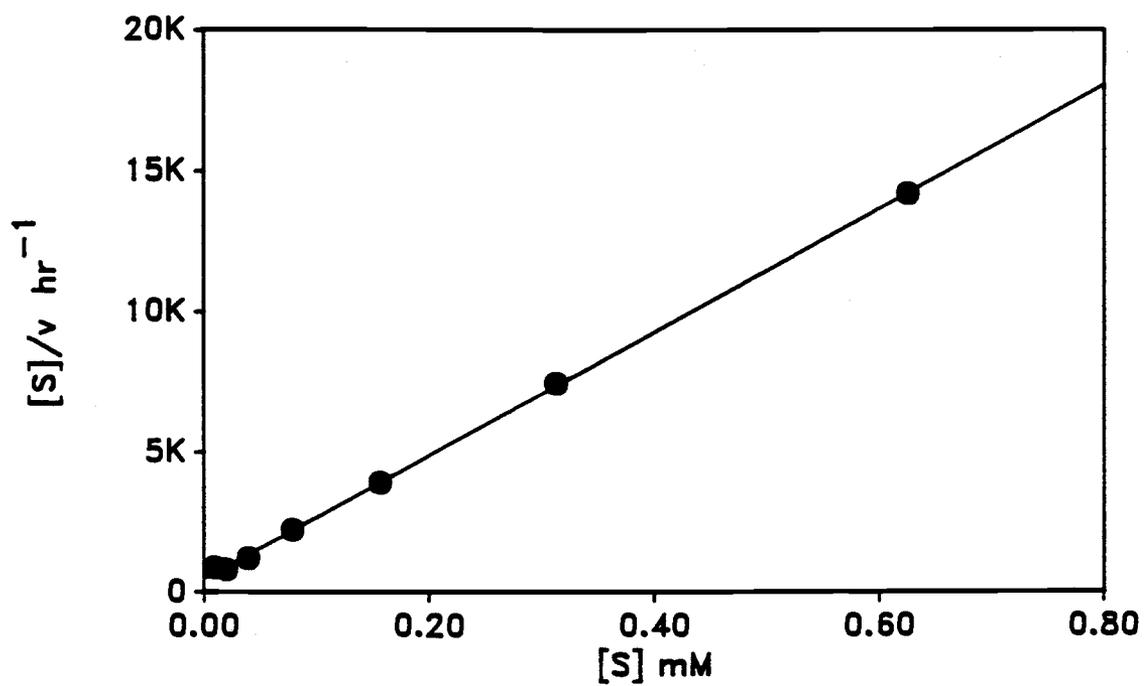
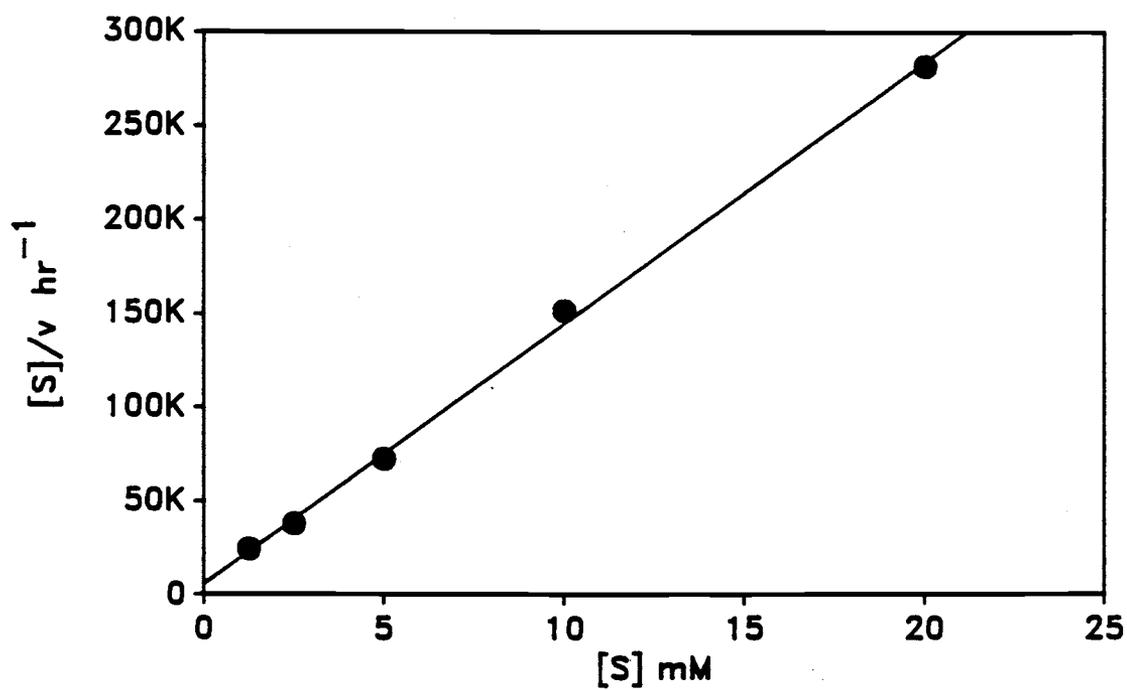


Figure 7: Michaelis-Menten plot of the fatty acid esterase substrate kinetics for starved cells.

Figure 7.

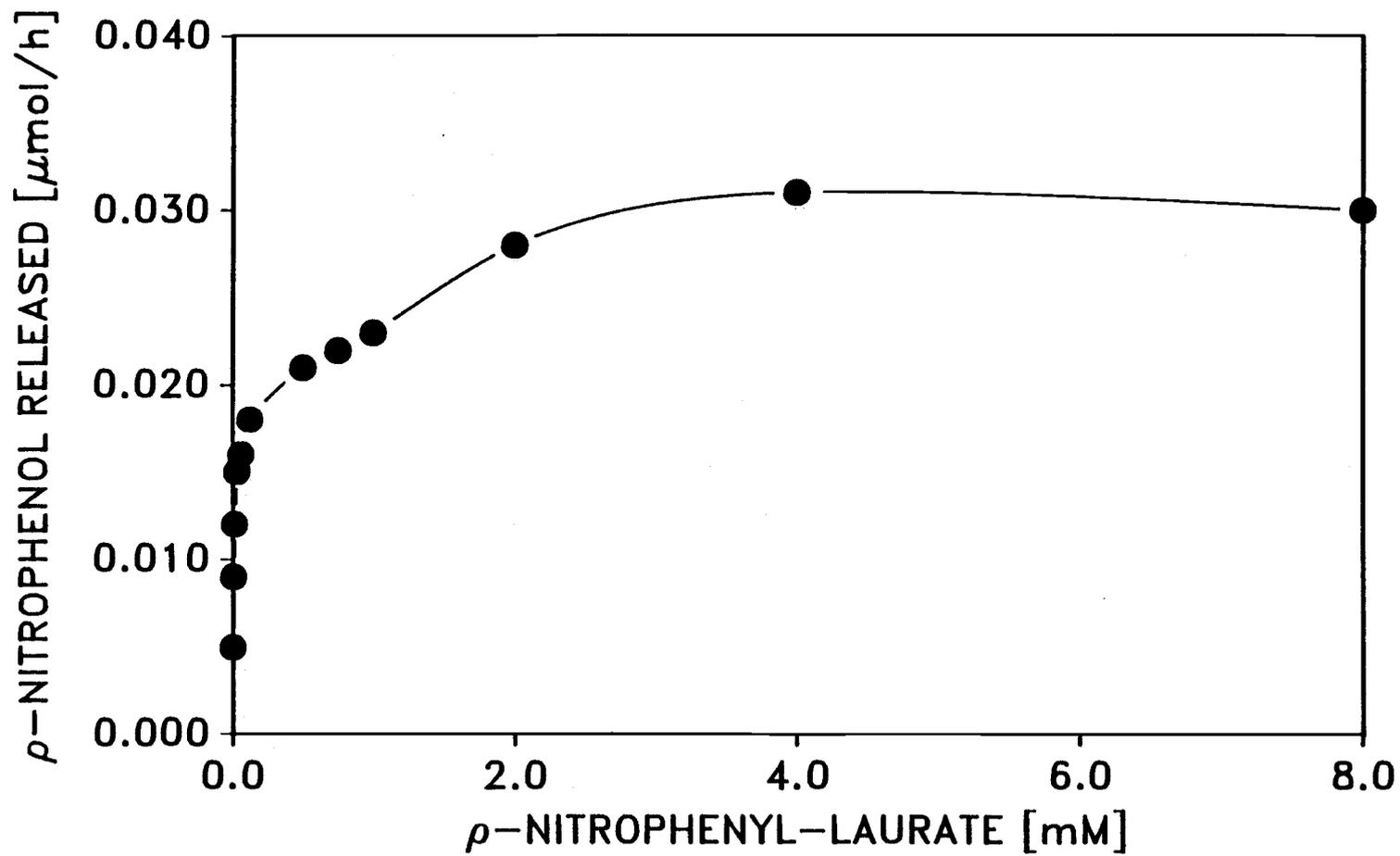


Figure 8: Hanes-Woolf transformation of the fatty acid esterase substrate kinetics data for starved cells. Low affinity system ( $K_m = 79 \mu\text{M}$ ) at the top, high affinity system ( $K_m = 22 \mu\text{M}$ ) at the bottom.  $[K = 10^3]$

Figure 8.

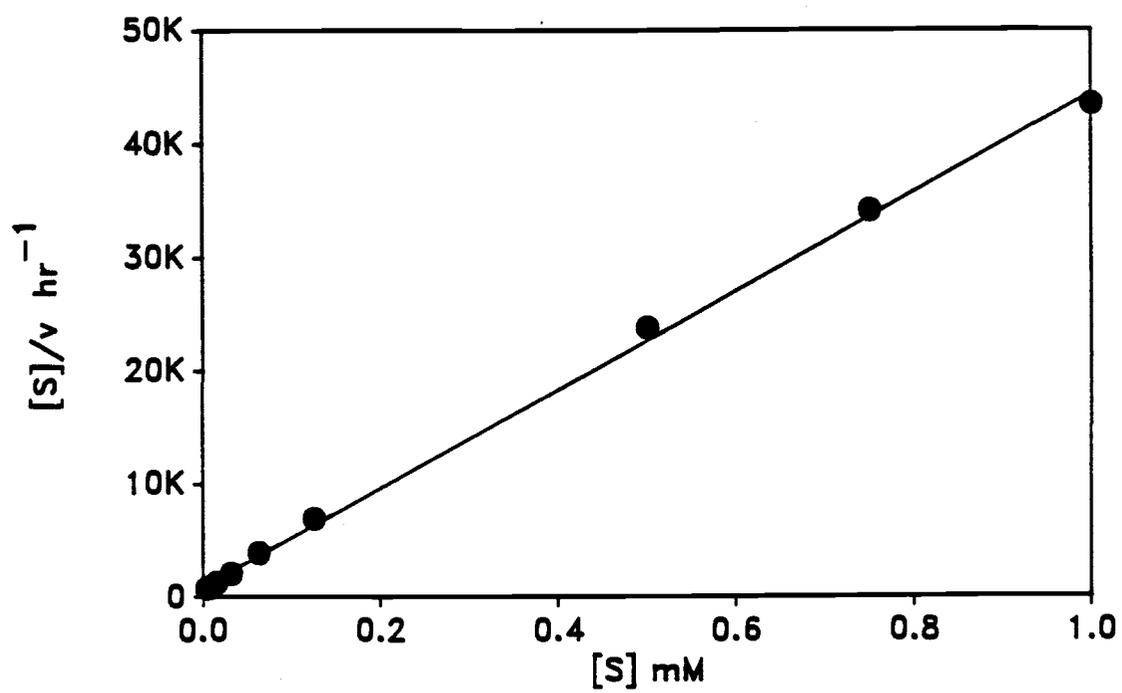
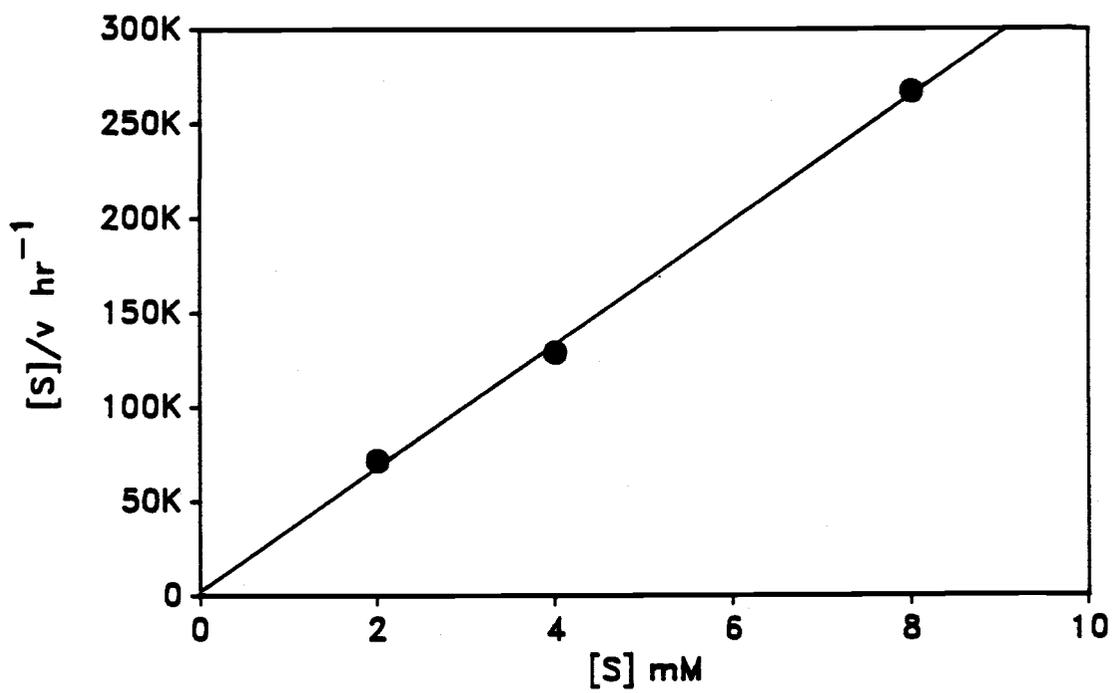


Figure 3. Although the pH optimum is at pH 9, ANT-300 has a broad plateau with almost optimal activity (6.5 nmol p-nitrophenol released per hour per ml compared to 9.7 nmol released at the pH optimum) between pH 6.5 and 8.5. Above pH 9 the activity dropped to the detection limit within one pH unit.

The relative activity (100% activity for C8 fatty acids) of the esterase for the utilization of p-nitrophenyl fatty acids with different chain lengths is demonstrated for starved and unstarved cells in Figure 4. The activity was highest in starved and unstarved cells for fatty acid esters with a chain length of 8 carbon atoms (C8). Acetate (C2) had no activity in unstarved cells while in starved cells the esterase utilized the substrate. The opposite trend was observed for myristate (C14) where the initial activity in unstarved cells decreased to zero in starved cells.

The enzyme kinetics experiments for the esterase revealed the most interesting feature of this enzyme. The Michaelis-Menten plot of p-nitrophenol released over the substrate concentration (Figure 5), shows the presence of two regions of saturation suggesting the presence of two enzyme systems. This becomes even more obvious in the Hanes-Woolf transformation of the plot (Figure 6). ANT-300 has a high affinity esterase with an apparent  $K_m$  of approximately 22  $\mu\text{M}$  and a low affinity esterase with  $K_m$  of 420  $\mu\text{M}$ . The true  $K_m$  values could not be calculated as there is interference between the two systems. Both esterases are maintained on starvation (Figure 7) but there is an increase in affinity for the low affinity esterase ( $K_m$  decreased to 79  $\mu\text{M}$ ) which is confirmed by the Hanes-Woolf transformation of the data

Figure 9: Phosphatase temperature profile for unstarved ANT-300 cells.

Figure 9.

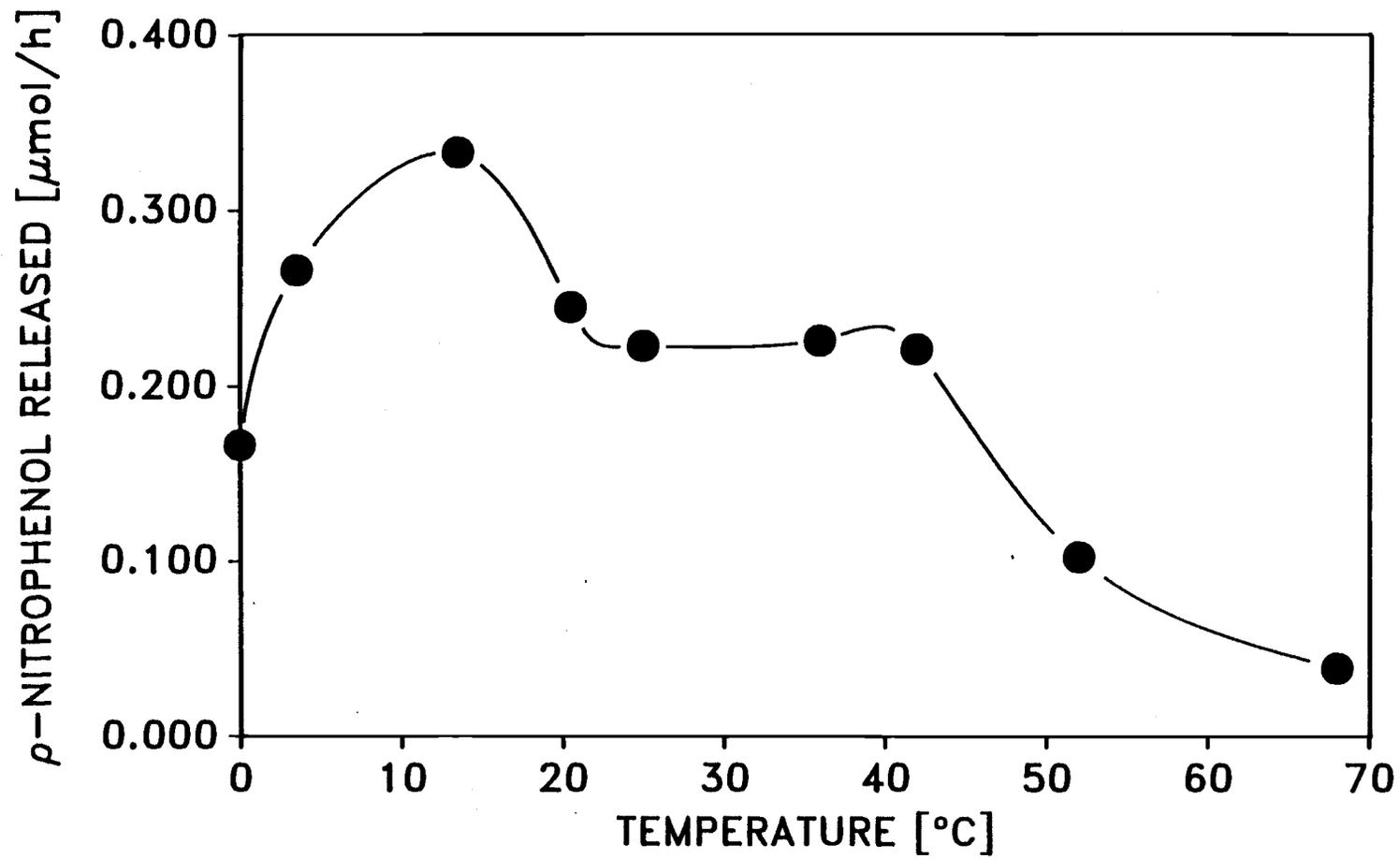


Figure 10: Michaelis-Menten plot of the phosphatase substrate kinetics data for unstarved cells.

Figure 10.

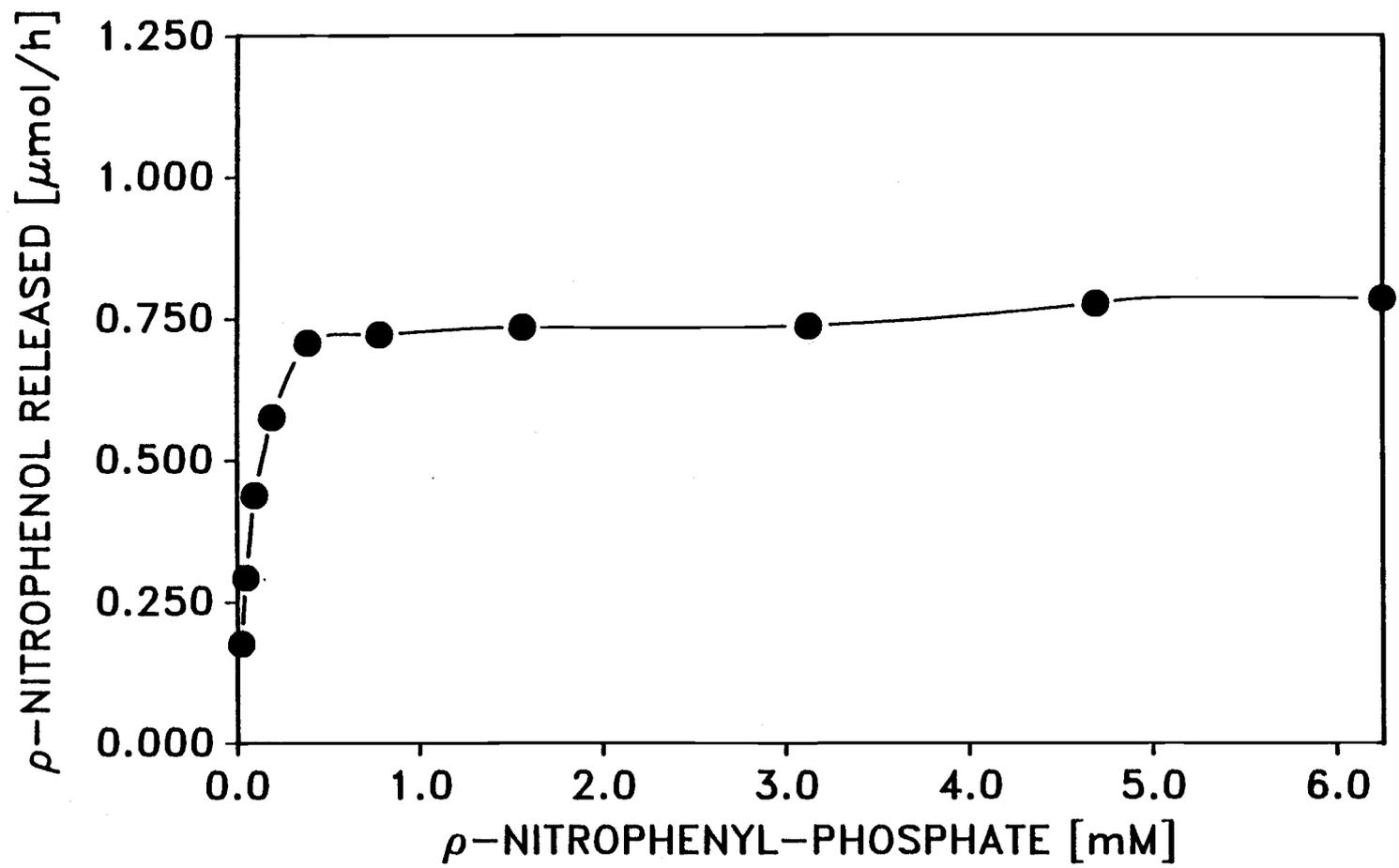


Figure 11: Hanes-Woolf transformation of the phosphatase substrate kinetics experiment for unstarved cells.

Figure 11.

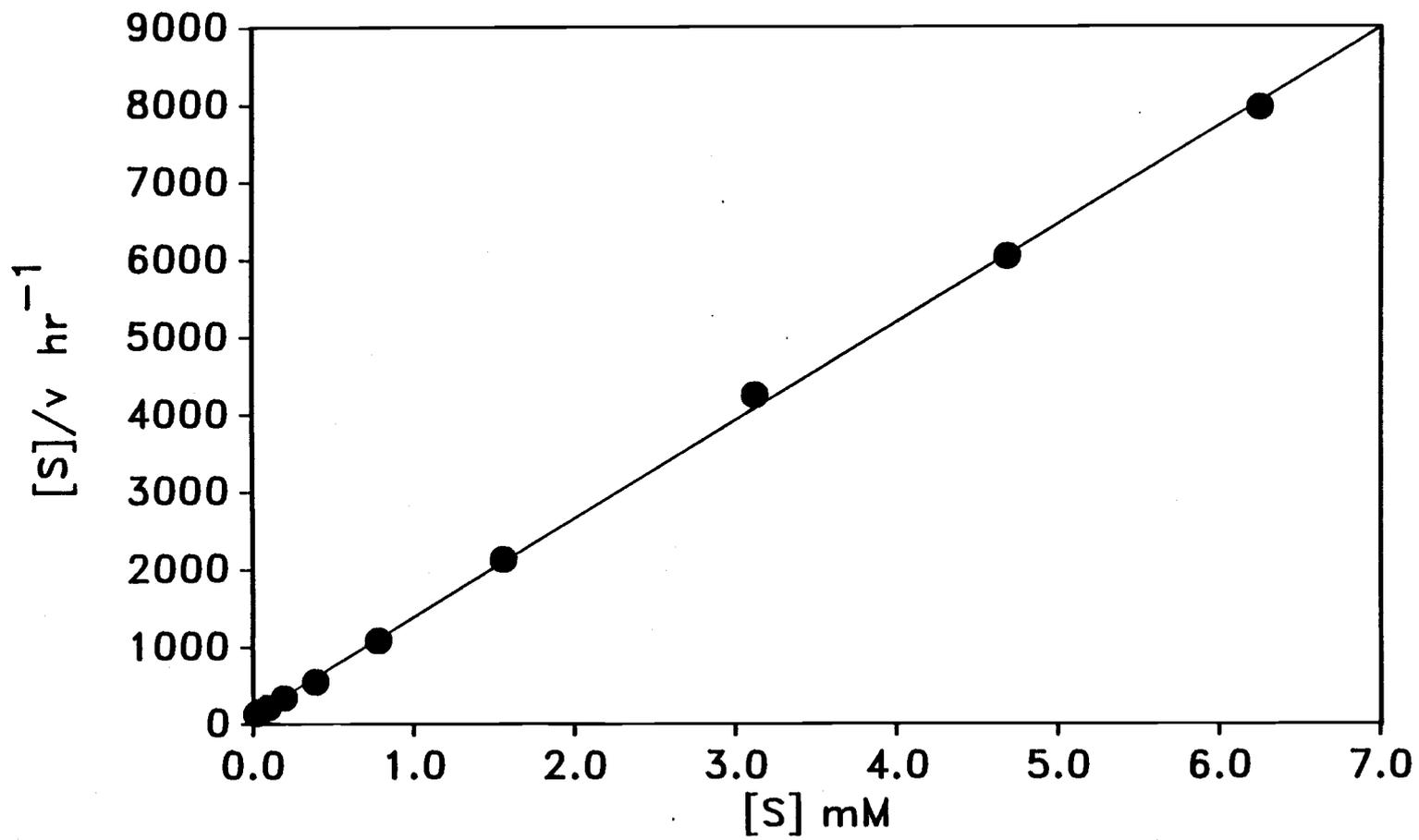


Figure 12: Michaelis-Menten plot of the phosphatase substrate kinetics data for starved cells.

Figure 12.

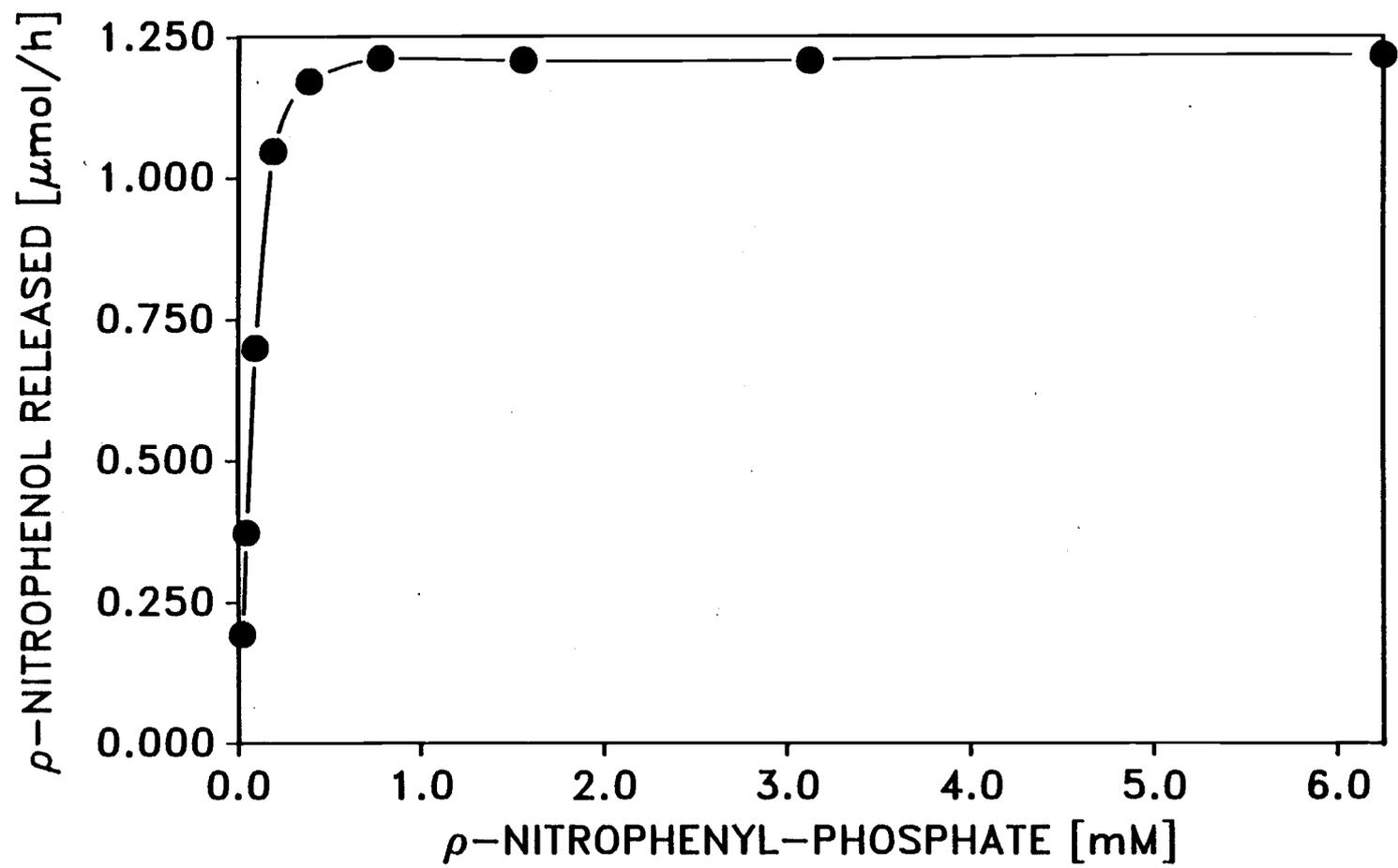
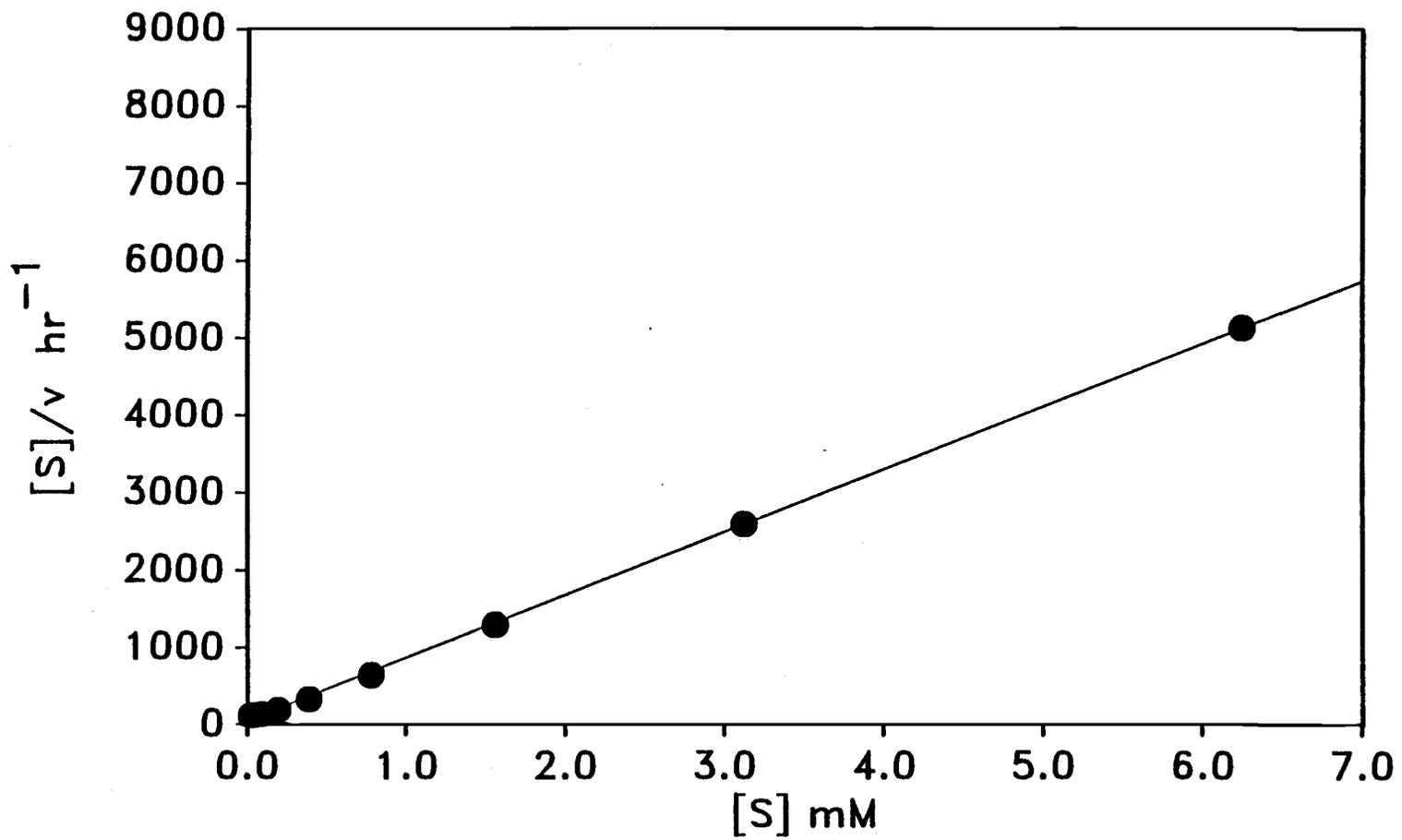


Figure 13: Hanes-Woolf transformation of the phosphatase substrate kinetics data for starved cells.

Figure 13.



(Figure 8). The  $K_m$  values were independent of cell densities of different batches of starved and unstarved cells, while the values for  $v_{max}$  differed with cell density of the culture examined. For this reason the data were not averaged. Sufficient precision of the data presented was determined by repeating each experiment at least twice.

### B. Phosphatase

ANT-300 produces an extracellular phosphatase with a temperature optimum of 13°C (Figure 9). Unfortunately it was not possible to determine the pH optimum for the enzyme there was a slow but steady increase in activity over the pH range examined and buffer solutions covering more alkaline pH ranges (pH  $\geq 11$ ) were unsuitable. The kinetics analysis of the phosphatase demonstrated a single region of saturation (Figure 10) and only one  $K_m$  value ( $K_m = 88 \mu M$ ) was calculated from the Hanes-Woolf transformation (Figure 11). The affinity of the enzyme towards its substrate increased on starvation (Figure 12) and for the Hanes-Woolf transformation of the same data see (Figure 13). A new  $K_m$  value of 60  $\mu M$  could be determined accordingly. The data behaved statistically the same way as the fatty acid esterase results.

## Results of the Starvation Experiments

### A. Viability Assessment

Due to an initial period of fragmentation of the cells during the first 48 hours of starvation, there was an increase in colony-forming units (CFU) during this time (Figure 14). Until day 57 an overall decrease in the number of viable cells could be observed which reached

$1.07 \times 10^7$  cells/ml. The direct counts with acridine orange, representing the total number of cells present, stabilized at  $2.29 \times 10^8$  cells per ml after fluctuations during the first 7 days of starvation. The percentage of viable cells from the total number of cells at day 57 is therefore 4.8%. Figure 14 represents the averaged values for CFU, direct count by acridine orange (AODC) and optical density ( $OD_{600}$ ) from the three independently starving cultures.

#### B. The Fatty Acid Esterase during the Starvation Experiments

Figure 15 shows the averaged results of the fatty acid esterase and phosphatase activities from the three starvation menstrooms as  $\mu\text{mol}$  p-nitrophenol released. The esterase activity increases during the whole experiment. Calculated on a per cell basis, the esterase activity increases slightly when calculated for direct and viable counts (Figure 16 and Figure 17).

#### C. The Phosphatase during the Starvation Experiments

The activity of the phosphatase (as  $\mu\text{mol}$  p-nitrophenol released) drops steeply during the first two days, levels between day two and six and decreases to its lowest value at day seven. After day seven the activity stabilizes (Figure 15). There was an increase in activity per viable cell that paralleled the one observed for the esterase (Figure 17). The phosphatase activity related to total cell counts fluctuates during the first week and then stabilizes after day 10 (Figure 16). A slight increase in activity could be observed.

As can be seen in Table 2, the utilization of different substrates

Figure 14: Colony forming units (CFU), direct counts by acridine orange (AODC) and optical density ( $OD_{600}$ ) for the starvation experiments. Vertical bars equal the standard error of the mean.

Figure 14.

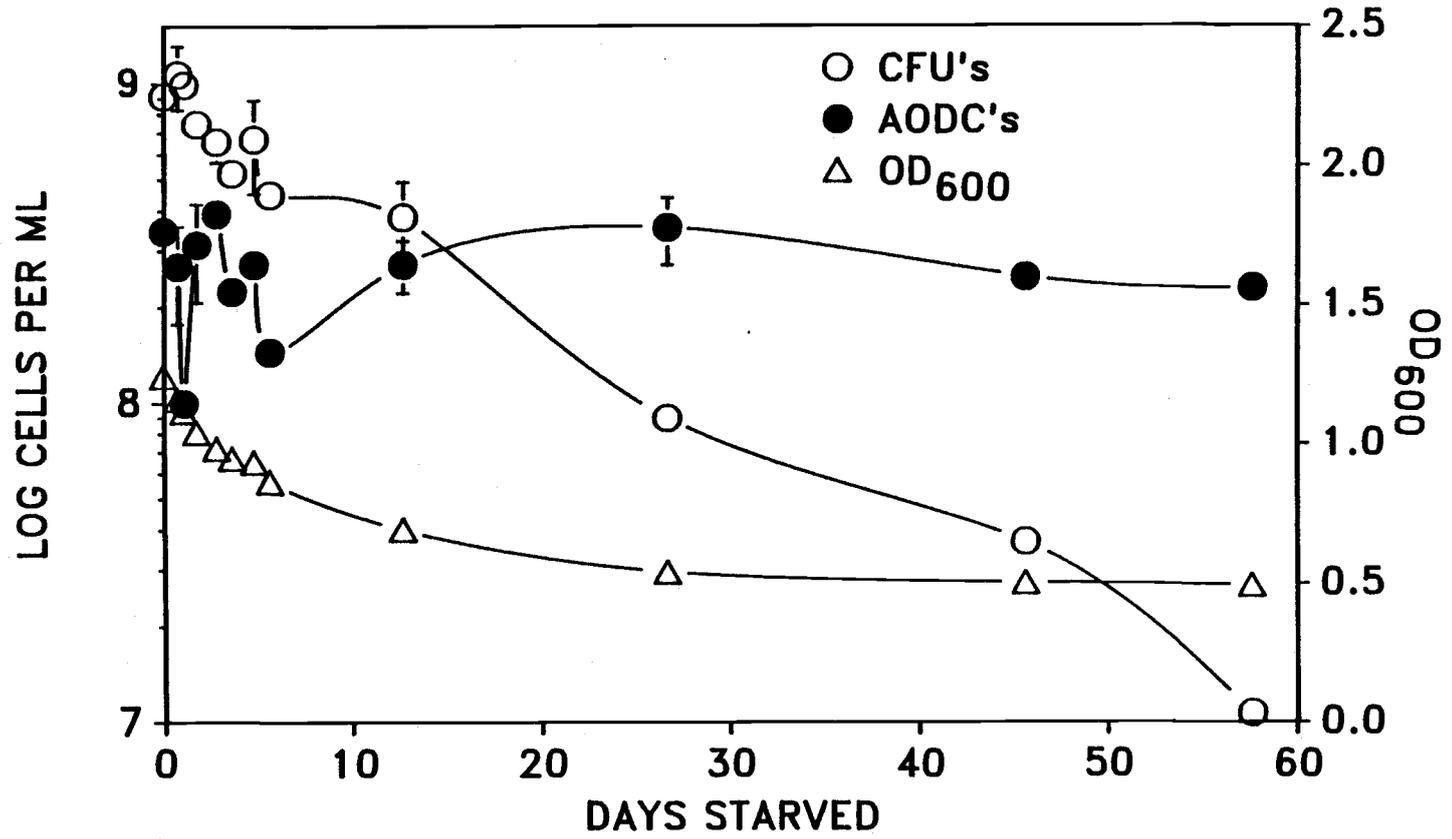


Figure 15: Fatty acid esterase and phosphatase activities on starvation expressed as  $\mu\text{M}$  p-nitrophenol released. Vertical bars equal the standard error of the mean.

Figure 15.

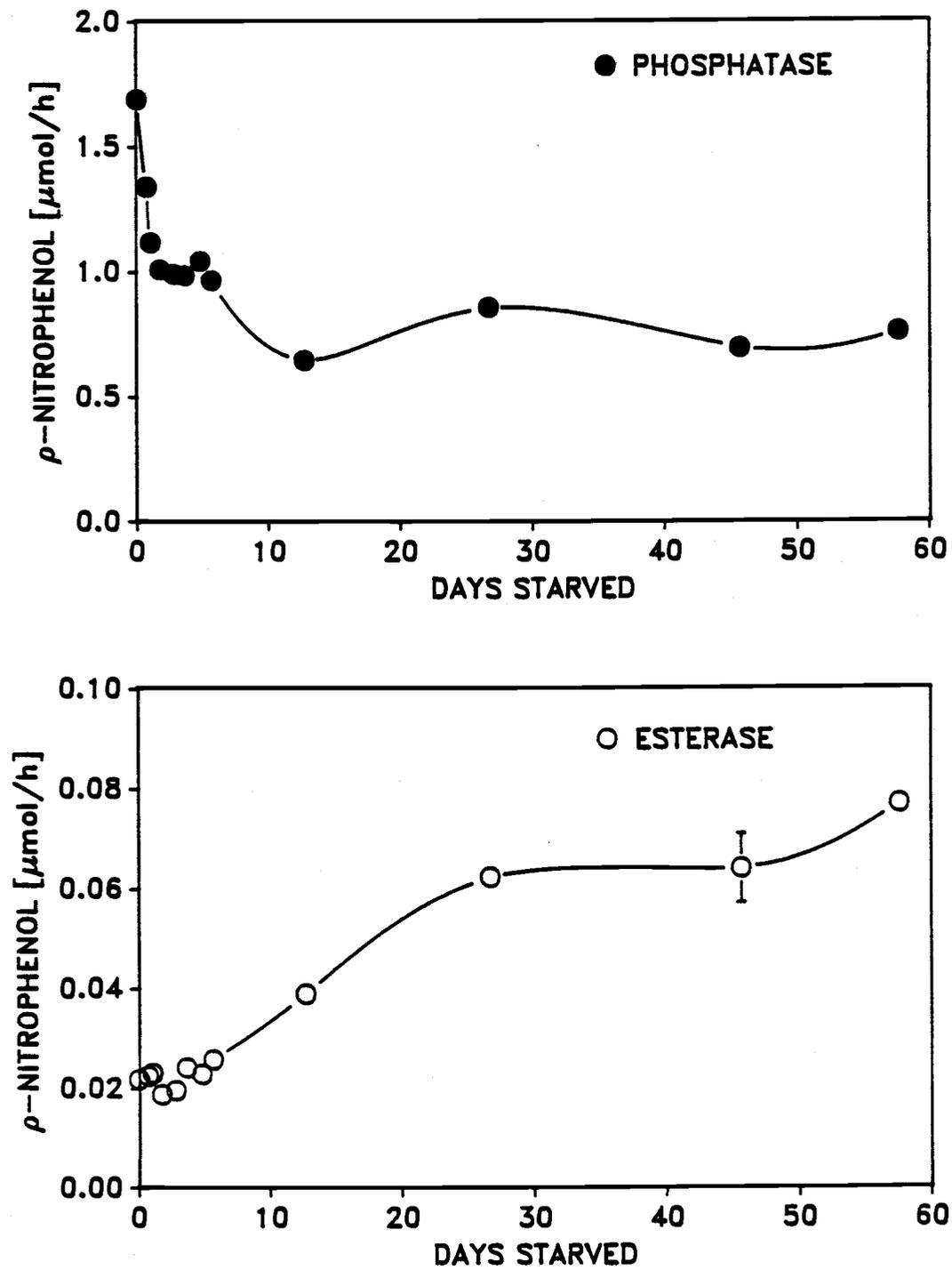


Figure 16: Fatty acid esterase and phosphatase activities per cell calculated for direct counts. Vertical bars equal the standard error of the mean.

Figure 16.

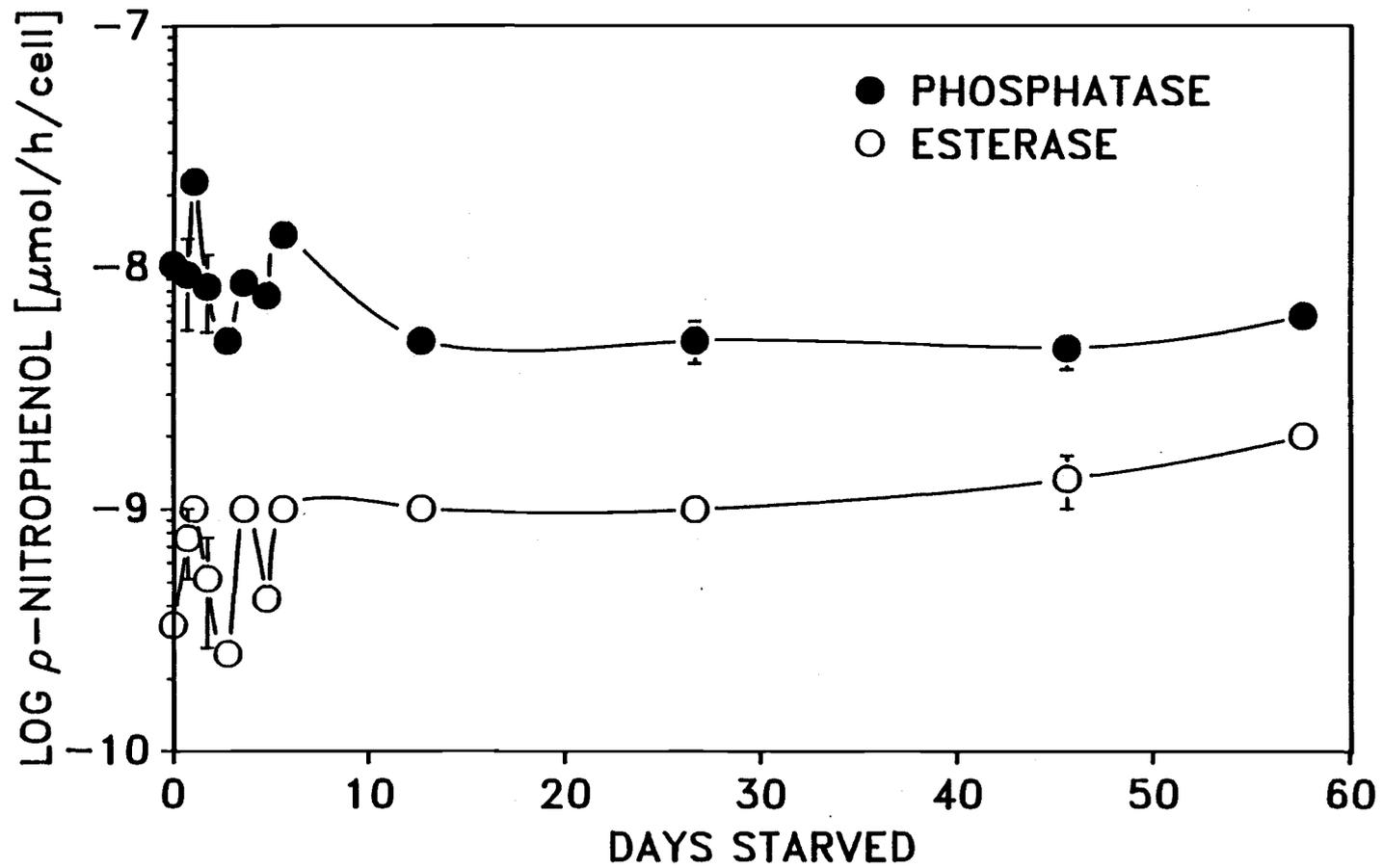
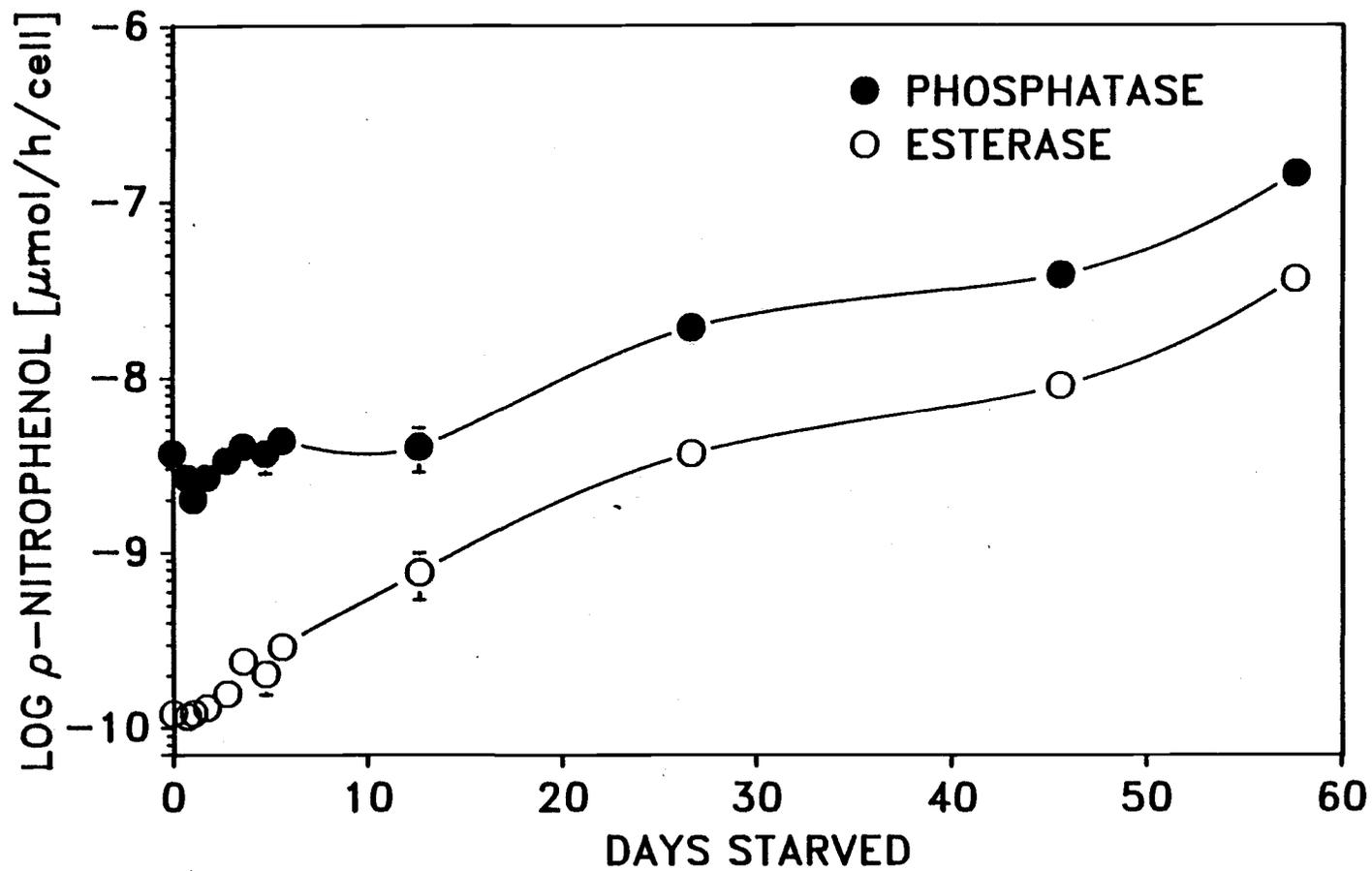


Figure 17: Fatty acid esterase and phosphatase activities per cell calculated for viable cells. Vertical bars equal the standard error of the mean.

Figure 17.



by the phosphatase does not change on starvation. For both starved and unstarved cells the activity is highest for AMP, followed by  $\beta$ -glycerophosphate and glucose-6-phosphate. No activity could be observed for inositol hexaphosphate, glucose-1-phosphate, and ATP.

Table 3. Utilization of different substrates by the phosphatase in starved and unstarved ANT-300 cells.\*

Substrate	$\mu\text{g PO}_4^{3-}/\text{l}$ released in %
Inositol hexaphosphate	0
$\beta$ -Glycerophosphate	23
Glucose-1-phosphate	0
Glucose-6-phosphate	3.5
AMP	100
ATP	0

\* As there was no difference in the relative percentage of  $\text{PO}_4^{3-}$  released in starved and unstarved cells, the results are presented as one data set.

#### D. Enzyme Activity in the Supernatant

The activities in the supernatant of both enzymes increased from approximately 10% at day 0 to 30% at day 9 for the phosphatase and 30% at day 53 for the fatty acid esterase. The activities for day 0 and day 57 were recalculated considering the activity in the supernatant. The value of day 57 was corrected with the value of day 53. With the corrected values the fatty acid esterase levels increased for viable cells and direct counts from  $1.1 \times 10^{-10}$  to  $9 \times 10^{-9}$  and from  $2.5 \times 10^{-10}$  to  $1 \times 10^{-9}$  respectively (activities expressed as p-nitrophenol released in  $\mu\text{mol/h/cell}$ ). The phosphatase activity increased for viable cells from an initial  $3 \times 10^{-9}$  to  $8.3 \times 10^{-8}$ , but decreased for direct counts

from  $8 \times 10^{-9}$  to  $4 \times 10^{-9}$  (activities expressed as  $\mu\text{mol p-nitrophenol}$  released /h/cell).

Table 4. Enzyme activity in the supernatant for starved and unstarved ANT-300 cells.

Time [days]	Percent activity in supernatant	
	Phosphatase	Fatty acid esterase
0	11.04	11.53
2	6.98	8.17
9	29.01	14.55
18	33.80	18.08
23	35.33	24.92
53	27.37	30.03

## DISCUSSION

ANT-300 is able to utilize a variety of substrates as sole carbon source. The nutritional spectrum of the organism is likely to be determined by the chemical nature of substrates available in the marine environment. Exudation of low molecular weight compounds seems to be a normal physiological function of healthy phytoplankton cells and is commonly interpreted as active release of photosynthate that accumulates when carbon fixation exceeds incorporation into new cell material (Mague et al., 1980; Fogg, 1983). Ittekkot (1982) reported the release of glucose, fructose, galactose, mannose and xylose as predominant sugars and of glutamate, serine, glycine, histidine, aspartate, threonine and lysine as predominant amino acids during a Chaetoceros bloom. These organic compounds were then taken up by heterotrophic microorganisms. Cole et al. (1982) emphasized the importance of photosynthetically produced DOC as a carbon source for planktonic bacteria. They showed that microbial biomass can be almost completely dependent on the uptake of algal exudates and that there was a seasonality in the productivity of the bacterioplankton that was correlated with times of high phytoplankton productivity. Hellebust (1974) summarized the excretion products of marine algae that are released as a result of unbalanced growth or aging and degeneration of the algae. The major carbohydrates released are glucose, mannose, galactose, fucose and arabinose. The utilization of arabinose, glucose and galactose was examined in the experiments presented here. ANT-300 was able to grow on glucose and galactose as the only carbon source in the presence or absence of yeast

extract. Arabinose was not utilized as a carbon source. No growth occurred in the presence of laminarin, which is a common polysaccharide in coastal marine waters (Raymont, 1980). ANT-300 showed growth on sterile-filtered maltose but lacked an  $\alpha$ -glucosidase. Maltose was therefore either taken up as a disaccharide or growth was due to glucose contamination of the substrate. The organism did not grow on cellobiose which corresponded to the absence of a  $\beta$ -glucosidase. Chitin, which is a very abundant polysaccharide in the marine environment, was readily utilized.

Algae can release nitrogenous materials as polypeptides and amino acids (Hellebust, 1974). Glutamate, aspartate, serine, glycine and alanine are the most common amino acids in the marine environment. ANT-300 was able to grow on glutamate and serine in the presence and absence of yeast extract but did not grow on pyruvate or succinate without the yeast extract supplement. Why yeast extract is required for some carbon sources while others can be utilized in its absence is not known. A possibility is that the breakdown of certain substrates requires one or more compounds present in yeast extract. The attempt to replace the yeast extract supplement with the vitamin solution or with either of its components was unsuccessful. Cell yields were generally lower without yeast extract, possibly because of less efficient substrate utilization.

ANT-300 was unable to hydrolyze DNA, RNA, starch, gelatin, and phospholipids, which indicates a lack of hydrolytic enzymes for more complex molecules. Fatty acid hydrolysis took place for all substrates offered except acetate. As lipids have by far the highest energy

content of all biomolecules and also a high potential as metabolic building blocks, they provide an excellent substrate under low nutrient conditions.

The absence of a sulfatase in ANT-300 would prevent the organism from taking up substrates that carry sulfate groups.

ANT-300 has an extracellular fatty acid esterase consisting of a low affinity and a high affinity enzyme system. Both systems are maintained during a starvation period of 57 days but the  $K_m$  of the low affinity system is decreased. Although the maintenance of an extracellular enzyme seems to be energetically unfavorable under nutrient limitation, previous research shows that it may be a crucial part of the survival strategy of the organism (Davis and Robb, 1985). It has been demonstrated that the lipid composition of starved bacteria changes remarkably during starvation. This includes changes in the membrane lipid composition (Mårdén et al., 1985). Oliver and Stringer (1984) reported a large neutral lipid fraction in ANT-300 which increased during long term starvation. There was also a tendency to increase the amount of monounsaturated C16 fatty acids. Malmcrona-Friberg et al. (1986) showed that during short term starvation (24 h) of a gram negative marine isolate the total lipid content of the cells decreased. Furthermore the ratio of monounsaturated to saturated fatty acids in the membrane decreased while the amount of short chain fatty acids increased. They also reported a decrease of monounsaturated C16 fatty acids on short term starvation which contradicts the results of Oliver and Stringer (1984). The fluidity of the membrane may play a crucial role in substrate uptake. Results presented by

Malmcrona-Friberg et al. (1986) suggest that increased membrane fluidity is more desirable at least for short term starvation survival because it aids substrate uptake. The importance of unsaturated fatty acids for the degree of membrane fluidity and the capabilities of microorganisms to survive nutrient deprivation, has been examined by Massa et al. (1988). They compared survival capabilities of an unsaturated fatty acid auxotroph when the organism was supplied with either oleic (cis 18:1) or linolenic acid (18:3). Although the number of colony forming units (CFU) declined faster for linolenic than for oleic acid grown cells, a higher degree of unsaturation is not conclusive for decreased starvation-survival capabilities of the organism as it was possible to increase the number of CFU after addition of respirable substrate. In unstarved cells microviscosity of the plasmamembrane was equal for oleic and linolenic acid grown cells. On starvation viscosity was lower for linolenic acid grown organisms. The fatty acid esterase described here displays optimal activity for substrates with a chain length of C8 for starved and unstarved cells. It is noteworthy that the relative activity for C16 saturated fatty acids almost doubles on starvation. This could indicate that together with the lipid composition of the cell which shifts more towards C16 monounsaturated fatty acids (Oliver and Stringer, 1984) the uptake of fatty acids with a chain length of C16 is also more pronounced. The increase in hydrolysis of p-nitrophenyl acetate could be due to utilization of this carbon source as a co-substrate on starvation, although ANT-300 was not able to grow on acetate as a sole carbon source. A drop in activity for myristate under nutrient deprivation may reflect a toxicity of this substrate. Torrella

and Morita (1982) showed that ANT-300 becomes chemotactic during the first days of nutrient deprivation, a capability which is possibly also influenced by the membrane composition. These results are contradicted by a report of Miller and Koshland (1977) who found that chemotactic response was independent of membrane fluidity. Due to their high energy content, lipids including membrane lipids (Amy et al., 1983) can also be utilized as energy sources. Poly- $\beta$ -hydroxybutyrate, for example, has been reported as a storage compound in some microorganisms and tends to disappear on starvation accordingly (Matin et al. 1979; Malmcrona-Friberg et al., 1986). The importance of lipids during the starvation process justifies the maintenance of two uptake systems even in the absence of substrate. Another explanation may be that nutrients in the ocean are generally scarce and their distribution is patchy. An enzyme that would have to be induced by the presence of substrate would therefore be too "slow" to respond to quickly changing nutrient concentrations efficiently (Azam and Ammerman, 1984). The high affinity system with its decreased  $K_m$  and the low affinity system would be able to take advantage of fluctuating levels of fatty acid esters in the water column. In their starvation experiments with ANT-300 Novitsky and Morita (1978b) showed that barotolerance of the organism increased with starvation - another feature that is influenced by membrane composition.

The relatively high temperature optimum of the fatty acid esterase suggests that a psychrophilic organism does not necessarily have to be psychrophilic in its enzymatic make-up to be successful from an evolutionary point of view. The pH optimum of pH 9 shows that the enzyme is well adjusted to the marine environment with its slightly

alkaline pH.

ANT-300 has an alkaline extracellular phosphatase with monophasic substrate kinetics. The enzyme's activity increases on starvation when it is reported as  $\mu\text{mol}$  p-nitrophenol released. If phosphorus is a limiting nutrient in seawater, the presence of a competitive enzyme system for the release of orthophosphate from organic compounds would be one of the parameters that decide the survival of a species in the water column. This would even be more pronounced under low nutrient conditions. Although the limiting qualities of phosphorus in the marine environment are still subject to discussions (Smith, 1984), competition experiments between algae and bacteria in chemostats have shown the superiority of the bacterial uptake systems in phosphorus-limited freshwater environments (Currie and Kalff, 1984a). Currie and Kalff found that phosphorus-starved bacteria were more successful in obtaining phosphorus than algae when both species were grown together in a chemostat. As the bacteria grew on the algal exudates as the only carbon source, they were carbon limited which resulted in increased excretion of organic phosphorus. Both algae and bacteria competed for this excreted phosphate. In a natural lake system almost all of the phosphorus taken up by algae was first passed through the bacterial fraction which then released a portion in the form of organic phosphate compounds (Currie and Kalff, 1984b). Remarkably, a fraction of the excreted phosphorus was rendered refractory to the action of the algal phosphatases. This shows that not the overall amount of phosphorus present may be the limiting factor, but rather its bioavailability (Bradford and Peters, 1987). The phosphatase described for ANT-300

utilizes AMP optimally. The phosphate in ATP is obviously less accessible which may be due to steric hindrance of the enzyme. No activity could be observed for inositol hexaphosphate which may have been due to the physical unavailability of this substrate after it precipitated in the reaction mix. As inositol hexaphosphate is not a common substrate in the marine environment its solubility properties would have little influence on the results of this experiment. Again maintenance of the enzyme system on starvation is advantageous as it guarantees a short reaction time to sporadic phosphate sources. The optimum temperature for the phosphatase is with 13.5°C closer to the optimum growth temperature of ANT-300 than that of the esterase. The substrate kinetics show that there is an increase in substrate affinity from  $K_m = 88\mu\text{M}$  to  $K_m = 60\mu\text{M}$  on starvation. High affinity uptake systems are an adaptation of the organism to low substrate concentrations and have been reported for ANT-300 by Geesey and Morita (1979).

The increase in enzyme activity that could be observed for the fatty acid esterase and the phosphatase is partly due to an increase of the activity in the supernatant. When this is considered there is an increase in activity for each enzyme when calculated for viable cells. For total counts the fatty acid esterase activity is still increased while the phosphatase activity decreases. This indicates that an increase or decrease in enzyme activity depends partly on the determination of a suitable frame of reference for the accumulated data. Kurath and Morita (1983) related cellular ATP levels observed on starvation for a marine Pseudomonas sp. to endogenous respiration. This was possible under the assumption that cells would have to respire to

produce ATP. In the case of the data presented here endogenous respiration would not be a good frame of reference as enzymes can also be associated with dead cells. In order to assess a decrease in activity for the phosphatase it would therefore be necessary to determine the fraction of activity attributable to living, dormant, or dead cells. To date this is not possible. An interesting approach would be to determine differences in the cell membrane which are likely to occur as mentioned earlier. Such differences could then result in different electrophoretic mobilities that would make "sorting" of the cells and determination of the enzyme activities associated with them possible. This method has already been employed for the separation of natural particles and cultured organisms by Gerritsen and Bradley (1987).

Microbial populations in aquatic environments consist to 97% of gram negative microorganisms. The ecological advantage for gram negative microorganisms lies in the association of their extracellular enzymes with the periplasmic space. Periplasmic hydrolases degrade polymeric substrates to monomeric compounds that are then taken up via specific uptake systems in the plasma membrane (Costerton et al. 1974). On starvation hydrolases as well as uptake systems would have to be maintained to ensure efficient scavenging capabilities in the presence of nutrients (Hoppe et al., 1988).

The maintenance of uptake systems under conditions of nutrient deprivation has been shown for mannitol in a marine Pseudomonas sp. and Vibrio sp. (Davis and Robb, 1985). The results presented here show that at least one of the hydrolytic enzymes examined increases its activity

on starvation and maintains biphasic enzyme kinetics. This corresponds well with the hypothesis that extracellular enzymes would also have to be present to supply the uptake systems with substrates when nutrients are encountered. Extracellular hydrolases are therefore generally conserved under conditions of nutrient deprivation.

The significance of the data presented here is also emphasized by a study of Amy and Morita (1983a) where 77% of the examined marine isolates underwent similar morphological changes than ANT-300. This may indicate the importance of this survival strategy in the marine environment, assuming that the organisms used in the study were representative of the open ocean environment.

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