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Joint Program
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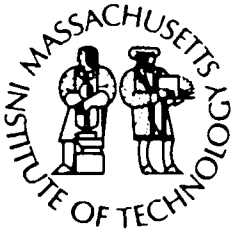

DOCTORAL DISSERTATION

Organic Nitrogen Utilization by Phytoplankton: the Role of Cell-Surface Deaminases

by

Brian P. Palenik

June 1989

WHOI-89-18

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Doctoral Dissertation

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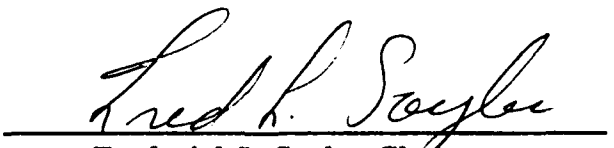

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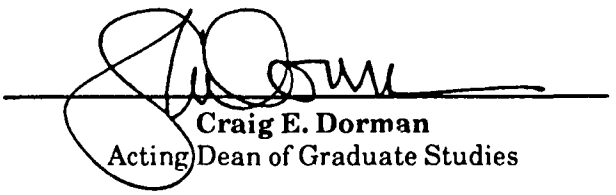

Craig E. Dorman
Acting Dean of Graduate Studies

ORGANIC NITROGEN UTILIZATION BY PHYTOPLANKTON: THE ROLE OF CELL-SURFACE DEAMINASES

by

BRIAN PHILLIP PALENIK

B.S.E., Princeton University
(1981)

Submitted in partial fulfillment of the
requirement for the degree of

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
and the
WOODS HOLE OCEANOGRAPHIC INSTITUTION

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ORGANIC NITROGEN UTILIZATION BY PHYTOPLANKTON: THE ROLE OF CELL-SURFACE DEAMINASES

by

Brian P. Palenik

ABSTRACT

Many phytoplankton can catalyze the decomposition of organic phosphates using cell-surface phosphatases and subsequently take up the phosphorus for growth. The nitrogen analogs, cell-surface deaminases, have not been reported in phytoplankton, except for a low affinity asparaginase. In fact, high affinity cell-surface L-amino acid and amine deaminases (oxidases) exist in at least three phytoplankton genera: Amphidinium, Pleurochrysis, and Prymnesium. One type of enzyme oxidizes L-amino acids, a second type primary amines; the endproducts are hydrogen peroxide, an organic product (an α-ketoacid or an aldehyde), and NH_4^+ which is assimilated by the cell.

The amino acid oxidases have half-saturation constants of about 0.2μM (0.1-0.45 μM range) for many L-amino acids. They do not oxidize D-amino acids, while glycine and L-serine, two amino acids commonly found in the environment, are not effective substrates. The amine oxidases studied both utilize ethanolamine, but differ in their specificity for other amines. The regulation of the amino acid and amine oxidases under different growth conditions and their inhibition by various reagents are discussed.

A method was developed for measuring H_2O_2 in seawater samples and was subsequently used to obtain field data showing dark production of H_2O_2. This phenomenon may have been due to the presence of organisms with cell-surface oxidases. Since cell-surface deaminases would not have been detected by standard field methods used to study amino acid and primary amine cycling, the existence of these enzymes suggests that phytoplankton may have a more important role in recycling organic nitrogen than currently believed. These results thus have wide-ranging implications for understanding nitrogen cycling; primary production; the geochemistry of H_2O_2, organic acids and aldehydes; and algal-invertebrate symbioses in aquatic environments.

Thesis supervisor: François M. M. Morel
Position: Professor, M.I.T.
Thesis co-supervisor: Oliver C. [illegible]riou
Position: Senior Scientist, W.H.O.I.

For my parents

The Fish

The first fish
I ever caught
would not lie down
quiet in the pail
but flailed and sucked
at the burning
amazement of the air
and died
in the slow pouring off
of rainbows. Later
I opened his body and separated
the flesh from the bones
and ate him. Now the sea
is in me: I am the fish, the fish
glitters in me; we are
risen, tangled together, certain to fall
back to the sea. Out of pain,
and pain, and more pain
we feed this feverish plot, we are nourished
by the mystery.

Mary Oliver

American Primitive

ACKNOWLEDGEMENTS

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I would like to thank my thesis committee--Neil Blough, Penny Chisholm, François Morel, John Stegeman, and Ollie Zafiriou--for providing laboratory space, equipment, potato chips, parties, and more importantly their sharpest critical eyes. Thanks also to Cindy Lee for her many helpful comments along the way and for reviewing the thesis as a whcle. My WHOI advisor Ollie Zafiriou deserves a special thanks for dragging me into the world of oceanography, as does my advisor François Morel for sharing his insights, friendship, and joie de vivre.

Thanks to Phil Gschwend (especially for the Swiss mountain analogy), Ellen Druffel, and Ed Sholkovitz for ushering me past the few signposts visible as one bushwacks towards a PhD.

Finally, I would like to acknowledge the help and support of the coworkers and friends that made the journey possible, even sometimes enjoyable: "the Research Group" in its various incarnations, Bob Hudson, Janet Hering, David Waite, Neil Price, Margie Roulier, Ginger Armbrust, Erik Zettler, Rob Olson, Tom Army, David Kieber, Ron Pearlstein, Kathleen Ledyard, Mark Solomon, Leslie Delong, Pam Kloepper-Sams, Lucia Susani, Bruce Woodin, John Waterbury, and Sheila Frankel.

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INTRODUCTION

Organic nitrogen is one of the major pools of nitrogen in the oceans. Its availability as a nitrogen source for phytoplankton, however, is controversial. Clearly some components of the organic nitrogen pool are readily utilized--particularly amino acids (See Wheeler, et al., 1974; Flynn and Butler, 1986, in chapter one)--but issues of transport affinity and competition with bacteria are still open. Less is known about the utilization of other components such as primary amines, proteins, DNA, humic and fulvic acids, etc.

The research presented here adds an additional dimension to the issue of organic nitrogen use by phytoplankton. Amino acids, primary amines, and perhaps some larger macromolecules can be utilized by phytoplankton without being transported into the cell through a "novel" mechanism. In this mechanism, the nitrogen containing compound is oxidized by cell-surface enzymes of the phytoplankton to produce H_2O_2, an organic product, and ammonium, which is assimilated by the cell. The enzymes responsible for this reaction are referred to as cell-surface deaminases or alternatively cell-surface oxidases.

Chapter one gives an overview of the thesis, highlighting its major findings and implications. The subsequent chapters present the details.

Chapters two and three discuss the presence of L-amino acid oxidases in *Pleurochrysis* species. Chapter two was written as an early report on the phenomenon of extracellular H_2O_2 production in *Pleurochrysis* species and is useful as a guide to the methods used in subsequent work. This chapter was published in Limnology and Oceanography (Palenik, B., O.C. Zafiriou, and F.M.M. Morel, 32:1365-

1369, 1987). Chapter three demonstrates the role of amino acids as electron donors for H_2O_2 production along with an investigation of enzyme function, specificity, inhibition, and regulation. Together, these chapters convinced me that science is like weaving a spider web. You put up one thread and then realize from there you have to leap two branches over. The final results can appear alternatively puzzling or strangely beautiful.

Chapter four is an investigation of L-amino acid oxidases in other phytoplankton genera. Chapter five extends the mechanism of cell-surface deamination to include primary amines such as ethanolamine and 4-aminobutyrate, and possibly primary amino groups on macromolecules such as peptides. These amine oxidases are investigated in the same phytoplankton genera that also possess cell-surface amino acid oxidases.

Chapter six presents a method for measuring H_2O_2 in the field. It also presents some preliminary evidence that oxidases are present in phytoplankton in the Sargasso Sea. This chapter was originally published in Limnology and Oceanography (Palenik, B., and F.M.M. Morel, 33:1606-1611, 1988).

Chapter seven presents a few additional speculations and suggestions for research not found elsewhere in the thesis.

Chapter 1.

Dissolved organic nitrogen use by phytoplankton: the role of cell-surface deaminases.

Many phytoplankton can catalyze the decomposition of organic phosphates using cell-surface phosphatases and subsequently take up the phosphorus for growth.[1] The nitrogen analogs, cell-surface deaminases, have not been reported in phytoplankton, except for a low affinity cell-surface asparaginase[2]. We show here that L-amino acid and amine deaminases (oxidases) exist in Prymnesium species typically found in coastal and brackish waters. One enzyme oxidizes L-amino acids, the other primary amines; the endproducts are H_2O_2, an organic product (an α-ketoacid or aldehyde), and NH_4^+ which is assimilated by the cell. These enzymes are also present in at least two other common phytoplankton genera, Amphidinium and Pleurochrysis[3]. Since the deaminases would not have been detected by standard field methods, their existence suggests that phytoplankton may have a more important role in recycling organic nitrogen than currently believed.[4] These results thus have wide-ranging implications for understanding nitrogen cycling, primary production, the geochemistry of organic acids and aldehydes, and algal-invertebrate symbioses in aquatic environments.

When L-alanine is added to a nitrogen-limited, axenic culture of Prymnesium parvum, a coastal and brackish water phytoplankton, hydrogen peroxide and an α-keto acid, pyruvate, are released into the

medium in a one to one ratio (Figure 1). The L-alanine is completely oxidized to these end products. The enzymes catalyzing this kind of reaction, amino acid oxidases, have been found and studied in a range of organisms; they release NH_4^+ as the third endproduct.[5]

The nitrogen released from amino acids by the enzyme of P. parvum can be taken up and used for growth. When ^{15}N labelled L-aspartic acid was added to a nitrogen-limited culture, the cells began to grow and the ^{15}N label was incorporated at rates typically found for H_2O_2 and pyruvate production, about 0.7-1.4 x 10^{-14} mol $cell^{-1}$ hr^{-1} (Figure 2).

The L-amino acid oxidase of P. parvum oxidizes many L-amino acids with three or more carbons, and where the R group of the amino acid is ($-CH_2X$). D-amino acids are not oxidized. The R substituent greatly affects the half-saturation constant for the substrate. Nitrogen-limited cultures have a half-saturation constant of approximately 0.2 μM for L-alanine ($R=CH_3$) and L-glutamate ($R=CH_2CH_2CO_2^-$), while the half-saturation constant for L-aspartate ($R=CH_2CO_2^-$) is significantly higher at about 2 μM. Glycine is not oxidized. Because of their known concentrations, we expect P. parvum to use mostly L-alanine, L-glutamate, L-leucine, L-lysine, and L-ornithine of the free amino acids found in the environment. Paradoxically, L-serine and glycine, two of the most abundant free amino acids, are not effective substrates. Perhaps their relative abundance is due to the removal of the other amino acids by amino acid oxidases in the environment.

Figure 1: Extracellular hydrogen peroxide and pyruvate production after addition of L-alanine (1μM final concentration) to a nitrogen limited culture of P. parvum (88,400 cells ml^{-1}): o , pyruvate; •, H_2O_2. Culture conditions are discussed in ref. 3. Pyruvate was measured on an HPLC system optimized for a-keto acids[6]. H_2O_2 was measured using published methods as discussed in refs. 3 and 14. Cell and alanine concentrations are higher than expected in the field by 1 to 2 orders of magnitude for ease of analysis.

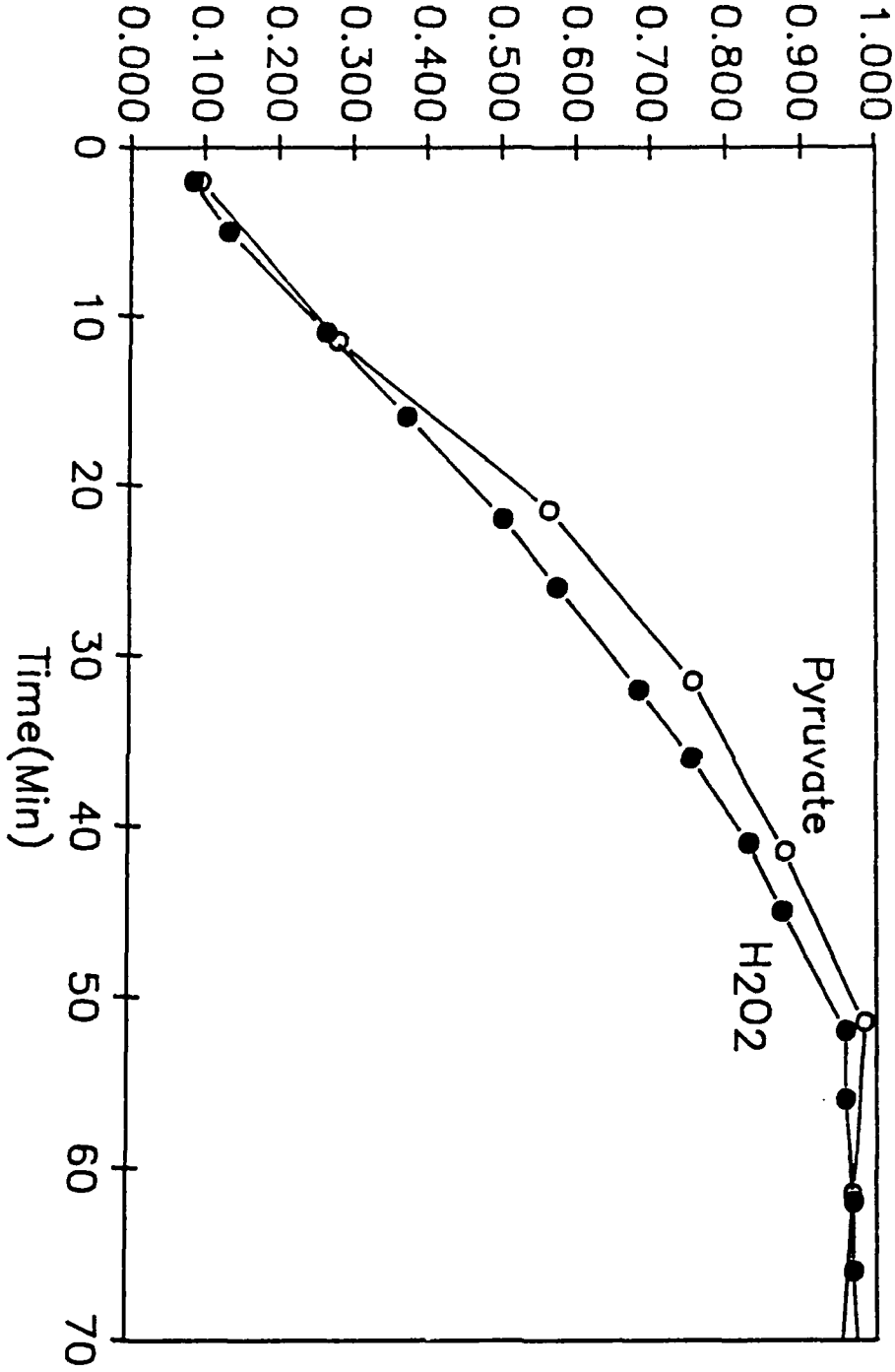

Endproduct (μM)
0.000
0.100
0.200
0.300
0.400
0.500
0.600
0.700
0.800
0.900
1.000
0
10
20
30
40
50
60
70
Time(Min)
Pyruvate
H2O2

Figure 2: ^{15}N incorporation (·)after addition of L-aspartic acid (final concentration 10.1μM, 98 atom % ^{15}N) to nitrogen limited P. parvum (74,600 cells ml^{-1}). Cell number after amino acid addition (●) .^{15}N ratio of filtered cells was measured using mass spectrometry by J. Nevins and J. McCarthy, Harvard University.

Cell Number (cells ml^{-1})
1.24E5
1.19E5
1.14E5
1.09E5
1.04E5
9.90E4
9.40E4
8.90E4
8.40E4
7.90E4
7.40E4
^{15}N incorporation (atom%)
40
30
20
10
0
0
4
8
12
16
20
24
Time (Hours)

When P. parvum cells are growing in exponential phase on excess NO_3^-, they show amino acid oxidase activities similar to those for nitrogen limited cells. This organism prefers to obtain ammonium by oxidizing amino acids rather than reducing nitrate. In contrast, cells growing on excess NH_4^+ (exponential phase) do not show oxidase activity.

P. parvum has a second cell-surface oxidase that catalyzes the deamination of primary amines, particularly ethanolamine. When ethanolamine is added to nitrogen-limited cultures, H_2O_2 (Figure 3) and glycolaldehyde (identified by HPLC[6]) were produced in the medium. The amine and L-amino acid oxidases are distinct enzymes since in the presence of excess L-alanine (20μM) to saturate the L-amino acid oxidase, ethanolamine additions result in further production of H_2O_2 (Figure 3). Under nitrate and nitrogen limited growth conditions, the saturated rates (Vmax) for the amine oxidase are about 0.7 and 1.4 x 10^{-15}mol $cell^{-1}$ hr^{-1}, respectively, about 10 to 20 percent of the saturated rates for the amino acid oxidase. Ethanolamine has been detected,[7] but its concentration in natural waters is poorly characterized . The specificity of the enzyme, which does not use several other naturally occurring amines such as 4-aminobutyrate, suggests that ethanolamine may be a principal amine used by P. parvum in the environment.

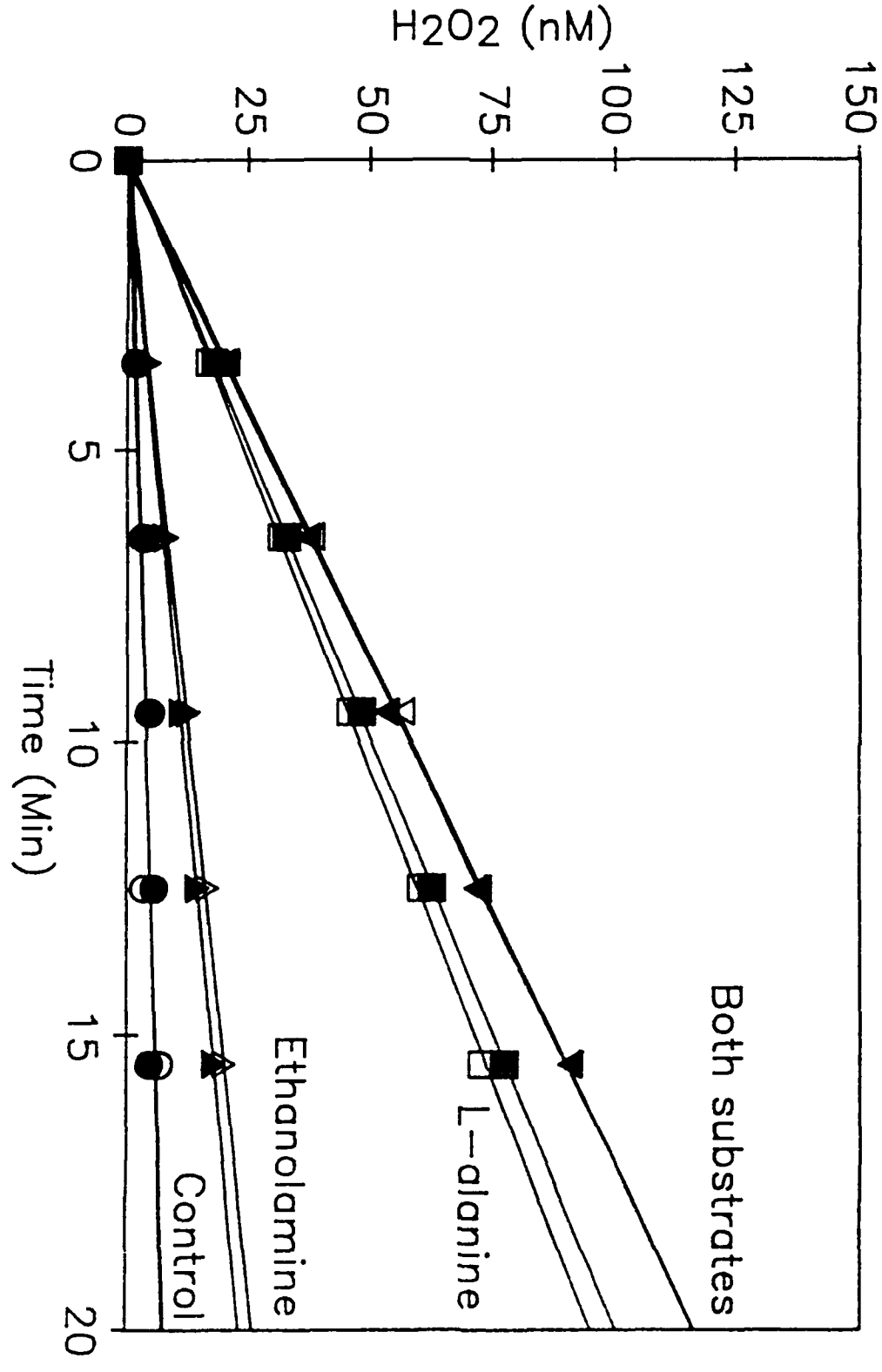
Cell surface deaminases have been found in two other phytoplankton genera. Pleurochrysis (also known as Hymenomonas) species are found in coastal regions and brackish waters[8]. One

species, Pleurochrysis carterae is often studied as a model calcifying phytoplankton (coccolithophorid) and has been shown to grow well on organic nitrogen sources.[9,10,11] An L-amino acid oxidase has been found in all four Pleurochrysis isolates examined[3]. In two isolates (including the isolate known as clone COCCOII) the enzyme was present when the organisms were growing on nitrate as their nitrogen source, while the other two isolates expressed the enzyme under nitrogen limited conditions only. The clone COCCOII of Pleurochrysis carterae also has a cell-surface amine oxidase, but only under nitrogen limited conditions, demonstrating the distinct character of this second oxidase. In this clone, the enzyme catalyzes the oxidation of a wide range of primary amines, including 4-aminobutyrate, ethanolamine, and the N-terminal glycines on peptides (glycylglycine). Other Pleurochrysis isolates have not been tested for amine oxidase activity.

Other species of phytoplankton expressing oxidase activity include Amphidinium carterae (Clone AMPHI) and Amphidinium operculatum (or klebsii, clone AMPHID), representatives of a common genus of dinoflagellates. AMPHID was originally isolated as an endosymbiont of the jellyfish, Cassiopea. These two organisms express the L-amino acid oxidase only under nitrogen-limited conditions, and AMPHID, at least, does not have an amine oxidase. Phytoplankton with cell-surface deaminases are thus common and from diverse evolutionary groups .

The use of organic nitrogen by phytoplankton is controversial. Based on laboratory and some field evidence, several authors have argued that phytoplankton are likely to use amino acids in the environment as a nitrogen source.[9,10,11] One counterarguement has been

Figure 3: H_2O_2 production by nitrogen limited P. parvum (40,000cells ml^{-1})after additions of 20μM L-alanine, 20μM ethanolamine, or both: ○ ●, no addition; △ ▲, ethanolamine only; □ ■, L-alanine only; ▽ ▼, both substrates. H_2O_2 production was measured as discussed in ref. 3.


H2O2 (nM)
0
25
50
75
100
125
150
Time (Min)
0
5
10
15
20
Both substrates
L-alanine
Ethanolamine
Control

that the half-saturation constants of amino acid transport systems reported to date in phytoplankton are relatively high (Kms of 0.4-150μM)[12], particularly compared with those in isolated bacteria or reported from uptake experiments in bulk samples from the field (Kms of 0.001-1μM).[13] In addition, field studies using ^{14}C or ^{3}H amino acid additions to natural waters show that the label typically ends up in bacteria.[14,15] Since the L-amino acid oxidases reported here have half-saturation constants of about 0.2μM (range of 0.1-0.45μM), they should be able to effectively utilize ambient free amino acids, which range from 0.02 to 0.5μM total concentration.[16] Because they would only transform radiolabelled amino acids into labelled α-keto acids which would be taken up by bacteria, the oxidases would not be detected by standard field methods for studying amino acid cycling.[14,15] In light of our findings, we believe the role of amino acids and other primary amines as a nitrogen source for phytoplankton and the issue of bacterial dominance of amino acid cycling needs to be reevaluated.

Two pieces of chemical evidence to date suggest that cell-surface L-amino acid (or amine) oxidases may be important in the environment. Dark, apparently biological, production of H_2O_2 has been reported in the Sargasso Sea at about 40m.[17] This production could have been the endproduct of an oxidase reaction. In addition, pyruvate, the endproduct of the oxidation of L-alanine, has been found to covary positively with alanine in oceanographic depth profiles (D. Kieber and K. Mopper, unpublished results). A peak of alanine and pyruvate typically occurs in the lower euphotic zone at the chlorophyll maximum, suggesting that this could be a particularly

active zone for amino acid cycling through oxidases. While these results are suggestive, simultaneous field measurements of dark production of H_2O_2, α-ketoacids, and aldehydes are clearly needed.

Phytoplankton growth rates in natural waters are difficult to quantify, but some evidence suggests that phytoplankton may be growing near a maximum rate of one division per day, even in oligotrophic environments.[18] Paradoxically, in oligotrophic regimes inorganic nitrogen concentrations appear too low to support this growth rate. It has been suggested that instead of relying on these low concentrations, phytoplankton may rapidly take up ammonium from difficult to detect micropatches (for example created by zooplankton excretion) that have much higher ammonium concentrations.[19] Our results suggest a second model: phytoplankton can also create their own ammonium "patches" by degrading amino acids and amines at their cell-surfaces.

If an organism like Pleurochrysis carterae were put in an oligotrophic region such as the Sargasso Sea at a depth with a 50nM "available" amino acid concentration [20], it could get enough nitrogen to support a growth rate of about 0.25 day^{-1} on these sources alone (assuming a 10um diameter cell, 3.3 x 10^{-13} moleN $cell^{-1}$, and enzyme kinetics reported for P. carterae).[3] Although the growth rate measurements are controversial, this is in the range of measured rates for phytoplankton in these environments. [21] The growth rate estimate of 0.25 day^{-1} is conservative in that evolutionary pressures in oligotrophic regimes may have resulted in higher affinity L-amino acid oxidases than we have measured, while other primary amines and the low

ambient ammonium concentrations would provide additional nitrogen for growth.

The existence of cell-surface deaminases may have implications in two other phenomenona: algal blooms and algal symbioses. Prymnesium and Amphidinium species have both been known to form blooms,[22,23] the former being particularly toxic to fish because of a toxin they produce that makes biological membranes leaky. The leakage of amino acids and other nutrients out of damaged cells may be the important ecological function of this toxin.

Amphidinium species are found in endosymbiotic relationships, often with pelagic hosts such as foraminifera and radiolaria.[24] Interestingly, L-alanine, an effective oxidase substrate, was identified as the amino acid most likely to be translocated from Cassiopea , a jellyfish, to its symbionts which can include Amphidinium species.[25] A Prymnesium-like phytoplankton has been identified as a symbiont of several species of acantharia.[26]

The cell-surface deaminases reported here produce H_2O_2 and small organic compounds (α-keto acids and aldehydes) extracellularly, while making nitrogen available to phytoplankton. The existence of cell-surface deaminases thus opens up perspectives on the cycling of H_2O_2 and small organic molecules and provides insights into the growth of phytoplankton in environments ranging from inside an invertebrate host to the expanses of oligotrophic ocean.

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Chapter 2.

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Limnol Oceanogr., 32(6), 1987, 1365–1369

Hydrogen peroxide production by a marine phytoplankter[1]

Abstract—*Hymenomonas carterae*, a calcified marine phytoplankter, produces hydrogen peroxide extracellularly. Hydrogen peroxide production by a washed cell suspension occurs in the dark and is inhibited by cell-impermeable protein modification reagents. A cell-surface redox enzyme is thus likely responsible for production of H_2O_2. The physiological function of this potentially toxic compound is unknown. If the production rate of $1–2 \times 10^{-14}$ mol cell^{-1} h^{-1} measured in cultures of *H. carterae* could be generalized, marine phytoplankton would be an important source of the hydrogen peroxide found in the marine environment.

[1] Funding for this work was provided by ONR Contract N-00014-80-C-0273 and NSF Grant OCE 83-17532.

Hydrogen peroxide occurs in surface ocean water at a steady state concentration of about 1 to 20×10^{-8} M (Van Baalen and Marler 1966; Zika 1984). This highly reactive species may have a profound influence on the chemistry and biological activity of the oceans. Hydrogen peroxide originates from wet and dry deposition (Zika et al. 1982; Thompson and Zafiriou 1983), is generated in photochemical reactions (Cooper and Zika 1983), and can also be leaked by phytoplankton, which produce it internally through light or dark reactions (Mehler 1951). In addition to these adventitious sources, organisms could produce

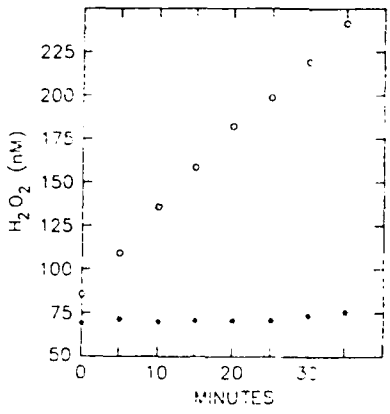

Fig. 1. Dark hydrogen peroxide production by *Hymenomonas carterae*. The data shown here represent typical reproducible results: O—in assay medium; *—in assay medium with catalase at 0.4 mg ml^{-1}.

H_2O_2 extracellularly by reducing oxygen enzymatically at their surface. The role of cell-surface redox enzymes found in a variety of cells is now controversial and receiving increasing attention.

Here we report the dark production of extracellular hydrogen peroxide by *Hymenomonas carterae*, a marine eucaryotic phytoplankter from the widely distributed class of Prymnesiophyceae. We also quantify production and provide evidence for the involvement of a cell-surface enzyme. This is the first evidence of cell-surface redox enzymes in eucaryotic phytoplankton. Subsequent evidence of other surface redox systems in algae has been reported by Jones et al. (1987).

We thank N. V. Blough, R. Olson, and S. W. Chisholm for advice and laboratory space during sections of this project; R. Hudson for discussions; S. Frankel for technical assistance; and B. Germanotta for manuscript preparation. *Hymenomonas carterae* (Cocco II) was provided by the Culture Collection of Marine Phytoplankton, Bigelow Laboratories.

Unialgal, axenic cultures of *H. carterae* were grown in f/10-enriched (Guillard and Ryther 1962) autoclaved Sargasso seawater, under constant light at 22°–23°C. Log phase cultures were harvested by centrifugation and diluted into 3 ml of assay medium in a quartz cuvette at a concentration of 6,000–7,000 cells ml^{-1} and counted by Coulter counter. Cells were also resuspended in 50 ml of assay medium (about 1 : 500 dilution) and recentrifuged before assaying for H_2O_2 production. This washing procedure did not significantly affect H_2O_2 production, indicating that a soluble extracellular enzyme was not responsible for the H_2O_2 produced. In addition, spent culture medium added to the assay medium did not show H_2O_2 production. The assay medium consisted of synthetic ocean water treated by passage through a leached Chelex column to remove trace metal contaminants and buffered with 0.36 mM borate to pH 8.2. The assay medium also included 13.3 μM hydroxyphenylpropionic acid (HPPA) and 0.033 mg ml^{-1} horseradish peroxidase (HRP) (Sigma, Type 6). Hydrogen peroxide production was measured by the H_2O_2—HRP catalyzed—oxidation of hydroxyphenylpropionic acid to a fluorescent dimer (excitation 320 nm, emission 404 nm) (Zaitsu and Ohkura 1980). The C.V. for H_2O_2 between replicate treatments is about 2.5%. All experiments discussed here were replicated at least once with the same results. Fluorescence increased as a function of time only in the presence of cells, HRP, and HPPA. For measuring dark production, cells were kept in the dark between fluorescence measurements. Cells kept in the dark for several hours before beginning the assay produced H_2O_2 at similar rates. The assay was calibrated by standard additions of H_2O_2 to the assay medium. Hydrogen peroxide was also assayed with scopoletin instead of hydroxyphenylpropionic acid; results were similar (Loschen et al. 1971).

Rates of hydrogen peroxide production varied with growth conditions but generally were 1–2 × 10^{-14} mol $cell^{-1}$ h^{-1} and were sensitive to added catalase, which competes with the analytical enzyme (HRP) for H_2O_2 (96% decrease at 0.4 mg ml^{-1} catalase; Fig. 1). Rates of H_2O_2 production did not change measurably upon illumination of cells during the assay (data not shown). In investigating the effects of several amino acids as potential electron donors, we found that only L-glutamate and L-glutamine enhanced H_2O_2 production (50% at 33 μM addition); D-glutamate did not. The difference between L and D isomers indicates that H_2O_2 production is a biochemical process. The glutamate and glutamine enhancement suggests a link

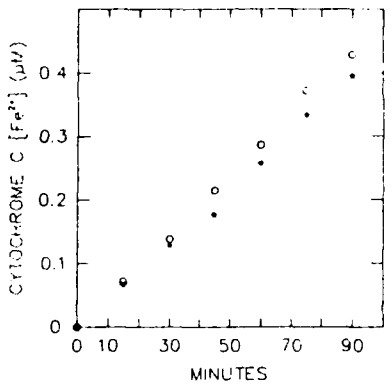

Fig. 2. Dark cytochrome C reduction by *Hymenomonas carterae*. The data shown here represent typical reproducible results: O—assay; *—assay and superoxide dismutase at 0.007 mg ml $^{-1}$.

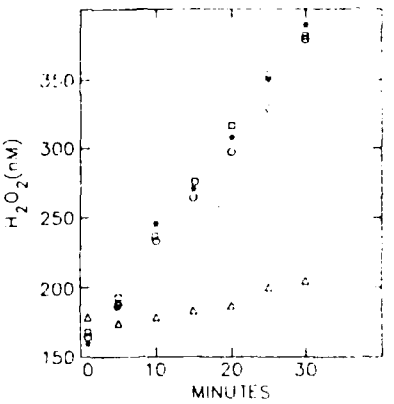

Fig. 3. Hydrogen peroxide production by ANDS-treated *Hymenomonas carterae*. ANDS was dissolved in 5 ml of *H. carterae* cell culture in each of two glass tubes to a concentration of 1 mM. One tube was exposed to 4 min of irradiation by a Kratos solar simulator. The other was kept in the dark. Two tubes without ANDS were treated in a similar manner. All four tubes were immediately diluted to 50 ml with autoclaved Sargasso seawater and centrifuged to harvest cells. Cells were assayed for H_2O_2 production as described. The data shown here represent typical reproducible results: *—dark, no ANDS; □—dark, ANDS; O—light, no ANDS; △—light, ANDS.

between H_2O_2 production and nitrogen metabolism.

We also assayed another species of phytoplankton, the marine diatom *Thalassiosira weissflogii*, for H_2O_2 production by both methods. The results were negative, showing that the H_2O_2 production of *H. carterae* is not simply an artifact of phytoplankton suspensions. It also shows that between-taxon differences exist in the production of H_2O_2 by eucaryotic phytoplankton.

We attempted to measure the production of extracellular superoxide anion (as a possible precursor of the H_2O_2 released by *H. carterae*) by assaying for ferricytochrome C reduction (Bielski 1984). *Hymenonomonas carterae* cultured and harvested as described was diluted to a concentration of 42,500 cells ml^{-1} into Chelexed, borate-buffered (pH 8.2) synthetic ocean water containing 20 μM ferricytochrome C (Sigma, Type 3). Cytochrome C reduction is plotted as the average of three determinations. Each determination is the absorbance at 550 nm minus absorbance at 500 nm (defined as delta) minus the average delta at $T = 0$. This procedure was followed to reduce the problems of light scattering by the cell suspension and to clarify data presentation. The rate of assayed cytochrome C reduction shown in Fig. 2 is 6.7×10^{-15} mol cell^{-1} h^{-1}, if an extinction coefficient of 21,000 cm^{-1} M^{-1} at 550 nm is assumed (Bielski 1984).

A saturation (at 40 μM ferricytochrome C) rate of about $1–2 \times 10^{-14}$ mol cell^{-1} h^{-1} was found for *H. carterae* depending on growth conditions. If this ferricytochrome C reduction were due to O_2^-, then superoxide dismutase (SOD) should lower the reduction rate by rapidly removing O_2^- (converting it to H_2O_2). Ferricytochrome C reduction was only slightly decreased by large additions of SOD, however, even though SOD retained its activity in the assay medium as shown using a xanthine-xanthine oxidase O_2^--producing system (McCord and Fridovich 1969). In addition, *H. carterae* did not reduce nitroblue tetrazolium, another commonly used scavenger of O_2^-. Thus, the bulk of the measured reduction of ferricytochrome C does not represent cellular release of O_2^-, and the relatively large H_2O_2 production by *H. carterae* cannot be due to production of extracellular O_2^-.

Production of extracellular hydrogen peroxide by cyanobacteria (Van Baalen 1965; Stevens et al. 1973) and by some freshwater eucaryotic phytoplankton species has been reported (Zepp et al. 1986). With the exception of a few freshwater cyanobacteria, H_2O_2 production has been observed to be light-dependent and is thought to represent

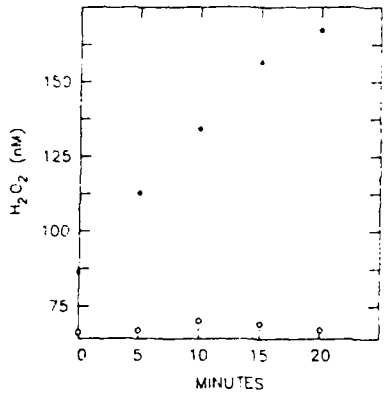

Fig. 4. Hydrogen peroxide production by DABS-treated *Hymenomonas carterae*. DABS was synthesized (Grebing et al. 1984) and used immediately; 5 ml of *H. carterae* cell culture was made up to 0.4 mM in DABS, incubated in the dark for 15 min, diluted to 50 ml with autoclaved Sargasso seawater, centrifuged, and assayed for H_2O_2. The control cells were treated in a similar manner without DABS addition. The data shown here represent typical reproducible results: *—control; O—0.4 mM DABS treatment.

leakage of photosynthetically reduced oxygen out of the cell. Hydrogen peroxide production by intact illuminated chloroplasts was first reported by Mehler (1951) and has been extensively studied. Dark production of extracellular H_2O_2 could result from oxygen reduction by other electron transport chains (e.g. by mitochondrial reduction of oxygen and diffusion out of the cell) (Forman and Boveris 1982; Frimer et al. 1983) or by enzymatic reduction of oxygen at the cell surface.

We looked for the involvement of cell-surface enzymes by using the mild, cell-impermeable alkylating reagents, ANDS (3-azido, 2,7 naphthalene disulfonate) and DABS (diazobenzene sulfonate), and the protease proteinase K. In the presence of UV light, ANDS produces a nitrene that inserts into C–H, O–H, and N–H bonds. ANDS has been used in this way to covalently label surface proteins with a fluorescent marker (Moreland and Dockter 1980). As shown in Fig. 3, a 4-min treatment with 1 mM ANDS in UV light inactivates about 80% of H_2O_2 production. Exposure of the cells to ANDS in the dark or to UV light alone had no effect. ANDS-UV treatment as above had only a small effect on cytochrome C reduction (≤20%, data not shown), suggesting that cytochrome C is reduced at a different site on the same enzyme or by a separate reducing system.

DABS has been extensively used to label surface proteins (Tinberg and Packer 1976). It reacts with nucleophilic sites, such as tyrosyl, histidyl, and sulfhydryl moieties. DABS synthesized (Grebing et al. 1984) and incubated with cells (0.4 mM for 15 min) completely inhibited both H_2O_2 production (Fig. 4) and cytochrome C reduction (data not shown).

H_2O_2 production was totally inhibited by treatment with proteinase K (20 μg ml^{-1}; Sigma) for 15 min before harvesting cells for the assay. Proteinase K is a nonspecific protease that is thought not to permeate into cells because of its large size.

H_2O_2 production (and cytochrome C reduction) by *H. carterae* is thus apparently due to the activity of cell-surface redox enzymes. As such, *H.carterae* may be a useful model for studying these enzymes (including final proof of their existence through isolation) and their implications for the marine environment. The presence and role of cell-surface redox enzymes in a variety of cells is being intensively studied (Crane et al. 1985). Examples include external iron reduction by higher plants (Bienfait 1985), the antibacterial functions of leucocytes (Iyer et al. 1961), and more recently the fungal degradation of lignin (Kelley and Reddy 1986). Production of hydrogen peroxide by *H. carterae* may similarly be involved in nutrient transport, antibacterial activity, the reduction or oxidation of necessary or toxic metals, or the mineralization of major nutrients such as nitrogen by degradation of marine organic materials.

Although photochemical reactions and rain inputs are currently considered the major sources of hydrogen peroxide in the marine environment, our results also indicate that biological production could be important. If, for example, 10^5 coccolithophorid cells $liter^{-1}$ (these organisms being present in many environments in a range of 10^2–10^7 cells $liter^{-1}$: Smayda 1980) produced H_2O_2 at the rate we measured for *H. carterae*, their total contribution would be 1–2 nM h^{-1}. A residence time of 5–200 h for biological H_2O_2 turnover is obtained, if we

assume 1–20 × 10^{-8} M H_2O_2 concentrations in seawater. This residence time is similar to estimates of H_2O_2 replacement by other processes, although all estimates are highly uncertain at this time.

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Chapter 3.

Amino acid utilization by marine phytoplankton: A novel mechanism

Abstract

An enzyme on the cell-surface of some marine phytoplankton, particularly the genus Pleurochrysis (Hymenomonas), oxidizes many L-amino acids to produce H_2O_2, ammonia, and an α-keto acid extracellularly. The ammonia is subsequently taken up and used for growth. The enzyme synthesized by P. carterae (clone CoccoII) has a half saturation constant of 0.25 μM assayed using L-α-aminobutyric acid as a model substrate. The enzyme activity is absent in NH_4^+-grown (logarithmic phase) cells, is present in NO_3^--grown (logarithmic phase) cells, and is enhanced 3.5 fold in nitrogen-limited cells. Some Pleurochrysis species show different patterns of regulation. The existence of this novel, high affinity mechanism for amino acid utilization, one that would not be detected by typical field methods, suggests that amino acids may be a more important source of nitrogen for phytoplankton than is currently believed.

The extent to which phytoplankton utilize free amino acids as a nitrogen source is controversial. Ambient concentrations of dissolved free amino acids in marine environments have been reported in the range of 0.02-5.0 μM (for example Lee and Bada, 1977; Garrasi, et al, 1979; Mopper and Lindroth, 1982; also see Fuhrman and Bell, 1985 on methodological issues). While these concentrations are often similar to or sometimes higher than those of inorganic nitrogen sources (NH_4^+, NO_3^-), amino acids have received far less attention (Sharp, 1983; Paul, 1983). Laboratory studies have shown that phytoplankton can grow on certain amino acids, in some cases as well or better than on inorganic sources (Pintner and Provasoli, 1963; Turner, 1979; Wheeler et al, 1974; Flynn and Butler, 1986). Field studies, on the other hand, usually conclude that amino acids are not significantly utilized by phytoplankton (Hoppe, 1976; Hollibaugh, 1976). Both laboratory and field studies usually assume explicitly or implicitly that amino acids are transported into the cells as the initial step in utilization.

In this paper we outline a novel mechanism by which some phytoplankton utilize amino acids as a nitrogen source. We were lead to it while investigating hydrogen peroxide production by phytoplankton (Palenik, et al, 1987; Palenik and Morel, 1988). In this mechanism, amino acids are oxidized by an enzyme at the cell surface of the organism to produce H_2O_2, an α-keto acid, and NH_4^+, with the ammonia subsequently used for growth. Transport of the amino acid is not the initial step of utilization, so that in using ^{14}C or ^{3}H labelled amino acids in either the field or the laboratory one could easily overlook this mechanism.

The L-amino acid oxidase described here is likely to be part of a class of enzymes in phytoplankton (e.g. asparaginases--Paul and Cooksey, 1979) that decompose organic nitrogen forms at the cell surface, thus making NH_4^+ available to the cell. These "cell-surface deaminases" are the nitrogen analog to cell-surface phosphatases long known to occur in phytoplankton.

Materials and Methods

Materials--Pleurochrysis carterae (clone Cocco II), Pleurochrysis sp. (156HYM), Pleurochrysis scherfelli (944-1), and Pleurochrysis sp. (UW398) were obtained from the Center for the Culture of Marine Phytoplankton (CCMP), West Boothbay Harbor, Maine. Pleurochrysis carterae (CoccoII-N) is a coccolithless clone isolated by the authors from CoccoII. Emiliania huxleyi (12-1) was isolated from the Sargasso Sea by the authors and is now available from the CCMP. Cultures were maintained in f/10 without silica (Guillard and Ryther, 1962) enriched tyndallized (heated/frozen/heated, as in Brand, 1983) Sargasso seawater under constant fluorescent (cool white) light at about 20 ^{o}C. To obtain nitrogen-limited cultures, cells were grown in f/10 without added nitrogen or silica until growth stopped. Growth of cultures was followed by chlorophyll fluoresence and cell counts were measured using a Coulter cell counter. Bacterial contamination of cultures was monitored with Difco marine broth 2216. All cultures were maintained and studied while free of bacterial contamination except UW398 and 12-1 in which bacterial contaminants were present during the assays.

Amino acids were obtained from Sigma Chemical and Aldrich except $^{14}C(U)$ L-leucine from ICN and ^{15}N-L-aspartic acid from Merck. Enzymes (proteinase K, trypsin, chymotrypsin, and horseradish peroxidase (HRP) Type VI) were obtained from Sigma. In experiments requiring low background amino acid concentrations, the HRP was prepared in UV-oxidized Corning distilled water on the same day it was used or it was dialysed by centrifugation in a Centricell (TM) unit obtained from Polysciences to concentrate it to 4 mg ml^{-1}. Assays in some cases used UV-oxidized synthetic ocean water without nutrients (Morel et al, 1979) as an assay medium with low amino acid background.

Methods--H_2O_2 production was assayed as in Palenik et al.(1987) by adding 50ul of a 2mM hydroxyphenylpropionic acid (Aldrich) stock and 1 to 5 μl (depending on experiment) of a 4mg ml^{-1} HRP stock to a 3ml aliquot of cell suspension or cells diluted into UV-oxidized medium in a quartz cuvette. 5μM L-α-aminobutyric acid or other amino acids were added to assay for L-amino acid oxidase activity. The sample fluoresence (320 nm excitation, 404 nm emission) of the sample was followed as a function of time in a Perkin-Elmer LS-5 spectrofluorometer. The assay for H_2O_2 was calibrated by standard additions of a μM H_2O_2 stock solution to the assay system.

α-Keto acid production by CoccoII was assayed on cultures spiked with L-alanine. 10ml samples were gravity filtered through 1.0 μm Nuclepore filters and the first 5ml of filtrate were collected and analyzed for pyruvate by D. Kieber using 2,4-dinitrophenylhydrazine derivatization and HPLC (Kieber and Mopper, 1986). H_2O_2 production was

simultaneously measured in a non-trapping assay on a Turner fluorometer (Palenik and Morel, 1988)

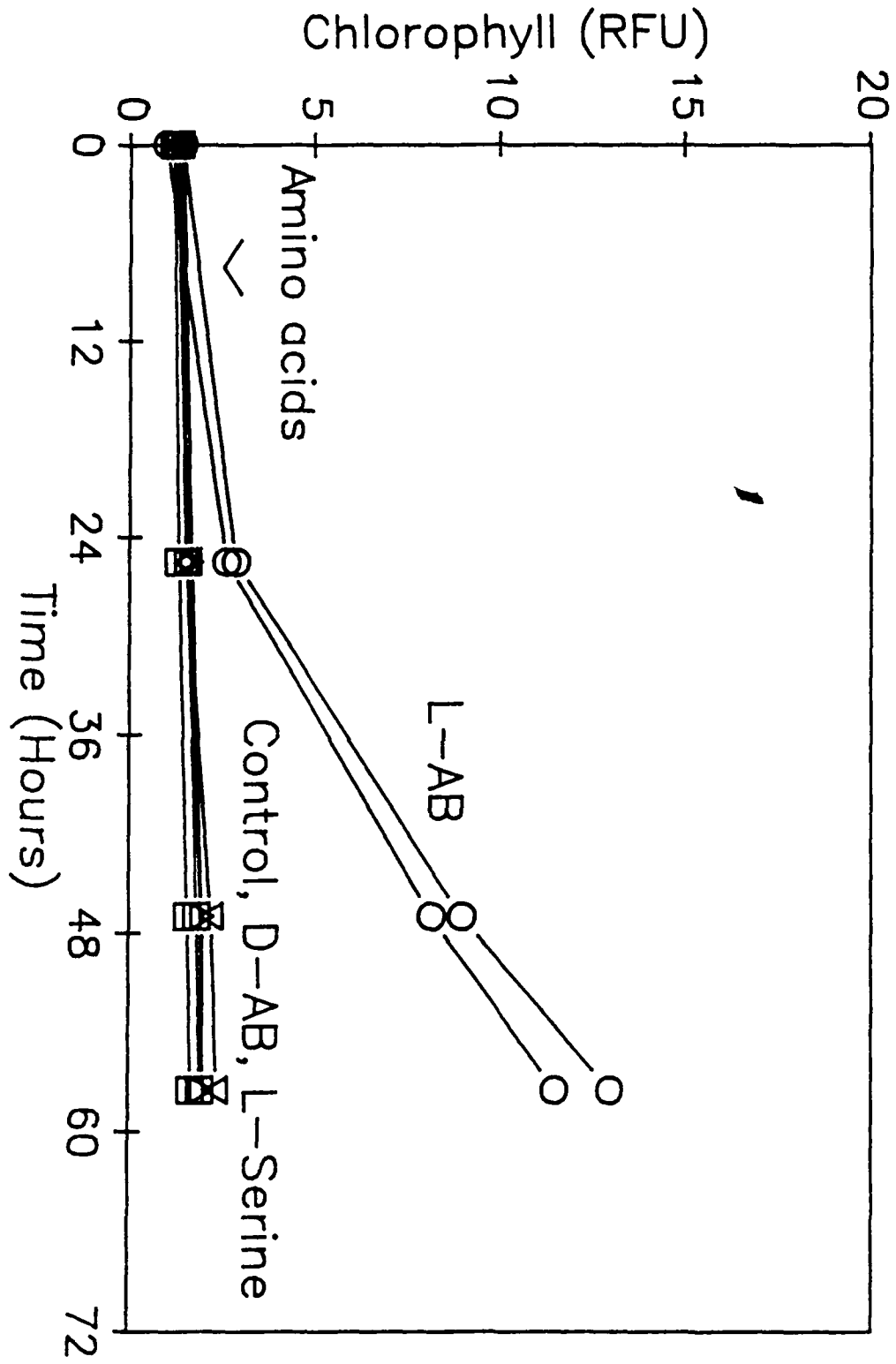
^{15}Nitrogen incorporation was measured by adding 9 μM ^{15}N-L-aspartic acid to nitrogen limited cultures. Approximately 10^7 cells were collected at subsequent times on precombusted glass fiber filters and analyzed by J. Nevins for ^{15}N using mass spectrometry.

Uptake of L-leucine was measured by adding 1 μCi ^{14}C(uniformly labelled, 270mCi mmole^{-1}) L-leucine to 25ml of nitrogen limited CoccoII culture at 3000cells ml^{-1}. Duplicate 2ml samples were gravity filtered through 3μm Nuclepore filters in 25mm Swinnex holders and before the cells were dry they were rinsed with about 5ml of synthetic ocean water (method developed by Hudson and Morel, 1988). The activity remaining on the filters was measured in a Beckman scintillation counter.

Results

Utilization and Oxidation of Amino Acids--When L-α-aminobutyric acid (50μM) is added to nitrogen limited cultures of P. carterae (CoccoII), the cells resume growth as measured by chlorophyll fluorescence (Figure 1). The final yield of cells is also proportional to the amount of added L-aminobutyric acid (data not shown). Neither D-α-aminobutyric acid nor L-serine at 50μM would support a rapid resumption of growth. L-serine did support a slow growth rate, however. These results indicate that CoccoII can efficiently utilize the model amino acid L-α-aminobutyric acid as a nitrogen source.

Figure 1: Growth of P.Carterae (Clone CoccoII) on amino acids. 50 μM substrate was added to duplicate (except control) nitrogen-limited cultures in 50 ml glass tubes. ▽ Control; ○ L-α-aminobutyric acid; △ D-α-aminobutyric acid; □ L-serine. L-serine supported slow but significant growth.


Chlorophyll (RFU)
0
5
10
15
20
0
12
24
36
48
60
72
Time (Hours)
Amino acids
L-AB
Control, D-AB, L-Serine

When L-aminobutyric acid is added to a sample of nitrogen-limited P. carterae (CoccoII), hydrogen peroxide is produced extracellularly (Figure 2). Furthermore, the rate of production of hydrogen peroxide initially increases with increasing amino acid additions. No H_2O_2 production by cells is found in the assay system in the absence of added amino acid if the horseradish peroxidase solution used in the assay is prepared on the day it is used or if it is purified by dialysis (Centricell TM centrifugation) before use. In diluted cultures, the diluting media must be UV-oxidized and stored before use in order to obtain low amino acid and H_2O_2 background concentrations (See Materials and Methods). The ubiquitous amino acid contamination in laboratories, and especially in enzyme reagents such as HRP, lead us to discover amino acid oxidation by phytoplankton, but also created major difficulties in studying the phenomenon.

A range of compounds were tested as substrates for oxidation by P. carterae. Only amino acids were found to support extracellular H_2O_2 production (See Figures 3a,3b,4). L-D-α-hydroxybutyric acid or n-butyric acid, both structurally similar to L-α-aminobutyric acid, were not effective (data not shown). Similarly, L-glutamate but not D-L-α-methyl-glutamate, in which a methyl group has replaced the oxidizable moiety, supports H_2O_2 production. The enzyme thus appears to be an amino acid oxidase. The products of this kind of enzymatic reaction are hydrogen peroxide and an imino acid which hydrolyzes to produce ammonia and an α-keto acid (Walsh, 1979).

The putative amino acid oxidase of CoccoII is relatively non-specific, producing hydrogen peroxide from L-alanine and most four carbon or longer L-amino acids with $-CH_2X-$ at the beta carbon (X is (C)arbon or (H)ydrogen, but not (O)xygen). It would not efficiently utilize glycine, L-serine, L-threonine, or L-proline of the amino acids tested (See Figures 3A,3B, and 4). D-amino acids do not lead to the production of H_2O_2. These results run parallel with the growth experiments presented above, where D-aminobutyric acid and L-serine do not support the rapid resumption of growth of nitrogen-limited CoccoII.

When 5μM L-alanine is added to a culture of nitrogen limited CoccoII, the corresponding α-keto acid, pyruvate, is produced in the medium. The recovery of pyruvate is essentially quantitative (Figure 5). When a 1μM L-alanine addition is made, pyruvate and H_2O_2 production occur with similar kinetics. Again pyruvate is essentially quantitatively recovered, but less H_2O_2 (70%) is recovered due to its decomposition in the medium, as seen in the later data points (Figure 5). No pyruvate production was found in the absence of added alanine. No pyruvate production occured after adding alanine to 3.0 μm filtered spent culture medium.

The oxidation of an amino acid to produce H_2O_2 and an α-keto acid liberates ammonia. To follow the uptake of this ammonia and to show that the growth of CoccoII on various amino acids is due to the amino acid nitrogen, we looked for direct ^{15}N incorporation from L-aspartic acid (^{15}N-98atom%). 9 μM labelled L-aspartic acid was added

Figure 2: L-α-aminobutyric acid-dependent hydrogen peroxide production by nitrogen limited CoccoII (9900 cells ml^{-1}). ○ 0 nM; ● 15nM; △ 30 nM; ▲ 60 nM. A linear regression line is drawn through the data for each treatment. Also shown is the lack of L-α-aminobutyric acid-dependent (4μM) H_2O_2 production by NH_4^+ (log phase) grown cells (5500 cells ml^{-1}) □ .

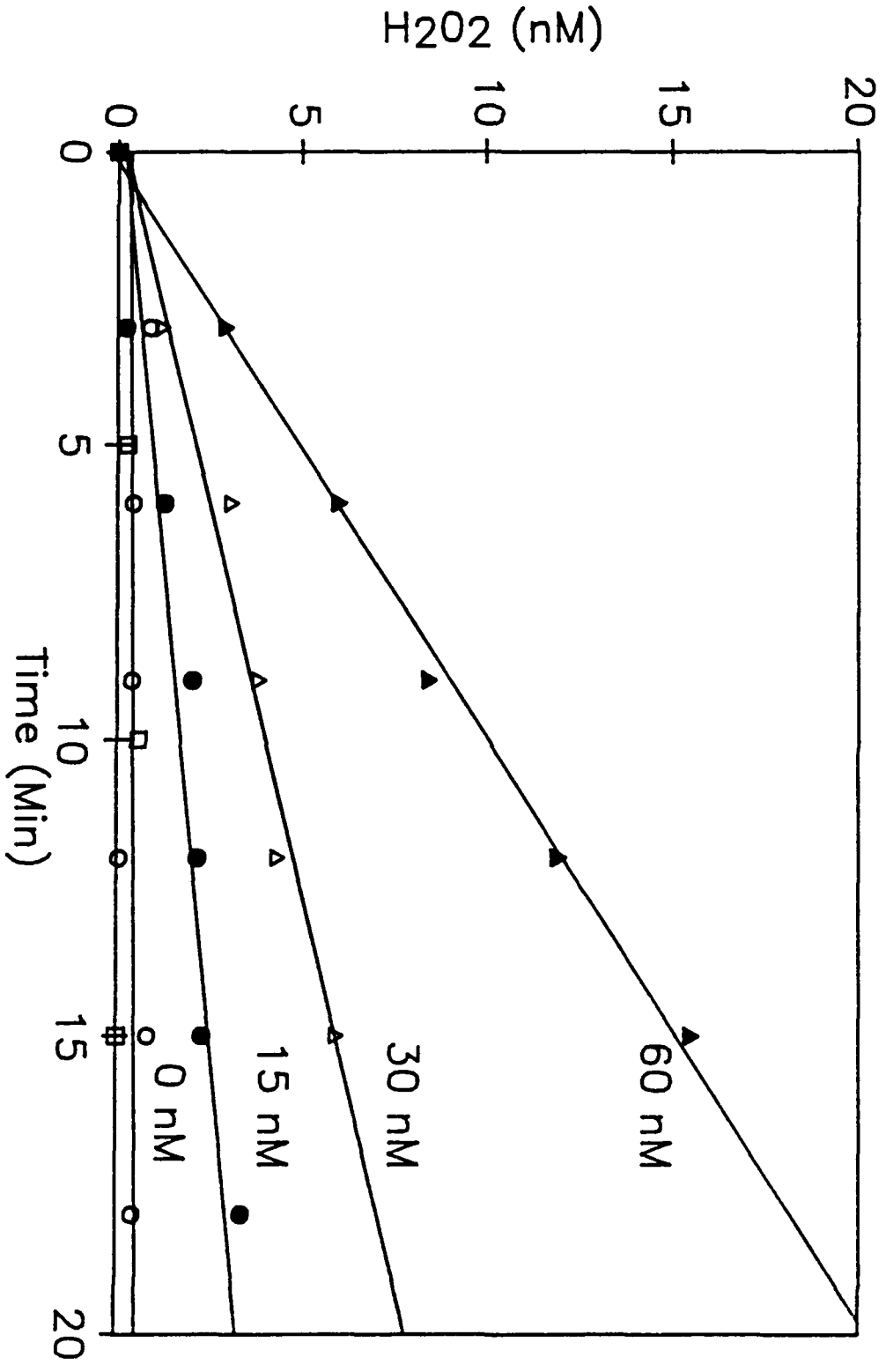

H2O2 (nM)
0
5
10
15
20
Time (Min)
0
5
10
15
20
0 nM
15 nM
30 nM
60 nM

Figure 3A: Amino acid-dependent (5μM) hydrogen peroxide production by CoccoII (23000 cells ml^{-1}). ○ L-alanine; △ L-α-aminobutyrate; ▲ L-serine; □ Glycine; ● Control(No addition). A linear regression line is drawn through the data for each treatment.

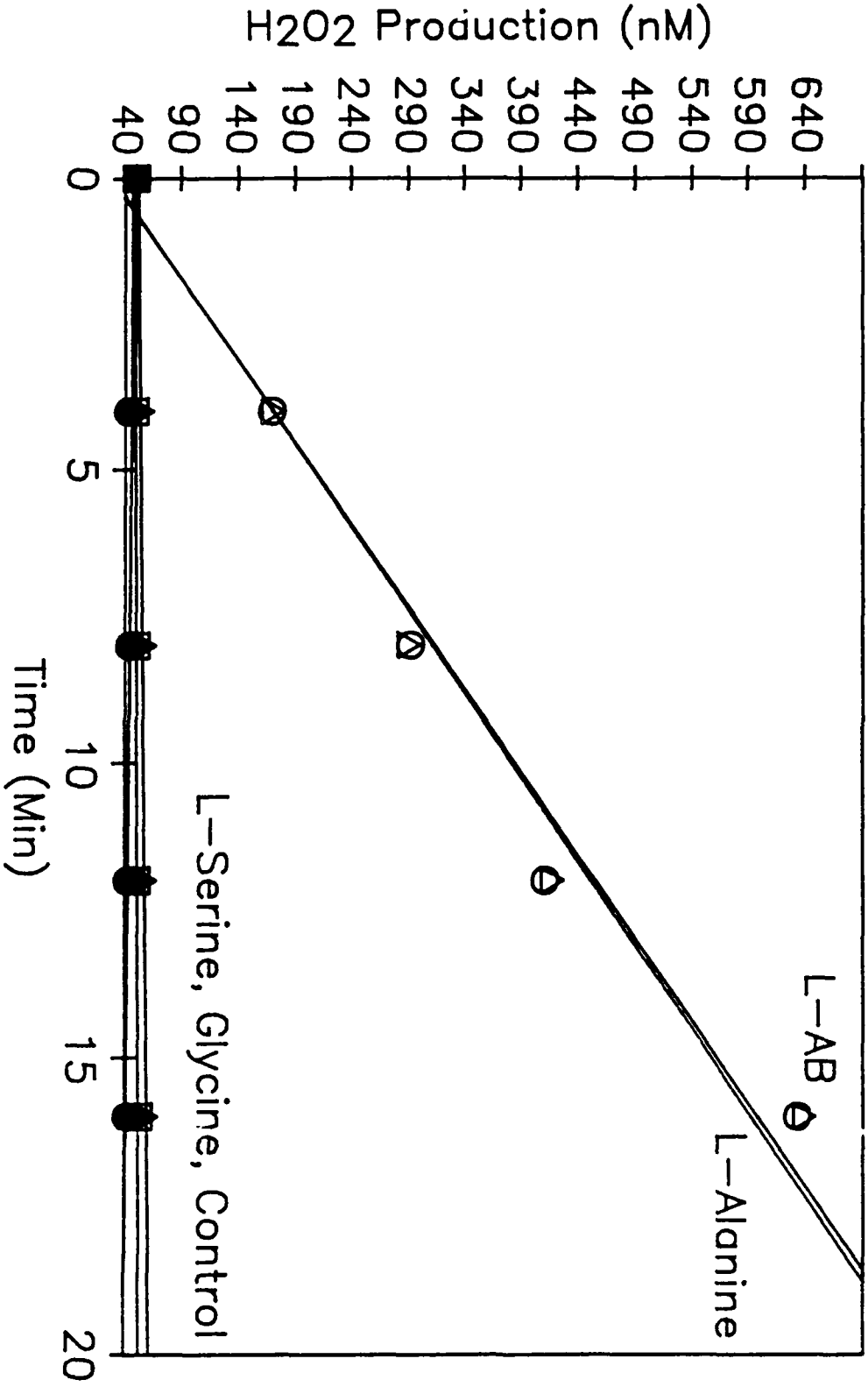

H2O2 Production (nM)
40
90
140
190
240
290
340
390
440
490
540
590
640
0
5
10
15
20
Time (Min)
L-AB
L-Alanine
L-Serine, Glycine, Control

Figure 3B: Amino acid-dependent (5μM) hydrogen peroxide production by CoccoII (5700 cells ml^{-1}) ▲ L-α-aminobutyric acid; ● L-methionine; △ L-threonine; □ D-α-aminobutyric acid; o Control.A linear regression line is drawn through the data for each treatment.

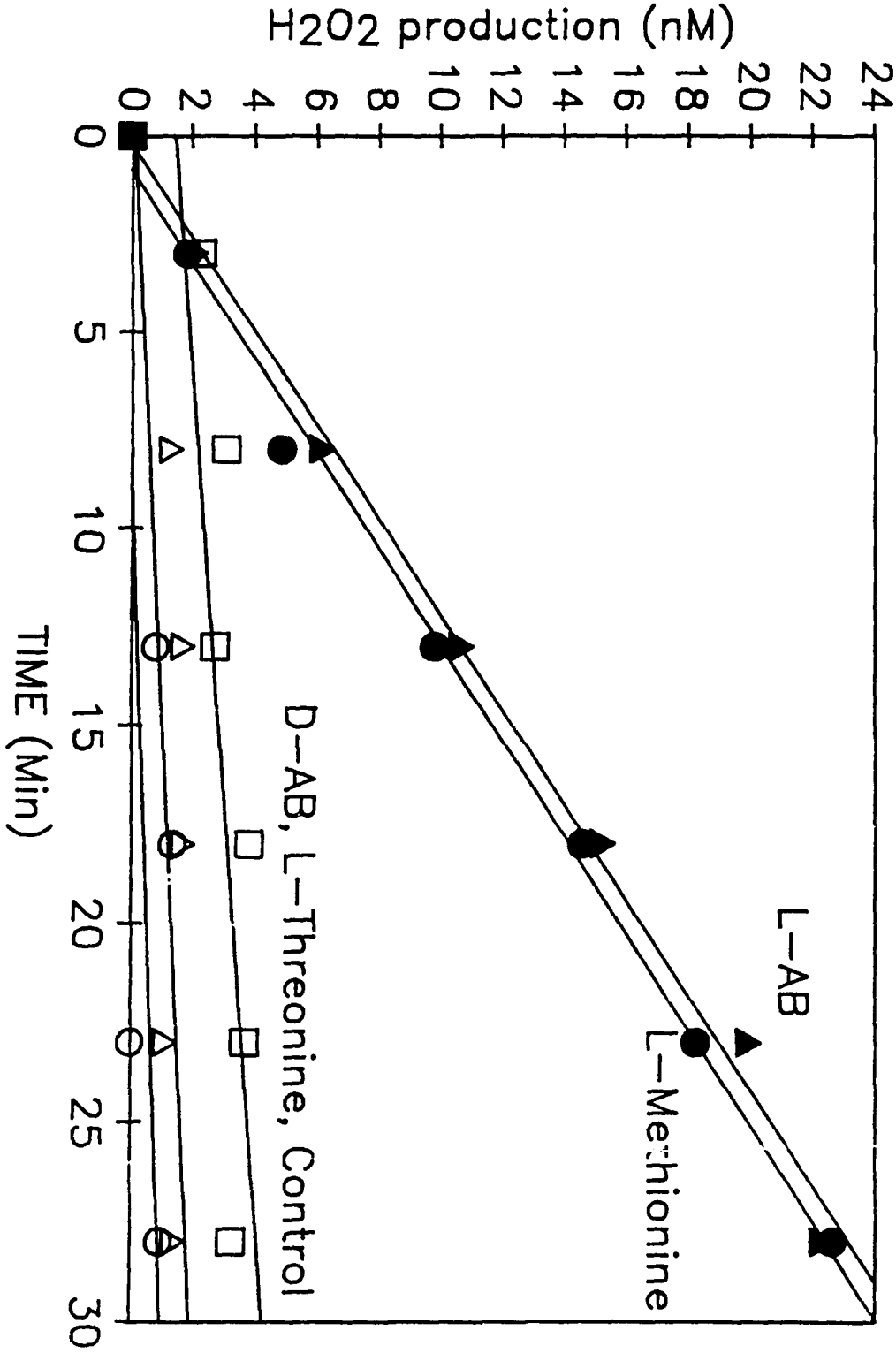
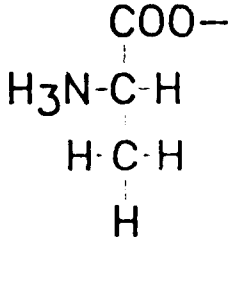
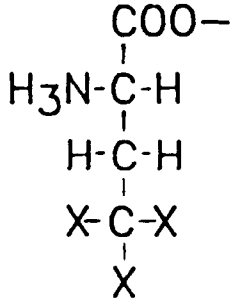

H2O2 production (nM)
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME (Min)
0
5
10
15
20
25
30
L-AB
L-Methionine
D-AB, L-Threonine, Control

Figure 4: Structure of some substances tested as oxidase substrates. The active site seems to require a $-CH_2X-$ group at the beta carbon, where X is H or C, but not O. Active: L-alanine, L-α-aminobutyrate, L-aspartate, L-glutamate, L-glutamine, L-homoserine, L-leucine, L-lysine, L-methionine, D-L-methionine, L-phenylalanine. Inactive : n-butyrate, D-L-α-hydroxybutyrate, γ-hydroxybutyrate, glycine, L-proline, L-serine (=< 1.7%), L-threonine, D-α-aminobutyrate, D-glutamate, D-L-α-methylglutamate.

ACTIVE

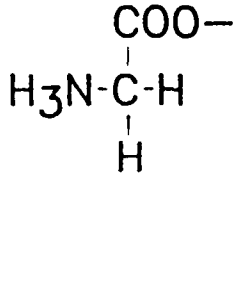


L—alanine

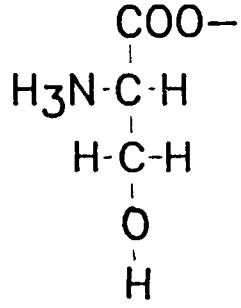


L—aminobutyrate, etc.

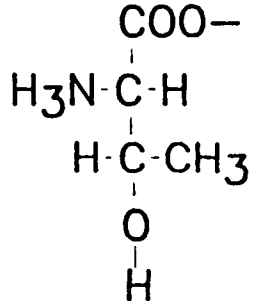
INACTIVE



Glycine

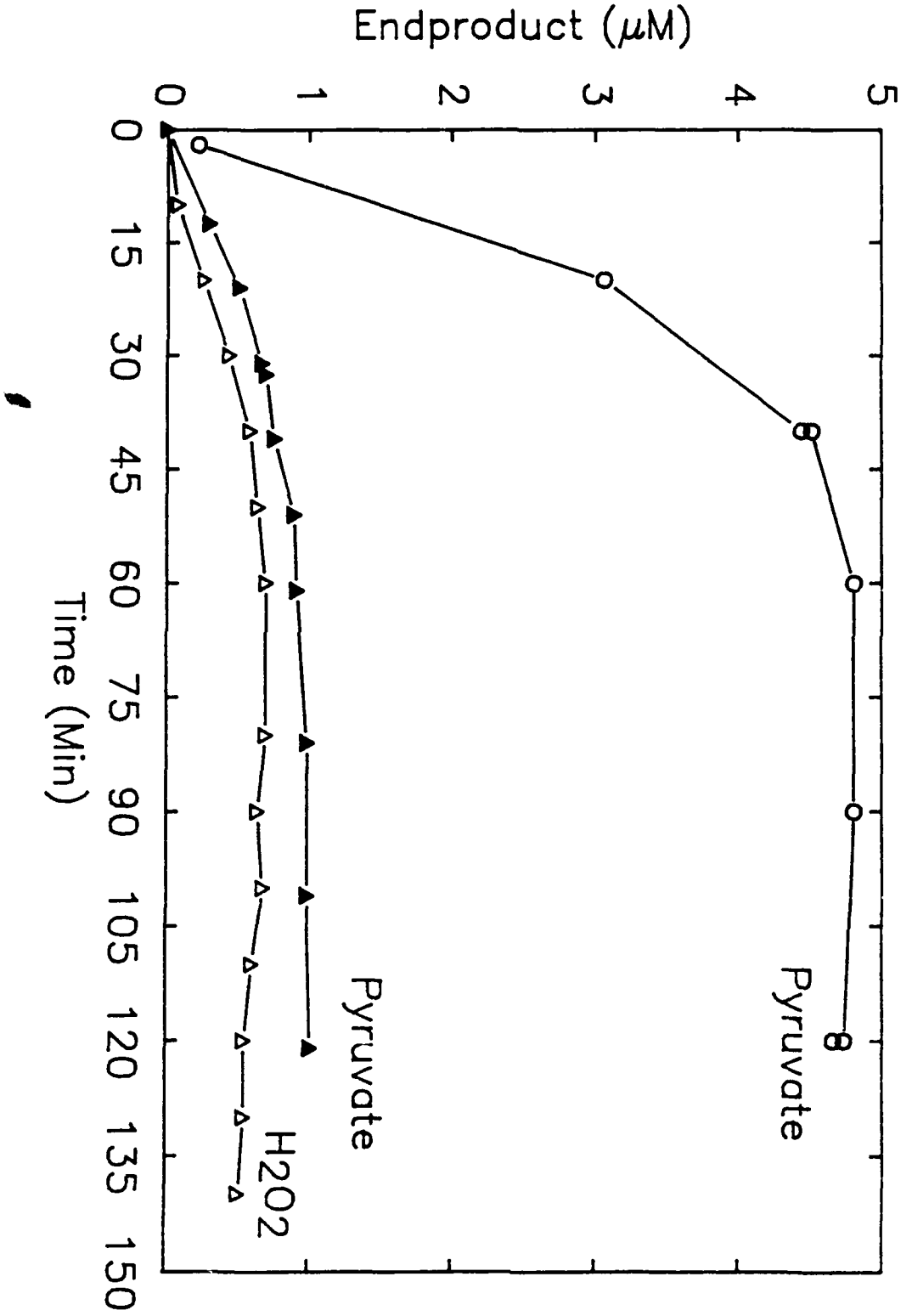


L—serine



L--threonine

Figure 5: α-Keto acid (pyruvate) production from addition of 5μM L-alanine addition to nitrogen limited CoccoII (72000 cells ml^{-1}) O. Simultaneous pyruvate and H_2O_2 production from 1μM L-alanine addition to nitrogen limited CoccoII (37000 cells ml-1) ▲, Pyruvate. △, H_2O_2.


Endproduct (μM)
0
1
2
3
4
5
0
15
30
45
60
75
90
105
120
135
150
Time (Min)
Pyruvate
Pyruvate
H2O2

to nitrogen limited CoccoII; the resulting ^{15}N incorporation (in atom% ^{15}N) is shown in Figure 6. The incorporation of ^{15}N occurs at an initial rate similar to that found for H_2O_2 and α-keto acid production (about $3x10^{-14}$mol cell^{-1} h^{-1}).

Enzyme Localization--The amino acid dependent production of H_2O_2 and α-ketoacids could be due to transport of the amino acids inside the cell followed by oxidation of the amino acids and export or leakage of H_2O_2 and α-ketoacids out of the cell. Alternatively the amino acids could be oxidized at the cell surface, leading to the production of H_2O_2 and α-ketoacids directly in the medium.

Evidence for the cell-surface rather than internal localization of the amino acid oxidase is found in the use of traditional cell impermeable protein modification reagents. We showed previously (Palenik, et al, 1987) that extracellular H_2O_2 production by CoccoII is inhibited by diazobenzenesulfonate and 3-azido,2,7 naphthalenedisulfonate, cell-surface alkylating reagents. Treatment of cells with a nonspecific protease proteinase K (20 μg ml^{-1} for 15 minutes) followed by washing inhibits amino acid dependent H_2O_2 production (Figure 7). Proteinase K is too large to be transported across the cell membrane so that in short treatments it only hydrolyzes cell surface proteins. The proteases trypsin and chymotrypsin were ineffective (data not shown) either because their specific hydrolysis sites were not accessible on the oxidase or because they may lose activity in seawater. Proteinase K treatment of cells also inhibited the subsequent L-amino acid oxidase activity (per

mg protein) of cell extracts, indicating that proteinase K is acting directly on the enzyme and not through a secondary effect (data not shown).

If the oxidase is located on the cell surface, then the uptake of ^{14}C labelled amino acids should be undetectable. When uniformly ^{14}C labelled L-leucine was added to cultures of P. carterae (CoccoII) no intracellular ^{14}C accumulation with time was detected (See Figure 8). H_2O_2 was produced by the same cells in the presence of an equivalent concentration of unlabelled L-leucine, however, and we can convert this H_2O_2 production into the amount of L-leucine we should have seen inside the cells if the oxidase were intracellular and all the α-keto acid remained inside (Also shown in Figure 8).

If the amino acid is oxidized internally and the α-keto acid is exported (see above), a steady state level of ^{14}C label (both from amino acid and α-keto acid) would develop inside the cells. There was no evidence for a steady state level of label in the cells, but given the detection limit of the method (about 100dpm) we can only say that in this experiment the internal concentration would have to be lower than about 50 μM.

The scenario of an internal oxidase would require at least three enzymes--one or more amino acid transport proteins, an L-amino acid oxidase, and an α-keto acid export protein. Because this scenario would contradict the cell-surface inhibitor data and is not consistent with the kinetics of H_2O_2 and α-ketoacid production, we conclude that the L-amino acid oxidase is a cell-surface enzyme.

Figure 6: ^{15}N incorporation (atom %N) after addition of 9μM L-aspartic acid (98 atom%) to nitrogen limited CoccoII. Cell number showed a corresponding 30% increase after 24 hours.

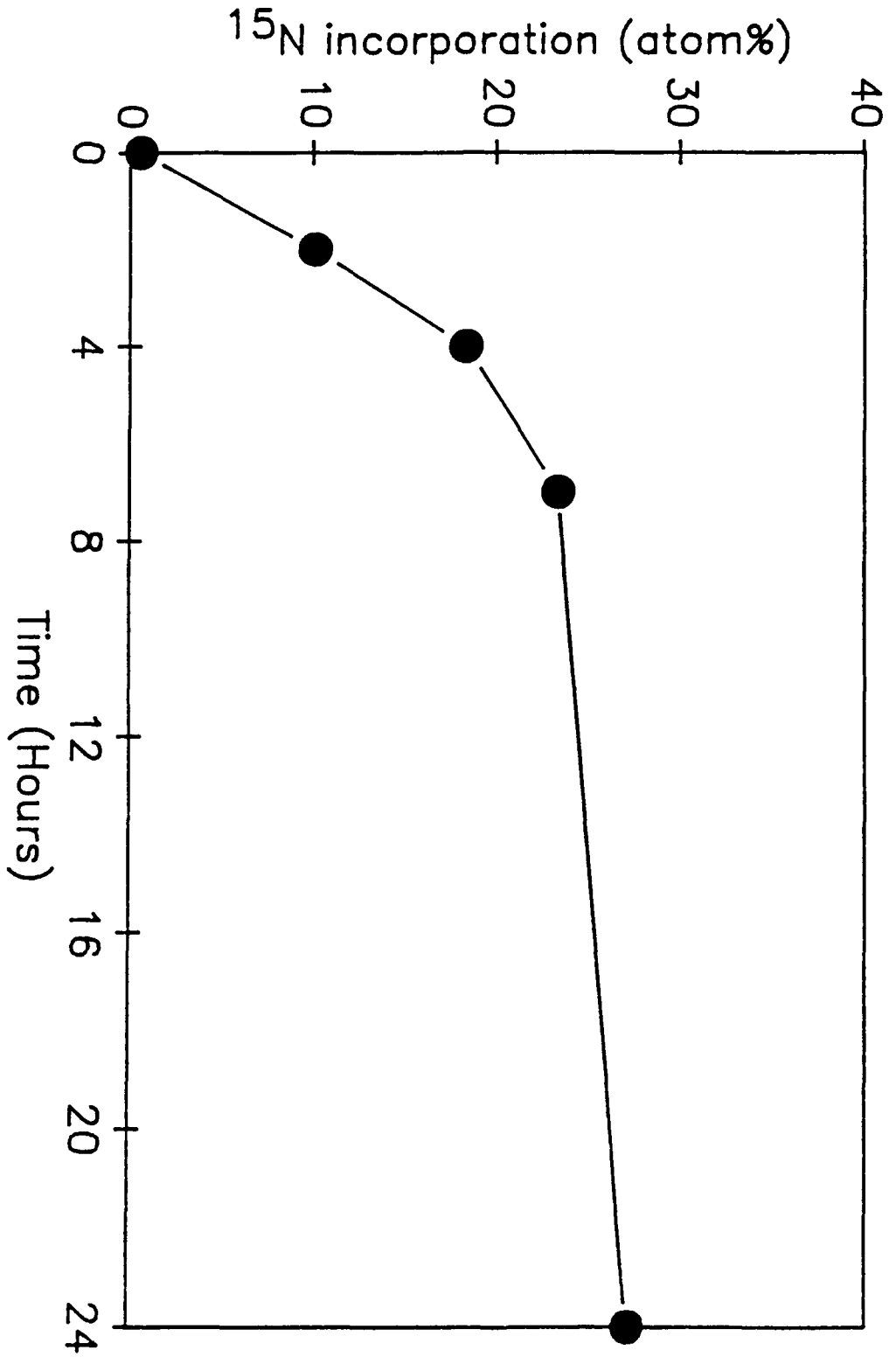

^{15}N incorporation (atom%)
0
10
20
30
40
Time (Hours)
0
4
8
12
16
20
24

Figure 7: Effect of Proteinase K treatment on L-amino acid oxidase activity of CoccoII. ○ Treated (20μg ml^{-1} for 15 minutes); ● Untreated.

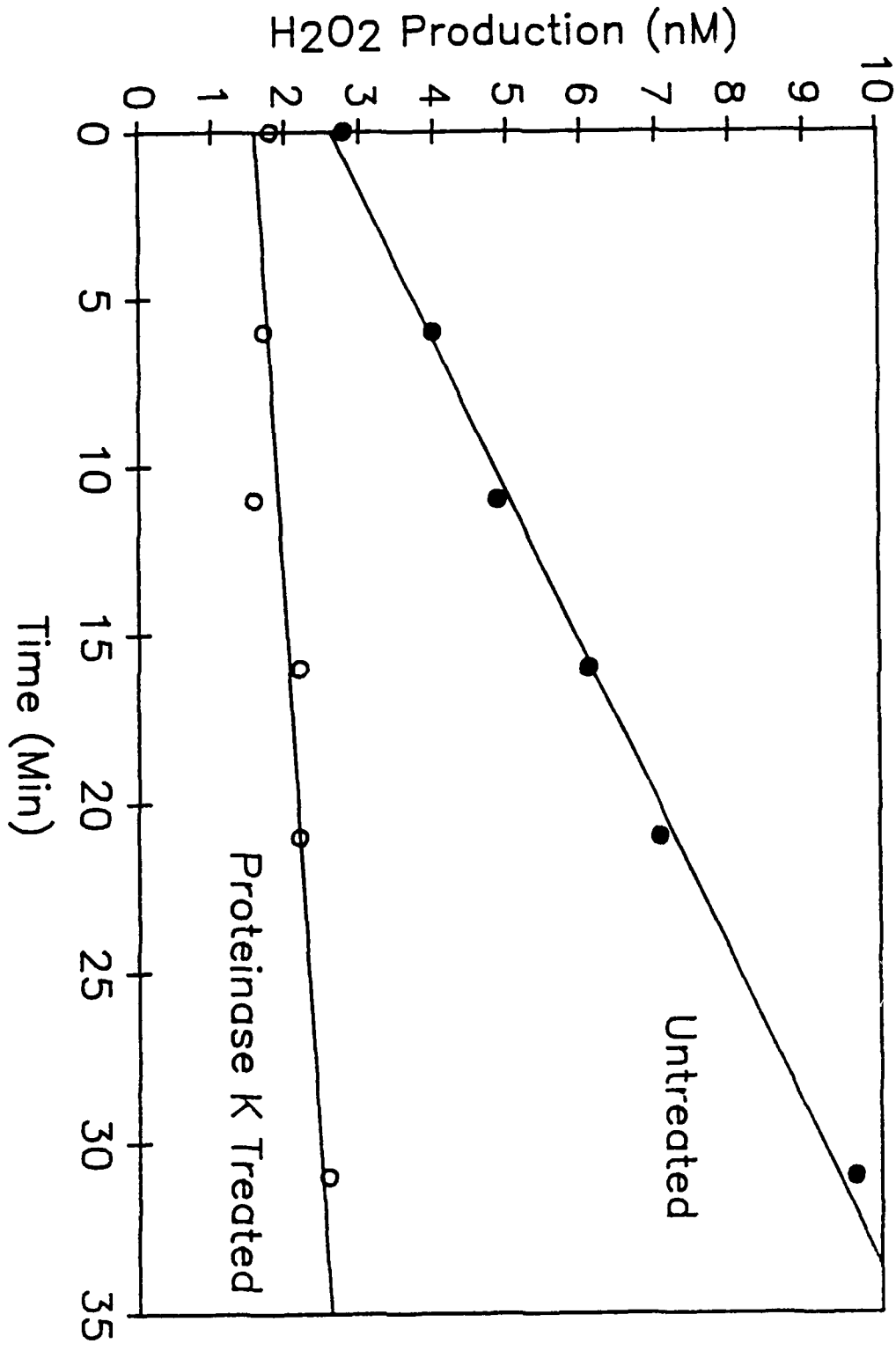
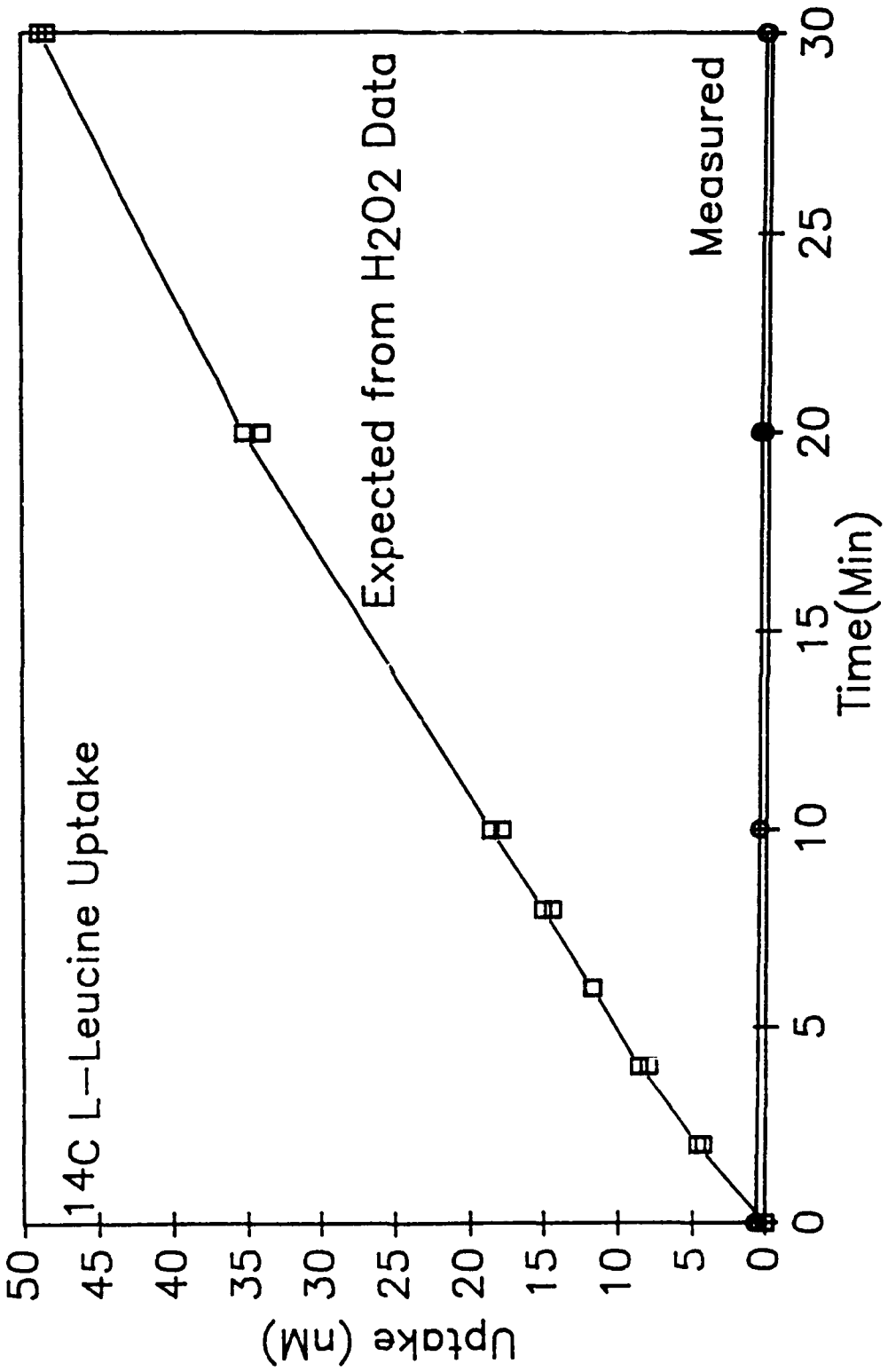

H2O2 Production (nM)
0
1
2
3
4
5
6
7
8
9
10
Time (Min)
0
5
10
15
20
25
30
35
Untreated
Proteinase K Treated

Figure 8: Uptake of ^{14}C-L-leucine by CoccoII. ○ duplicate measurements; ——— line through average of duplicates; □ uptake expected if all measured H_2O_2 produced represented α-keto acid retained inside cells.


14C L-Leucine Uptake
Expected from H2O2 Data
Measured
Uptake (nM)
Time(Min)
0
5
10
15
20
25
30
35
40
45
50

Enzyme Kinetics and Regulation--When CoccoII is growing in log phase with NH_4^+ as the nitrogen source (f/10 medium, see Materials and Methods), no L-amino acid oxidase activity is detected (Figure 2). The amino acid oxidase activity for cells growing on NO_3^- in log phase batch cultures is saturable with a Vmax of 1.6×10^{-14} mol cell^{-1} hr^{-1} (Figure 9). The half saturation constant Km is about 0.25 μM, when the data are analyzed by an Eadie-Hofstee (single reciprocal) plot. When cells reach nitrogen limitation in batch culture, the activity increases about 3.5 fold to a Vmax of 5.6×10^{-14} mol cell^{-1} hr^{-1} (Figure 9).

The enzyme saturation data under nitrogen-limited conditions can not be fit by a Michaelis-Menten type model, and instead the diffusion limited model of Pasciak and Gavis (1974) was used. This model explicitly incorporates the mathematics of diffusion to cell surfaces which becomes important when Vmax is relatively high and Km is relatively low. A diffusion limited model curve along with a predicted Michaelis-Menten curve, both assuming a Km of 0.25μM (as for the NO_3^- log phase data) are shown in Figure 9. For the diffusion limited model a 0.25 μM Km provides a better fit than 0.2 or 0.3 μM (not shown). The Vmax of 5.76×10^{-14} mol cell^{-1} hr^{-1} used in the models was measured at 5 μM L-aminobutyric acid. A cell diameter of 10 μm and an amino acid diffusion constant of 1.5×10^{-5} cm^2 sec^{-1} was assumed. The diffusion limited model used here treats these organisms as nonmotile, partly for simplicity and because on microscopic examination they were not actively swimming under the nitrogen-limited conditions assayed.

As mentioned above, Cocco II growing in log phase on NH_4^+ shows no amino acid oxidase activity. Ammonia could be repressing synthesis of active enzyme (transcriptional or translational regulation) or inhibiting its activity (allosteric regulation as typically seen for nitrate reductase). To begin to examine the mechanism of this effect, a nitrogen limited culture of Cocco II was spiked with 71 μM NH_4^+ and the enzyme activity was followed in time. One ml aliquots were added to 2ml UV oxidized seawater and the final cell concentration and H_2O_2 production (saturated) rate were measured. These data were used to calculate the production rate per cell. The results from this experiment are shown in Figure 10. The assayed enzyme activity in a constant volume remained constant, but declined slowly over the next 30 hours when normalized to cell number because the cell numbers began to increase as the cells were released from nitrogen limitation . In the control there was no change in cell number, saturated production rate, or rate per cell, the last shown in Figure 10. These results support the model of a stable cell-surface enzyme which is not synthesized in the presence of excess ammonia. The enzyme is gradually diluted from the cell surface as the cells grow.

Enzyme Distribution--All five Pleurochrysis isolates tested exhibited L-amino acid oxidase activity (Table 1). In two, Pleurochrysis carterae (CoccoII) and Pleurochrysis scherfelli (944-1), the enzyme is expressed when the cells are in logarithmic phase growth with high nitrate concentrations (f/10) as the nitrogen source. The activity increases about 3.5 fold when the cells become nitrogen limited in batch cultures. Three isolates P. carterae (156HYM), P.

Figure 9: Kinetics of L-α-aminobutyrate oxidation by preconditioned CoccoII. ▼o▼ NO_3^- log phase; △ N-limited. • • • Michaelis-Menten curve fitted to NO_3^- log phase data; • • • Michaelis-Menten curve for N-limited data, assuming Km =0.25 μM and a measured Vmax; —— Paschiak and Gavis model curve for N-limited data assuming Km=0.25 μM and a measured Vmax.

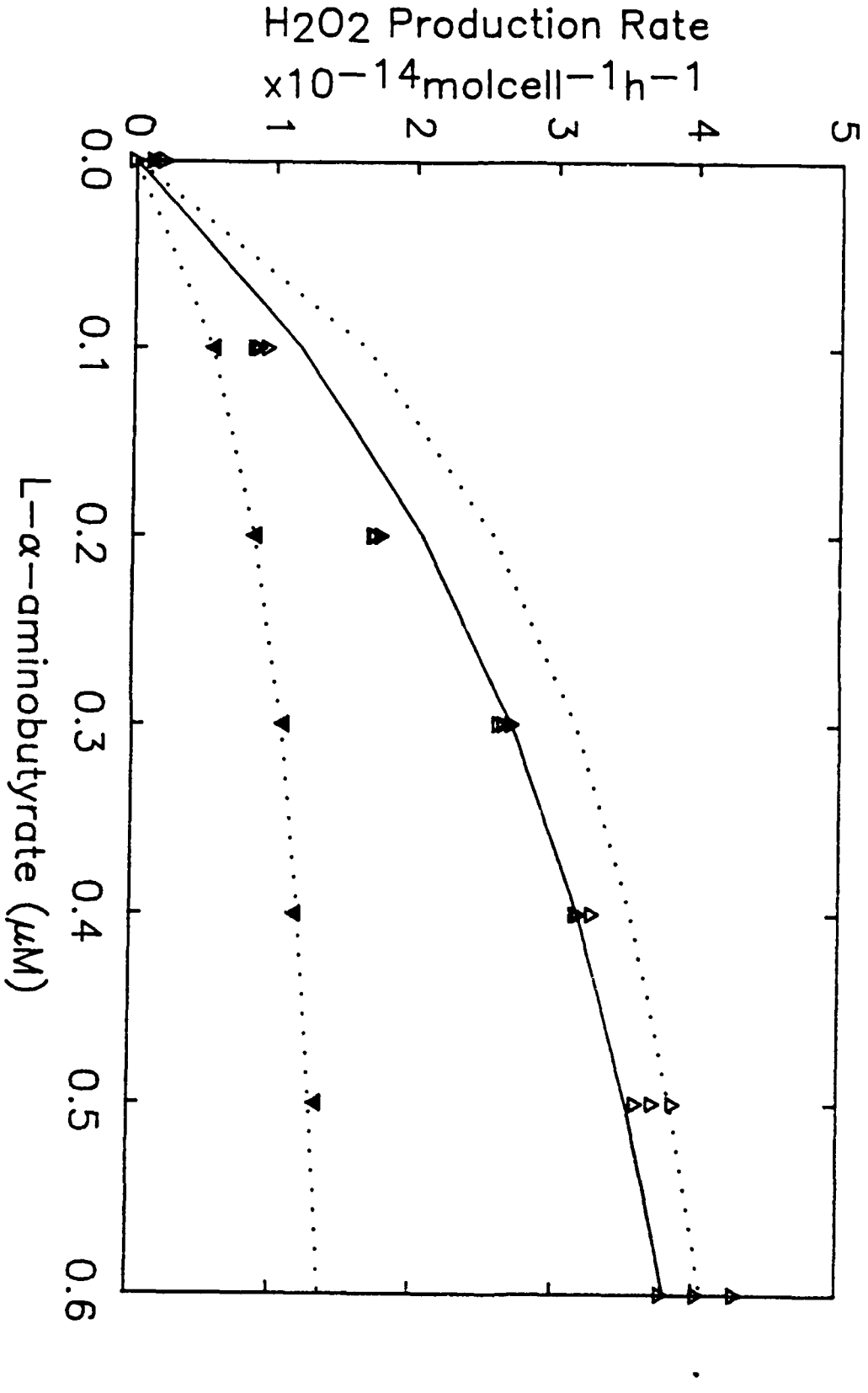

H2O2 Production Rate
x10−14molcell−1h−1
0
1
2
3
4
5
0.0
0.1
0.2
0.3
0.4
0.5
0.6
L−α−aminobutyrate (μM)

Figure 10: Kinetics of the repression of oxidase activity by addition of 71μM NH_4^+ to nitrogen limited *CoccoII* culture. □ assayed cell concentration (cells ml^{-1}); ○ assayed activity (nM min^{-1}); △ assayed activity (x10^{-14} mol $cell^{-1}$ h^{-1}); ▲ control activity (x10^{-14} mol $cell^{-1}$ h^{-1}).

cellsml^{-1}x10^{3}
H2O2 (nMmin^{-1})
H2O2(x10^{-14}molcell^{-1}hr^{-1})
Time (Hours)
0
4
8
12
16
20
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34

carterae (CoccoII-N), and Pleurochrysis sp. (UW398) show little or no L-amino acid oxidase activity in log phase growth on high (f/10) nitrate concentrations. Under nitrogen limitation in batch culture, L-amino acid oxidase activity appears. The Pleurochrysis sp. isolate (UW398) was not axenic. Because bacterial contamination of CoccoII cultures did not affect the measured activities (data not shown), we can assume that the results for UW398 are accurate. The amino acid oxidase activity was assayed in all cases with L-aminobutyric acid; we have not examined whether or not the other Pleurochrysis isolates have the same half saturation constant or amino acid specificity as CoccoII.

We have isolated and tested another coccolithophorid, Emiliania huxleyi from the Sargasso Sea (now 12-1, CCMP) for L-amino acid oxidase activity. No activity was found under log phase growth with nitrate or under nitrogen limitation. No activity was found in field samples from a 1988 E. huxleyi summer bloom in the Gulf of Maine (data not shown). Thus the cell-surface L-amino acid oxidase is not apparently a feature of all coccolithophorids.

Discussion

The possibility that some phytoplankton may have a type of amino acid oxidase was first proposed by Algeus (1948). He found that Scenedesmus obliquus released ammonia when grown on glycine as a nitrogen source. The mechanism of this effect or the enzyme location was not demonstrated, however. An internal amino acid oxidase has been

Organism	Nitrogen Status	Rate ($x10^{-14}$ mol $cell^{-1}$ hr^{-1})
CoccoII	Nitrate	1.6
CoccoII	Nlim	5.6
CoccoII-N	Nitrate	<0.03
CoccoII-N	Nlim	1.9
944-1	Nitrate	0.57
944-1	Nlim	2.0
156HYM	Nitrate	0.04
156HYM	Nlim	5.1
UW398	Nitrate	<0.03
UW398	Nlim	3.3
12-1	Nitrate	0
12-1	Nlim	0

TABLE 1: L-amino acid oxidase regulation. Saturated oxidase rate constants (Vmax) are shown for Pleurochrysis isolates and one Emiliania huxleyi isolate (12-1). Nlim refers to cells in f/10 medium without added nitrogen assayed typically one day after growth stops. Nitrate refers to cells growing in log phase on nitrate. The coefficient of variation of replicate cultures was about 23%. The detection limit of the method is about 0.03×10^{-14} mol $cell^{-1}$ h^{-1} with the cell concentrations typically used in the assays.

reported in some cyanobacteria (Meyer and Pistorius, 1987). Amino acid oxidases have been studied in a range of other organisms, such as Neurospora, venomous snakes, etc. Neurospora can produce an extracellular but not cell-surface bound L-amino acid oxidase (Sikora and Marzluf, 1982). We have shown here that Pleurochrysis (previously Hymenomonas) species have a cell-surface L-amino acid oxidase.

Pleurochrysis carterae (CoccoII) can grow on L-aminobutyric acid (Figure 1) as its sole nitrogen source, and has been shown in the literature to be able to grow well on other amino acids (Pintner and Provasoli, 1963; Wheeler et al, 1974; Turner, 1979) although these reports are contradictory for some amino acids. These contradictions may be due to different clones or experimental conditions used. The mechanism of utilization for many amino acids is their initial oxidation to produce H_2O_2 (Figure 2), NH_4^+, and an α-keto acid (Figure 5). The NH_4^+ is transported into the cell for growth, either by the same enzyme complex or by a separate NH_4^+ transport protein. The α-keto acid and any breakdown products remain outside the cell as shown by using L-alanine (Figure 5) or $^{14}C(U)$ L-leucine (Figure 8) as oxidase substrates. The L-amino acid oxidase is accessible to inhibitors that act at the cell surface such as diazobenzenesulfonate, 3-azido-2,7-napthalenedisulfonate and Proteinase K (Figure 7 and Palenik et al, 1987)

Phytoplankton amino acid transport proteins have reported half-saturation constants of 0.4-150 μM (often toward the higher end--recently reviewed in Flynn and Butler, 1986), sometimes leading to the speculation that amino acid uptake by phytoplankton is not an

important process in the environment (Paul, 1983). Reported bacterial and field measurements of half saturation constants for amino acid utilization are about 0.01 to 1.0 μM (Billen, 1984). With a half-saturation constant of about 0.25 μM (for L-aminobutyric acid, Figure 9), the oxidase has a relatively high affinity for amino acids, in the range that allows it to compete effectively with bacterial uptake. While total and individual amino acid concentrations are highly variable, amino acids that would be commonly utilized by the enzyme (L-lysine, L-aspartate, L-glutamate, L-alanine) are typically about 30-50% of the total free amino acid pool or at about 0.1 μM combined concentration (Garrasi et al, 1979). It thus appears that Pleurochrysis species are well equipped to utilize amino acids at environmentally relevant concentrations.

An interesting aspect of the enzyme system is that under nitrogen limitation, the combination of a high Vmax and low Km depletes cell-surface concentrations and appears to result in the cell utilizing amino acids at rates limited by diffusion to the cell surface. The model of Pasciak and Gavis was formulated to describe this kind of situation. In their terminology the P value (=14.4 pi R D K_m/V_{max} where R is the cell radius in cm and D is the diffusivity of the nutrient) is 1.47 for the amino acid oxidase of CoccoII under nitrogen limitation, less than 2 and thus indicating diffusion limitation (Pasciak and Gavis, 1974). The ability of an organism to perform a cell surface reaction at diffusion limited rates represents the tuning of an enzyme system to reach the limits of the physically possible.

Why would an organism utilize a cell-surface amino acid oxidase rather than take up the amino acid? An advantage of the latter is the ability to incorporate the amino acid directly into protein, thus saving the energy required to synthesize a carbon skeleton. A disadvantage is that it might require several proteins to transport the different classes of amino acids (neutral, acidic, basic) in order to process the same range of amino acids as the oxidase. There are also unknown factors such as the relative affinities and stabilities of the two kinds of systems that make a "cost-benefit" analysis difficult at this time. The cell surface oxidase of CoccoII seems to be quite stable (Figure 10) which may be one of its advantages. One particularly important unresolved issue in such an analysis is whether the NH_4^+ produced by the oxidase is ever free to diffuse away.

The distribution of cell-surface oxidases in the environment is unknown. They would be missed by current methods for measuring amino acid utilization, because these typically involve spiking samples with ^{14}C or ^{3}H labelled amino acids and looking for incorporation of the label with autoradiography or size-fractionation (Hollibaugh, 1976; Hoppe, 1976). As shown here, these methods would not measure oxidase activity as the radiolabel is not transported into the cell (Figure 8). A similar difficulty with the field use of radiolabelled amino acids has been noted before by Stephens and North (1971) who showed ^{14}C excretion by Platymonas given radiolabelled amino acids. Since the conclusion that bacteria are the major consumers of amino acids in the environment is based primarily on radiolabel studies and on comparison of half saturation constants (see above), this conclusion must now be

considered suspect. In at least some environments, particularly oligotrophic regimes, phytoplankton will probably control amino acid cycling.

Assaying for oxidase activity could utilize the measurement of amino acid dependent H_2O_2 or α-ketoacid production, or possibly immunochemical methods. The presence of what appears to be significant biological (rather than photochemical) H_2O_2 production in some environments (Palenik and Morel, 1988) could be explained by L-amino acid oxidases. This H_2O_2 may be involved in subsequent oxidation of high molecular weight dissolved organic carbon (possibly absorbed to the high surface area of the coccoliths) and the release of more free amino acids.

The cell-surface L-amino acid oxidase described here is a clear example of what is likely to be a class of enzymes that process organic nitrogen sources at the cell-surface and liberate ammonia. Paul and Cooksey (1979), for example, have reported a cell-surface asparaginase in Chlamydomonas, although the enzyme did not seem to have particularly high affinity (110 μM) for its substrate. We have preliminary evidence that CoccoII may have a cell-surface amine oxidase in addition to the amino acid oxidase reported here. Cell-surface phosphatases that liberate phosphorus from organic phosphates have long been known to occur in phytoplankton (Kuenzler and Perras, 1965). Cell-surface deaminases are the nitrogen analog that some phytoplankton use to actively recycle organic nitrogen rather than relying on other organisms to provide inorganic forms.

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Chapter 4.

Comparison of Cell-Surface L-Amino Acid Oxidases From Several Marine Phytoplankton

The presence of a cell-surface L-amino acid oxidase in Pleurochrysis species was reported in Chapter 3. If this were the only genus of phytoplankton with the enzyme, its overall ecological importance would be minimal. It is clearly important to examine the distribution of this enzyme in other phytoplankton, and to do this a number of species were assayed for enzyme activity. These organisms were either available in our culture collection or were selected from the collection of the Center for Culture of Marine Phytoplankton based on published reports of their utilization of amino acids. As a result of this work, the enzyme was found in two other phytoplankton genera, Amphidinium and Prymnesium, suggesting its likely ecological significance. Chapter 4 discusses these two cases and compares them with the enzyme from Pleurochrysis carterae. The enzymes are surprisingly similar across such evolutionary distances.

Materials and Methods

Materials--Pleurochrysis carterae (clone COCCOII), Prymnesium parvum (PRYM), Prymnesium calathiferum (CHANG1), Amphidinium carterae (AMPHI), Amphidinium operculatum (AMPHID), Symbiodinium microadriaticum (HIPP), Pavlova gyrans (MPPAV), and Micromonas pusilla

(DW8) were obtained from the Center for Culture of Marine Phytoplankton (CCMP), Bigelow Laboratories, West Boothbay Harbor, Maine, 04575. Isochrysis galbana (ISO), Emiliania Huxleyi (12-1) and Thalassiosira weissflogii (ACTIN) were already in culture, but can be obtained from CCMP. A Tetraselmis species (4B-9) was isolated from the Sargasso Sea at 32N 62W. Aiptasia pallida, a marine and brackish water anenome, was obtained from Carolina Biological Supply House and maintained according to accompanying instructions.

Amino acids, bovine serum albumin (BSA), pyruvate, and hydroxyphenylpropionic acid were obtained from Sigma Chemical or Aldrich. The highest purity stocks available were obtained, typically 98-99+% pure. Horseradish peroxidase Type VI was obtained from Sigma. 2,4-Dinitrophenylhydrazine was obtained from Sigma and recrystallized before use (Kieber and Mopper, 1986).

Methods--Cultures were maintained in f/10 without silica (Guillard and Ryther, 1962) enriched coastal (Woods Hole, MA) or occasionally Sargasso seawater. The seawater was 0.45μm filtered and sterilized by microwaving (Keller et al., 1988), autoclaving, or pasteurization (Brand et al., 1983) with little obvious effect on enzyme activities. Cultures were maintained under constant fluorescent (cool white) light at about 18-20 ^{o}C. Nitrogen limited cultures were obtained by leaving out nitrogen (nitrate) additions or making small (f/200) additions and using the cells after growth stops. Growth of cultures was followed by chlorophyll fluorescence on a Turner fluorometer and cell counts were measured using a Coulter cell counter. Bacterial contamination was typically monitored with Difco marine broth 2216, and occasionally by plating cultures on f/10

enriched agar or by microscopic examination. The cultures which are the major focus of this study (PRYM, COCCOII, and AMPHID) were maintained and studied while free of bacterial contamination. Of the other cultures listed above, MPPAV, ACTIN, and AMPHI were also axenic, while the others were bacterized.

α-Keto acid production by cultures was assayed using the method of Kieber and Mopper (1986) as described in Palenik and Morel (1989a). Simultaneously, H_2O_2 production was measured in a "nontrapping" mode by taking 2ml subsamples at intervals, diluting to 20ml with low H_2O_2 Sargasso seawater, adding 0.5ml hydroxyphenylpropionic acid (2mM) and 10μl freshly prepared horseradish peroxidase (about 4mg ml^{-1}) and measuring the fluorescence (proportional to H_2O_2) of the sample. H_2O_2 was also measured in a "trapping " mode by removing a 20ml sample, adding the H_2O_2 reagents, and following fluorescence (H_2O_2) with time. In both cases H_2O_2 was measured on a Turner fluorometer (Palenik and Morel, 1988). For other assays of H_2O_2 production, the measurements were made with a Perkin-Elmer LS-5 fluorometer as in Palenik and Morel (1989a)

The symbiotic dinoflagellates (Symbiodinium sp.) in Aiptasia pallida were isolated based on the procedures of McLaughlin and Zahl (1959). The tenacles of four hosts were briefly homogenized in a tissue grinder and filtered through cheesecloth. The material was diluted with 0.2μm filtered Sargasso seawater and centrifuged to collect the cells. The cells were resuspended and filtered through 25μm Nitex. The cells were centrifuged and the pellet again resuspended in seawater. The cells were then assayed for H_2O_2 production (72,000 cells ml^{-1}) in the presence and absence of L-

alanine, and, as a blank, in the absence of HRP. A slow rate of nonspecific fluoresence increase was observed.

Results

The following organisms did not show L-amino acid oxidase activity under nitrogen limited or other conditions tested: Thalassiosira weissflogii (ACTIN), Micromonas pusilla (DW8), Isochrysis galbana (ISO), Pavlova gyrans (MPPAV), Emiliania huxleyi (12-1), or a Tetraselmis sp. (4B-9). However, Amphidinium carterae (AMPHI), A. operculatum (or klebsii, AMPHID), Prymnesium parvum (PRYM), and P. calathiferum (CHANG1) showed oxidase activity and were studied in more detail.

Because AMPHID was originally isolated as an endosymbiont of the jellyfish, Cassiopea, the presence of L-amino acid oxidase was investigated in members of the other major group of endosymbiotic dinoflagellates, Symbiodinium sp. The enzyme was not found in Symbiodinium microadriaticum (clone HIPP), an organism in culture. It was not found in dinoflagellates (Symbiodinium sp.) freshly isolated from an anenome, Aiptasia pallida (See Methods). Although genetic differences exist between Symbiodinium species, these results suggest that L-amino acid oxidases are not present in this genus.

When certain L-amino acids are added to preconditioned AMPHID, PRYM, or COCCOII cultures, H_2O_2, NH_4^+, and an α-keto acid are produced due to the presence of a cell-surface L-amino acid oxidase (Palenik et al, 1989; Palenik and Morel, 1989a). For example, when 1μM L-alanine is added to a nitrogen-limited culture of Prymnesium parvum (PRYM),

H_2O_2 and pyruvate are produced in a 1:1 molar ratio when the H_2O_2 produced is "trapped" (Figure 1--See Materials and Methods) When H_2O_2 is measured without trapping, less H_2O_2 is measured due to the breakdown of H_2O_2 in the culture. A similar result is seen for a 0.5μM addition of L-alanine to a nitrogen limited AMPHID culture (Figure 1). In this case, hydrogen peroxide trapping was begun at 20 minutes, so some H_2O_2 was lost. H_2O_2 decomposition is apparently more rapid than for PRYM cultures. Pyruvate and H_2O_2 production from L-alanine has been reported for COCCOII (Palenik and Morel, 1989a).

The third product of the amino acid oxidase is NH_4^+, which is taken up and used for growth. Thus we would expect organisms to grow on amino acids as a nitrogen source which are substrates of the oxidase. When 50μM sterile filtered L-aspartic acid and L-alanine are added to nitrogen limited cultures of PRYM (Figure 2) and AMPHID (Figure 3) the organisms begin to grow. Growth on these two amino acids is slower than on ammonia, particularly for AMPHID. L-serine supports some slow growth in both organisms, but is a better source of nitrogen for PRYM. As discussed later these results parallel the enyzyme kinetics for these substrates.

Neither PRYM nor AMPHID could grow on a protein, bovine serum albumin (BSA), at 10 mg l^{-1} as a nitrogen source (Figure 2 and 3). Pleurochrysis carterae, various clones of which utilize amino acids for growth (Wheeler et al., 1974; Turner, 1979; Palenik and Morel, 1989a), also could not grow on BSA (Figure 4). Interestingly, if bacteria are added to axenic cultures of COCCOII, this organism could

Figure 1: Hydrogen peroxide and pyruvate production from L-alanine. After addition of 1μM L-alanine to nitrogen-limited PRYM culture (88,400 cells ml^{-1}): △ ,pyruvate; ○ , H_2O_2, trapped; □ , H_2O_2, untrapped. After addition of 0.5 μM L-alanine to nitrogen limited AMPHID culture (41,600 cells ml^{-1}): ▲ ,pyruvate; ● , H_2O_2, trapped after 20 minutes; ■ , H_2O_2, untrapped.

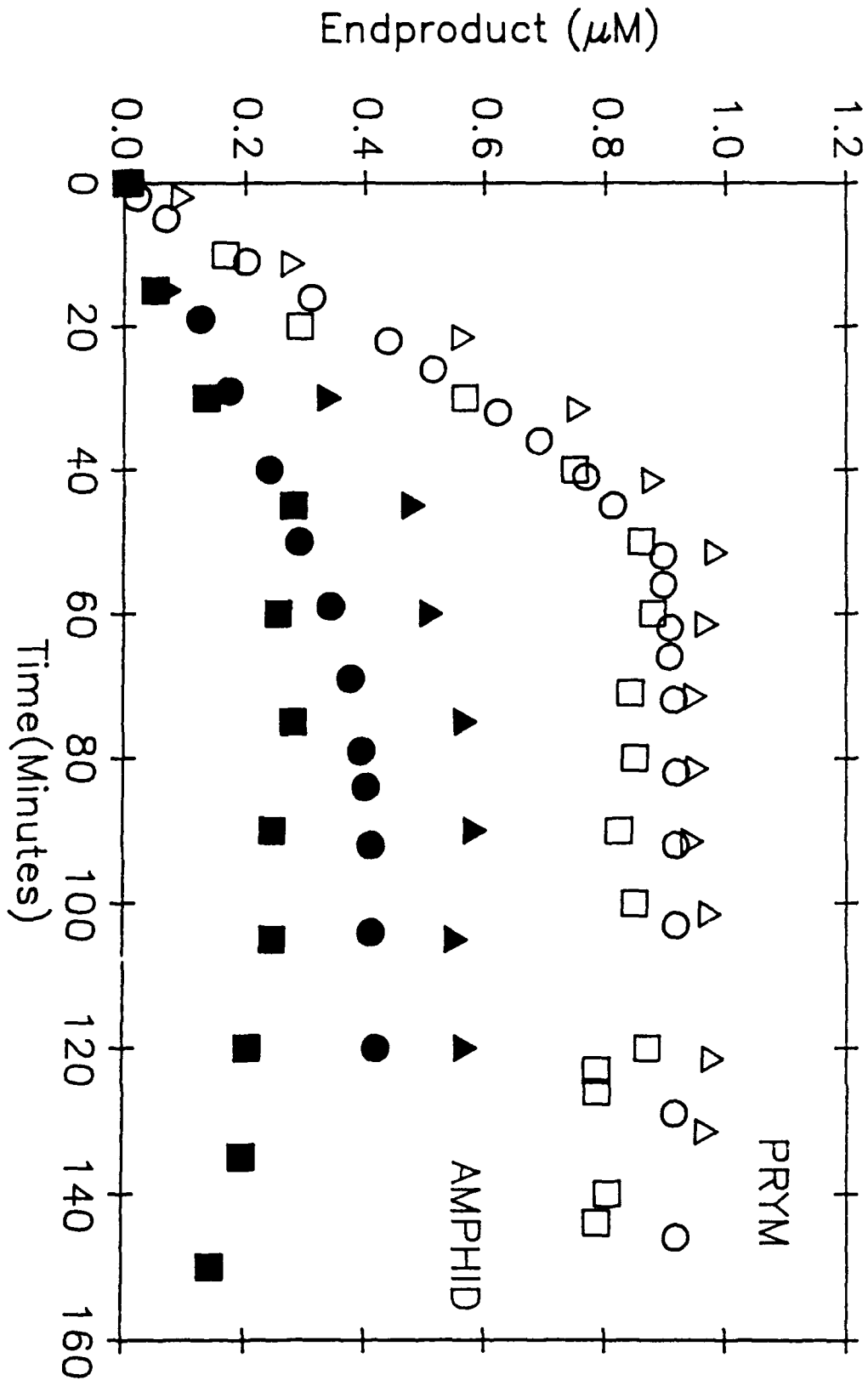

Endproduct (μM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
20
40
60
80
100
120
140
160
Time(Minutes)
PRYM
AMPHID

Figure 2: Growth of PRYM after 50μM amino acid or 0.01 mg ml^{-1} BSA additions to nitrogen-limited cultures (as the change in Chlorophyll from its initial value at the time of nitrogen addition). △ , NH4+; □ L-alanine; ○ L-aspartate; ▲ L-serine; ● No addition; ■ BSA.

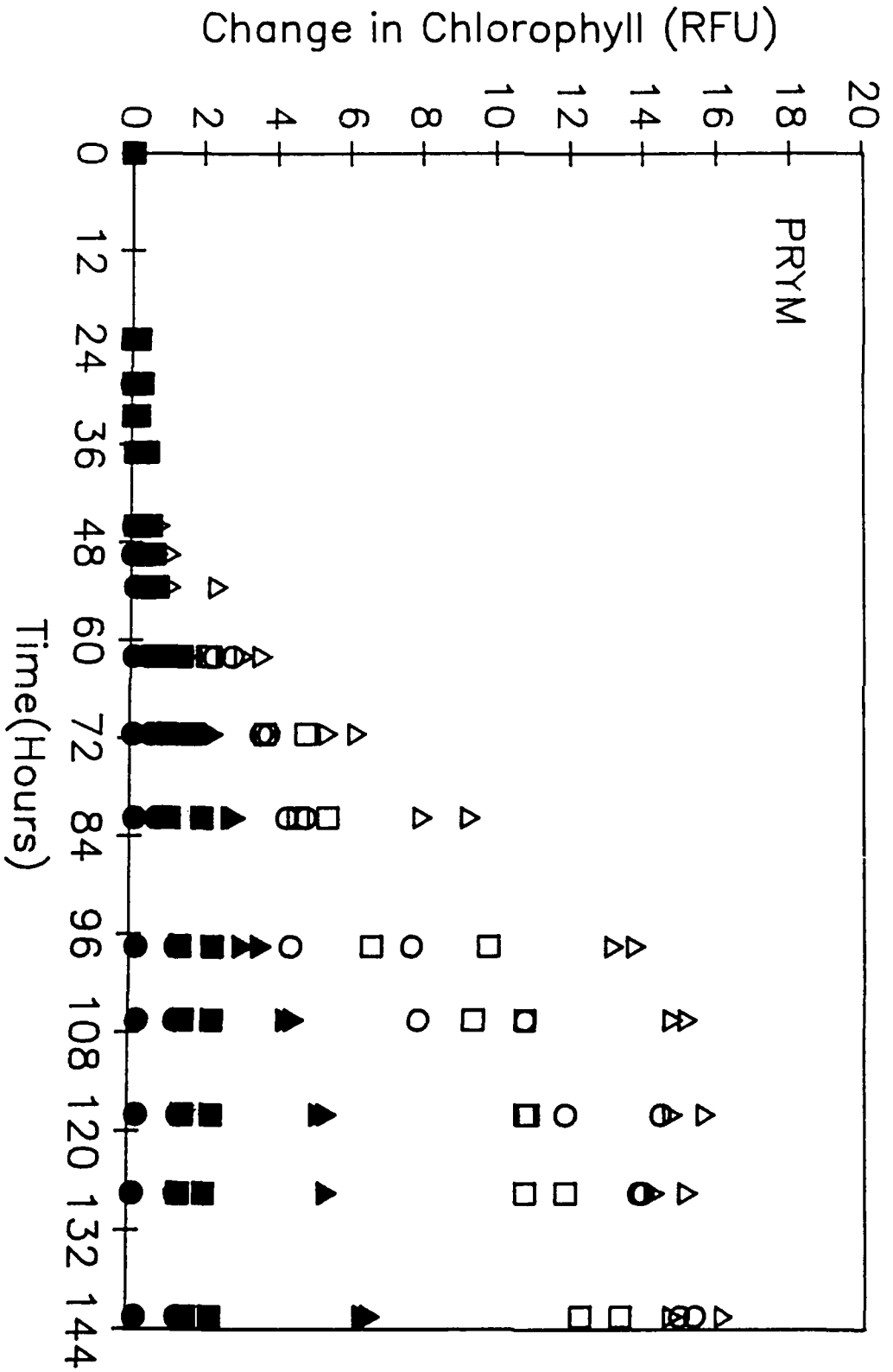

Change in Chlorophyll (RFU)
0
2
4
6
8
10
12
14
16
18
20
PRYM
Time(Hours)
0
12
24
36
48
60
72
84
96
108
120
132
144

Figure 3: Growth of AMPHID after 50μM amino acid or 0.01 mg ml^{-1} BSA additions to nitrogen limited cultures. Symbols as for Figure 2.

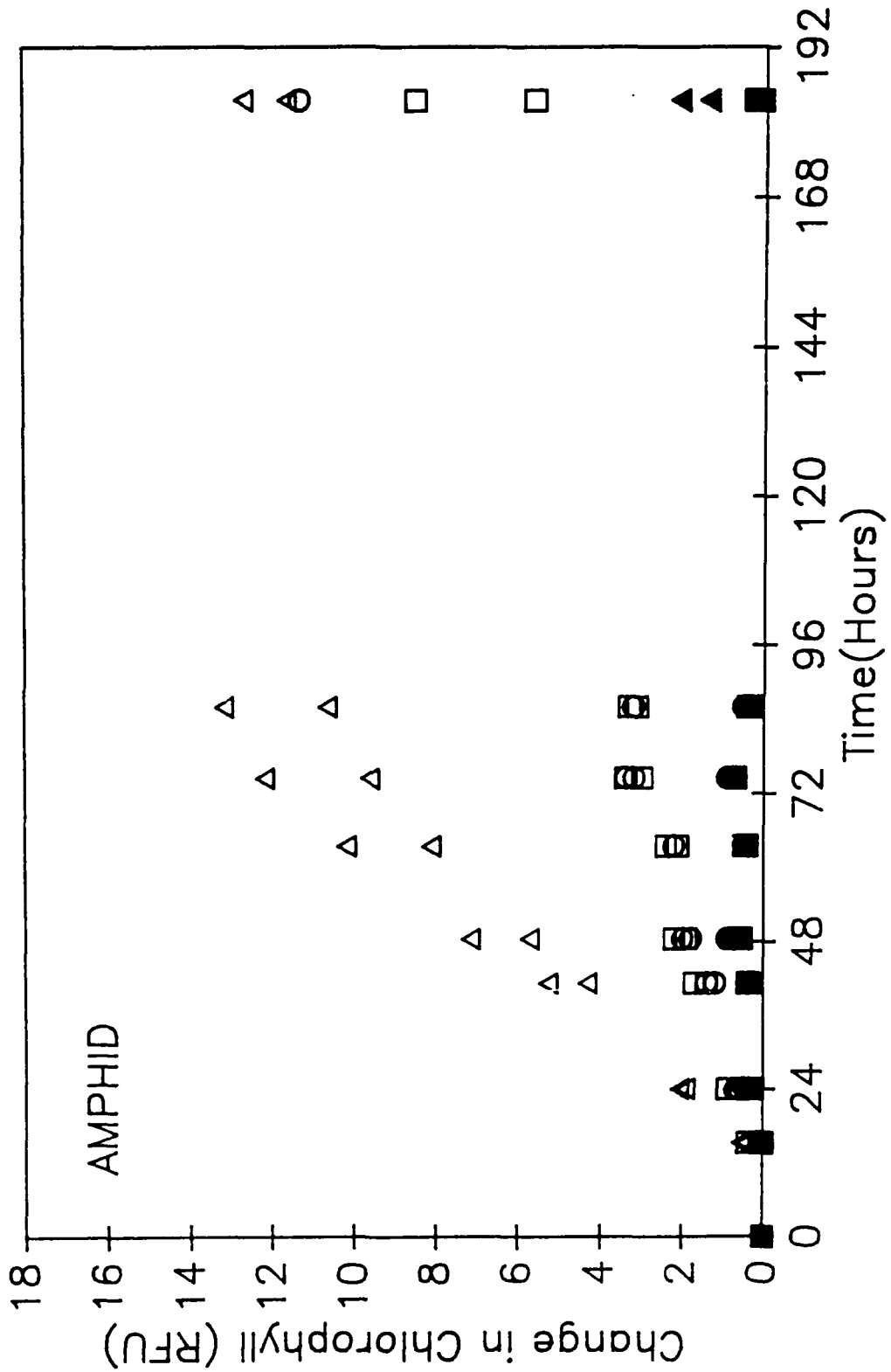
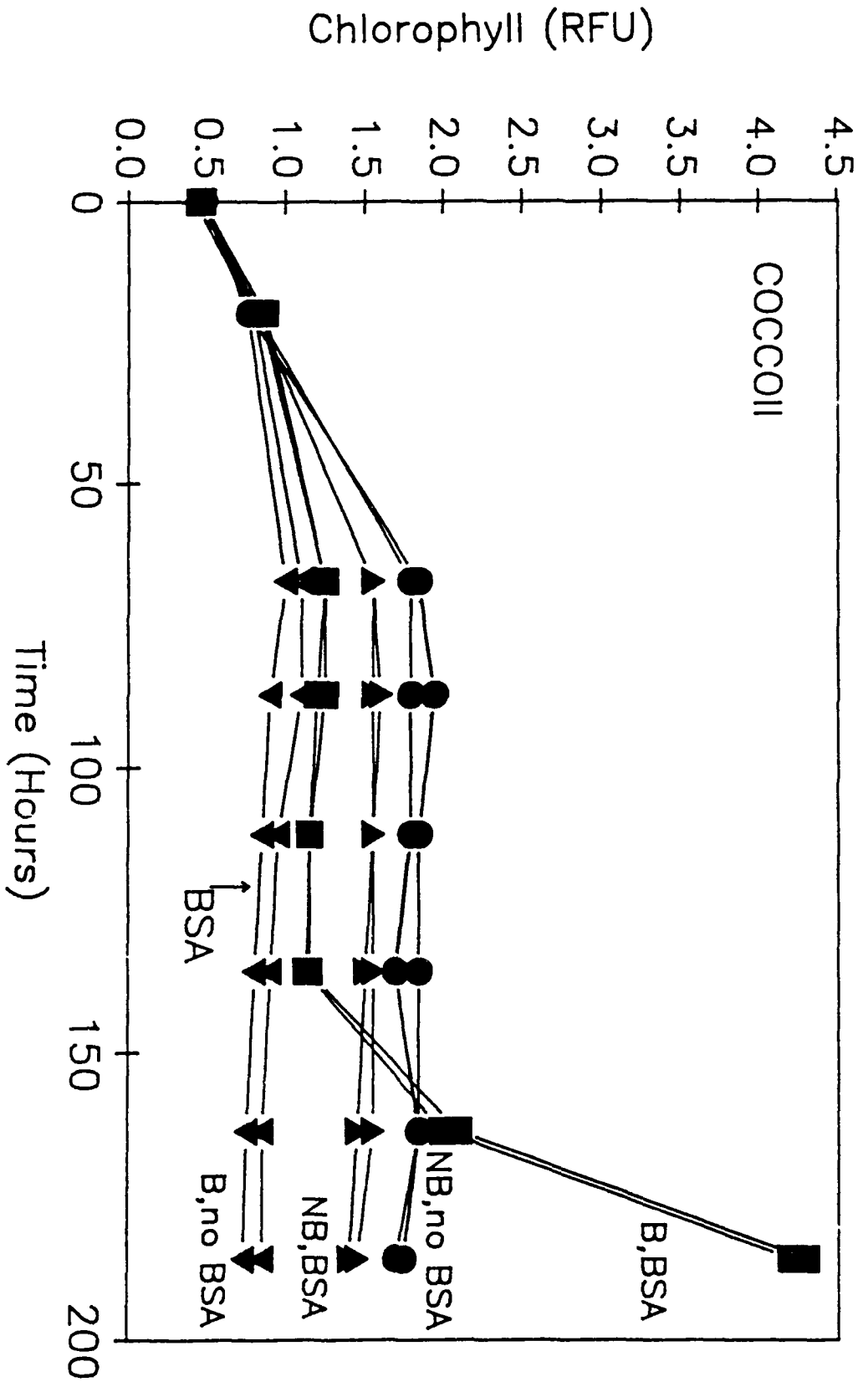

AMPHID
Change in Chlorophyll (RFU)
Time(Hours)
0
2
4
6
8
10
12
14
16
18
0
24
48
72
96
120
144
168
192

Figure 4: Growth of COCCOII with and without bacteria after addition of 0.006 mg ml^{-1} BSA to nitrogen limited cultures: ● , no bacteria, no protein; ▲ no bacteria, protein; ▼ bacteria, no protein; ■ bacteria, protein.


Chlorophyll (RFU)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0
50
100
150
200
Time (Hours)
COCCOII
BSA
B,BSA
NB,no BSA
NB,BSA
B,no BSA

grow on BSA as a nitrogen source (Figure 4). These bacteria were taken from a bacterized culture of COCCOII. They were not identified, and it is not known whether or not the growth of COCCOII is due to the release of NH_4^+ or hydrolysis of the albumin to amino acids by the bacteria.

To ensure that the growth of organisms on the various amino acids was not due to contamination or other artifacts, the incorportation of ^{15}N label from L-aspartic acid (^{15}N-98 atom%) has been measured. Incorporation occurs at rates similar to that of the oxidation of amino acids (Palenik and Morel, 1989a; Palenik et al., 1989).

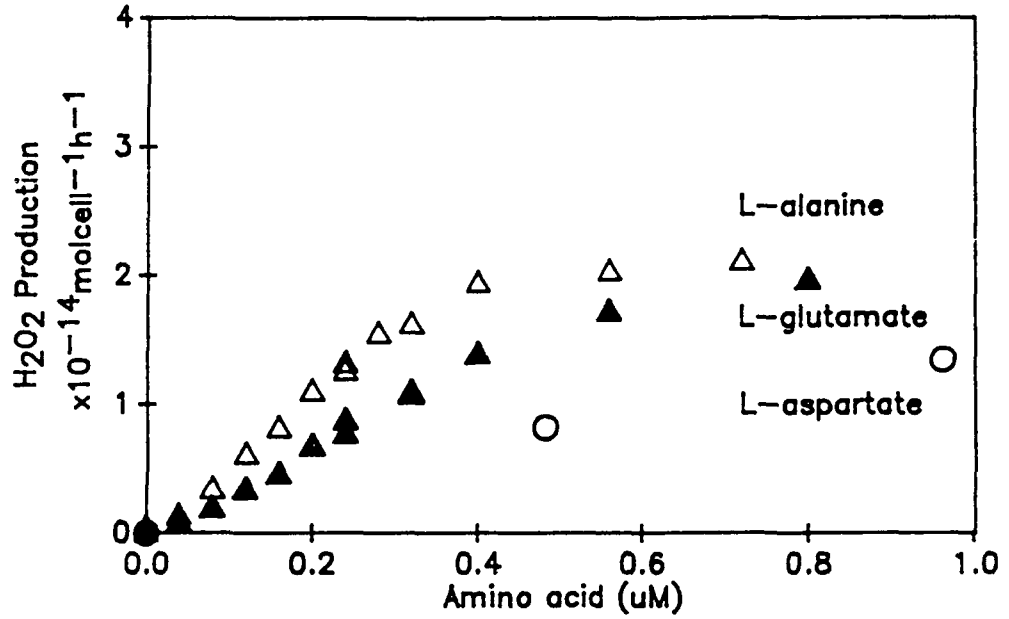
The kinetics of H_2O_2 production from various amino acids was investigated for COCCOII, PRYM, and AMPHID. The initial rates of H_2O_2 production were investigated as a function of amino acid concentration for L-alanine, L-α-aminobutyrate (PRYM only), L-aspartate, L-glutamate, and L-serine. These results are shown in Figures 5, 6, and 7. The half-saturation constants for L-alanine, L-glutamate, and L-α-aminobutyrate are approximately 0.2μM (range of 0.1-0.5) for the three organisms. The half-saturation constants for L-aspartic acid are higher for all organisms, at 1, 2, and 2μM for COCCOII, PRYM, and AMPHID, respectively. Since the data do not always conform to Michaelis-Menten kinetics due to diffusion limitation (Palenik and Morel, 1989a) and perhaps nonsteady state effects due to the enzyme itself, the use of linear transformation and regression analysis would be inappropriate. Instead a half-saturation constant was estimated as

the concentration that yielded approximately a half-saturated hydrogen peroxide production rate.

At 5μM concentration, AMPHID and PRYM, but not COCCOII, showed slow but detectable rates of H_2O_2 production from L-serine. PRYM oxidizes serine at rates that provided significant ammonia for growth (Figure 2). Serine or its oxidation product hydroxypyruvate was not irreversibly inhibiting the active site of COCCOII (as a suicide substrate), a possible mechanism for the lack of serine oxidation by this enzyme. These results indicate that although the L-amino acid oxidases are quite similar, some subtle distinctions exist in the characteristics of their active sites.

Treatment of COCCOII with the nonspecific protease, proteinase K, inactivates (80-100% inhibition) the cell-surface L-amino acid oxidase (Palenik and Morel, 1989a). Treatment of PRYM cultures under similar conditions (0.1mg ml^{-1} for 15 minutes followed by dilution and centrifugation to collect the cells) has no effect on the oxidase activity (Figure 8). Treatment of AMPHID cultures with proteinase K has some effect on oxidase activity (approximately 40% inhibition). It seems that the oxidases of PRYM and AMPHID are less "exposed"; they may have sugar residues covalently attached to exposed amino acids in the protein, for example, that protect these sites from proteases. Treatment of PRYM with DABS, another cell surface inhibitor, appeared to lyse the cells and was not used further.

Figure 5: Initial rates of H_2O_2 production by COCCOII at varying amino acid concentrations. △,L-alanine; ▲, L-glutamate; ○ ,L-aspartate; ● ,L-serine. Figures show the same data at different amino acid concentration scales.


H_2O_2 Production
$x10^{-14}$molcell$^{-1}h^{-1}$
L–alanine
L–glutamate
L–aspartate
Amino acid (uM)
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4

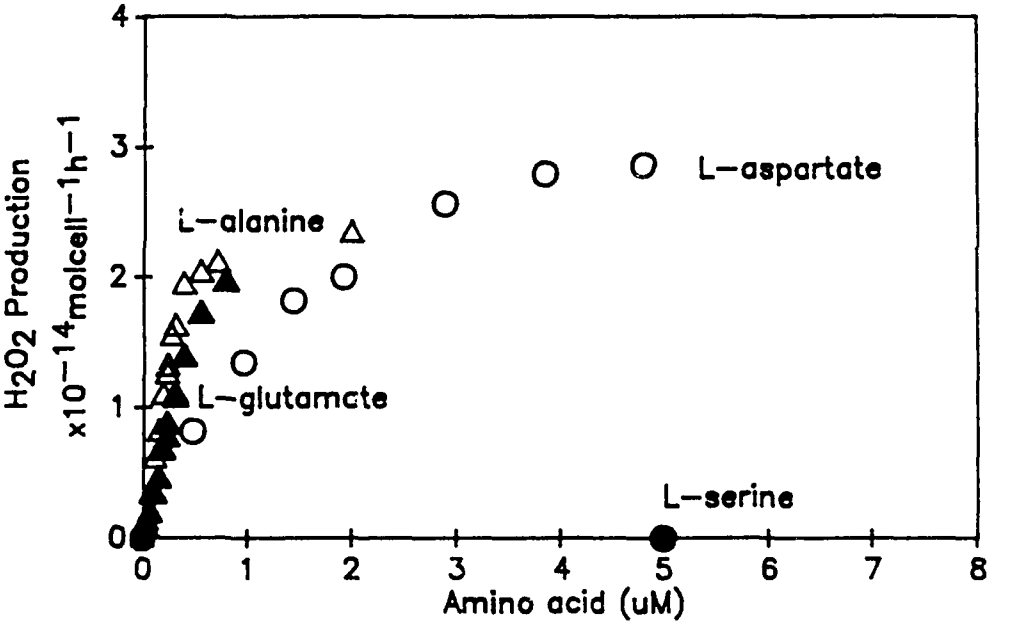
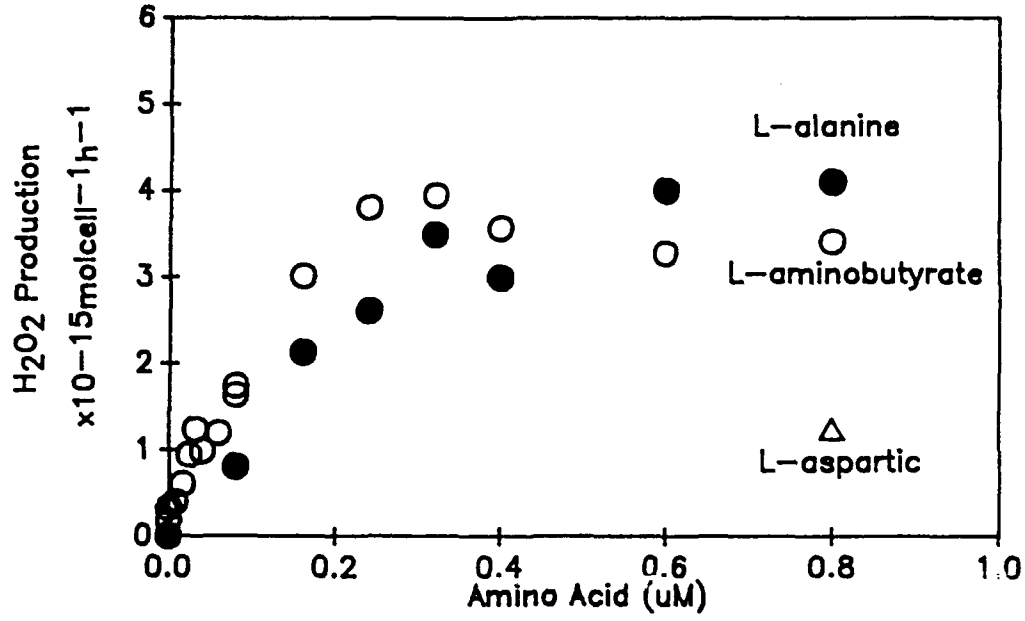

H_2O_2 Production
$x10^{-14}$molcell$^{-1}h^{-1}$
L–aspartate
L–alanine
L–glutamcte
L–serine
Amino acid (uM)
0
1
2
3
4
5
6
7
8

Figure 6: Initial rates of H_2O_2 production by PRYM at varing amino acid concentrations. ● , L-alanine; ○ , L-aminobutyrate; △ ,L-aspartate; ▲ ,L-serine. Figures show the same data at different amino acid concentration scales.


H_2O_2 Production
x10−15molcell−1h−1
Amino Acid (uM)
L−alanine
L−aminobutyrate
L−aspartic
0
1
2
3
4
5
6
0.0
0.2
0.4
0.6
0.8
1.0

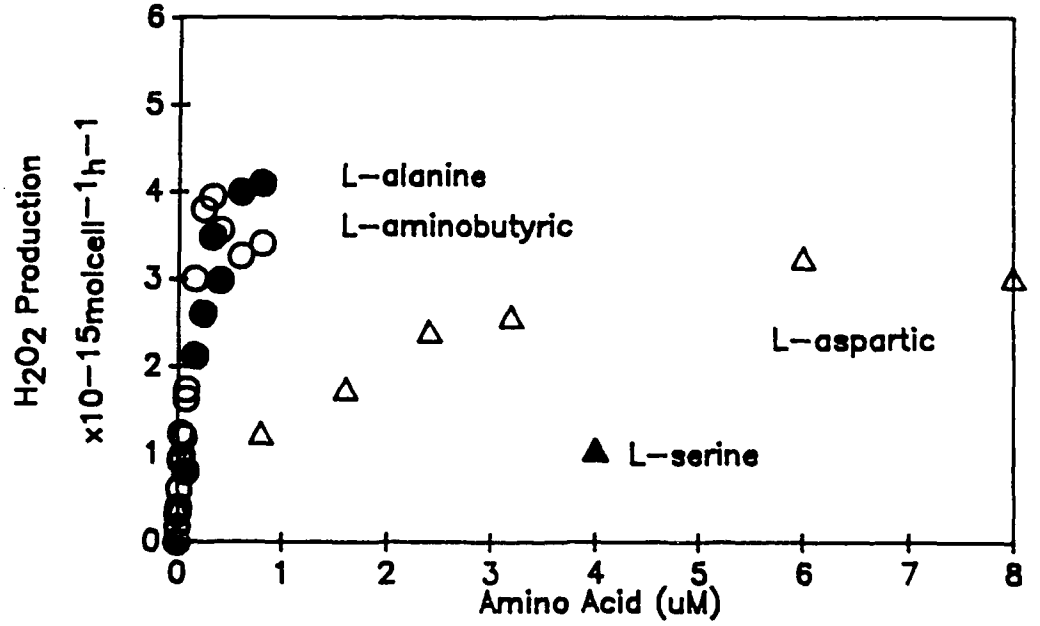
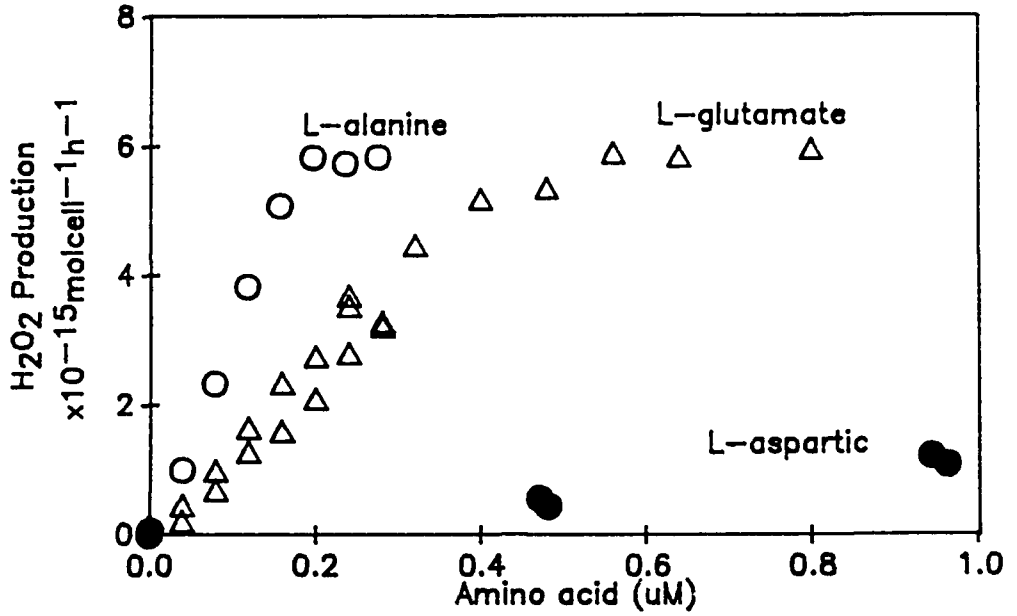

H_2O_2 Production
x10−15molcell−1h−1
Amino Acid (uM)
L−alanine
L−aminobutyric
L−aspartic
L−serine
0
1
2
3
4
5
6
7
8

Figure 7: Initial rates of H_2O_2 production by AMPHID at varing amino acid concentrations. ○ ,L-alanine; △ ,L-glutatmate; ● ,L-aspartate; ▲ ,L-serine. Figures show the same data at different amino acid concentration scales.


8
6
4
2
0
H_2O_2 Production
$x10^{-15}$molcell$^{-1}h^{-1}$
L–alanine
L–glutamate
L–aspartic
0.0
0.2
0.4
0.6
0.8
1.0
Amino acid (uM)

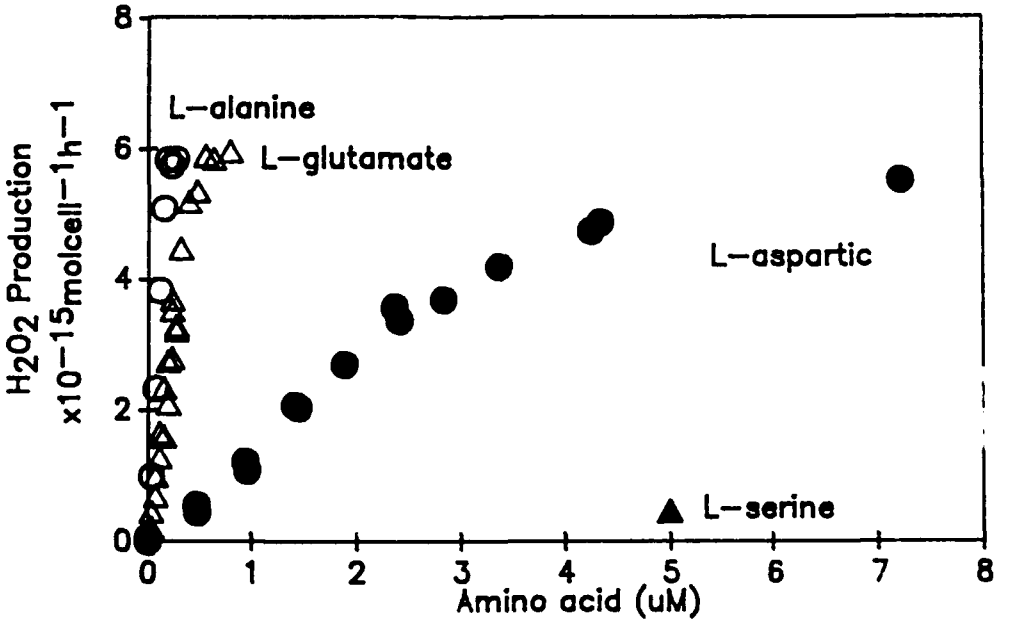
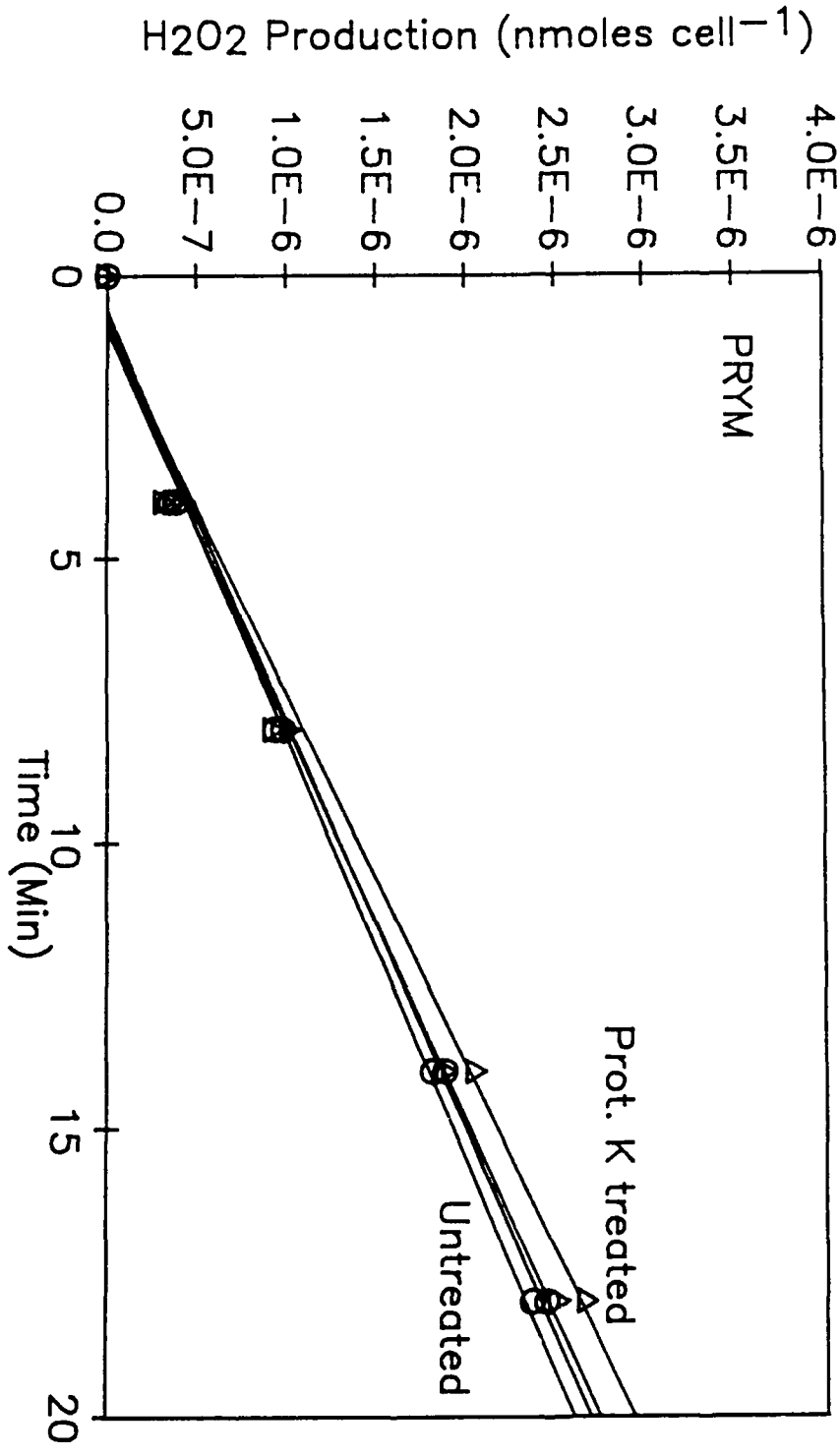

8
6
4
2
0
H_2O_2 Production
$x10^{-15}$molcell$^{-1}h^{-1}$
L–alanine
L–glutamate
L–aspartic
L–serine
0
1
2
3
4
5
6
7
8
Amino acid (uM)

Figure 8: Effect of proteinase K treatment on PRYM whole cell amino acid oxidase activity. △ , prot. k treated;○ , untreated. The H_2O_2 production is normalized to cell concentration.


H2O2 Production (nmoles cell−1)
4.0E−6
3.5E−6
3.0E−6
2.5E−6
2.0E−6
1.5E−6
1.0E−6
5.0E−7
0.0
0
5
10
15
20
Time (Min)
PRYM
Prot. K treated
Untreated

After treatment of PRYM with the detergent lithium dodecyl sulfate (LDS--1%), followed by high speed centrifugation (120,000g, 1.5 hours), the L-amino acid oxidase activity is present in the pellet fraction (Figure 9). Treatment of COCCOII in a similar manner, also resulted in activity in the pellet only. These results indicate that the enzyme in these organisms is either cell wall or membrane bound, and that it is also stable in 1% LDS, a treatment that denatures most proteins. For AMPHID, 1% LDS treatment destroyed oxidase activity, however (Figure 9).

L-amino acid oxidase activity has been found in three phytoplankton genera to date: Pleurochrysis, Prymnesium, and Amphidinium. The enzyme activity in these organisms was investigated under conditions of excess nitrate (f/10--NO_3^- exponential phase, batch culture), under nitrogen limited conditions, and in cells grown in excess ammonia (f/10--NH_4^+ exponential phase, batch culture). Table 1 summarizes the saturated rate constants for L-amino acid oxidase activities, typically assayed with 20μM L-alanine, for various organisms under the different nitrogen regimes.

The Amphidinium species tested showed oxidase activity only under nitrogen limited conditions. AMPHID has a much higher activity (per cell) than AMPHI. Prymnesium calathiferum also shows activity only under nitrogen limited conditions, and the activity is quite low. In Prymnesium parvum the enzyme is expressed under excess nitrate and nitrogen limited conditions; there is little enhancement under the latter conditions. Pleurochrysis caterae also shows enzyme activity under excess nitrate conditions and its Vmax increases several fold

under nitrogen limitation. No organism showed oxidase activity when growing in the presence of excess ammonia.

Figure 9: Effect of 1% LDS treatment and ultracentrifugation on amino acid oxidase activity. PRYM: ● , 10μl pellet; ○ , 50μl supernatant. AMPHID: ▲ ,10μl pellet; □ , 50μl supernatant.

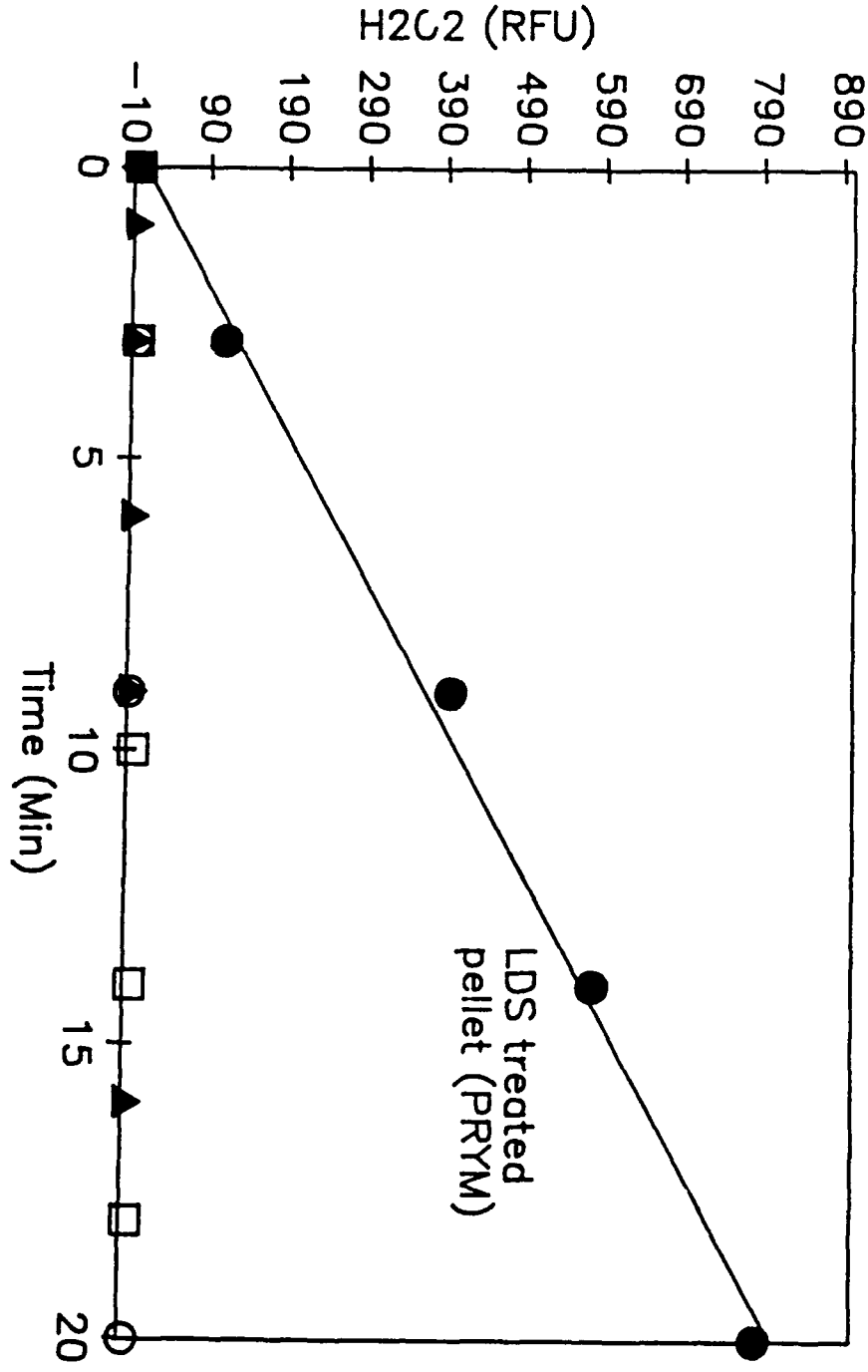

H2O2 (RFU)
−10
90
190
290
390
490
590
690
790
890
0
5
10
15
20
Time (Min)
LDS treated
pellet (PRYM)

Table 1: Saturated rate constants (Vmax) for L-amino acid oxidases from several phytoplankton ($x10^{-15}$ mol $cell^{-1}$ hr^{-1})

Organism	NH4+	NO3-	Nlim
Amphidinium carterae (AMPHI)	N.D.	N.D.	1.3
Amphidinium operculatum (AMPHID)	N.D.	N.D.	8.5
Pleurochrysis carterae (COCCOII)	N.D.	12	30-56
Prymnesium parvum (PRYM)	N.D.	6.5	7.3
Prymnesium calathiferum (CHANG1)	--	N.D.	0.1

N.D.: not detectable
--: not assayed
The rates are averages of two or more measurements of the saturated rate constant, typically assayed using L-alanine (20μM). The coefficient of variation of replica e cultures was found previously to be about 23 %.

Discussion

The presence of L-amino acid oxidase activity in three separate phytoplankton genera suggests its ecological importance. The oxidases of the three organisms studied in detail appear to be quite similar, especially given the evolutionary distances separating dinoflagellates (AMPHID, AMPHI) and prymnesiophytes (COCCOII, PRYM, and CHANG1). They have half-saturation constants of about 0.2μM (0.1-0.45μM range) for the "ideal" amino acid substrates (where the R group is $-CH_2(H)$, $-CH_2(CX_3)$, but not $CH_2(OH)$. Amino acids likely to be oxidized by this enzyme in the environment are L-alanine, L-glutamate, L-leucine, L-lysine, and L-ornithine. L-aspartic acid has a higher half-saturation constant for the oxidase, but in some environments will still be an important nitrogen source because of its relatively high concentrations.

Two common amino acids in natural waters, L-serine ($R=CH_2OH$) and glycine (R=H), are not effective substrates. On the one hand this is puzzling, because one would expect the oxidases to have evolved to utilize common amino acids. If however, phytoplankton amino acid oxidases control amino acid distributions in natural waters, then glycine and serine concentrations would be high because they are not oxidized by these enzymes. While previous research on the uptake of ^{14}C labelled amino acids suggested that bacteria control amino acid concentrations (for example Hoppe, 1976; Hollibaugh, 1976), the results reported here reopen the question of the relative importance of bacterial or phytoplankton utilization of amino acids.

When one looks closely, there are clearly differences among the enzymes. AMPHID and PRYM oxidize L-serine at slow but detectable rates, while COCCOII does not. Similarly, the oxidase of COCCOII shows a higher affinity (lower Km) for L-aspartic acid than the other two enzymes. Since L-serine and L-aspartic acid vary by substitutions at the beta-carbon, it appears that these differences reflect variations in the active site. The enzymes also show different sensitivities to treatment with proteinase K or LDS (Figures 8 and 9). These differences are likely to reflect differences in higher order structure or protecton by sugar residues (glycosylation).

Phytoplankton regulate the expression of their L-amino acid oxidase depending on the nitrogen conditions of the medium (Table 1). The nitrogen-limited conditions in our experiments were obtained at the beginning of the plateau phase in (nitrogen limited) batch cultures. These conditions are useful for testing phytoplankton cultures for the presence of the oxidase. It would be instructive to utilize continuous cultures with varying NO_3^-, NH_4^+, and amino acid ratios to ascertain under what precise conditions the amino acid oxidases are synthesized.

Given our previous results with Pleurochrysis species and isolates, and the comparison of the two Amphidinium and the two Prymnesium species reported here, it seems that within any phytoplankton genus with the enzyme, a range of regulation patterns will be found. These patterns would presumably be due to evolutionary pressures associated with the organic nitrogen (amino acid) conditions in the specific organism's environment.

None of the organisms tested could use high concentrations of bovine serum albumin as a nitrogen source except in the presence of bacteria. COCCOII can utilize N-terminal glycines on peptides, however (Palenik and Morel, 1989b). The organisms with amino acid oxidases thus do not possess cell-surface or soluble extracellular proteases that would convert protein to amino acids and thus enhance the amino acid concentrations at cell-surfaces. Such proteases have been reported in only a few phytoplankton such as Ochromonas and Nitzschia (Pringsheim, 1951, 1952), however their presence has not been thoroughly investigated.

Marine bacteria have been shown to have cell-surface or soluble proteases (Amano et al., 1982), but the contribution of protein hydrolysis by these enzymes to the pool of free amino acids is unknown. The work of Hollibaugh and Azam (1983) suggests that the amino acids and peptides from bacterial proteolysis are taken up too rapidly to equilibrate with the soluble pool. A range of interactions between phytoplankton and bacteria may occur in natural waters, perhaps some involving competition for amino acids derived from bacterial proteolysis. Others may involve cooperative interactions in which bacteria hydrolyze proteins to recycle nitrogen for the phytoplankton, and phytoplankton produce α-ketoacids and other exudates for bacteria. For this reason, the growth of bacterized but not axenic cultures of COCCOII on BSA (Figure 4) are intriguing.

The major portion of dissolved organic nitrogen is neither free nor combined amino acids, but is uncharacterized (Sharp, 1983). Recent reports suggest that this pool is even larger than previous estimates (Suzuki and Sugimura, 1985). The importance of cell-surface enzymes or

cell-surface catalyzed mechanisms for breaking down these nitrogen forms should not be overlooked. These mechanisms, if they exist, could enhance the cell-surface concentration of primary amines and ammonia beyond that of the bulk solution.

Cell-surface L-amino acid oxidases have been found in two prymnesiophytes and one dinoflagellate genus. The presence of amino acid oxidases, possibly cell-surface, in some chlorophytes is likely, if the early work of Algeus (1948a,b) is correct. His results suggested that Scenedesmus species might be deaminating glycine extracellularly. A D-amino acid oxidase (location unknown) may occur in Chlorella species (Wikstroem et al., 1982). From the results to date, diatoms appear to have amino acid transport systems (reviewed in Flynn and Butler, 1986) rather than oxidases. Prokaryotic phytoplankton (Synechococcus) could potentially have cell-surface oxidases; they are already known to have internal ones (Meyer and Pistorius, 1987). Our preliminary testing of these organisms, however, was inconclusive. Since many phytoplankton are brought into culture by enriching with inorganic nitrogen sources, culture collections may be biased against species with oxidases or deaminases in general. The potential buildup of H_2O_2 and α-keto acids with large amino acid enrichments may also be a problem. Finding additional species with amino acid oxidases should thus involve both screening existing culture collections and the isolation of new species by gradual amino acid enrichments of natural samples. The three phytoplankton genera discussed here are likely to represent only some of the possible variations on the common theme of cell-surface deamination.

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Chapter 5.

Amine oxidases of marine phytoplankton

In addition to the L-amino acid oxidases reported previously, Prymnesium parvum and Pleurochrysis carterae possess enzymes capable of oxidizing certain primary amines to NH_4^+, H_2O_2, and an aldehyde. The oxidation of amino acids to obtain ammonia thus appears to be a general mechanism applicable to primary amines. Results from the study of these enzymes in the phytoplankton genera Pleurochrysis, Prymnesium, and Amphidinium are presented here. These results suggest that amines may also be important sources of nitrogen for phytoplankton growth.

Materials and Methods

Amines used is this work were obtained from Aldrich and were typically 98-99+% pure. Other reagents were obtained as described in chapters 3 and 4.

The methods used are the same as described in Chapters 3 and 4, except that glycolaldehyde, the endproduct of ethanolamine oxidation by amine oxidases, was measured by derivatization with 2,4-dinitrophenylhydrazine and HPLC (Kieber and Mopper, 1986) with the help of D. Kieber.

Results

The phytoplankton with active cell-surface L-amino acid oxidases (Palenik and Morel, 1989) were assayed for amine-dependent H_2O_2 production. This latter activity was initially assayed with 20μM ethanolamine and with 20μm ethanolamine in the presence of 20μm L-alanine to saturate the L-amino acid oxidases. The phytoplankter, Amphidinium operculatum (or klebsii, clone AMPHID) possesses an L-amino acid oxidase, but did not show detectable amine (ethanolamine) oxidase activity (Figure 1). In Prymnesium calathiferum (CHANG1), the amino acid oxidase activity is very low, and the amine oxidase activity undetectable.

In the presence of 20μM ethanolamine, nitrogen limited cultures of Pleurochrysis carterae (clone COCCOII), produce H_2O_2 in the medium (Figure 2). The same rate of hydrogen peroxide production from ethanolamine (as found by difference) also occurs in the presence of a concentration of L-alanine (20μM) that saturates the L-amino acid oxidase of this organism (Figure 2). This additional H_2O_2 production is thus due to a distinct oxidase. Cultures in exponential phase on nitrate do not show ethanolamine dependent H_2O_2 production (Figure 2) further proof that this is a distinct oxidase.

Ethanolamine dependent H_2O_2 production was also found in Prymnesium parvum (clone PRYM), a brackish and coastal water phytoplankter (Palenik, et al, 1989).

The organic product from the oxidation of ethanolamine by an amine oxidase is glycolaldehyde ($HOCH_2CHO$). In the presence of 10μM added ethanolamine both nitrogen-limited COCCOII and PRYM produced glycolaldehyde in the medium. No glycolaldehyde production occurred in

the absence of added ethanolamine. The rate of production (one point estimate) was $9x10^{-15}$mol cell^{-1} h^{-1} and $1.8x10^{-15}$mol cell^{-1} h^{-1} for COCCOII and PRYM, respectively. The rate for PRYM was what would be expected from the saturated rate constants assayed using H_2O_2 production, while the rate for COCCOII was about a third of what would be typically expected for similarly conditioned cells (See below).

Several primary amines were tested as substrates for H_2O_2 production by these oxidases (Figure 3). For COCCOII, ethanolamine, ethylamine, glycylglycine, 4-aminobutyric acid, putrescine, 3-amino-1-propanol, and L-ornithine supported H_2O_2 production, but not alanylalanine, serylglycine, tryptophanylglycine, D-glucosamine, D-galactosamine, or beta-alanine (most shown in Figure 4). For PRYM, utilizable amines were ethanolamine, ethylamine, and 3-amino-1 propanol, but not glycylglycine, 4-aminobutyric acid, serylglycine, or L-ornithine (most shown in Figure 5). The two amine oxidases thus show somewhat different specificities, with the enzyme of PRYM apparently specific for ethanolamine-like amines, and the enzyme for COCCOII utilizing a wider range of substrates.

To test the utilization of primary amines as nitrogen sources for growth, sterile filtered solutions of ethylamine, ethanolamine, 4-aminobutyrate, L-ornithine, glycylglycine, and NH_4^+ were added at 50μM concentrations to nitrogen limited cultures of COCCOII and PRYM (Figures 6 and 7). Most amines which were shown to produce H_2O_2 above, also supported the growth of these organisms. An exception was ethylamine. While it is oxidized by the amine oxidases, it or its endproduct (acetaldehyde) appear to be toxic to COCCOII at 50μM concentrations. For PRYM, ethylamine growth was variable (see

Figure 1: Lack of hydrogen peroxide production from ethanolamine by nitrogen limited AMPHID culture (65,000 cells ml^{-1}). ○ , no addition; ● , 20μM L-alanine; Δ , 20μM ethanolamine; ▲ , both substrates at 20μM.

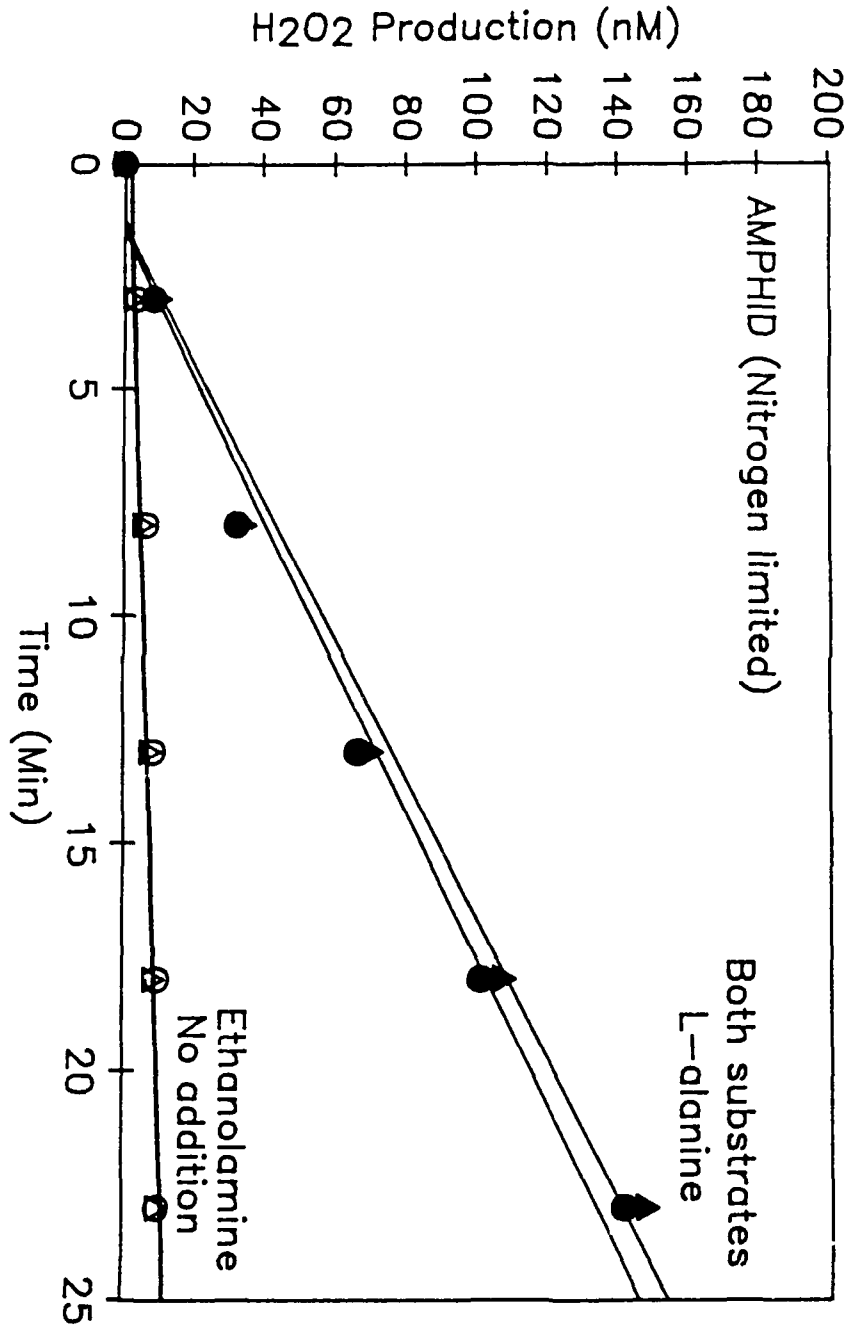
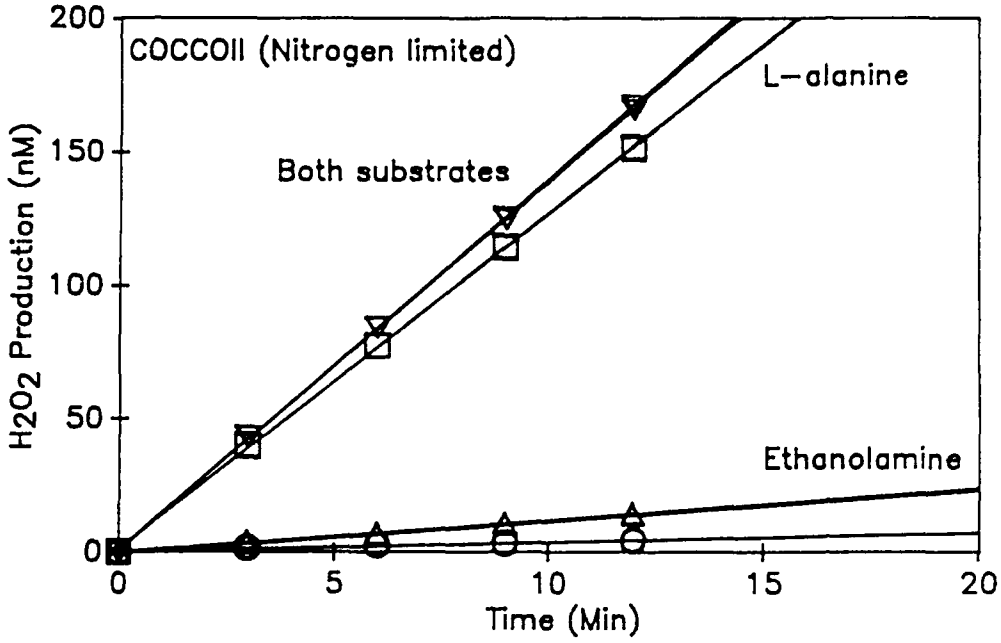

H2O2 Production (nM)
0
20
40
60
80
100
120
140
160
180
200
0
5
10
15
20
25
Time (Min)
AMPHID (Nitrogen limited)
Both substrates
L-alanine
Ethanolamine
No addition

Figure 2: Duplicate measurements of hydrogen peroxide production from ethanolamine. Top--Nitrogen limited COCCOII (24800 cells ml^{-1}). ○ , no addition; △ , 20μM Ethanolamine; □ , 20μM L-alanine; ▽ , both substrates at 20μM. Bottom--Nitrate grown exponential phase COCCOII (21,200 cells ml^{-1}). Symbols as above.


COCCOII (Nitrogen limited)
L–alanine
Both substrates
Ethanolamine
H_2O_2 Production (nM)
Time (Min)
0
50
100
150
200
0
5
10
15
20

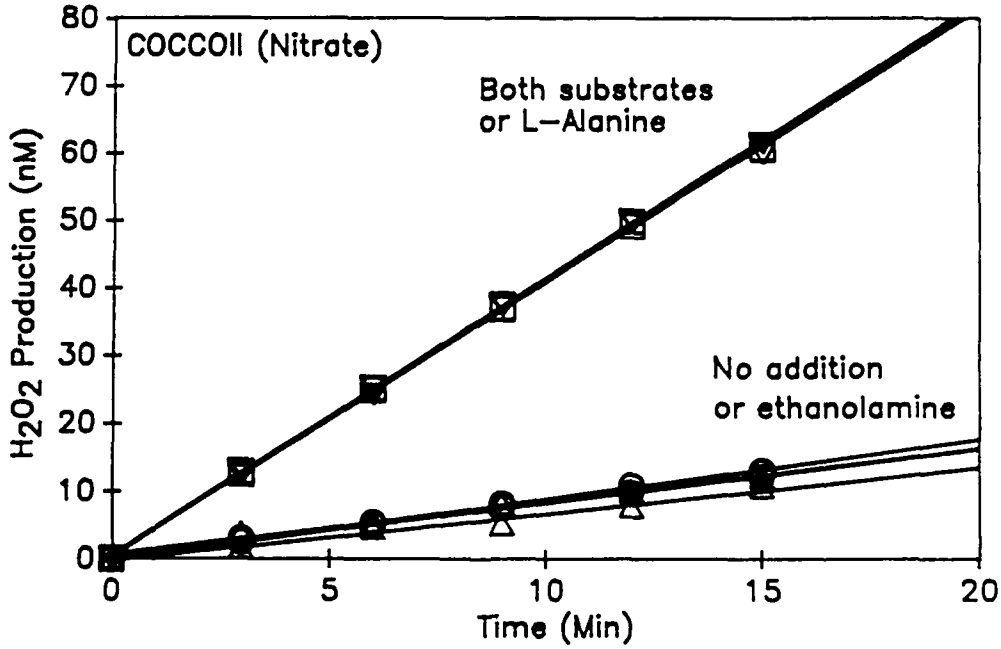
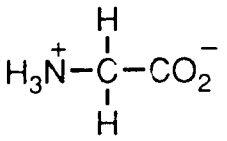
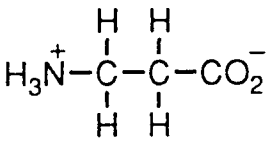

COCCOII (Nitrate)
Both substrates
or L–Alanine
No addition
or ethanolamine
H_2O_2 Production (nM)
Time (Min)
0
10
20
30
40
50
60
70
80
0
5
10
15
20

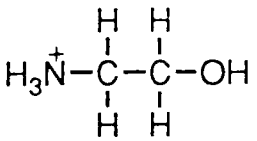
Figure 3: Structures of possible substrates for amine oxidases in natural waters.



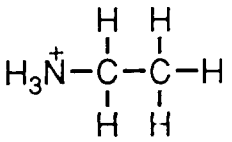
GLYCINE



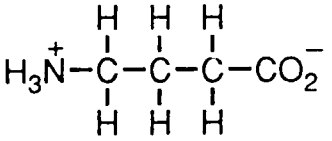
β-ALANINE



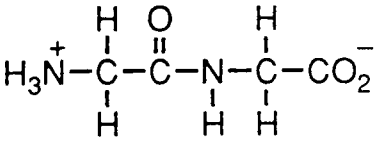
ETHANOLAMINE



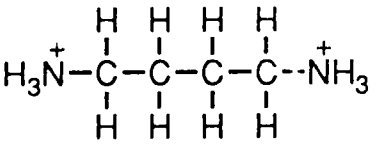
ETHYLAMINE



4-AMINOBUTYRATE



GLYCYLGLYCINE



PUTRESCINE

POSSIBLE AMINE OXIDASE SUBSTRATES

Figure 4: Hydrogen peroxide production from several amines at 20μM: COCCOII (32,000 cells ml^{-1})-- ● , Milli-Q water addition (duplicates); □ ,ethylamine; ◇ , 4-aminobutyrate; ○ , ethanolamine(duplicates); ▲ , serylglycine; △ , 3-amino-1-propanol; ▼ , beta-alanine; ▽ , glycylglycine(duplicates); ◆ , D-glucosamine.

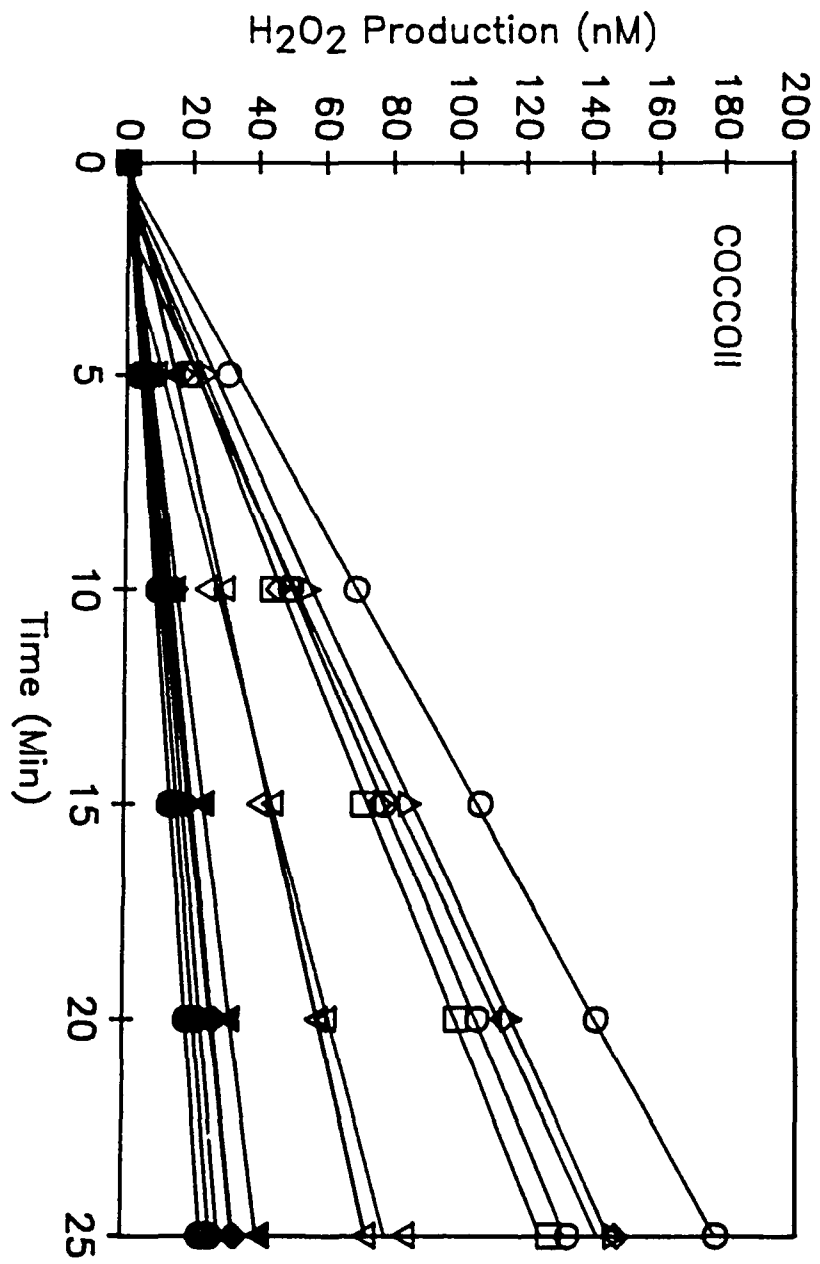

H_2O_2 Production (nM)
0
20
40
60
80
100
120
140
160
180
200
Time (Min)
0
5
10
15
20
25
COCCOII

Figure 5: Hydrogen peroxide production from several amines at 20μM: PRYM (105,500 cells ml^{-1})-- ● , Milli-Q water addition; ◇ , 4-aminobutyrate; ▲ , 3-amino-1-propanol; □ , ethylamine; ○ , ethanolamine; ▽ , glycylglycine; △ , serylglycine.

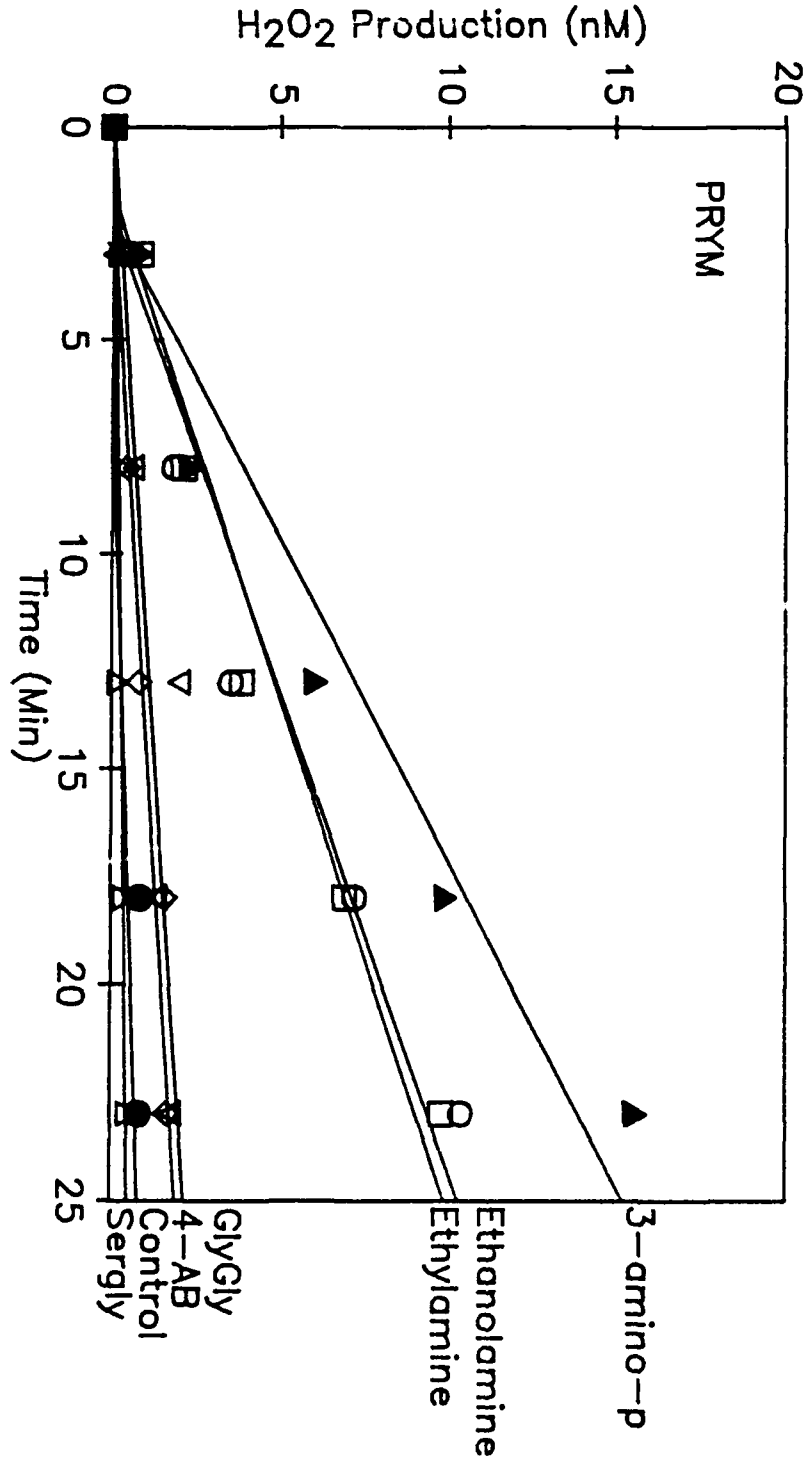

H2O2 Production (nM)
0
5
10
15
20
PRYM
Time (Min)
0
5
10
15
20
25
3-amino-p
Ethanolamine
Ethylamine
GlyGly
4-AB
Control
Sergly

Figure 6: Growth (chlorophyll fluorescence, duplicate tubes) of nitrogen limited COCCOII after addition of 50μM sterile filtered amines; □ , no addition; ● , NH_4^+; ▽ , L-ornithine; ○ , ethylamine; △ , ethanolamine; ▲ , glycylglycine; ■ , 4-aminobutyrate.

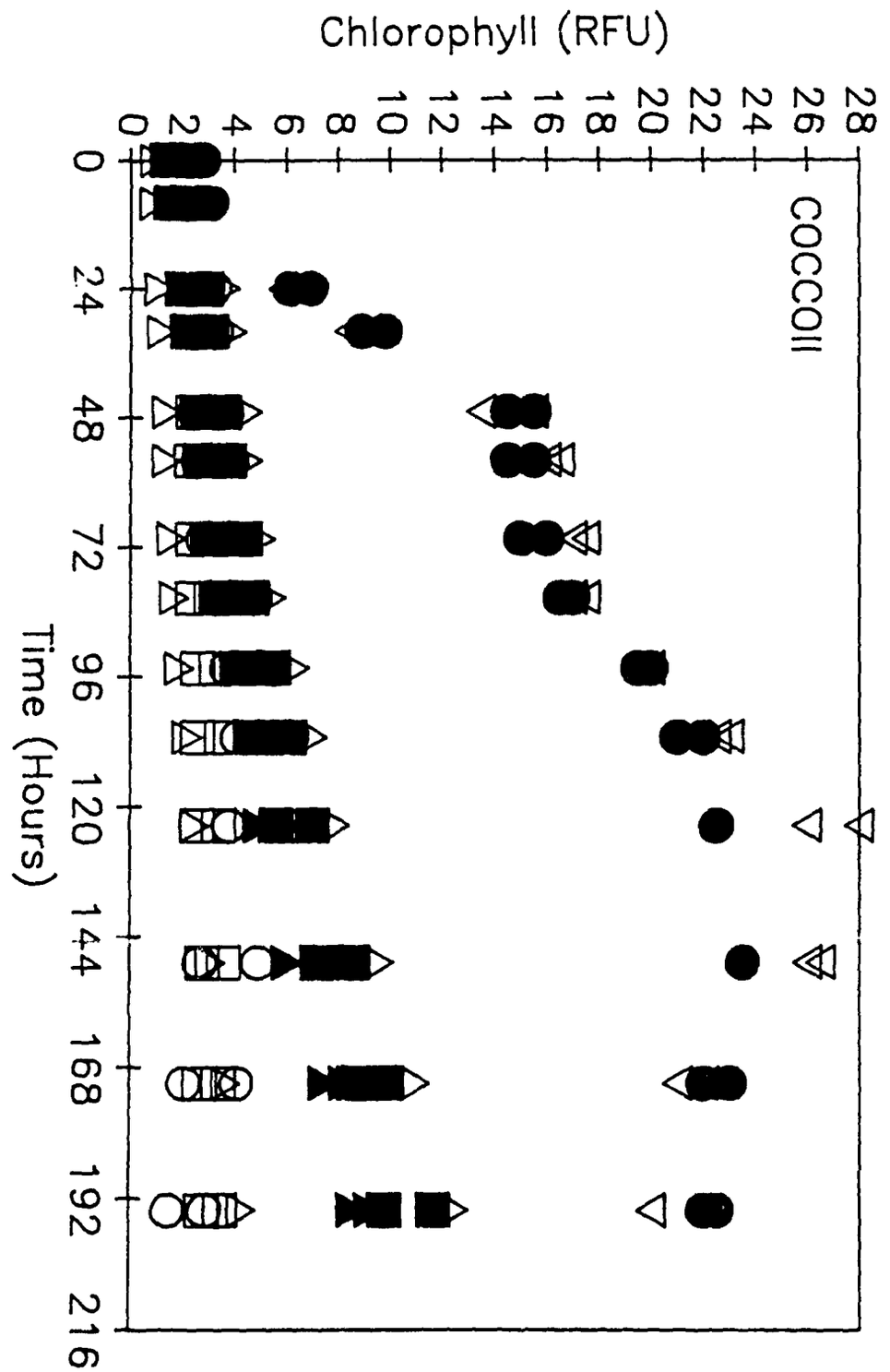
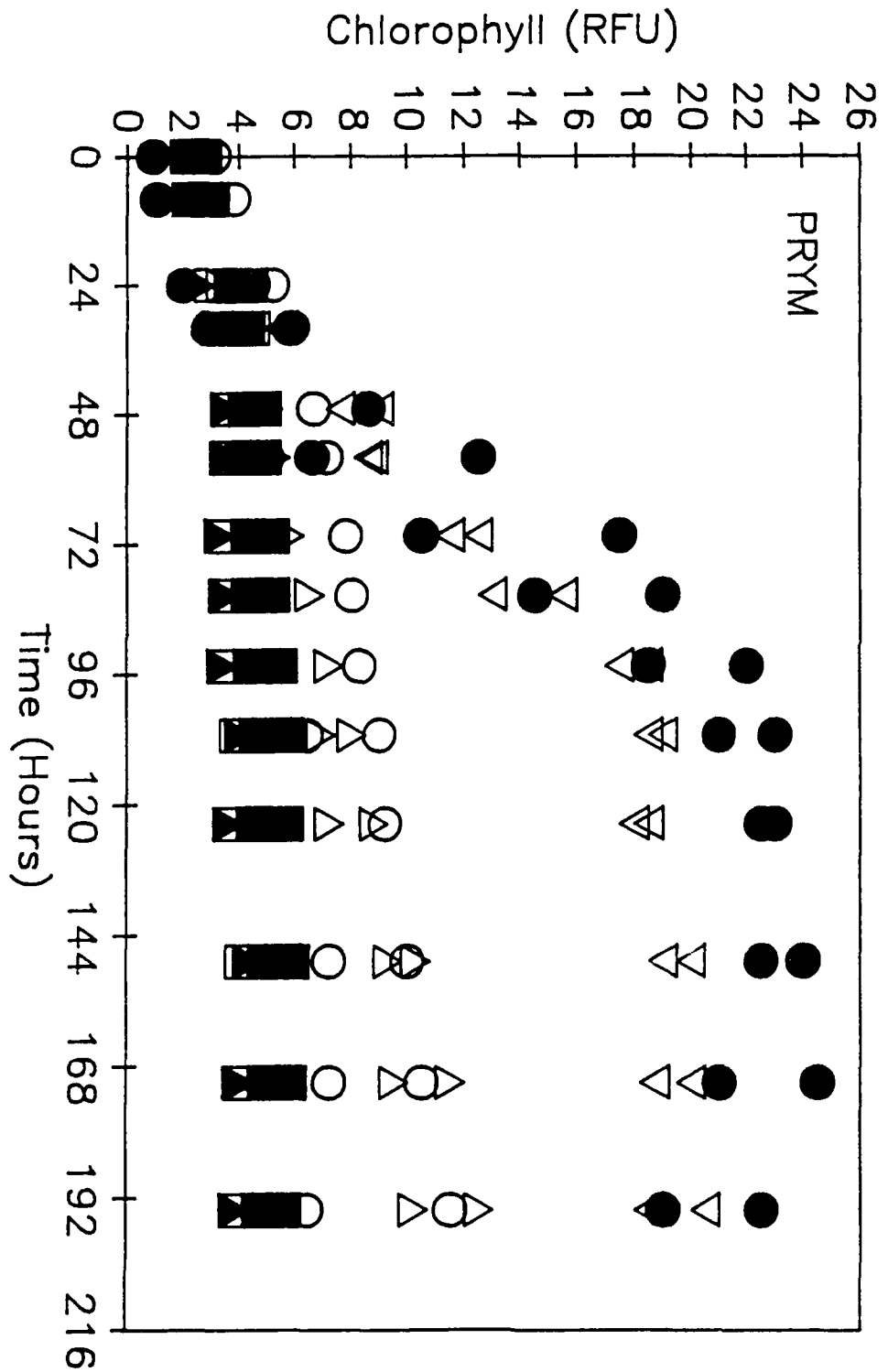

Chlorophyll (RFU)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Time (Hours)
0
24
48
72
96
120
144
168
192
216
COCCOII

Figure 7: Growth (chlorophyll fluorescence) of nitrogen limited PRYM after addition of 50μM sterile filtered amines. Symbols as Figure 6.


Chlorophyll (RFU)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Time (Hours)
0
24
48
72
96
120
144
168
192
216
PRYM

duplicates). Colonies appeared to form on the glass tube walls, an unusual morphology for this organism. COCCOII grew as well on ornithine as ammonia, but only attained a little more than half the theoretical yield. Since this compound is being oxidized by both the amino acid and the amine oxidases, the endproduct of one enzyme may not be available to the other. The resulting endproducts require further analysis.

The regulation of these enzymes under exponential NH_4^+, exponential NO_3^-, and nitrogen limited batch cultures was investigated for COCCOII, PRYM, CHANG1, and AMPHID. The enzyme saturated rate constants under the various conditions along with the constants for the accompanying L-amino acid oxidases (Palenik and Morel, 1989) are shown in Table 1. The most important results are that PRYM has an amine oxidase even in the presence of excess nitrate, and the enzyme activity is enhance under nitrogen limitation. COCCOII shows amine oxidase activity only under low nitrogen conditions.

Table 1 : Enzyme saturated rate constants for the amine oxidase of preconditioned cultures as compared to the rates for the respective L-amino acid oxidase ($x10^{-15}$ mol $cell^{-1}$ h^{-1}).

Organism	Preconditioning		
	NH4+	NO3-	Nlim
Amphidinium operculatum (AMPHID)			
L-amino acid oxidase	ND	ND	8.5
Amine oxidase	ND	ND	ND
Prymnesium parvum (PRYM)			
L-amino acid oxidase	ND	6.5	7.3
Amine oxidase	ND	0.7	1.4
Prymnesium calathiferum (CHANG1)			
L-amino acid oxidase	--	ND	0.1
Amine oxidase	ND	ND	ND
Pleurochrysis carterae (COCCOII)			
L-amino acid oxidase	ND	12	30-56
Amine oxidase	ND	ND	3

ND: not detectable
--: not assayed

Discussion

Amine oxidases, directly analogous to the L-amino acid oxidases reported previously, are found in the two marine phytoplankton, Prymnesium parvum (PRYM), and Pleurochrysis carterae (COCCOII). Both enzymes produce H_2O_2 (Figure 2 and Palenik et al, 1989) and glycolaldehyde as extracellular endproducts when the enzymes are assayed with ethanolamine. The third endproduct, NH_4^+, is used as a nitrogen source; the two phytoplankton can in general grow on amines such as ethanolamine that are substrates for the oxidase (Figures 6 and 7). Ethylamine is an exception: it is oxidized, but it or its endproduct appears to be toxic to COCCOII and supports variable growth for PRYM. L-ornithine did support some additional growth of COCCOII after its apparent use as an amino acid nitrogen source, but then growth (fluorescence increase) stopped, perhaps due to limitation by a nutrient other than nitrogen.

The saturated rate constants for the amine oxidases are 10-20% of that for the amino acid oxidases. Growth rates on amines are similarly lower than for NH_4^+ or amino acids as nitrogen sources. Apparently the role of these oxidases is to augment other available nitrogen sources with nitrogen from amines. It is possible, however, that some phytoplankton may have evolved to take advantage more specifically of amines in natural waters, and these organisms would obviously have higher enzyme activities.

The amine oxidase of PRYM appears to be relatively specific for ethanolamine. Such an enzyme has been reported previously in some bacteria, but it did not oxidize ethylamine (Narrod et al., 1964; Blackwell et al., 1976). Other reports have shown that bacteria can

grow on related amines (ethylamine) as nitrogen sources by an unknown mechanism (Bicknell and Owens, 1980). The concentrations of ethanolamine in natural waters are not known, but since phosphatidylethanolamine is a major bacterial lipid, ethanolamine probably is a component of the soluble organic nitrogen pool. The existence of an ethanolamine oxidase as reported here also suggests that ethanolamine is present.

There may be some connection between the toxins of Prymnesium parvum, which are known to bind to phosphatidylethanolamine groups on cell surfaces (Padilla and Martin, 1973) and its ethanolamine oxidase. One can speculate that as part of its toxicity, P. parvum may produce an extracellular phospholipase D, an enzyme that would hydrolyze phosphatidylethanolamine in membranes to produce ethanolamine in solution.

The amine oxidase of COCCOII is relatively nonspecific; it appears to require a methylene group adjacent to the amino group (H_3N^+-CH_2-R). In addition, carboxylated amines must have longer carbon chains. For example, glycine (R=CO_2^-) and beta-alanine (R=$CH_2CO_2^-$)are not oxidized, but 4-aminobutyrate (R=$CH_2CH_2CO_2^-$) is oxidized. Glycylglycine is oxidized, suggesting that the enzyme can oxidize N-terminal glycines on most peptides; however at 20μM concentration glycylglycine is oxidized more slowly than the other oxidizable amines tested. In general the oxidase thus seems developed to utilize a range of primary amines that might be found in natural waters. These results explain the previous reports of the use of glycylglycine (Turner, 1979), and 4-aminobutyrate (Pintner and Provasoli, 1963) as nitrogen sources by P. carterae .

The primary amino groups other than amino acids and a few amines are poorly characterized in natural waters. They are present at about 5-10 times the amino acid concentrations as measured by amine reactive reagents such as fluorescamine (North, 1975; Billen, 1981). These reagents react with a wider range of moeities than are substrates of the oxidases reported here; it is thus difficult to estimate what fraction of this large pool is actually available as a nitrogen source for phytoplankton. Some specific amines have been measured in seawater such as beta-alanine, 4-aminobutyrate, and L-ornithine at low but significant levels in the dissolved (Mopper and Lindroth, 1982) and particulate (Lee and Cronin, 1982) organic nitrogen pools. Putrescine utilization by bacteria in seawater has received some attention (Hoefle, 1984), but concentration levels are not well characterized.

One interesting finding in this regard is that heterotrophic microflagellates may release amino acids and primary amines when feeding on bacteria (Andersson, et al, 1985). Active cycling of amines probably occurs in natural waters just as it does for amino acids.

The amine oxidases reported here are examples of a general mechanism by which phytoplankton utilize organic nitrogen. In this mechanism, the substrate is oxidized at the cell-surface to produce NH_4^+, H_2O_2, and an aldehyde. This may be an important mechanism for the production of small organic aldehydes in natural waters. At a more general level these are examples of how phytoplankton use cell-surface enzymes to actively degrade organic forms of nitrogen and phosphorus to more utilizable forms, particularly NH_4^+ and PO_4^-. The challenge is to now expand our definition and quantitation of available nutrient

pools beyond NH_4^+, NO_3^-, and urea to include the other nitrogen compounds phytoplankton are using.

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Chapter 6.

Dark production of H_2O_2 in the Sargasso Sea.

Introduction

This chapter reports on a sensitive method for measuring hydrogen peroxide in seawater, especially in field samples. It also shows that dark production of H_2O_2, possibly due to the action of oxidases, can be found in the environment.

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Abstract—Hydrogen peroxide is introduced into the marine environment by wet and dry deposition and is produced there by photochemical reactions. Dark production, particularly by the biota, has not been demonstrated in situ. To investigate dark H_2O_2 production, we adapted a method of measuring H_2O_2 for use in the field. The method uses the enzyme-catalyzed oxidation by H_2O_2 of hydroxyphenylpropionic acid to a fluorescent dimer; its detection limit is about 3 nM. Results of a field study in the Sargasso Sea (32°N, 62°W) confirmed several general characteristics of H_2O_2 distributions—a surface maximum and a diel cycle in surface waters. Data from the Sargasso Sea also demonstrated that dark, probably biological, production was occurring at significant rates at depths of 40–60 m.

Hydrogen peroxide occurs in surface ocean water at a concentration of about 10–200 nM (Van Baalen and Marler 1966; Zika 1984). As an important intermediate in redox processes involving oxygen, this reactive species may profoundly influence the chemistry and biological activity of the oceans, particularly through its interaction with transition metals and organic compounds. The interaction of H_2O_2 with the biota may also occur directly, for example, through a recently demonstrated activity as a chemotactic agent (Haeder 1984; Nultsch and Kumar 1984). Elucidation of the role of H_2O_2 in marine systems, however, still requires a better understanding of its distribution and dynamics.

Hydrogen peroxide in the marine environment can be introduced via wet and dry deposition (Zika et al. 1982; Thompson and Zafiriou 1983) and can originate from photochemical reactions (Cooper and Zika 1983). It can also be produced by the biota. We have recently reported on the production of H_2O_2 by a marine coccolithophorid in the dark (Palenik et al. 1987). In order to investigate the importance of biological sources of H_2O_2 in the marine environment, we adapted a method of measuring H_2O_2

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for use in the field. Results with this method in the Sargasso Sea (32°N, 62°W) are presented, including evidence that dark, probably biological, production of H_2O_2 occurs at this site.

Hydrogen peroxide oxidizes substituted phenols in the presence of the enzyme horseradish peroxidase (HRP). The coupled phenol product has a characteristic fluorescence, and the system can be used to measure H_2O_2 in aqueous samples (Guilbault et al. 1968). The phenol hydroxyphenylacetic acid, for example, has been used to quantify H_2O_2 in rain (Lazrus et al. 1985) and in freshwaters (Zepp et al. 1988). The survey of Zaitsu and Ohkura (1980) showed that hydroxyphenylpropionic acid (HPPA) was the best phenol for assaying for H_2O_2 in selected buffers due to its rapid formation of a highly fluorescent dimer. It was chosen in this study.

The dimer fluorescence has an excitation maximum at 320 nm and an emission maximum at 415 nm in seawater. For field analysis of H_2O_2, a Turner Designs model 10 fluorometer was equipped with the appropriate lamp (10-049) and filters: a UG-11 and a 34A Wratten filter (Ealing Optics and Sequoia-Turner, respectively) as excitation filters; a GG-400 and a 410-nm interference filter (Ealing Optics) as emission filters.

To measure H_2O_2, we added 50 μl of a 2 mM HPPA (Aldrich) stock in Milli-Q water to 20 ml of sample. The small residual fluorescence, if any, was blanked and 10 μl of HRP stock (4 mg ml^{-1}; Sigma, type 6) were added. Fluorescence was recorded on a strip-chart recorder. The method was calibrated by standard additions from a 10^{-5} M H_2O_2 solution prepared immediately before use from a calibrated H_2O_2 stock. We assumed that a calibration curve obtained at one sample depth was valid for the complete water column. The method will also measure some organic peroxides, but they have never been detected in seawater (Zika et al. 1985*a*). Other potential interferences are discussed by Guilbault et al. (1968), such as transition metals at environmentally irrelevant concentrations. The presence of humic or other fluorescent materials at a site will affect the calibration (Zepp et al. 1988).

The method as described appears to have a slightly better detection limit (3 nM) than the scopoletin-based H_2O_2 assay (5 nM, Zika et al. 1985*b*). The chemical basis of the method is also better understood (Guilbault et al. 1968).

Samples were collected with 5-liter Niskin or 10-liter Go-Flo bottles on Kevlar line or occasionally with surface casts of a linear-polyethylene flask. Most samples (all H_2O_2 incubations) were transferred into 250-ml linear-polyethylene bottles prewashed by successive overnight treatments with 1 N NaOH, 4 N HCl, and Milli-Q H_2O. Bottles were rinsed with sample before use. Analysis began immediately and took a few minutes for each sample. Incubating bottles were kept in the dark at ambient laboratory temperature, about 22°–24°C. Samples were occasionally gravity filtered through rinsed Nuclepore filters (1-μm pore size) directly from one incubation bottle into a second.

Samples from 40 to 60 m were also enriched with f/50 nutrients (Guillard and Ryther 1962) and incubated in acid-washed glass tubes for later study and subculturing in the laboratory, usually in continuous light (100 μEinst m^{-2} s^{-1}) and at 20°C. Subsequent transfers of small volumes (about 0.5 ml) of these cultures were made into about 25 ml of 0.2-μm, sterile-filtered, 40-m Sargasso seawater enriched with f/50 nutrients added aseptically. Cultures were treated aseptically after collection. Cultures were assayed for dark H_2O_2 production as outlined by Palenik et al. (1987), but without a cell-washing step.

In a calibration curve made by standard additions of H_2O_2 to natural seawater samples, fluorescence increased linearly with H_2O_2 addition up to at least 0.175 μM total H_2O_2 (Fig. 1). There was no HRP-dependent fluorescence in the absence of H_2O_2, as shown by the measurement of 130-m samples. The detection limit of the technique as developed on the Turner fluorometer was about 3 nM. The C.V. was typically 2% for repeated measurements at the 50-nM level, though it can be better or worse depending on operating conditions.

With this method, H_2O_2 in the Sargasso Sea (32°N, 62°W) showed distributions similar to those found in other areas (Zika et al. 1985*a*,*b*). The maximal concentration

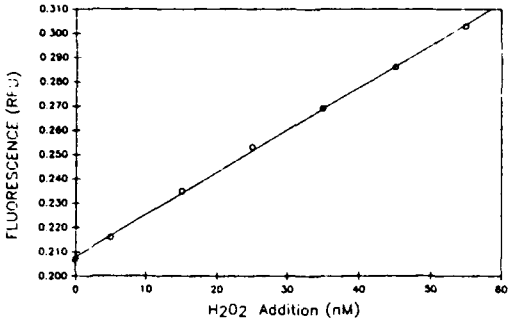

Fig. 1. Calibration of H_2O_2 assay using standard additions to a water sample (32°N, 62°W, 40 m). A regression line is drawn through the data points.

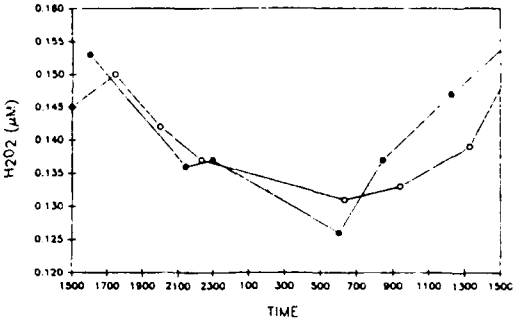

Fig. 3. Hydrogen peroxide concentration as a function of time for a 48-h diel study (○—1st 24 h; ●—2nd 24 h).

always found in surface samples, and concentrations decreased with depth to undetectable levels at about 130 m. All six complete and five partial H_2O_2 profiles measured were quite similar (e.g. Fig. 2), apparently following the temperature profile, with most variability in the surface values.

Surface concentrations exhibited a diel cycle with a late afternoon maxima at 1600 hours (Fig. 3). The amplitude of the variation (peak to trough) was about 20% of the maximal concentration, similar to amplitudes found in oligotrophic waters of the Gulf of Mexico (Zika et al. 1985*a*). The similarity between H_2O_2 distributions and dynamics in the Sargasso Sea and those found elsewhere suggest their general validity and bolster our confidence in the analytical method.

To investigate biological production in the Sargasso Sea, we incubated samples in the dark. The H_2O_2 concentrations in bottles were measured immediately after collection and then at intervals (Figs. 4–6). In all incubation profiles *net* (dark production minus dark consumption) H_2O_2 production was clearly occurring in some incubation bottles, demonstrating a sharp peak of production at 40–60 m in the thermocline. Within the 40–60-m range, however, there was some variability in the peak. In Fig. 4 it occurs at 50–60 m and in Figs. 5 and 6 it occurs at 40 m. The nitrite maximum also showed changes in depth (80–100-m range), but there were insufficient data to see if these effects were correlated (data not shown). Production of H_2O_2 in the peak was usually fastest in the first 1–2 h. The maximal initial rate of net hydrogen peroxide production was 11 nM h^{-1} (Fig. 4, 50 m).

Over a whole incubation period, the av-

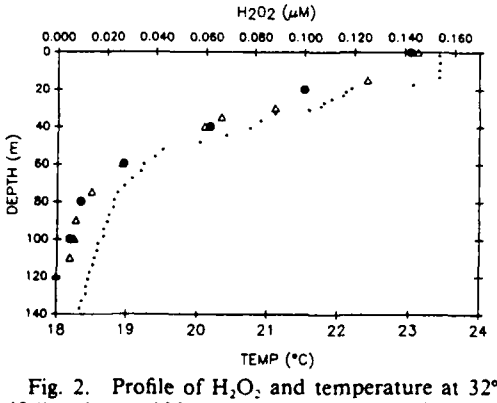

Fig. 2. Profile of H_2O_2 and temperature at 32°N, 62°W, 19 June 1987 (△—1500 hours; ●—1930 hours; •—temperature).

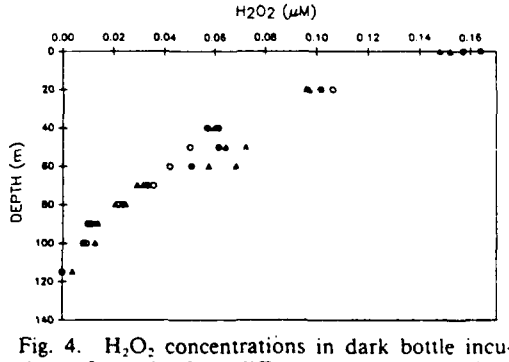

Fig. 4. H_2O_2 concentrations in dark bottle incubations of samples from different depths. Incubations began at 0800, 14 June 1987 (○—0 h; ●—1 h; △—3.5 h; ▲—8.5 h).

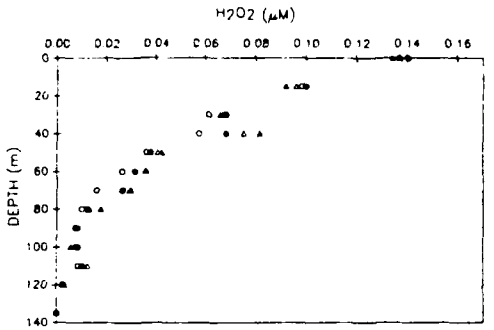

Fig. 5. As Fig. 4, but incubations began at 0940 hours, 17 June 1987 (○—0 h; ●—2.5 h; △—5.25 h; ▲—8 h).

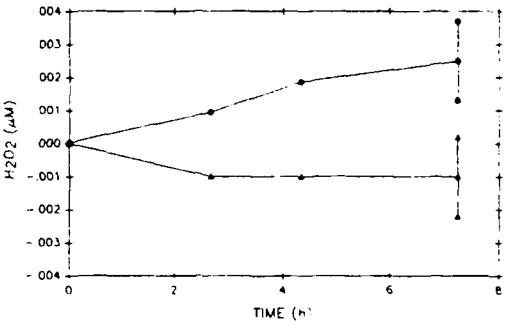

Fig. 7. H_2O_2 concentration changes from their initial values in dark bottle incubations of a sample from 40 m. Incubations began at 1315 hours, 21 June 1987. Filtration caused a 3 nM loss of H_2O_2 so that the samples did not have the same initial concentration (●—unfiltered; ▲—1-μm Nuclepore-filtered). Error bars represent 1 SD on either side of the measurement.

erage net dark H_2O_2 production in the "peaks" was typically 1–3 nM h^{-1}. These rates provide an estimate of the minimal total H_2O_2 production rates at those depths. In comparison, the net rate of surface H_2O_2 increase during the day was about 3 nM h^{-1} (Fig. 3). Net dark H_2O_2 decomposition occurred in some surface samples.

The localized nature of this phenomenon suggests that biota are the most likely sources of dark H_2O_2 production. The production peaks did not correspond, however, with the chlorophyll maximum (about 90 m) or the *Synechococcus* maximum (about 70–90 m). Perhaps a specific organism at 40–60 m or an organism in a specific physiological state at that depth was producing H_2O_2. Hydrogen peroxide production could also be occurring at faster rates at other depths but local decomposition rates could be keeping pace, leading to no apparent dark H_2O_2 production.

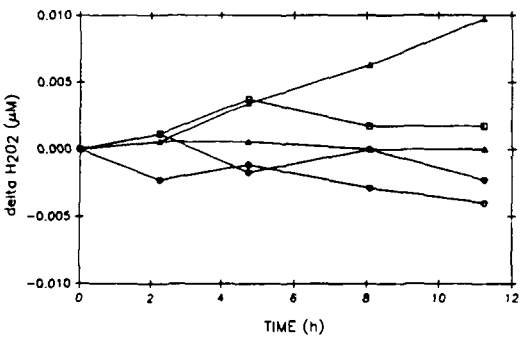

Fig. 6. H_2O_2 concentration changes from their initial values in dark bottle incubations. Incubations began at 0600 hours, 21 June 1987 (○—0 m; ●—30 m; △—40 m; ▲—50 m; □—60 m).

Filtering a sample through a 1-μm Nuclepore filter stopped net dark H_2O_2 production in the filtrate (Fig. 7) the one time we were able to perform this experiment. This result implicates eucaryotic or large procaryotic organisms. It is possible, of course, that auto-oxidation of organic material occurring on large particles produces H_2O_2. Filtration itself caused a 3 nM loss of H_2O_2, the reason for which is not known. The rate of net hydrogen peroxide production in the unfiltered sample was also relatively low, barely above the detection limit, but it represents another independent observation of H_2O_2 production at 40 m.

Further evidence for biological production of hydrogen peroxide was obtained from f/50 nutrient-enriched samples brought back to the laboratory. A few of them produced H_2O_2 in the dark when assayed as described above. Small volumes of these samples were transferred into 40-m Sargasso seawater that was 0.2-μm filtered and enriched with f/50 (transfer medium) and assayed 2–3 weeks after transfer. Subsequent transfers of these cultures were performed in a similar manner (Fig. 7). In each case there was measurable H_2O_2 production, but there was none in transfer medium alone (Fig. 8). The second transfer appeared to contain only marine *Synechococcus* and presumably in situ bacteria. Dark H_2O_2 production thus was

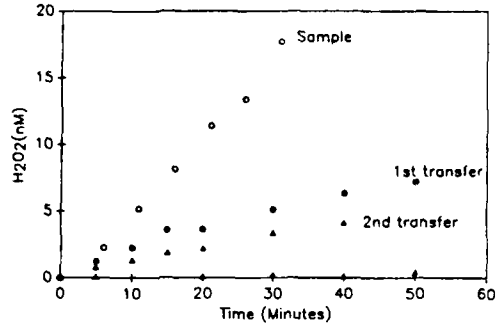

Fig. 8. H_2O_2 production by 3 ml of nutrient-enriched 40-m Sargasso seawater and by subsequent transfers. H_2O_2 is plotted as production beyond the initial amount present in the sample. Samples were assayed 2–3 weeks after enrichment or transfer. The initial sample was grown at 18°C; subsequent transfers were grown at 20°C. Sample from 40 m, 21 June 1987 (●—first transfer; △—second transfer; ▲—0.2-μm-filtered and f/50-enriched 40-m seawater assayed with the first transfer).

present two transfers and 2 months after its observation in the Sargasso Sea, a situation that strongly implicates a biological phenomenon.

We have no way of knowing that the putative organism producing H_2O_2 in the transfers was also producing H_2O_2 at our study site, but to date the capacity for dark H_2O_2 production does not seem to be widespread. Dark H_2O_2 production has been reported for a few freshwater species of *Synechococcus*, but not for the few marine species so far examined (Stevens et al. 1973).

We were able to clone out several unialgal but not axenic isolates of *Emiliania huxleyi*, a marine coccolithophorid, from nutrient-enriched samples brought back to the laboratory (identified by R. R. L. Guillard). These isolates did not produce H_2O_2 in the dark, contrary to our hypothesis that it might be a general characteristic of coccolithophorids (Palenik et al. 1987).

In conclusion, significant rates of dark H_2O_2 production were found in the Sargasso Sea, particularly in the 40–60-m depth range. This production of H_2O_2 was most probably biological due to its localized nature, its removal by filtration, and its presence in samples brought back and cultured in the laboratory. Our results are the first reported demonstrations of dark H_2O_2 production and imply that efforts to model H_2O_2 dynamics at a site must consider dark production or specifically rule out its presence.

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Chapter 7.

Further research.

The response of phytoplankton to nitrogen limitation is a fascinating physiological problem with important implications for field studies of nitrogen cycling or nutrient cycling in general. Amino acid and amine oxidases are ideal systems for probing, at the molecular level, the cell's response to nitrogen limitation: they are one of the few known systems in phytoplankton that are induced by nitrogen limitation, and they are easy to assay. Furthermore, the presence of genetic variations in the different isolates will provide valuable clues to the regulatory mechanisms involved.

A similar kind of study has been carried out in the fungus *Neurospora crassa*. There it is believed that a single protein, the **nit2** gene product, will bind to DNA and initiate or enhance transcription of nitrogen scavenging proteins, including an L-amino acid oxidase (Grove, G. and G. A. Marzluf. 1981. J. Biol. Chem. 256: 463-70). Under nitrogen replete conditions, glutamine levels inside these cells are high, and glutamine specifically binds to the **nit2** product and prevents it from binding to DNA. Perhaps a similar system is present in phytoplankton.

Clearly additional work on the presence of L-amino acid oxidases in the three "environments" mentioned in Chapter 1 would be valuable. These include algal blooms, algal-invertebrate symbioses, and

oligotrophic regimes. To examine the first environment, the common bloom forming dinoflagellates (and other phytoplankton) should be assayed in the laboratory and the field for cell-surface oxidase activity. For the second, the flatworm *Amphiscolops langerhansi* is easy to raise in the laboratory and contains endosymbiotic *Amphidinium* species, making it an ideal host in which to examine the role of oxidases in endosymbiotic relationships. In the third example, the intial observations of H_2O_2 in the Sargasso Sea make it the obvious place to look for amino acid and amine oxidases, although any oligotrophic environment would be worth examining. I hope that the research presented in this thesis provides the conceptual framework and the analytical tools for studying cell-surface deaminases in these environments.

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16. Abstract (Limit: 200 words)

Many phytoplankton can catalyze the decomposition of organic phosphates using cell-surface phosphatases and subsequently take up the phosphorus for growth. The nitrogen analogs, cell-surface deaminases, have not been reported in phytoplankton, except for a low affinity asparaginase. In fact, high affinity cell-surface L-amino acid and amine deaminases (oxidases) exist in at least three phytoplankton genera: *Amphidinium*, *Pleurochrysis*, and *Prymnesium*. One type of enzyme oxidizes L-amino acids, a second type primary amines; the endproducts are hydrogen peroxide, an organic product (an α-ketoacid or an aldehyde), and NH_4^+ which is assimilated by the cell. The characteristics (Km, Vmax, inhibition by various reagents) and the regulation of these enzymes are discussed. A field method for measuring H_2O_2 is presented which was used to obtain preliminary data suggesting that cell-surface oxidases may be present in the environment. The implications of these results for understanding nitrogen cycling; primary production; the geochemistry of H_2O_2, organic acids and aldehydes; and algal-invertebrate symbioses in aquatic environments are discussed.

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