

UNIVERSITY OF ILLINOIS

May 4 19 93

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Brett Edward Close

ENTITLED Studies on Biological Halogenation: I. Salt Induction
Studies of Methyl Chloride Transferase from the Plant
Batis maritima and II. The Effect of Nutrient
Availability on the Expression and Activity of
Chloroperoxidase from the Fungus *Caldariomyces fumago*

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Biochemistry

Crowell Hoza

Instructor in Charge

APPROVED

Robert M. King

HEAD OF DEPARTMENT OF Biochemistry

Studies on Biological Halogenation:

**I: Salt Induction Studies of Methyl Chloride
Transferase from the Plant *Batis maritima***

and

**II: The Effect of Nutrient Availability on the
Expression and Activity of Chloroperoxidase
from the Fungus *Caldariomyces fumago***

by

Brett E. Close

THESIS

for the
DEGREE OF BACHELOR OF SCIENCE

IN

BIOCHEMISTRY

College of Liberal Arts and Sciences
University of Illinois
Urbana, Illinois

1993

TABLE OF CONTENTS

Part One: Salt induction studies of methyl chloride transferase from the plant *Batis maritima*.

<u>Section</u>	<u>Page</u>
I. Introduction	1
A. <i>Batis maritima</i>	1
B. Methyl chloride transferase	1
C. Project Goal	2
II. Materials and Methods	5
A. <i>Batis maritima</i> plants	5
1. Collection	5
2. Culturing	5
B. Methyl Chloride Transferase Analysis	7
1. Whole Leaf Assays	7
2. Ground Leaf Assays	8
3. Methyl Bromide Quantification	9
4. Protein Determination	9
III. Results	10
A. Whole Leaf Assays	10
B. Ground Leaf Assays	10
IV. Discussion	17
A. Whole Leaf Assays	17
B. Ground Leaf Assays	17
C. Methyl chloride transferase	18
D. Conclusion	19
V. References	20
VI. Acknowledgements	21

Part Two: The effect of nutrient availability on the expression and activity of chloroperoxidase from the fungus *Caldariomyces fumago*.

I. Introduction	23
---------------------------	----

A.	<i>Caldariomyces fumago</i>	23
B.	Chloroperoxidase	23
C.	Project Goal	24
II.	Materials and Methods	26
A.	<i>C. fumago</i> Preparation	26
B.	Bottle Culture Preparation	26
C.	CPO Production Preparation	27
D.	MCD Assay	28
III.	Results	29
A.	Effect of Iron on CPO Production	29
B.	Succinate as a Carbon Source	29
C.	Effect of Amino Acids on CPO Production	29
IV.	Discussion	33
A.	Effect of Iron	33
B.	Succinate as a Carbon Source	34
C.	Effect of Amino Acids	34
D.	Conclusion	35
V.	References	36
VI.	Acknowledgements	37
VII.	Appendix	38

Part I: Salt Induction Studies of Methyl Chloride
Transferase from the Plant *Batis maritima*

I. Introduction

A. Batis maritima

Batis maritima is a succulent, halophytic plant of the Batidaceae family that is indigenous to Brazil, the southwestern United States, Hawaii, and Mexico (1). Although it is able to grow in landlocked areas, *Batis maritima* is more commonly found to grow in low lying coastal areas that are frequently flooded with sea water. Since *Batis maritima* is frequently in a hypersaline environment, it is confronted with an interesting problem of how to get rid of the excess chloride that the plant collects during the process of water uptake. Most plants do not have systems for excretion of excess nutrients and waste products through their stems (2). The presence of activity of these excretion systems in the roots of halophytic plants, like *Batis maritima*, would be a futile and energetic waste for the plants due to the hypersaline environment surrounding the plant. However, it appears that *Batis maritima* has evolved a way to control its internal chloride concentration through the activity of an enzyme called methyl chloride transferase.

B. Methyl chloride transferase

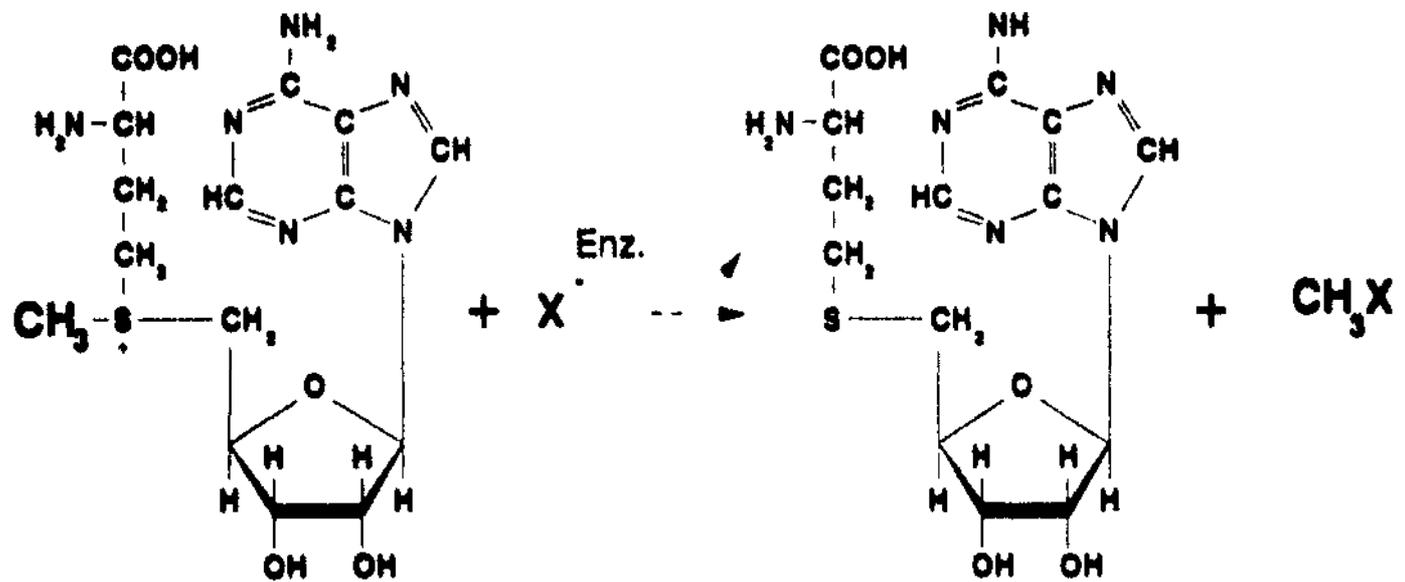
Methyl chloride transferase activity was first described by Wuosmaa and Hager (3) in *Endocladia muricata*, a marine red algae, and *Mesembryanthemum crystallinum*, a coastal succulent. The enzyme isolated from these two species was found to catalyze the reaction of S-adenosyl methionine and halide ion to S-adenosyl homocysteine and methylhalide. This is shown in Figure 1. The

presence of methyl chloride transferase in *Batis maritima* certainly would provide a plausible hypothesis for the mechanism of excretion of excess chloride from the plant for osmoregulatory purposes. Since the enzyme would produce gaseous methyl chloride as a product, the methyl chloride would simply exit through the top of the plant during its normal process of transpiration. Previous studies have shown that the most prevalent halohydrocarbon species in the upper atmosphere is methyl chloride, and it is widely believed that biological synthesis is largely responsible for the global emission rate of 5×10^6 tons of methyl chloride per year (4). For example, in vivo synthesis of methyl halides by marine macroalgae and phytoplankton has been reported (5). Also, a recent survey of methyl chloride production by marine algae in the Monterey Bay area of California by Wuosmaa and Hager (3) has suggested a broad distribution of the methyl chloride transferase enzyme. Considering the global distribution of many algal species and *Mesembryanthemum crystallinum*, the suggested methyl chloride synthesis rate of 5×10^6 tons per year seems quite reasonable.

C. Project Goal

The aim of this thesis project is to ascertain whether or not methyl chloride transferase expression is inducible by halide. If this is correct, higher halide concentrations should exact an elevated level of enzyme synthesis and activity. Long range goals are to clone the methyl chloride transferase gene and integrate the gene into the soybean genome in order to make

soybeans more salt tolerable.



S-Adenosyl Methionine

S-Adenosyl Homocysteine

X = Cl, Br, or I

Figure 1: Schematic view of the methylation reaction carried out by methyl chloride transferase.

II. Materials and Methods

A. Batis maritima plants

1. Collection

With the help of Edward Glenn of the University of Arizona's Environmental Research Laboratory in Tucson, a collecting trip to the halophyte fields at the Intercultural Center for the Study of Deserts and Oceans (CEDO) in Puerto Penasco, Sonora, Mexico was arranged. Peggy Boyer, curator of CEDO, identified the halophytic species that were collected. The *Batis maritima* plants were collected from an intertidal flat three miles southeast of CEDO. This area typically floods once daily with high tide water from the Sea of Cortez. The root systems of the plants were preserved as much as possible. The plants were wrapped in wet newspaper and placed in a refrigerator for transport.

2. Culturing

Upon return, the plants were placed in 6 1/2 inch plastic pots equipped with drain holes that were filled with washed and autoclaved Mason County, Illinois river sand. The plants were then placed in the Gottfried S. Fraenkel Memorial Greenhouse of the Department of Entomology at the University of Illinois. The greenhouse was kept at an average humidity between 60% and 75%. The average temperature range of the greenhouse was between 21 and 26 °C. The greenhouse was equipped with lights that illuminated the greenhouse and were timed to be operating for 16.8 hours per day. This represents a typical summer day.

The procedure and watering schedule followed was similar to that described in Glenn and O'Leary (6). The plants were irrigated by placing the pots in a plastic tub and flooding it with nutrient solution twice daily, three days a week. The plants remained in the nutrient solution until the top layer of soil in the pots was visibly damp. The nutrient solution recipe was obtained by personal communication with Ed Glenn. The nutrient solution consisted of a micronutrient solution and a macronutrient solution. The micronutrient stock solution consisted of 2.3 mM MoO_3 , 270 mM H_3BO_3 , 76 mM $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$, 4.8 mM $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, and 9.1 mM $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. The total volume of the micronutrient stock solution was 450 mL. The macronutrient solution consisted of 1mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1mM KH_2PO_4 , 0.5 mM KNO_3 , and 2.9 mM $\text{Ca}(\text{NO}_3)_2$. The total volume of the macronutrient solution was 24 L. Also added to the macronutrient solution was 60 mL of 3.25% liquid chelated iron (Fertilome). From the micronutrient stock solution, 3.6 mL was added to the 24 L macronutrient solution. All common chemicals, except those specifically stated, were obtained from the Biochemistry stockroom.

The pH of the nutrient solution was found to be 3.1. All efforts to raise the pH to the recommended level of 6.3 (7) without precipitating nutrients in the solution failed. Therefore, it was used as is.

This nutrient solution was used on the plants until they had established roots and grown to a larger size. The nutrient solution was replaced once a week with fresh solution. Also, the

plants were flushed with fresh water biweekly to prevent the accumulation of crystallized nutrients. Once experimentation was ready to begin the bulk nutrient solution was reduced in volume, and a series of nutrient solutions enriched with varying concentrations of NaCl were made. The NaCl concentrations used were 0 mM, 180 mM, 360 mM, 540 mM, and 720 mM. Also prepared was a pure solution of sea salts (Sigma) at a concentration of 40 g/L. The salinity of the nutrient solutions given the respective plants was raised 180 mM every three days (6) until the final desired concentration was reached in order to prevent shock to the plants. Two plants were raised at each nutrient salt concentration. The schedule of watering, flushing, and nutrient solution replacement remained the same as before. The use of the sea salt solution was discontinued due to yellowing and wilting of the plants watered with the solution. Clearly, the plants that live in the intertidal flats receive vital nutrients from the soil they reside in, not solely the sea water.

B. Methyl Chloride Transferase Analysis

1. Whole Leaf Assays

One leaf from each plant at the respective salt concentrations were plucked from the plants and weighed on a Mettler analytical balance to determine the weight. Each leaf was subsequently chopped up with a spatula and added to a 5 mL gas tight serum bottle. Next, 3 mL of 500 mM KBr was added to the serum bottle and then capped. Bromide ion was used in both the whole leaf and ground leaf assays for two reasons. One is

that bromide ion is simply a better nucleophile than chloride ion. The other reason bromide ion was used was to separate the enzymatic activity using the exogenously added bromide as substrate from the activity using the endogenous chloride.

The reaction mixtures were allowed to incubate at room temperature for two hours. Using a gas tight 1 mL Hamilton luer lok syringe equipped with a 1 1/2", 25 gauge needle, 1 mL headspace gas samples were removed from each bottle and analyzed by gas chromatography for methyl bromide. Gas samples were run on a Varian 3700 gas chromatograph interfaced with a Spectra Physics 4290 computing integrator and equipped with a Chromosorb 101 180 cm x 0.2 cm glass column packed with 60/80 mesh carbopack. The injector temperature was set at 250 °C, while the detector temperature was set at 350 °C. At a column temperature of 140 °C and nitrogen carrier gas flow rate of 30 mL/minute, methyl bromide had a retention time of 1.55 minutes.

2. Ground Leaf Assays

Similar to the technique described above, one leaf was plucked from each plant at each salt concentration. Each leaf was then completely ground, using a mortar and pestle, in 1.5 mL of 250 mM potassium phosphate buffer at a pH of 8.0. The 1.5 mL of cell suspension was then added to a 5 mL serum bottle. To the cell suspension was added 750 µL of a 1 mg/mL S-adenosyl methionine (SAM)/2 M KBr solution. The serum bottle was then capped and allowed to incubate at room temperature for two hours. Headspace gases were analyzed exactly as above.

3. Methyl Bromide Quantification

Methyl bromide production was quantified by relating peak areas from unknown samples to samples from identically prepared serum bottles, minus leaves, containing known amounts of methyl bromide. Independent standard curves were constructed for the whole leaf assay and the ground leaf assay. Activities were expressed as picomoles of methyl bromide produce per minute.

4. Protein Determination

The protein concentrations were determined only for the ground leaf assay in order to express enzymatic activity as specific activity (activity per milligram of protein). The protein assay used was a BioRad DC protein assay kit, using Bovine Serum Albumin as a standard. Absorbencies were read at 750 nm.

III. Results

A. Whole Leaf Assays

The results of the whole leaf assays on the plants watered with various nutrient enriched salt solutions are shown in Figures 2 through 6. A definite trend of increasing methyl chloride transferase activity is seen as the NaCl content of the nutrient solutions was increased. This observation corroborates the hypothesis that methyl chloride transferase expression is induced by elevated NaCl levels. The fact that very little methyl chloride transferase activity was seen in the assays from the plants watered with 0 mM further supports the hypothesis that *Batis maritima* has developed this enzyme to control the cytosolic halide ion concentration for osmoregulatory purposes.

B. Ground Leaf Assays

Figures 7 through 11 show the results of the assays of ground leaf extracts from the plants grown on differing NaCl concentrations. These assays display an explicit trend of higher enzymatic activity in leaf extracts from plants grown on nutrient solutions enriched with increasing NaCl concentrations. This observation, in conjunction with the results from the whole leaf assays, presents very strong evidence that methyl chloride transferase expression is induced by the presence of halide ion.

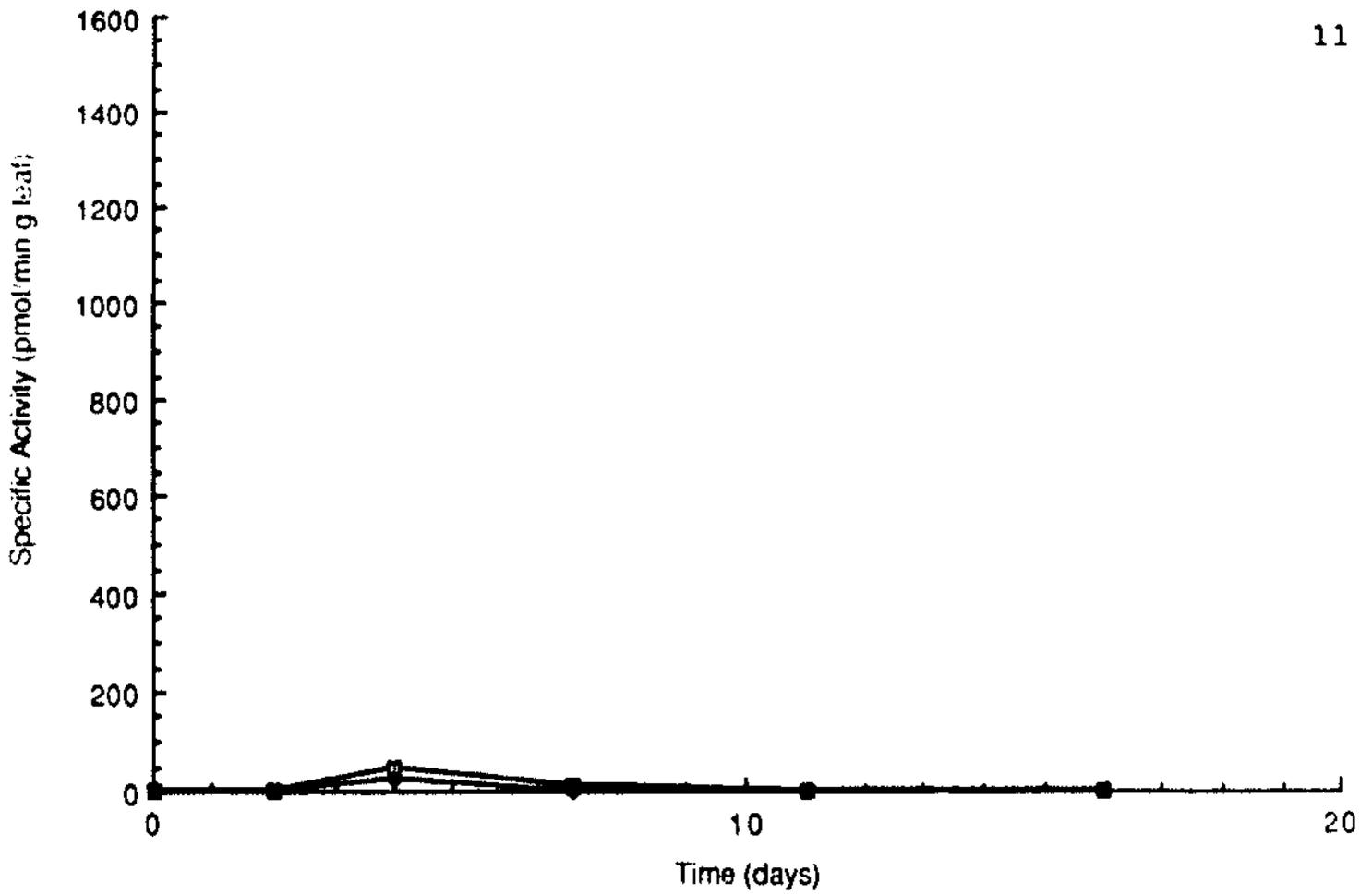


Figure 2: Plot of methyl chloride transferase specific activity over time for whole leaf assays from plants watered with 0 mM salt.

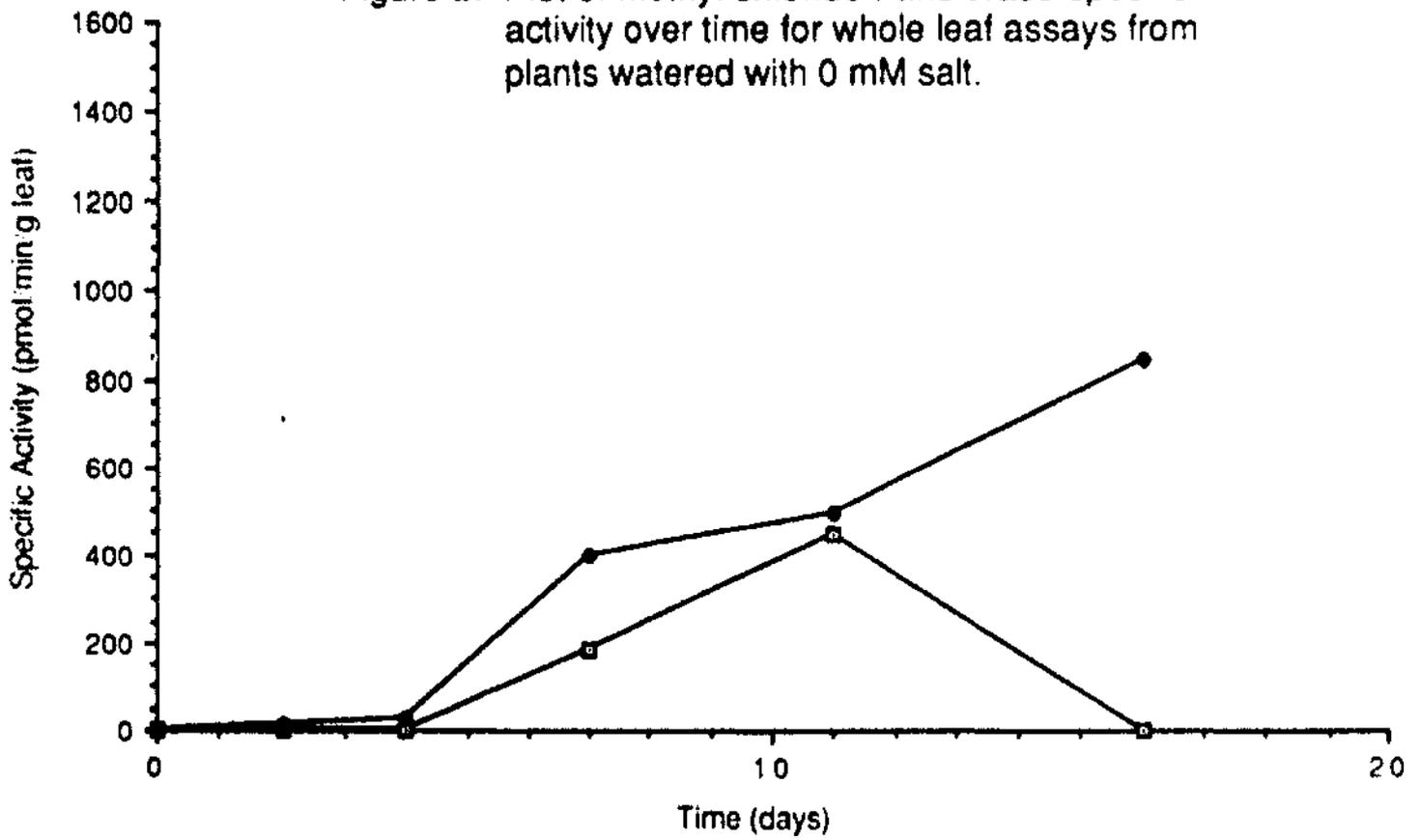


Figure 3: Plot of methyl chloride transferase specific activity over time for whole leaf assays from plants watered with 180 mM salt.

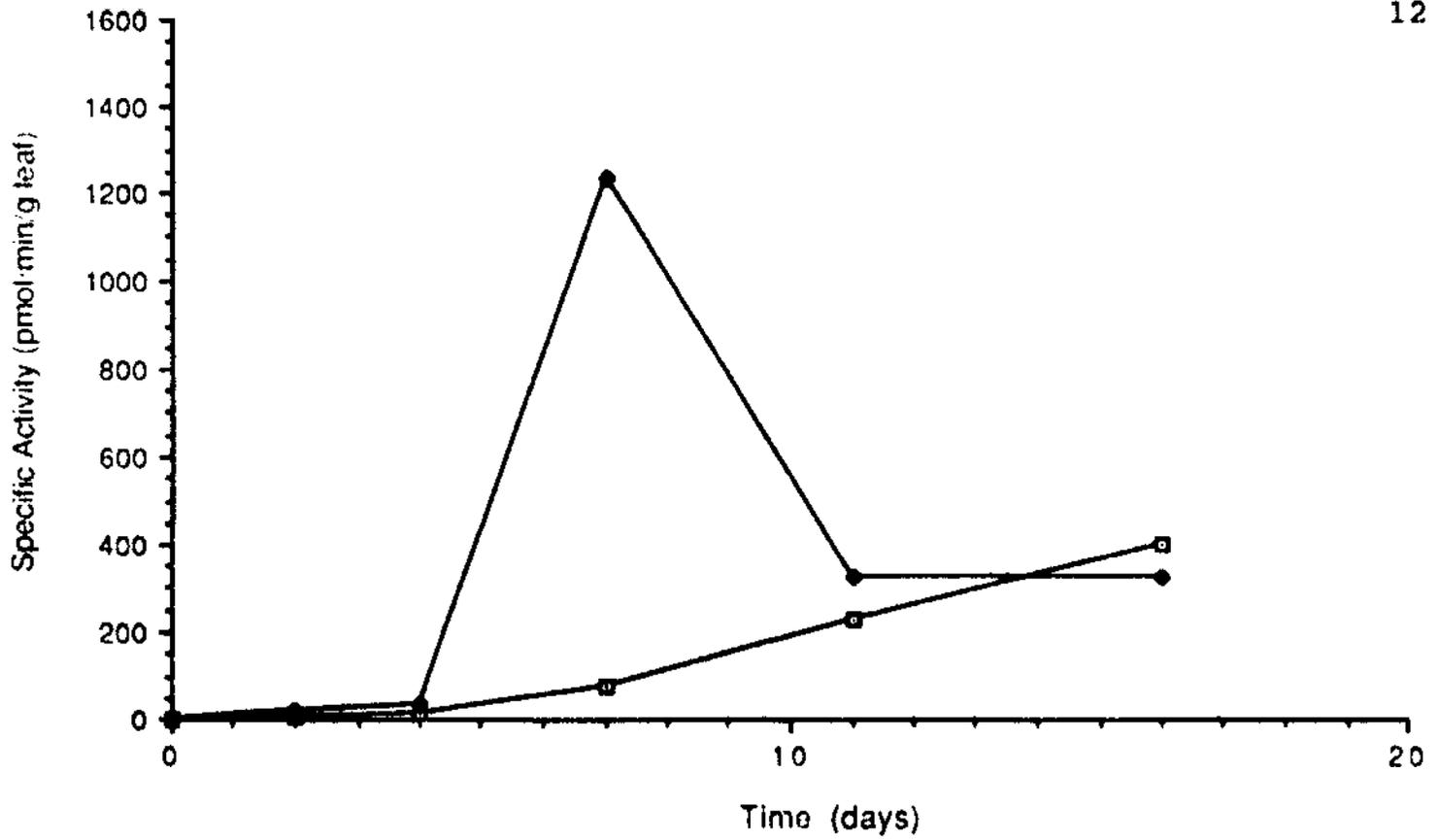


Figure 4: Plot of methyl chloride transferase specific activity over time for whole leaf assays from plants watered with 360 mM salt.

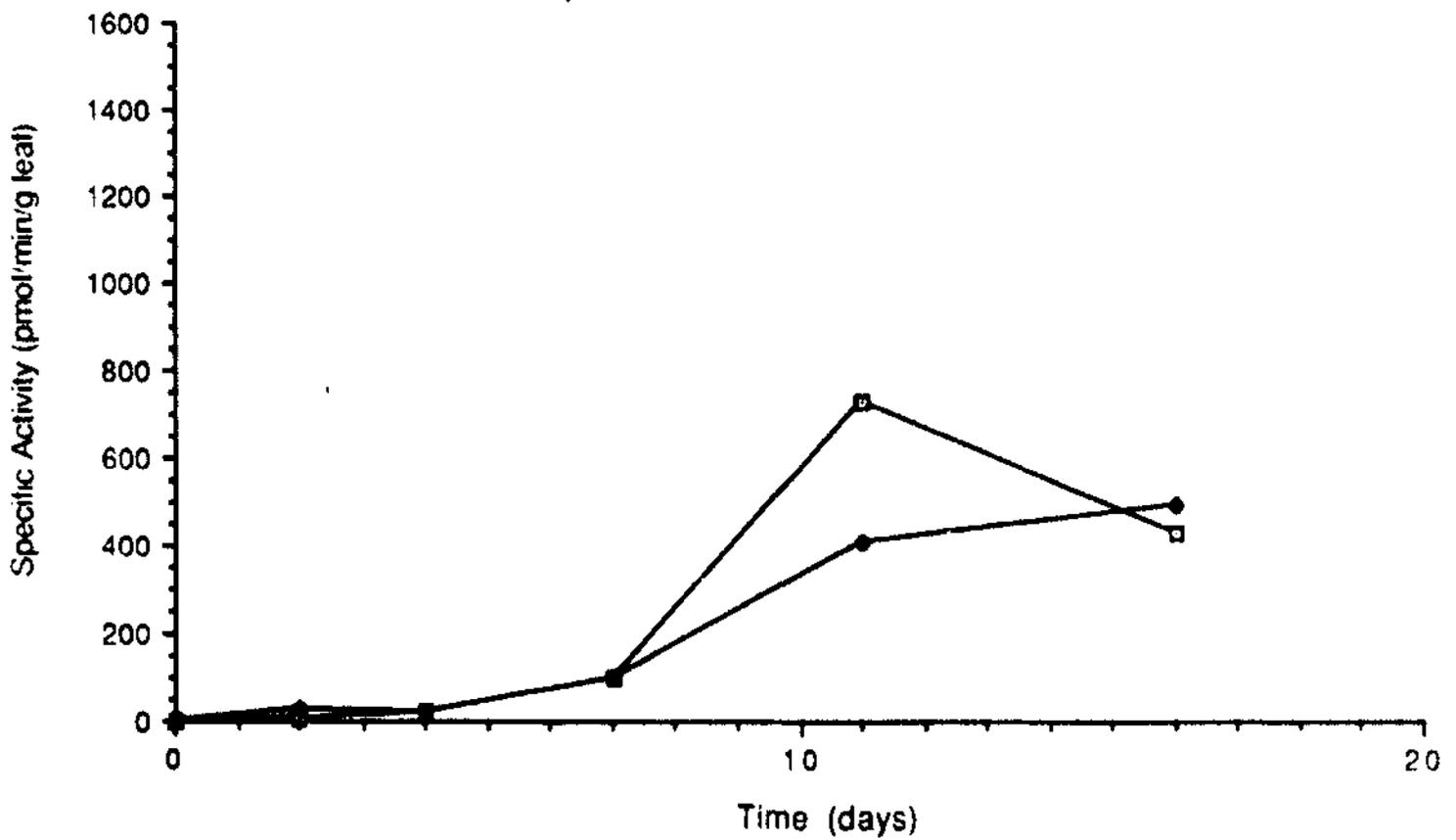


Figure 5: Plot of methyl chloride transferase specific activity over time for whole leaf assays from plants watered with 540 mM salt.

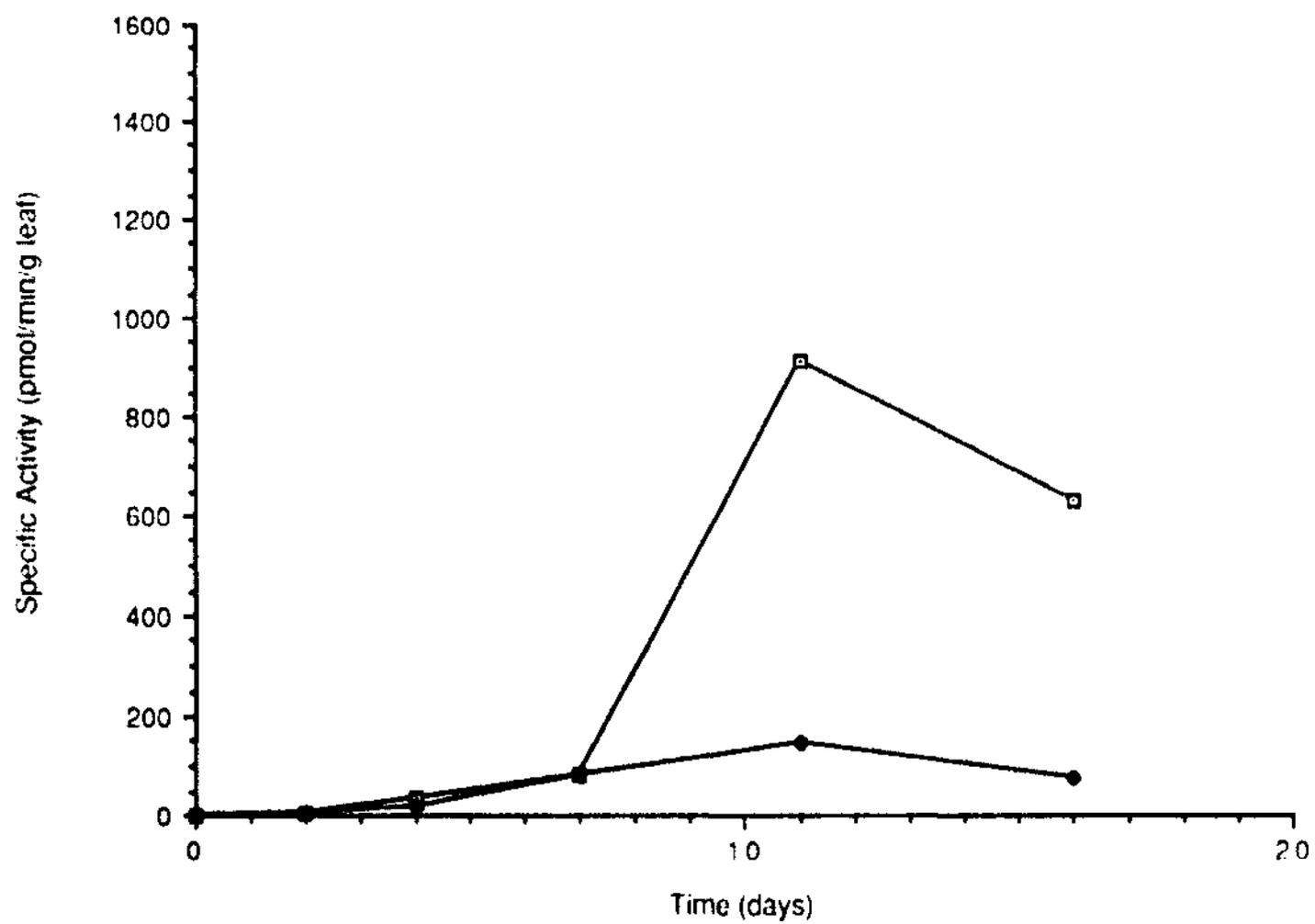


Figure 6: Plot of methyl chloride transferase specific activity over time for whole leaf assays from plants watered with 720 mM salt.

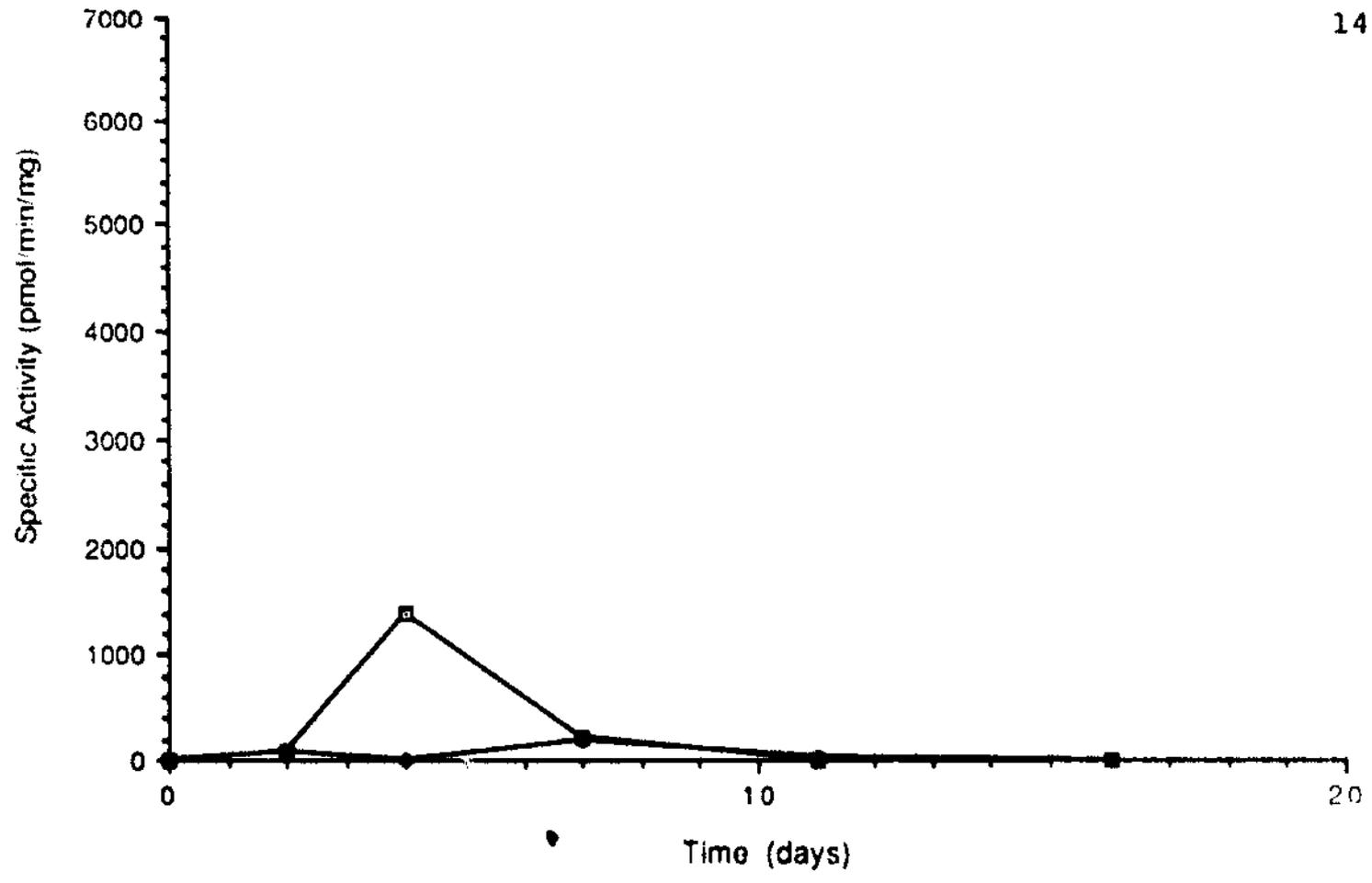


Figure 7: Plot of methyl chloride transferase specific activity over time for ground extracts from plants watered with 0 mM salt.

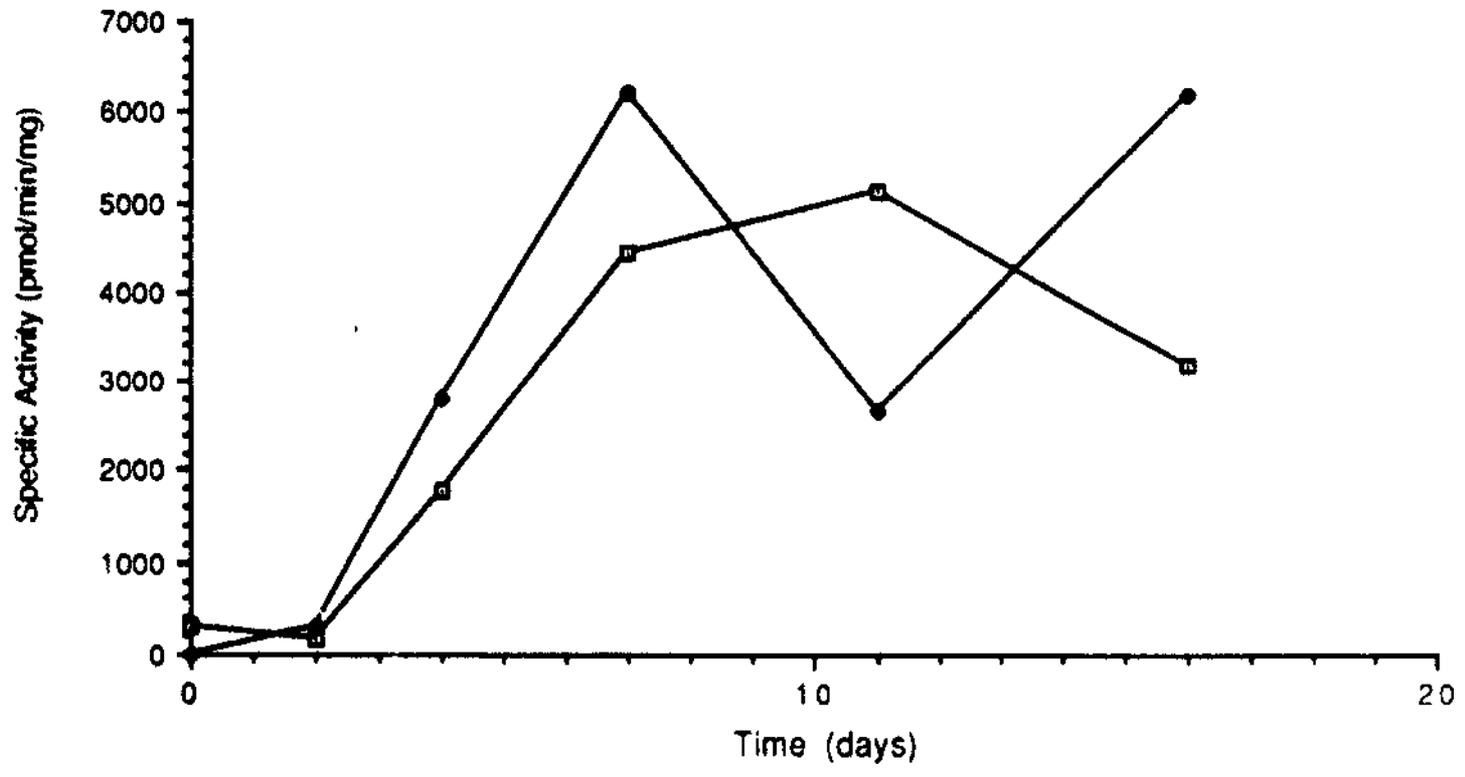


Figure 8: Plot of methyl chloride transferase specific activity over time for ground extracts from plants watered with 180 mM salt.

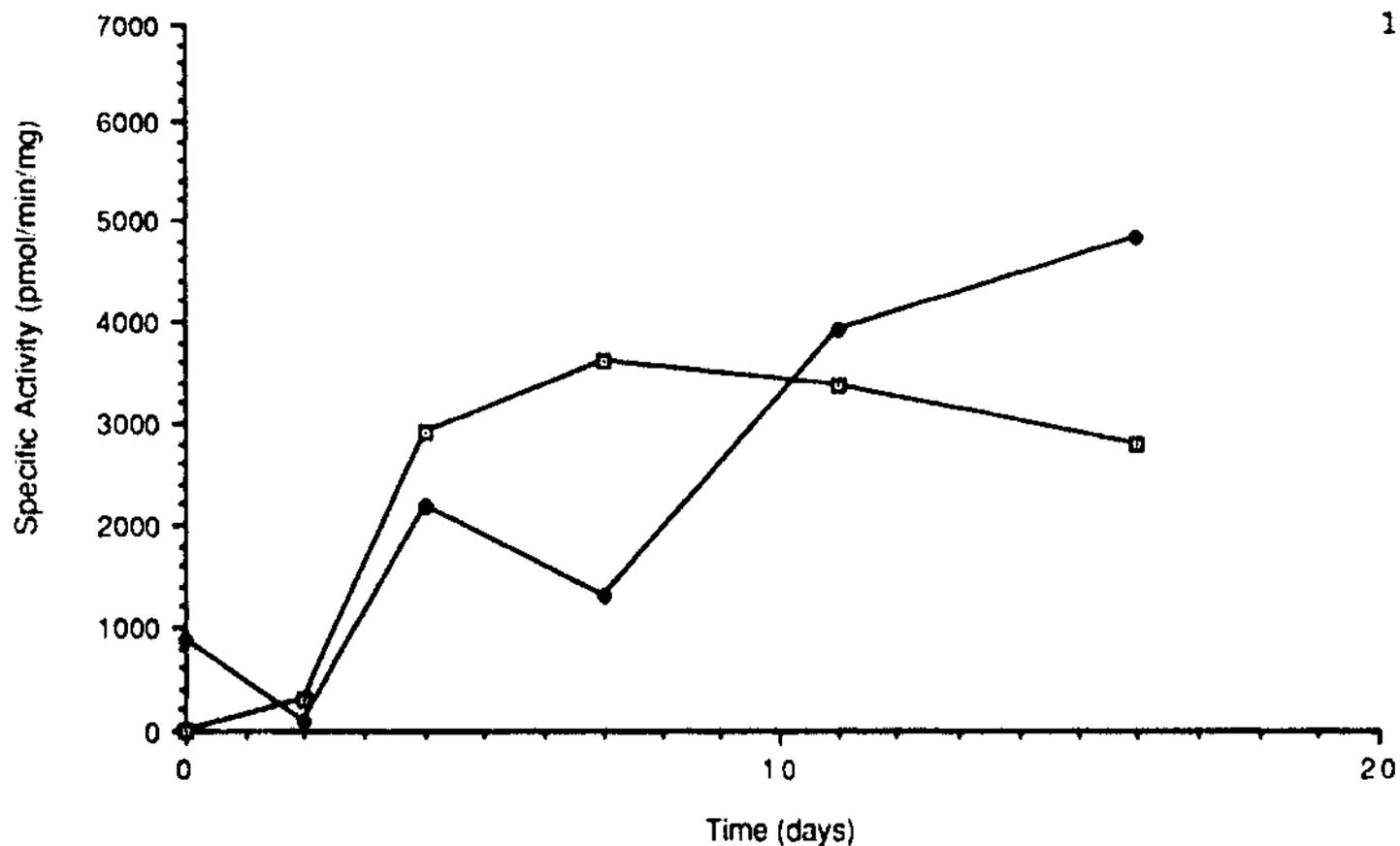


Figure 9: Plot of methyl chloride transferase specific activity over time for ground extracts from plants watered with 360 mM salt.

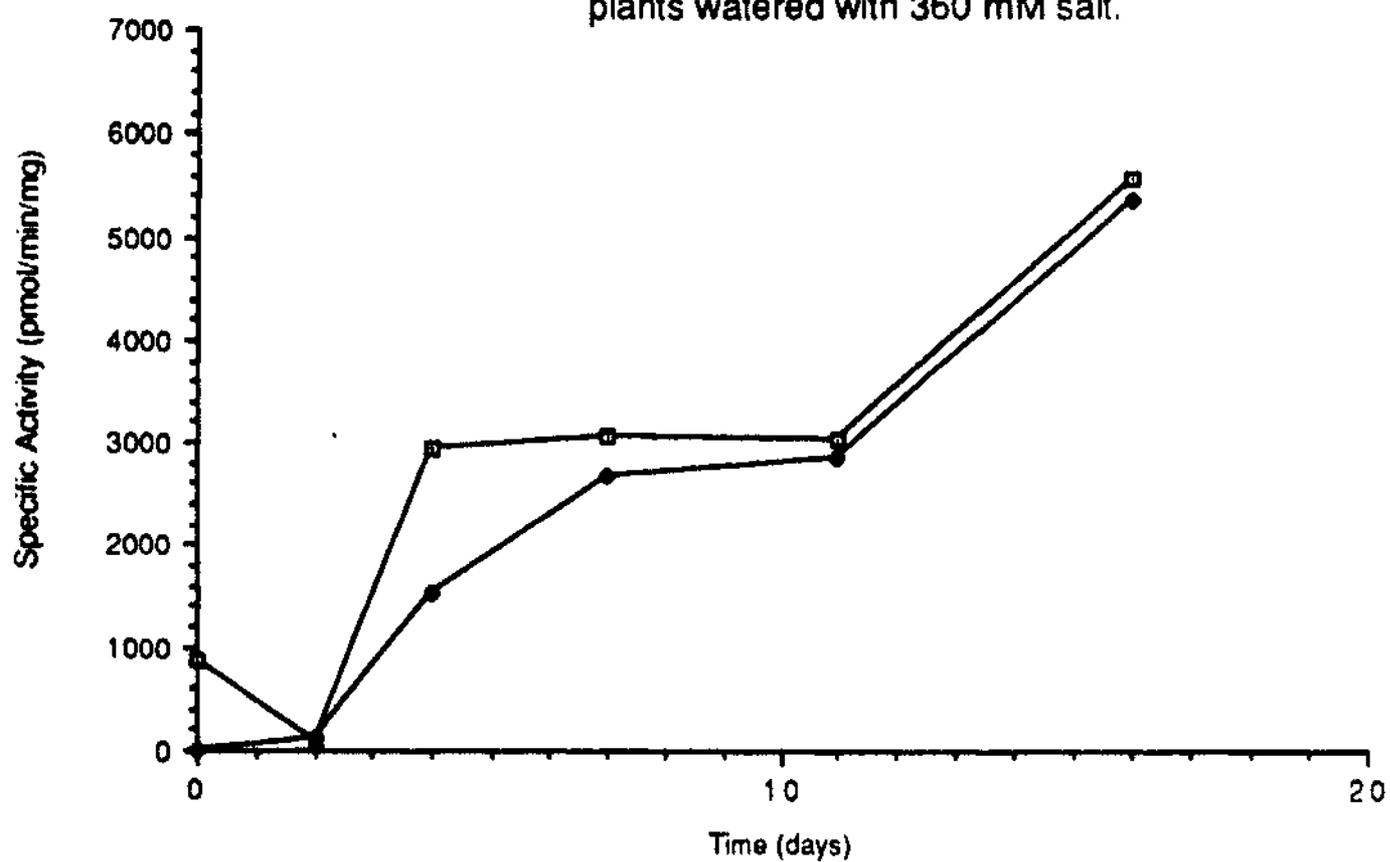


Figure 10: Plot of methyl chloride transferase specific activity over time for ground extracts from plants watered with 540 mM salt.

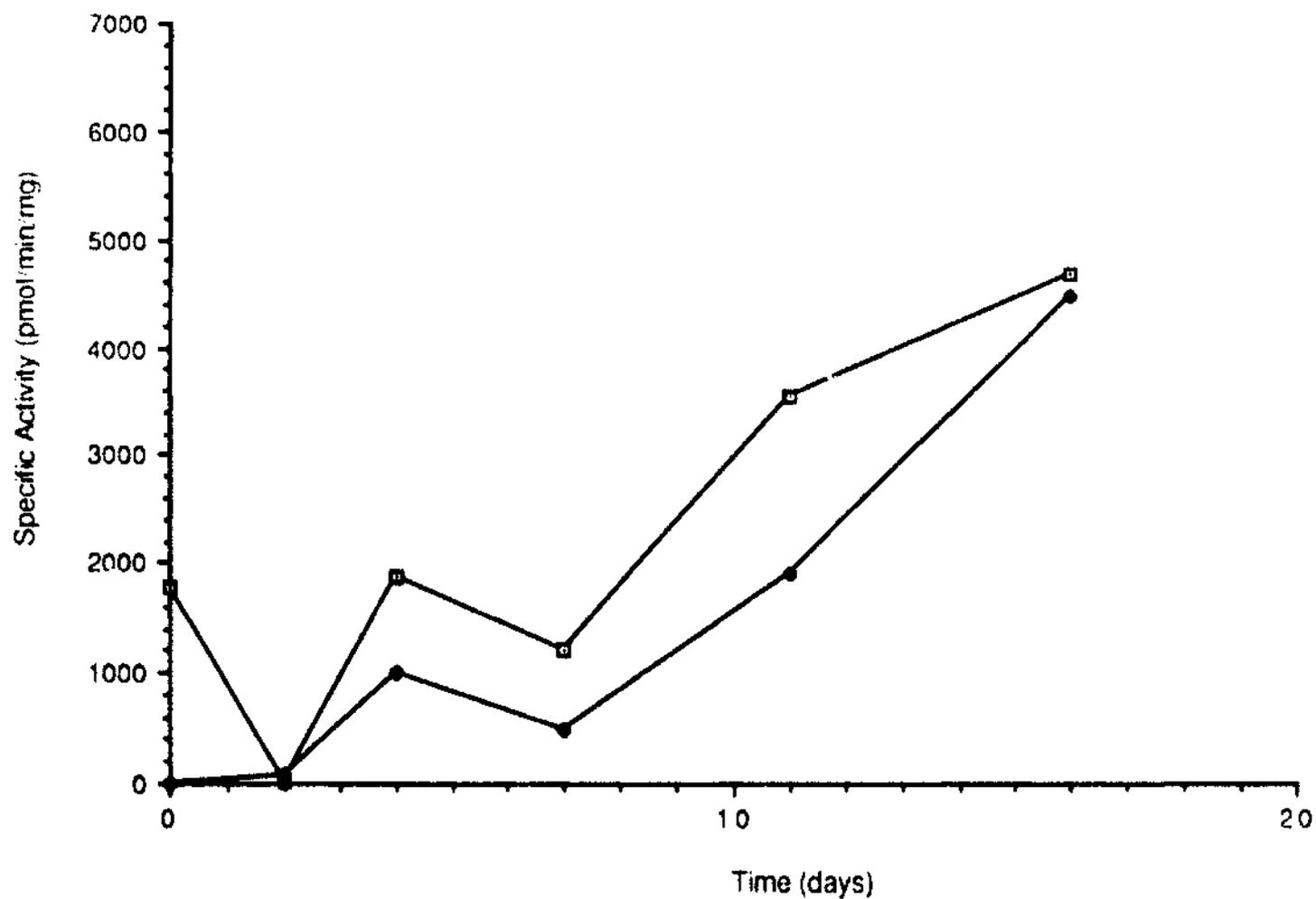


Figure 11: Plot of methyl chloride transferase specific activity over time for ground extracts from plants watered with 720 mM salt.

IV. Discussion

A. Whole Leaf Assays

Overall, the whole leaf assays worked well for at least one of the two plants at each salt concentration. However, even in the assays that did work well there were fluctuations in the specific activities from day to day. At times, the specific activity would be discernable one day, and then zero the next. The reason the specific activities fluctuated so much is due to the preparation of the leaves for assay. In the whole leaf assays, the leaves were disrupted manually by using a spatula. This was meant to disrupt the plant cells to allow the bromide ions to enter the plant cells and be used as substrate in the enzymatic reaction. S-adenosyl methionine, the other substrate in the reaction, is endogenous to most living organisms. Therefore, none was added to the reaction mixture. Even though there were some fluctuations in the assay results, the effect of increased salt concentrations on enzymatic activity is clearly interpretable from the figures.

B. Ground Leaf Assays

The assays using completely ground leaf extracts worked quite well. Figures 7 through 11 undoubtedly confirm the hypothesis of methyl chloride transferase being induced by higher concentrations of salt in the nutrient solutions. The problem of specific activity variability from day to day was not as prominent in the ground leaf assays. However, there were occasions when specific activities were unexpectedly low. This

is most likely due to the error in statistical sampling of leaves from one plant to the next. The reaction mixtures that had lowered enzymatic activity probably contained extracts from leaves that were not fully mature or were under environmental stresses and, therefore, not turning on the gene for methyl chloride transferase in order to deal with the stress that it was under at that time. Overall, the assays worked very well to show the effect of increased salt levels on expression and activity of methyl chloride transferase.

C. Methyl chloride transferase

In general, higher NaCl concentrations elicited high enzymatic activities. The highest enzymatic activities were seen in the ground leaf extracts. Their specific activities ranged from 88 to 6215 picomoles of methyl bromide per minute per milligram of protein. This compares favorably with other methyl transferase enzymes, which have specific activities in the range of 166 to 5800 picomoles per minute per milligram of protein (8). The highest enzymatic activities were seen in the ground leaf extracts from the plants watered with nutrient enriched salt solutions of 180 mM concentration. Interestingly, this corresponds to the exact salt concentration that Glenn and O'Leary (6) found *Batis maritima* to have the fastest relative growth rate at 180 mM salt and the lowest at 0 mM and 720 mM salt. My own personal observations confirm this to be true. Perhaps these halophytic plants have evolved to be salt dependent instead of solely salt tolerant.

D. Conclusion

Clearly, all observations point to methyl chloride transferase expression being induced by increased halide concentrations. More research needs to be done in order to isolate and clone the gene for the enzyme. It would be of immeasurable benefit to people in arid, semi-arid, and coastal regions of the world if researchers could introduce the methyl chloride transferase gene into soybeans and be able to make viable soybeans that could be raised in the hostile salt environments of coastal and desert regions.

V. References

1. Jacobsen, H. (1974) Lexicon of Succulent Plants. Blandford Press Ltd., London, 110-111.
2. Wessells, N. K. and Hopson, J. L. (1988) Biology. Random House, New York, New York, 694.
3. Wuosmaa, A. M. and Hager, L. P. (1990) *Science*. 249, 160-162.
4. Rasmussen, R. A., Rasmussen, L. E., Khalil, M. A., and Dalluge, R. W. *J. Geophys. Res.* 85, 7350.
Crutzen, P. J., Heidt, L. E., Krasnee, J. P., Pollock, W. H., and Seiler W. (1979) *Nature*. 282, 253-256.
5. Gschwend, P. M., MacFarlane, J. K., and Newman, K. A. (1985) *Science*. 227, 1033.
Manley, S. L. and Dastoor, M. H. (1987) *Limnol. Oceanogr.* 32, 709.
Harvey, G. R., quoted in Chameides, W. L. and Davis, D. D. (1980) *J. Geophys. Res.* 85, 7383.
6. Glenn, E. P. and O'Leary, J. W. (1984) *Plant, Cell and Environ.* 7, 253-261.
7. Nicholls, J. E. (1990) A Beginner's Guide to Hydroponics. Running Press, Philadelphia, 59-100.
8. Hitt, M. M., Wu T., Cohen, G., and Linn, S. (1988) *J. Biol. Chem.* 263, 4392.
Glick, J. M. and Leboy, P. S. (1977) *J. Biol. Chem.* 252, 4790.
Eicher, D. C., Raber, N. K., Shumard, C. M., and Eales, S. J. (1987) *Biochemistry* 26, 1639.

VI. Acknowledgements

First, I would like to thank Dr. Lowell Hager for allowing me to perform undergraduate research in his laboratory. This experience has helped me to learn from my mistakes and build upon the knowledge gained from them. I would also like to thank May Berenbaum and the Department of Entomology at the University of Illinois for the use of their greenhouse. Also, I would like to thank Edward Glenn at the Environmental Research Laboratory at the University of Arizona in Tucson for setting up the trip to CEDO in Puerto Penasco, Mexico and for making all the arrangements for the bringing back of live plants. Thank yous are also in order for Peggy Boyer and her husband at CEDO. Their hospitality and help in identifying all the halophytic species collected will not be forgotten. Last, but certainly not least, I would like to express my extreme gratitude and indebtedness to Anne Marie Wuosmaa. Her help with every aspect of this project was crucial to the positive results seen in the end. Without Anne, my research would have come to a grinding halt every time there were problems with the gas chromatograph. However, Anne was always there to remedy the problem. I learned a lot from Anne, and I think that I made a good friend along the way. I want to thank Anne from the bottom of my heart for all her help and kindness. I wish her and Dave all the luck in the world in their future endeavors.

Part Two: The Effect of Nutrient Availability on
the Expression and Activity of Chloroperoxidase
from the fungus *Caldariomyces fumago*

I. Introduction

A. Caldariomyces fumago

Caldariomyces fumago is a filamentous fungus that was first described in 1849 by Montagne and placed in the genus *Caprodium* (1). Since then, *C. fumago* has assumed many different rubrics. However, the current classification is Fungi (kingdom), Eumycota (division), Deuteromycotina (sub-division), Hyphomycetes (class), Moniliales (order), *Caldariomyces* (genus), and *fumago* (species) (2). Though the history and taxonomy may interest some, the Hager lab is interested in *C. fumago* for another reason. When *C. fumago* mycelium is grown on a simple and inexpensive medium that contains inorganic salts and a specific sugar, the fungus will secrete an enzyme called chloroperoxidase (CPO). Protein purity in the liquid media has been judged to be 90 to 95% of the total extracellular protein material (3). *C. fumago* grown in culture bottles or in other continuous expression systems commonly secretes CPO into the culture medium at levels between 500 mg/L and 1 g/L (3). CPO, which can catalyze a number of different reactions, is discussed in more detail below.

B. Chloroperoxidase

While studying the chlorine metabolism of several fungi, Clutterbuck et al. (4) discovered the production of caldariomycin (2,2-dichloro-1,3-pentanediol) by *C. fumago*. Subsequently, caldariomycin was found to be peroxidatively chlorinated by CPO (5). In 1966 CPO was purified by Morris and Hager and shown to have a molecular weight of 42,000 daltons, is heavily

glycosylated, and has a single prosthetic heme group (iron protoporphyrin IX) (6). The heme exhibits a Soret absorbance peak at 400 nm with an extinction coefficient of $85,000 \text{ M}^{-1} \text{ cm}^{-1}$ (3). CPO has been shown to halogenate numerous compounds using chloride, bromide, and iodide (7). However, bashing the myth of enzymes having extensive substrate specificities, CPO has been shown to have catalase and halo-peroxidase activity (8), along with epoxidation (9), N-demethylation (10), and sulfoxidation activities (11). Currently, the Hager lab, in conjunction with Dr. Joshua Wand at the University of Illinois, is in the process of deducing the three dimensional structure of CPO using NMR.

C. Project Goal

Originally, the production of CPO was performed using media that contained glucose and malt as the carbon sources (4)(5). In 1981, Pickard (12) observed that removal of malt from the media and substitution of fructose for glucose resulted in increased production of CPO. Recently, it was shown that expression of CPO is regulated at the transcriptional level, with glucose inhibiting and fructose stimulating mRNA production (13). All aspects of the culturing of *C. fumago* and media components have been well defined except for iron concentration. Many people have tried various iron concentrations, but no one has attempted to find the optimal concentration of iron that will exact the highest rate of CPO expression. It is the goal of this project to find at what iron concentration, if any, that the optimal rate of CPO synthesis occurs. Along with this experiment, alternative

carbon sources will be explored to see the effect that they have on expression of CPO.

11. Materials and Methods

A. C. fumago Preparation

In a sterile hood, the mycelial carpet of a stock culture of *C. fumago* grown on a 3% Potato Dextrose Agar (PDA) plate was lifted off with a sterile spatula and placed inside a 350 mL Mason jar. To the jar, 250 mL of 10% Glucose/Yeast/Salts (GYS) media was added. (See the Appendix for the recipes of all media). Glucose is used to grow mycelia on the PDA plates because the fungus appears to be more viable in the presence of glucose while residing on the PDA plates (14). The jar was then fitted with a lid containing a homogenizer protruding through the lid. The whole unit was then attached to a 115 V Sorvall Omni Mixer connected to a variable voltage selector. The mycelia were ground for 10 seconds at 45 volts. The lid was removed and a series of fresh 3% PDA plates were inoculated with the homogenate. The plates were then allowed to grow at room temperature for five days or until covered with a carpet of mycelium.

B. Bottle Culture Preparation

Once the culture had formed a thick carpet over the agar surface, the mycelia were once again lifted off a plate and placed in the jar containing 250 mL of 10% Fructose/Yeast/Salts (FYS). Fructose was the sugar of choice when grinding the mycelia to put in the culture bottles because fructose stimulates, while glucose inhibits, the production of CPO mRNA (13). The mycelia were once again ground at 45 volts for ten

seconds. 125 mL of the homogenate was used to inoculate a 490 cm² (108.7 x 143.9 mm) sterile tissue culture roller flask (Corning) lined with autoclaved miracloth (Calbiochem). The lid of the roller flask was equipped with a Millex FG₅₀ 0.2 μm (50 mm) male/male air filter (Millipore). Twelve additional bottles were inoculated with 125 mL of mycelial homogenate each. All bottles were placed in a 19 °C room on a cell production roller apparatus (Bellco) which was set at a rate of 2 R.P.M.

C. CPO Production Preparation

After four days, all of the mycelial cells in the bottles had stuck to the miracloth. The FYS was then drained from the bottles and replaced with media solutions in each bottle as follows:

- #1--125 mL of 10% FS and 0 g/L (0 μM) of iron solution.
- #2--125 mL of 10% FS and 0.01 g/L (36 μM) of iron solution.
- #3--125 mL of 10% FS and 0.02 g/L (72 μM) of iron solution.
- #4--125 mL of 10% FS and 0.03 g/L (108 μM) of iron solution.
- #5--125 mL of 10% FS and 0.04 g/L (144 μM) of iron solution.
- #6--125 mL of 10% FS and 0.1 g/L (360 μM) of iron solution.
- #7--125 mL of 10% FS and 0.2 g/L (720 μM) of iron solution.
- #8--125 mL of 10% FS and 0.4 g/L (1440 μM) of iron solution.
- #9--125 mL of 10% FS and 0.6 g/L (2160 μM) of iron solution.
- #10--125 mL of 10% Succinate and salts (SS) of 0 g/L iron.
- #11--125 mL of 10% SS and 0.02 g/L (72 μM) of iron solution.
- #12--125 mL of amino acids and 0 g/L of iron solution.
- #13--125 mL of amino acids/salts and 0.02 g/L iron solution.

The bottles were then returned to the 19 °C room and placed on the roller apparatus. Lastly, the air filters on the lids were attached to an oxygen tank set at a pressure of 1.8 lbs/in².

D. MCD Assay

Assays on the series of bottles were begun the day following the addition of the CPO production media. The standard assay used is a spectrophotometric assay where a decrease in absorbance is followed. Monochlorodimedon (MCD) is a strong chromophore ($\epsilon = 1.22 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) while dichlorodimedon (DCD) is a weakly absorbing chromophore ($\epsilon = 30 \text{ M}^{-1} \text{ cm}^{-1}$) (15). Chloroperoxidase chlorinates MCD to DCD, therefore, causing an absorbance decrease. Stock solutions of 0.16 mM MCD (Sigma)/20 mM KCl in 10 mM Potassium phosphate buffer at pH 2.75 and 120 mM H₂O₂ were made. In a quartz cuvette with a light path of 1 cm, 3 mL of the MCD/KCl solution and 50 μL of the H₂O₂ were mixed. The cuvette was then placed in a Cary 219 uv/vis spectrophotometer. Under standard assay conditions of a range of 2, a chart display of 5, and a wavelength of 278 nm, the MCD/H₂O₂ solution had an absorbance of approximately 2. Then, 10 μL of the CPO medium from a roller bottle was added to the cuvette using a plastic mixing rod, and the decrease in absorbance was measured. From the slope of the line (abs. change/time), taking into account any dilution made, the activity of CPO in milligrams per liter can be calculated (see Appendix).

III. Results

A. Effect of Iron on CPO Production

Figures 12a and 12b show the effect of the various concentrations of iron on the production of CPO. A general trend of increasing CPO activity with increasing iron concentration can be seen. Higher iron concentrations elicited CPO production that was accelerated in comparison to lower iron concentrations. One unexpected result seen was that the bottle with no exogenous iron added to the media exhibited a noticeable increase in CPO activity after a few days. The explanation for this effect will be given later. No iron concentrations in this experiment were found to demonstrate any toxicity toward *C. fumago* or the production of CPO. The bottles were only assayed nine days due to time constraints.

B. Succinate as a Carbon Source

Figure 13 shows the effect of using succinate as a carbon source on the production of CPO. Clearly, the production of CPO by *C. fumago* when succinate is the sole source of carbon is attenuated. Even in the presence of 0.02 g/l. iron, CPO production is very limited.

C. Effect of Amino Acids on CPO Production

As with the case of using succinate as the carbon source, using amino acids instead of fructose does not lead to high CPO production. Adding inorganic salts and iron to the amino acids had a minimal effect on the production of CPO. This can be seen in Figure 14.

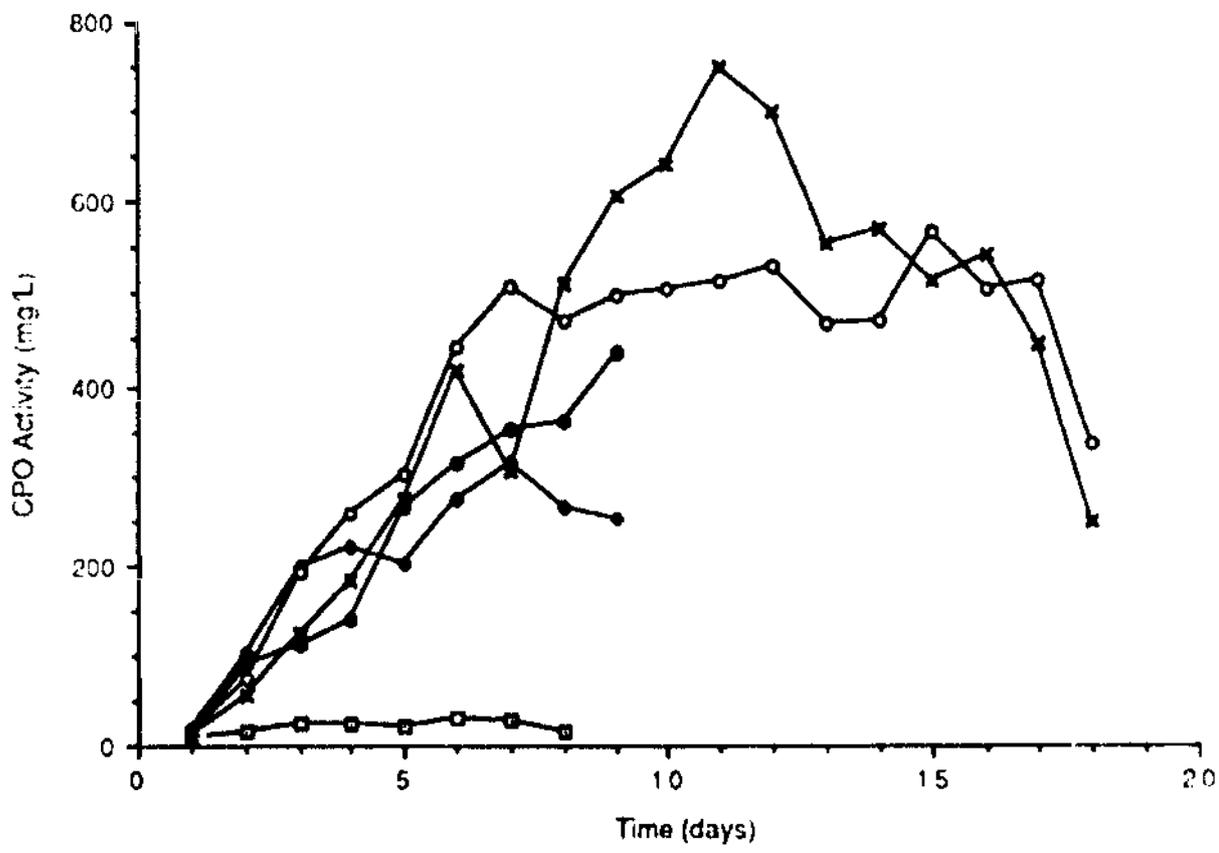


Figure 12a: Plot of CPO Activity over time as a function of iron concentration.

- 0 g/L Fe
- 0.01 g/L Fe
- 0.02 g/L Fe
- ◆— 0.03 g/L Fe
- 0.04 g/L Fe

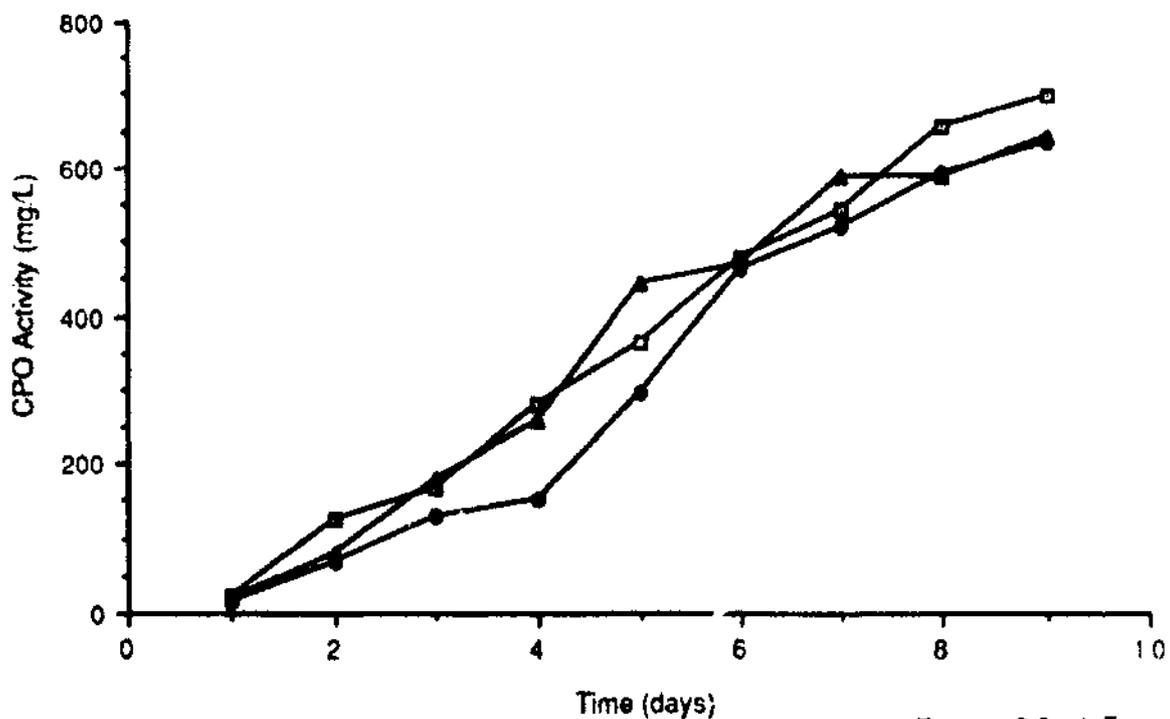


Figure 12b: Plot of CPO Activity over time as a function of iron concentration.

- 0.2 g/L Fe
- ◆— 0.4 g/L Fe
- 0.6 g/L Fe

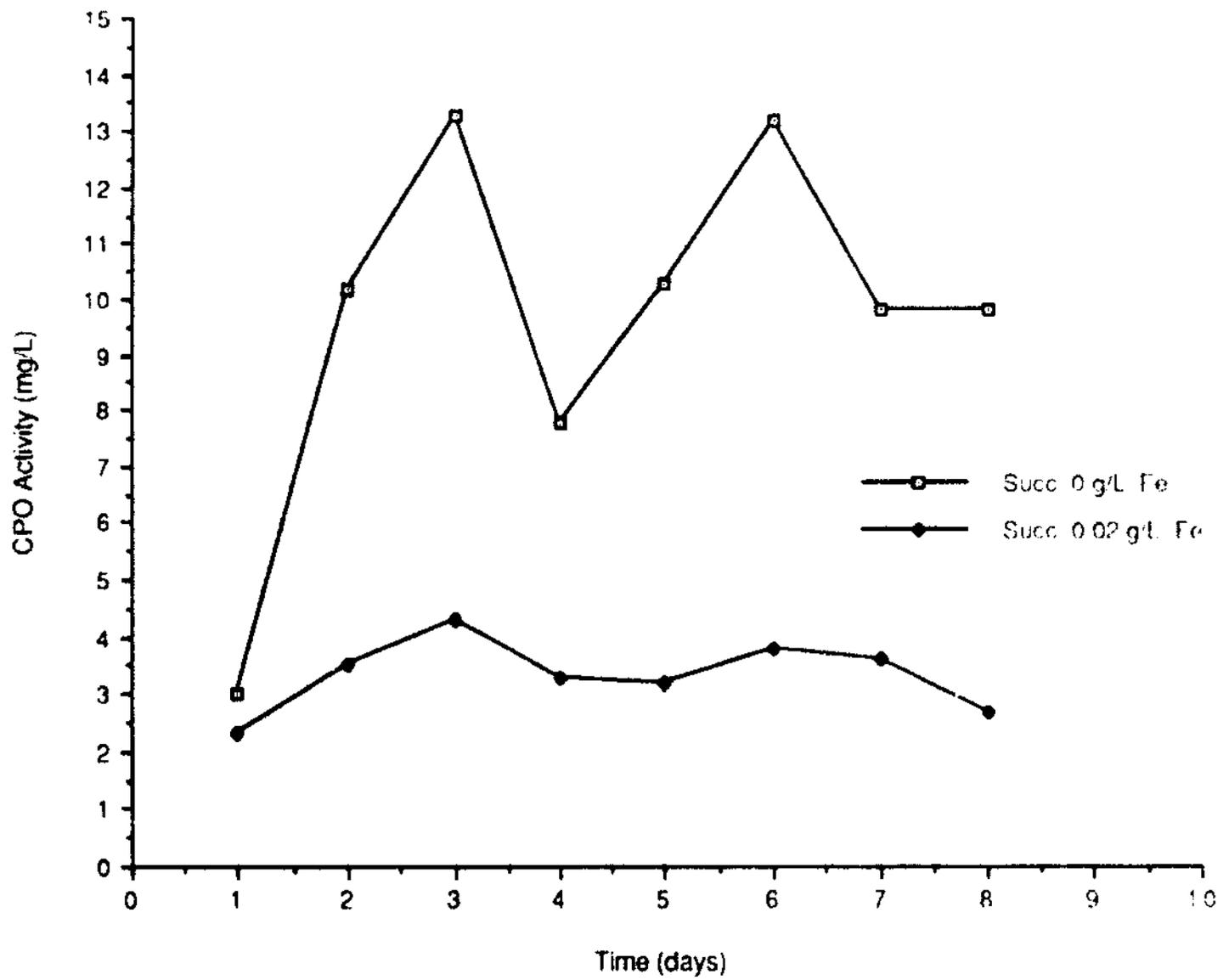


Figure 13: Plot of CPO activity over time for bottles with succinate as the sole carbon source.

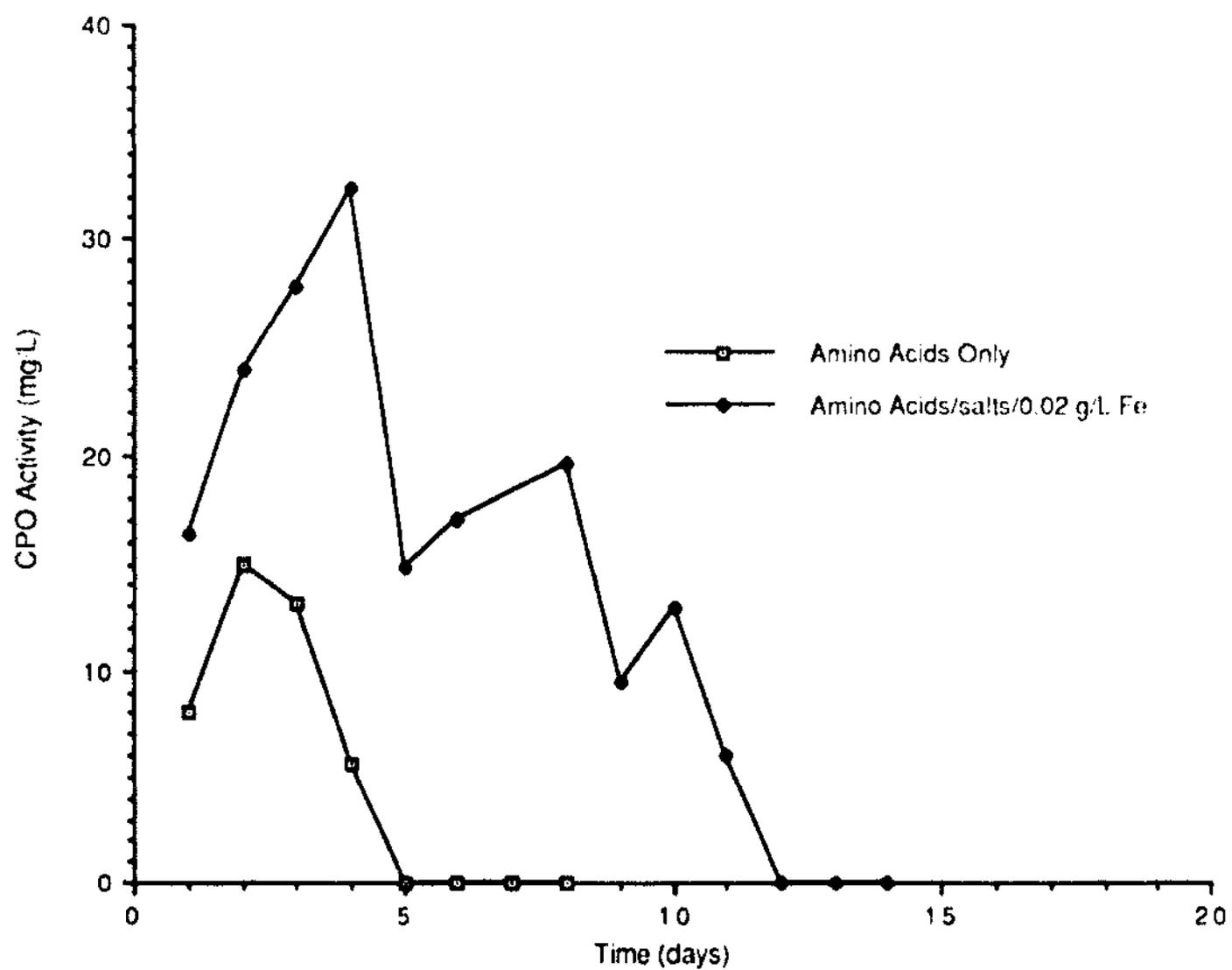


Figure 14: Plot of CPO activity over time for bottles with amino acids as the nutrient source.

IV. Discussion

A. Effect of Iron

As stated before, the CPO production and rate of production followed the general paradigm of increasing with increasing concentrations of iron. This can be rationalized by concluding that since CPO is an iron protoporphyrin IX heme containing enzyme, an increase in the availability of iron could make an increase in CPO production feasible. This appears to be what could be happening. Knowing little about what controls the initiation of transcription at the CPO gene, one could postulate that *C. fumago* may have an environmental sensing system that detects an increase in iron availability. That, in turn, could turn on transcription at the CPO gene, and possibly at other ferric heme protein genes.

Although bottles with higher iron concentrations did show significant amounts of CPO activity, a discernible amount was also seen in the bottle that contained no exogenous iron. This can be easily explained. The medium used to grind the fungus up is FYS. Communication with Sigma Chemical (16) led to the finding that the yeast extract has a 3.3 mg/L contaminant of iron. This is huge in comparison to the 1 part per million contaminant of iron in the inorganic salts in the medium. Thus, the increase in CPO production in the bottle with 0 g/L iron is most likely due to the uptake of the iron contaminant while in the stage of sticking to the wall of the bottle. After the FYS is drained and FS is added, the iron is used to make CPO and

other iron containing proteins.

One last source of iron for the fungus in the bottle with no added iron is the death of other fungal cells, and the subsequent release of its cytoplasmic components. Cells could take up free iron from the dead cell or degrade the dead cells iron containing proteins.

B. Succinate as a Carbon Source

Figure 13 shows the effect of using succinate as the sole source of carbon. Very little CPO was made during the course of this experiment. Also, one could visibly see that the growth of the mycelia was severely retarded. This can be easily understood. Succinate is a highly oxidized compound. If *C. fumago* produces its reducing equivalents for oxidative phosphorylation through a Krebs cycle, then only one mole of FADH_2 and one mole of $\text{NADH} + \text{H}^+$ are made in the oxidation of succinate to oxaloacetate. These reducing equivalents effectively represents 5 moles of ATP. If fructose is used as the carbon source, it will be isomerized into glucose, and then proceed through glycolysis, the Krebs cycle, and oxidative phosphorylation. This will lead to the generation of approximately 38 moles of ATP. Clearly, the use of succinate cannot supply enough ATP to the fungus to fuel all of its biosynthetic reactions. This is plainly seen in the appearance of the growing mycelia.

C. Effect of Amino Acids

Growth of the mycelia on a medium that contained amino

acids as the source for the cells biosynthetic starting molecules did not yield high CPO production even when salts and iron were added. One could explain this effect by saying that *C. fumago* does not have the correct enzymes or enzymatic pathways to change the amino acids into the metabolites that are need. However, previous attempts at growing *C. fumago* on amino acids in order to produce CPO have been successful. It is not clear the reasons why this result could not be replicated in this experiment.

D. Conclusion

Nearly all iron concentrations explored in this project yielded CPO activities in the 500 mg/L to 1 g/L range, except for the 0 - 0.01 g/L iron concentrations. Higher iron concentrations resulted in CPO being produced at accelerated rates. However, a toxic level of iron could not be found. More research needs to be done on the control of the CPO gene to find out the exact environmental controls that turn on the gene. It would be interesting to know if the level of iron does play a significant role in the control of the gene.

V. References

1. Friend, R. J., (1965) *Trans. British Mycol. Soc.* 48, 371-375.
2. Hanlin, R. T. and Ulloa, M. (1979) Atlas of Introductory Mycology. Hunter Publishing Co., Winston-Salem, N.C.
3. Patterson, W. (1993) Doctoral thesis. University of Illinois. In print.
4. Clutterbuck, P. W., Mukhopadhyay, S. L., Oxford, A. E., and Raistrick, H. (1940) *Biochem. J.* 34, 664-677.
5. Shaw, P. D. and Hager, L. P. (1961) *J. Biol. Chem.* 263, 1626-1630.
6. Morris, D. R. and Hager, L. P. (1966) *J. Biol. Chem.* 241, 1763-1768.
7. Hager, L. P., Morris, D. R., Brown, F. S. and Eberwein, H. (1966) *J. Biol. Chem.* 241, 1769-1777.
Thomas, J. A. and Hager, L. P. (1969) *Biochem. Biophys. Res. Commun.* 35, 444-450.
8. Thomas, J. A., Morris, D. R. and Hager L. P. (1970) *J. Biol. Chem.* 245, 3129-3142.
9. McCarthy, M. B. and White, R. E. (1983) *J. Biol. Chem.* 258, 9153-9158.
10. Kedderis, G. L., Koop, D. R. and Hollenberg, P. F. (1980) *J. Biol. Chem.* 255, 10174-10182.
11. Geigert, , J., DeWitt, S. K., Neidleman, S. L., Lee, T. D., Daliotos, D. J. and Moreland, M. (1983) *Biochem. Biophys. Res. Commun.* 116, 82-85.
12. Pickard, M. A. (1981) *Can. J. Microbiol.* 27, 1298-1305.
13. Axley, M. J., Kenigsberg, P. and Hager, L. P. (1986) *J. Biol. Chem.* 261, 15058-15061.
14. Patterson, W. Unpublished observations.
15. Blanke, S. (1989) Doctoral thesis. University of Illinois.
16. Craig Krausz's communication with Sigma.

VI. Acknowledgements

I would like to thank Dr. Lowell Hager for giving me the opportunity of performing undergraduate thesis research in his lab. I believe I have learned a lot from the experience and this will help me in my future years in graduate school. I want to also thank the following:

Annette Dexter for always being interested in how things are progressing.

Anne Wuosmaa for her optimistic attitude.

Craig Krausz for his friendship.

Qin Zong for always letting me use the Cary first when he had planned to use it and for his friendliness.

Dr. Pawel Osmulski for all of his help.

Dr. Byron Bertagnolli for his suggestions and his off the wall, and sometimes downright strange, sense of humor.

Finally, I would like to express my extreme gratitude to Eric Allain. Eric took the time out of his busy schedule to show me everything that I had to do in this project and never appeared to mind. Eric was always there to answer any question I had, no matter how trivial it seemed. Congratulations to Eric for winning the award for best presentation at the 1993 University of Illinois Prospective Graduate Student Conference. Thank you Eric for your friendship and all your help.

VII. Appendix

<u>Abbreviation</u>	<u>Contents</u>
FYS	10% Fructose 0.5% Yeast Extract 0.2% NaCl 0.2% MgSO ₄ * (H ₂ O) 0.2% NaNO ₃ 0.2% KH ₂ PO ₄ pH 5.5
GYS	10% Glucose 0.5% Yeast Extract 0.2% NaCl 0.2% MgSO ₄ * (H ₂ O) 0.2% NaNO ₃ 0.2% KH ₂ PO ₄ pH 5.5
FS	10% Fructose 0.2% NaNO ₃ 0.2% MgSO ₄ * (H ₂ O) 0.2% KH ₂ PO ₄ X g/L iron solution pH 5.5
SS	10% Succinate 0.2% MgSO ₄ * (H ₂ O) 0.2% NaNO ₃ 0.2% KH ₂ PO ₄ X g/L iron solution pH 5.5
Amino Acid Solution	100 mg/L Alanine 50 mg/L Arginine 150 mg/L Asparagine 30 mg/L Cysteine 200 mg/L Glutamine 40 mg/L Glycine 20 mg/L Histidine 50 mg/L Isoleucine 70 mg/L Leucine 70 mg/L Lysine 20 mg/L Methionine 50 mg/L Phenylalanine 50 mg/L Proline 150 mg/L Serine 100 mg/L Threonine 30 mg/L Tryptophan 130 mg/L Valine pH 5.5

Amino Acid Solution Plus Salts same [amino acids]
 0.2% $\text{MgSO}_4 \cdot (\text{H}_2\text{O})$
 0.2% KH_2PO_4
 0.2% NaNO_3
 X g/L iron
 pH 5.5

Iron Solution

Dissolve 0.1 grams of $\text{FeSO}_4 \cdot (7\text{H}_2\text{O})$ in 5mL of sterile deionized water. Sterile filter using a 10 cc luer lok disposable syringe attached to a 0.2 μm (25 mm) surfactant free cellulose/acetate syringe filter (Nalgene). Add to the roller bottle a volume of stock iron solution to yield desired final iron concentration in media.

CPO Activity Calculations

$$\frac{\Delta \text{ abs.}}{\text{time}} \times \frac{1}{\text{dilution}} \times 1140^{\text{a}} \text{ mg sec L}^{-1} \text{ cm}^{-1} \times 1 \text{ cm}^{\text{b}} = \text{activity in mg/L}$$

^a Blanke, S. (1989) Doctoral thesis. University of Illinois.

^b Length of the lightpath.