RELATIONSHIP BETWEEN CHLOROPHYLL AND CHLOROPHYLLASE ACTIVITY DURING GREENING AND "GLUCOSE-BLEACHING" OF CHLORELLA PROTOTHECOIDES

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ABSTRACT

RELATIONSHIP BETWEEN CHLOROPHYLL AND CHLOROPHYLLASE ACTIVITY DURING GREENING AND "GLUCOSE-BLEACHING" OF CHLORELLA PROTOTHECOIDES

By

Víctor Guillermo Ganoza

It has been shown that chlorophyll synthesis or degradation in the alga Chlorella protothecoides can be induced by varying the nitrogen to glucose ratio in the culture medium.

Chlorophyll synthesis was found to precede increase in chlorophyllase activity by 2-4 hours. During chlorophyll degradation chlorophyllase activity remained at high levels, suggesting a degradative role.

Inhibitor experiments indicate that chlorophyllase is synthesized in the cytoplasm. Methyl pyropheophorbide a, a competitive inhibitor of the enzyme, showed no effect on chlorophyll synthesis or degradation but affected the chlorophyllase activity pattern in both cases.

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By

Víctor Guillermo Ganoza

A THESIS

Submitted to
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1974

DEDICATION

This thesis is dedicated to my parents

Elena and Guillermo

and to Marylee

who gave me the support, encouragement

and love necessary to make this degree

a reality.

ACKNOWLEDGMENTS

I wish to express my sincerest appreciation to my academic advisor and committee chairman, Dr. Roger F. McFeeters. His aid and guidance were invaluable in the completion of this research.

I would also like to thank Drs. Clifford L. Bedford and Richard W. Luecke, committee members.

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CHAPTER I

INTRODUCTION

Although chlorophyllase was discovered more than half a century ago little progress has been made in assessing its true role in chlorophyll metabolism. Many investigators have studied changes in chlorophyllase activity in relation to chlorophyll synthesis or degradation in different plants and lower organisms. Differences in techniques and results have failed to clarify the *in vivo* role of chlorophyllase.

Hase and coworkers have carried out extensive investigation on the chlorophyll metabolism of the alga *Chlorella protothecoides* (Aoki and Hase, 1964; Shihira-Ishikawa and Hase, 1964; Matsuka and Hase, 1969). Chlorophyll synthesis and degradation in the alga are affected by the nitrogen to glucose ratio in the culture medium. By appropriately varying this ratio, it is possible to obtain cultures that synthesize or degrade chlorophyll. Hase's group, however, was concerned with the mechanisms that trigger the processes of synthesis or degradation rather than specifically with the degradation or synthesis of chlorophyll.

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Chiba et al. (1967) studied the changes in chlorophyllase activity of the alga in relation to chlorophyll synthesis and degradation. They reported that an increase in chlorophyllase activity precedes the appearance of chlorophyll. Rapid synthesis of chlorophyll occurred parallel to an increase in the activity of the enzyme. During chlorophyll degradation, a decrease in enzyme activity parallel to the rate of chlorophyll degradation was observed. Their results are expressed on the basis of a fixed packed cell volume. This causes some problems in reporting the data, especially when cell division occurs during the course of the experiments.

By combining the investigations of Hase and coworkers with those of Chiba et al., much knowledge can be gained on the nature and role of the enzyme in the cell. Using metabolic inhibitors it is possible to eliminate synthesis of undesirable components that could mask the relationship between chlorophyll and chlorophyllase. More specific inhibitors, such as specific inhibitors of the enzyme, could provide definite information as to the true role of the enzyme in vivo.

This study extends the work done by Chiba et al. (1967). Changes in chlorophyll and chlorophyllase activity are reported on the basis of a liter of culture

as well as on a fixed cell number. The per liter expression allows one to compare changes expressed on a constant base, while the fixed cell number expression allows comparison with Chiba's results. Metabolic inhibitors were added to cultures during synthesis and degradation of chlorophyll. Their effects on the processes of synthesis and degradation of chlorophyll, as well as their effect on chlorophyllase activity during these processes were studied.

CHAPTER II

LITERATURE REVIEW

While trying to isolate crystals formed in leaf sections treated with alcohol, Willstätter and Stoll (1913) discovered chlorophyllase. Chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) catalyzes *in vitro* the removal of the phytol group from chlorophyll *a*, chlorophyll *b*, their respective pheophytins, and other phytol esters.

Chlorophyllase is widely distributed in nature. It appears that it is present in all chlorophyll containing plants. An extensive study of its distribution in higher plants has been carried out by Mayer (1930). Chlorophyllase has also been found in gymnosperms and ferns (Willstätter and Stoll, 1913), in purple bacteria (Holden, 1963), and in several families of algae (Barret and Jeffrey, 1964).

The enzyme occurs in all parts of the plant. However, stems, roots, and seeds have a much lower activity than leaves (Holden, 1963). Ardao and Vennesland (1960) suggest that chlorophyllase is localized

in a chlorophyll-lipoprotein complex. Stobart and Thomas (1968) indicated that chlorophyllase of Kalanchoë tissue cultures may be located in lipoprotein aggregates of the stroma.

Chlorophyllase is insoluble in water. It is usually extracted from plant tissues by using detergents or suitable organic solvents (Holden, 1961; Shimizu and Tamaki, 1962). Ogura (1969) successfully extracted chlorophyllase of young tea leaves with water. However, as the leaves matured, chlorophyllase became more difficult to extract.

Since chlorophyllase substrates are also insoluble in water, the reaction has been commonly carried out in an aqueous acetone medium (Willstätter and Stoll, 1913). Klein and Vishniac (1961) devised a method that allows the reaction to proceed in an aqueous medium by using detergents to solubilize substrates. This procedure has been used by Stobart and Thomas (1968) and by McFeeters et al. (1971).

Chlorophyllase in Chlorophyll Synthesis

Although the reaction catalized by chlorophyllase in vitro normally favors pigment hydrolysis, a few investigators have successfully reversed it. Willstätter and Stoll (1913), in their original description of chlorophyllase, reported synthesis of chlorophyll

from chlorophyllide and phytol using an air-dried meal of Heracleum leaves. Shimizu and Tamaki (1963) were able to synthesize chlorophyll from chlorophyllide and phytol and pheophytin from pheophorbide and phytol. Chiba et al. (1967) synthesized chlorophyll from methyl chlorophyllide and phytol. Ellsworth (1971; 1972) reported synthesis of pheophytin both by direct esterification of pheophorbide and by transesterification of methyl pheophorbide with phytol.

Holden (1961) found that chlorophyllase activity increased when etiolated bean seedlings were exposed to the light and chlorophyll synthesis began. Shimizu and Tamaki (1963) followed seasonal changes in tobacco leaves and found that chlorophyllase activity paralleled chlorophyll synthesis and degradation. Chiba et al. (1967) found similar results in cells of C. protothecoides. Ellsworth and Aronoff (1968) and Aronoff et al. (1971; 1972) described a mutant of Chlorella which could not form chlorophyll, but instead formed chlorophyllide a. Chlorophyllase activity in the mutant cells was 20 - 25% of that of the wild type.

Reduction in the chlorophyll content of various fruits parallels decrease in chlorophyllase activity (Sudyina, 1963). Sudyina (1963) also studied chlorophyllase activity in leaf sections of box elder (Acer negundo L.) and showed that activity was highest in

greening sections, somewhat lower in the dark green sections, and lowest in the albino parts. These results tend to link chlorophyllase with chlorophyll synthesis.

Indirect support for a synthetic role of chlorophyllase has been given by Park et al. (1973). They found that phytol can be demonstrated in yellow leaves in quantities comparable with those of green leaves only after saponification. They assumed that the phytol ester compound in yellow leaves is a fragment of ring IV of the chlorophyll molecule.

Chlorophyllase as a Degradative Enzyme

Looney and Patterson (1967) have reported increased activity of chlorophyllase during the respiration climacteric of apples and bananas. Rhodes and Wooltorton (1967) reported identical findings in apples. During the climacteric chlorophyll degradation occurs. Ogura (1969) reports that chlorophyll of young tea leaves increases while chlorophyllase activity is decreasing. Ziegler and Schanderl (1969) found that chlorophyllase activity in a mutant of Chlorella increases while photobleaching. During dark bleaching chlorophyllase activity increased and pheophorbides accumulated. Bailiss (1970) has reported an increase

in chlorophyllase activity and chlorophyll degradation in cucumber cotyledons infected with cucumber mosaic virus.

Based on these and other similar observations, some authors suggest that the first step of chlorophyll degradation is hydrolysis of chlorophyll by chlorophyllase (Ziegler and Schanderl, 1969).

The data in the literature do not give firm support for either a synthetic or degradative role for chlorophyllase. Certainly additional information on the relationship of chlorophyllase activity to chlorophyll metabolism will be required before the biological role of chlorophyllase can be determined.

The Greening and Bleaching of *Chlorella protothecoides*

Shihira-Ishikawa and Hase (1964) found that pigmentation of cells of *C. protothecoides* depends upon the nitrogen/glucose (N/G) ratio of the culture medium. At high N/G ratios, green cells with fully developed chloroplasts are obtained. Low N/G ratios give bleached (white) cells with degenerated chloroplasts. At intermediate N/G ratios, light green to yellow cells may develop. Upon transferring to a new medium with appropriate N/G ratio, cells of all colors are interconvertible.

Fructose, galactose, glycerol, and acetate also cause chloroplast degeneration. This degeneration is enhanced by darkness and delayed by light (Shihira-Ishikawa and Hase, 1964; Takashima et al., 1964; Aoki and Hase, 1965). Bleaching of the cells has been shown to be an aerobic process requiring oxidative phosphorylation (Matsuka and Hase, 1965). This led them to suggest that an 0_2 depending step is needed for chlorophyll degradation to occur in the living cells.

Greening has been shown to be light dependent (Shihira-Ishikawa and Hase, 1964). However, "etiolated" cells which synthesize a small amount of chlorophyll in the dark are produced at appropriate N/G ratios (Aoki and Hase, 1964). Greening of these cells differs from greening of normal bleached cells in that no lag period in chlorophyll synthesis occurs upon exposure to light (Aoki and Hase, 1964). From these results, Ochiai and Hase (1970) infer that the light effect is of two types. A long-term effect during which formation of δ-amino levulinic acid is initiated, and a short-term effect during which chlorophyll is formed. The light induction of chlorophyll formation in bleached cells appears to be mediated by nonchlorophyllous photoreceptors which are most sensitive to blue and yellow light (Sokawa and Hase, 1967).

Actinomycin, a DNA-directed RNA synthesis repressor, inhibits the light independent phase more strongly. Glucose represses RNA and protein synthesis during the light-dependent phase (Shihira-Ishikawa and Hase, 1965).

Aoki and Hase (1965) and Aoki, Matsubara, and Hase (1965) showed that no "new" cell formation was necessary for greening or bleaching of the cells but synthesis of nucleic acids and protein were essential for greening.

Effects of Antimetabolites on the Formation of Chlorophyll by C. protothecoides

Chlorophyll synthesis of C. protothecoides has been inhibited by various antimetabolites. Actinomycin C, a suppressor of RNA synthesis, can inhibit chloroplast development in bleached cells if applied before provision of urea and light (Aoki and Hase, 1964). Mytomycin C, an inhibitor of cell division, has no effect on chlorophyll synthesis or degradation (Aoki and Hase, 1965; Aoki, Matsubara, and Hase, 1965). Uncouplers or oxidative phosphorylation have been found to inhibit chlorophyll degradation in bleaching cells (Matsuka and Hase, 1965). Inhibition of greening in C. protothecoides has also been shown by 5-fluorouracil, acridine orange, dihydrostreptomycin (Aoki and Hase, 1965),

chloramphenicol (Aoki, Matsubara, and Hase, 1965; Aoki, Matsuka, and Hase, 1965), and cycloheximide (Matsuka and Hase, 1968).

Bleaching has been inhibited by cycloheximide. Partial inhibition of bleaching has been shown by chloramphenicol, puromycin, and ethionine (Matsuka and Hase, 1968).

Chloramphenicol

Chloramphenicol has been found to inhibit protein synthesis of chloroplast ribosomes due to its preferential binding to this type of ribosome (Anderson and Smillie, 1960; Margulies and Brubaker, 1970). Margulies (1968) reported that a particular protein fraction, normally present in small amounts, increased after illumination of chloramphenicol-treated leaves, but an overall inhibition of protein synthesis occurs. It appears that chlorophyll synthesis inhibition is a secondary effect of protein synthesis inhibition (Ben Shawl and Markus, 1969).

Bleaching of *C. protothecoides* is not totally inhibited by chloramphenicol (Aoki, Matsuka, and Hase, 1965; Matsuka and Hase, 1968). However, greening of algal cells is at least partially inhibited (Aoki, Matsuka, and Hase, 1965; Czygan, 1966; Hoober and Siekevitz, 1968; Smith-Johansen and Gibbs, 1972).

Cycloheximide

Cycloheximide has been shown to inhibit protein synthesis in the cytoplasmic ribosomes (Mahler et al., 1968; Hoober and Siekevitz, 1968). Other effects of cycloheximide include inhibition of chloroplast DNA synthesis (Drilica and Knight, 1971), inhibition of chloroplast membrane formation in Chlamydomonas reinhardi (Hoober and Siekevitz, 1968), inhibition of cell division in Euglena (Bishop and Smillie, 1970), and nuclear DNA synthesis in C. pyrenoidosa (Wanka and Moors, 1970).

Glucose assimilation by cells of C. protothecoides was inhibited and no bleaching observed if cycloheximide was added to the culture at the same time as glucose. If added later, bleaching continued for a short period before it stopped completely (Matsuka and Hase, 1969).

CHAPTER III

MATERIALS AND METHODS

Culture and Propagation of Chlorella protothecoides

Chlorella prothotecoides (ACC #25) was obtained from the University of Indiana algal culture collection (Bloomington, Indiana). The culture was maintained on a proteose agar slant (Starr, 1964) at 4°C.

To propagate the culture, the agar slant was incubated with 5 ml of the basal inorganic medium described by Shihira-Ishikawa and Hase (1964) containing 0.5% urea and 1% glucose. The composition of a liter of medium is: KH_2PO_4, 0.7g; K_2HPO_4, 0.3 g; $MgSO_4$, 0.1465 g; $FeSO_4 \cdot 7H_2O$, 3 mg; thiamine hydrochloride 10 µg, Arnon's "A_5" mineral solution, 1 ml. A liter of Arnon's "A_5" mineral solution contains H_3BO_3, 3.86 g; $MnCl_2 \cdot 4H_2O$, 1.18 g; $ZnSO_4 \cdot 5H_2O$, 0.222 g; $CuSO_4 \cdot 5H_2O$, 0.079 g; Mo_2O_3, 0.0187 g (Mitsuda et al., 1970). Glucose and urea are added as indicated to give greening or bleaching conditions (Shihira-Ishikawa and Hase, 1964). Phosphates were sterilized separately from the rest of the medium to avoid precipitation and cloudiness of

the medium. Thiamine hydrochloride was filtered through a sterilized 0.45 mm Millipore filter to avoid degradation by heat.

The agar slant with 5 ml propagation medium was incubated at room temperature for 48 hours under continuous room light. The cell suspension was then transferred to a 500 ml erlenmeyer flask containing 200 ml of the propagation medium. The culture was placed in the dark and shaken on a reciprocal shaker at a rate of 110 strokes per minute. Temperature was kept constant at 24°C. After 48 hours, cells were transferred to a Fernbach flask containing 1.5 l of propagation medium. Incubation was continued in the dark under the same conditions. Aereation of the Fernbach flask was found to be necessary to obtain optimal growth. Air was pumped through a sterilized 0.45 mm Millipore filter with a Cenco Pressovac 4 Pump (Central Scientific Company, Chicago, Illinois) and humidified by bubbling it through sterilized water. The air outlet of the Fernbach flask was made out of glass tubing with three consecutive "U" bends to avoid bacterial contamination. Aereation rate was approximately 60 ml/min. After 72 hours, cells were harvested by centrifuging (3-4 min., at 8000 x g) under sterile conditions. The cells, after two washings with sterilized distilled water, were

ready for subsequent transfers. Most cells at this time had a yellow color, but a few were yellow-green.

Bleaching Experiments

The yellow cells were transferred to basal medium containing 0.5% urea and grown in the light (900 lux at the culture surface) until a deep green color was obtained (chlorophyll content was then 0.15 mg per 10^9 cells or greater). Agitation, aereation and temperature conditions remained the same as earlier described. The green cells were harvested under sterile conditions, washed twice with sterile distilled water and transferred to basal medium containing 1% glucose. The culture was then kept in the dark. All other conditions remained constant. The set-up for the experiment required two Fernbach flasks linked in series with each containing 1.5 l of medium. Bleaching was initiated at the point where cells were transferred to the basal medium containing 1% glucose. Samples were taken at appropriate intervals to determine dry weight, number of cells, chlorophyll content, and chlorophyllase activity.

Greening Experiments

The yellow cells obtained after the initial propagation period were harvested as earlier described. The cells were then transferred to basal medium

containing 1% glucose and incubated in the dark for 48 hours under standard conditions of aereation, agitation, and temperature. At the end of this period, the cells were harvested and suspended in basal medium containing 0.5% urea. Incubation was done under illumination (900 lux at the culture surface) with all other conditions remaining constant. Samples were taken at appropriate intervals and analyzed in the same way as for the bleaching experiments.

Cell Counts

Cells were counted by using an improved Levy-Housser counting chamber (C. A. Hausser and Son, Philadelphia, Pennsylvania). Cell numbers are expressed as number of cells per milliliter.

Chlorophyll Content

Eighty milliliters of culture suspension were centrifuged and the supernatant discarded. The cells were then suspended in 1-2 ml of acetone. The suspension was filtered through a fritted glass funnel layered with 0.6 g sea sand. The cells and sand were then transferred to a mortar and ground. The sea sand was used as an abrasive. The powder resulting from grinding of the cells was suspended in acetone and filtered through a fine fritted glass funnel. This procedure of grinding and filtering was repeated at least twice

and until no more chlorophyll could be extracted. All of the filtrates were combined, made up to 10 ml and used to determine chlorophyll content.

Readings of the extract were made at 664, 647, and 750 nm with a Beckman DU spectrophotometer equipped with a Gilford absorbance indicator. The 750 nm reading was subtracted from 664 and 647 measurements to correct for light scattering. Equations developed by Ziegler and Egle (1965) were used.

$$\text{Chlorophyll } \underline{a} \text{ (mg/l)} = 11.78\ A_{664} - 2.29\ A_{647}$$

$$\text{Chlorophyll } \underline{b} \text{ (mg/l)} = 20.05\ A_{647} - 4.77\ A_{664}$$

These equations are for 80% acetone solutions. Since large quantities of lipid material would precipitate under these conditions, it was necessary to use c.a. 100% acetone solutions and correct the absorbance reading for 80% solutions. The absorbance readings were divided by 1.04 (Ziegler and Egle, 1965).

Results were expressed in mg per liter of culture and as mg per 10^9 cells depending on how the data were reported.

Enzyme Determination

The powder remaining from the chlorophyll extraction was further extracted for 24 hours in the cold with 3 ml of 0.012 M acetate - 0.06 M phosphate -

0.012 M borate buffer, pH 7.50 containing 0.5% Triton X-100. The powder was then separated by centrifugation at 27,000 x g for 10 min. Activity of the supernatant was measured by the procedure of McFeeters et al. (1971). The reaction is run in an aqueous medium (0.012 M acetate - 0.06 M phosphate - 0.012 M borate buffer pH 7.50, containing 0.2% Triton X-100). Pheophorbide a is separated from pheophytin a by shaking an aliquot of the reaction mixture in a 60:40 hexane - acetone solution with enough KOH to raise the pH of the aqueous phase to 8.5. The pheophorbide and the enzyme remain in the lower aqueous acetone phase while the pheophytin is transferred to the hexane phase. Absorbance readings of the lower phase were done at 750 and 667.5 nm (red absorption maximum for pheophorbide a). Reactions were run for 2 hours. Samples were taken every 40 minutes.

Results were expressed as nmoles of pheophytin a hydrolized per hour per liter of culture. In the case of the 10^9 expressions, results were expressed as nmoles of pheophytin a hydrolyzed per hour.

Inhibition Experiments

The effects of chloramphenicol (Sigma Chemical Company), cycloheximide (Calbiochem), and methyl pyropheophorbide a on chlorophyll content and chlorophyllase activity of greening and bleaching cells were studied.

Chloramphenicol

Green and white cells were incubated in the dark for 15-16 hours in the basal medium containing chloramphenicol (2×10^{-2}M). The green cells were kept in the dark and glucose was added (final concentration 1%) to the medium. The white cells were transferred to the light and urea was added to the medium (final concentration 0.5%). Control cultures without inhibitor, but otherwise treated in the same way, were carried out at the same time. Conditions of aereation, temperature and agitation were the same as described for bleaching and greening.

Samples were taken at appropriate intervals after the addition of urea or glucose and analyzed for cell number, dry weight chlorophyll and chlorophyllase.

Cycloheximide

Cells were treated in the same way as with chloramphenicol except that cycloheximide (5.3×10^{-5}M) was used as the inhibitor.

Methyl Pyropheophorbide a

Both green and white cells were incubated in the dark for 24 hours. At the end of this period methyl pyropheophorbide a, suspended in a 2% Triton X-100 solution, was added to the culture.

Methyl pyropheophorbide a dissolved in 80:20 ether-acetone and was suspended in a 2% Triton X-100 solution. The solvents were then evaporated at low pressure. The concentration of methyl pyropheophorbide a was 5000 nmoles/ml of Triton X-100 solution. The Triton X-100 solution with methyl pyropheophorbide a was then added to the cultures to give a final concentration of 0.02% Triton X-100 and 500 nmoles methyl pyropheophorbide a/ml in the culture. An equivalent amount of 2% Triton X-100 without methyl pyropheophorbide was added to the control culture. Samples were taken periodically after addition of the compound. To measure chlorophyll and methyl pyropheophorbide a content of the cells, the equations described by Vernon (1960) were used. Since the visible absorption spectra of pheophytin a and methyl pyropheophorbide a are identical, it was assumed in this experiment that the pheophytin a concentration calculated with the equations was in fact methyl pyropheophorbide a.

Methyl pyropheophorbide a was prepared from leaves of Ailanthus altissima. Methyl chlorophyllides were prepared by the method described by Holt and Jacobs (1954) using methanol rather than ethanol. The method is based on transesterification of phytol with methanol catalyzed by chlorophyllase. Methyl chlorophyllides a and b were converted to pheophorbide a and b

and separated by their difference in HCl number. Porphyrins of low HCl number were first washed out of an ether solution of the pigments with 10% HCl. This treatment converted the chlorophyllides to pheophorbides. The methyl pheophorbide *a*, formed during the first HCl extraction, was then extracted with 17.5% HCl. Methyl pheophorbide *b* remained in solution. Methyl pheophorbide *a* was suspended in a 50:50 benzene-petroleum ether solution and chromatographed on a sugar column with the same solution.

Pyrolysis was carried out according to the method of Pennington *et al.* (1964). A sample of methyl pheophorbide *a* (50-100 mg) is dissolved in 10 ml of pyridine and heated in an evacuated ampule at 100°C for 24 hours. Purity of the compound was tested by thin layer chromatography. The compound was then transferred to ether, dried, and suspended in 80:20 ether-acetone to give a concentration of 300 nmoles/0.05 ml. Concentration was measured by using molecular absorption data given by Pennington *et al.* (1964) for ether. They reported the molecular absorption coefficient for methyl pyropheophorbide *a* at 667 nm to be 52,000 M^{-1} cm^{-1}.

CHAPTER IV

RESULTS

Greening Under Normal Conditions

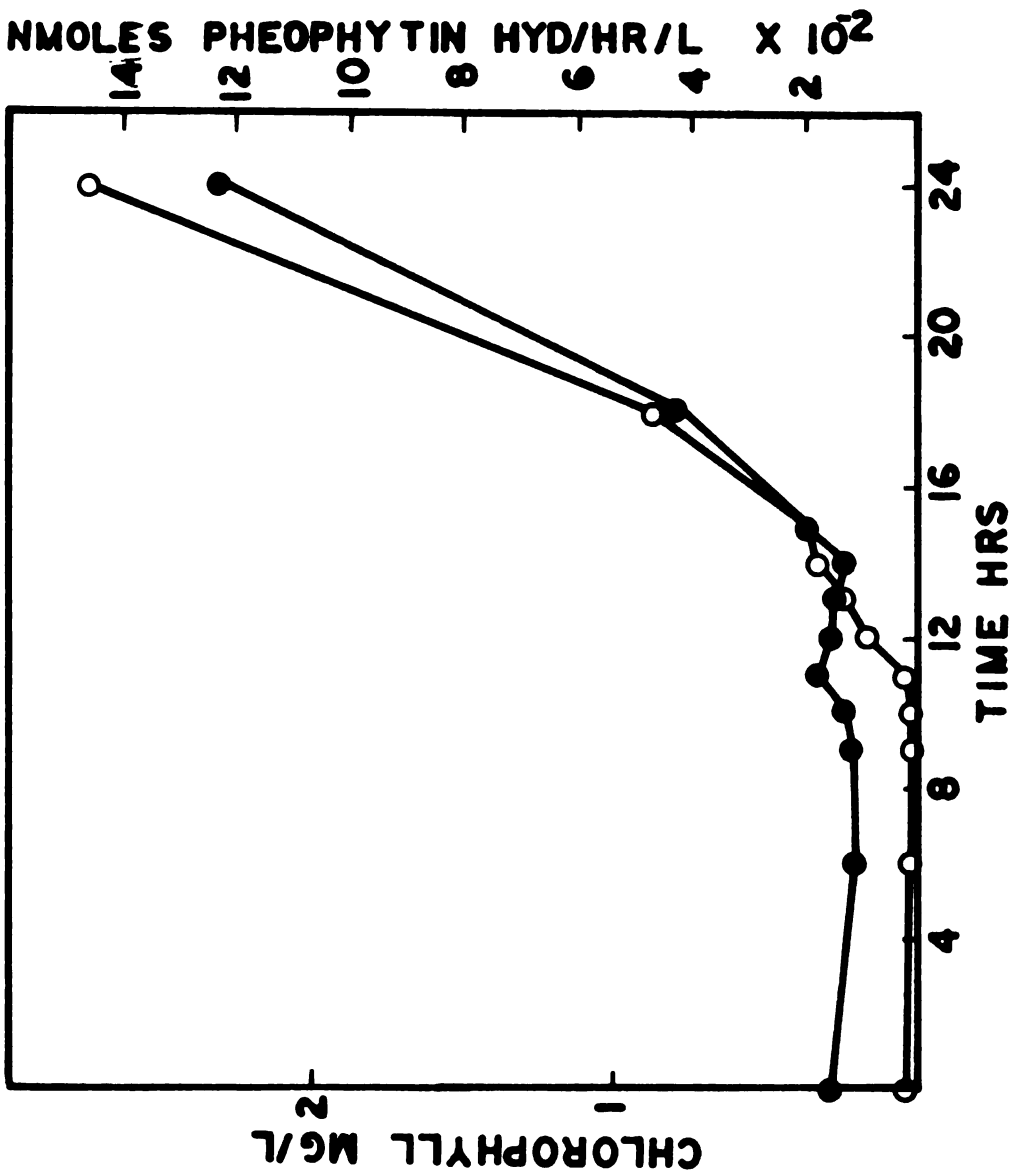
Greening of the cells was achieved by transferring previously bleached cells into basal medium containing 0.5% urea and exposing them to continuous light.

Figure 1 shows the changes in chlorophyll content and chlorophyllase activity after urea was added and cells were placed in the light. The results are expressed on the basis of a liter of culture. It can be seen that a lag of approximately 4 hours occurs between the time chlorophyll begins to increase and the time chlorophyllase activity increases appreciably. However, there is a measurable amount of chlorophyllase activity in the totally bleached cells. It is possible that this level of activity may account for the synthesis of chlorophyll during the lag period. The lag period varied throughout the greening experiments from 0 to 12 hours.

The experiments were stopped as soon as the culture appeared deep green in color regardless of

Figure 1.--Chlorophyll and chlorophyllase activity changes per liter of culture in greening cells of C. protothecoides. Greening of white cells was achieved by culturing in basal medium containing 0.5% urea as nitrogen source.

O———O chlorophyll, ●———● chlorophyllase


NMOLES PHEOPHYTIN HYD/HR/L X 10⁻²
14
12
10
8
6
4
2
TIME HRS
4
8
12
16
20
24
CHLOROPHYLL MG/L
2
1

chlorophyll content or chlorophyllase activity. It was expected that any relationships between chlorophyllase and chlorophyll synthesis would be apparent by the time the culture was green.

Figure 2 shows the changes in chlorophyllase activity and chlorophyll content expressed per 10^9 cells. The lag between the time of beginning chlorophyll synthesis and increase in chlorophyllase activity is no longer apparent. When results are expressed in this way, they appear similar to those described by Chiba et al. (1967).

Bleaching Under Normal Conditions

Bleaching of the cells was achieved by transferring green cells to basal medium containing 1% glucose and incubating them in the desk. Figure 3 shows changes in chlorophyll content and chlorophyllase activity after the addition of glucose and removal from the light on a per liter of culture basis. There is little loss of chlorophyllase activity during the time when most of the chlorophyll is degraded.

Results in Figure 4 focus on the same variables but on the basis of 10^9 cells. The results again appear very similar to those reported by Chiba et al. (1967), while those in Figure 3 are clearly different. There was a four-fold increase in cell number during the

Figure 2.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a greening culture of C. prototheocoides. Conditions used were the same as in Figure 1.

O———O chlorophyll, ●———● chlorophyllase

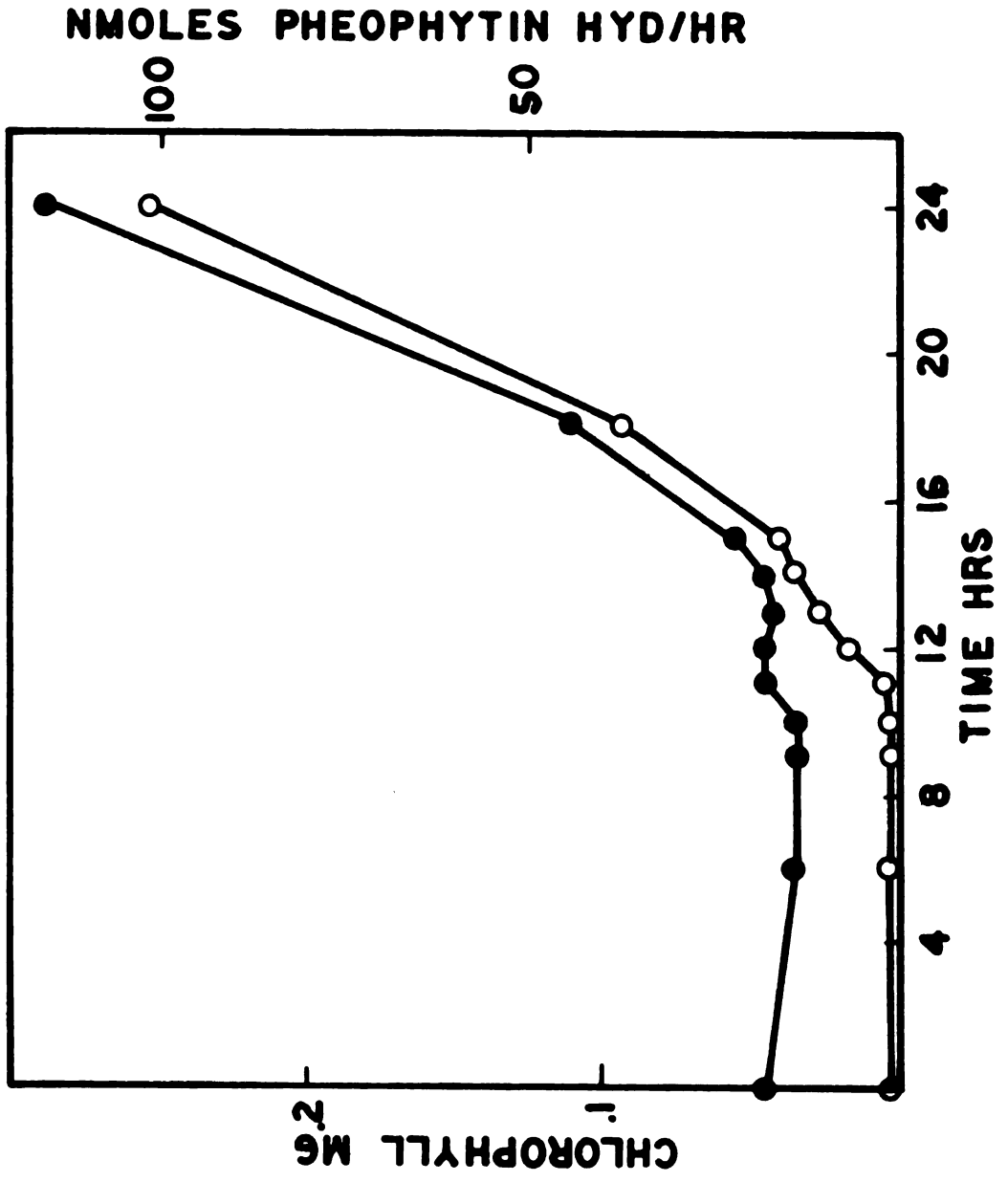

NMOLES PHEOPHYTIN HYD/HR
100
50
24
20
16
12
8
4
TIME HRS
2
1
CHLOROPHYLL MG

Figure 3.--Chlorophyll and chlorophyllase activity changes per liter of culture in bleaching cells of C. prototheocoides. Bleaching was achieved by transferring green cells into medium containing 1% glucose as a carbon source.

O———O chlorophyll, ●———● chlorophyllase

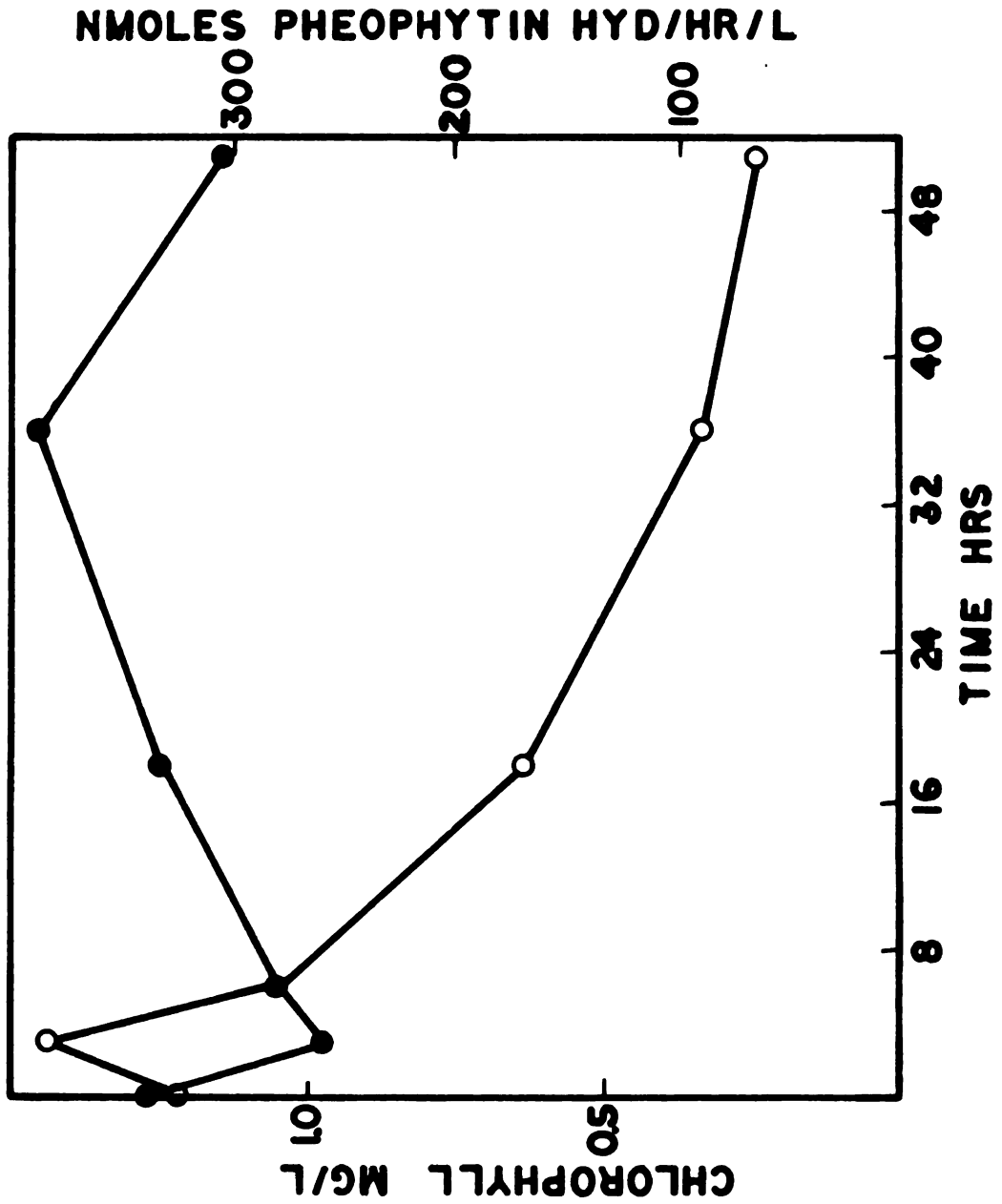
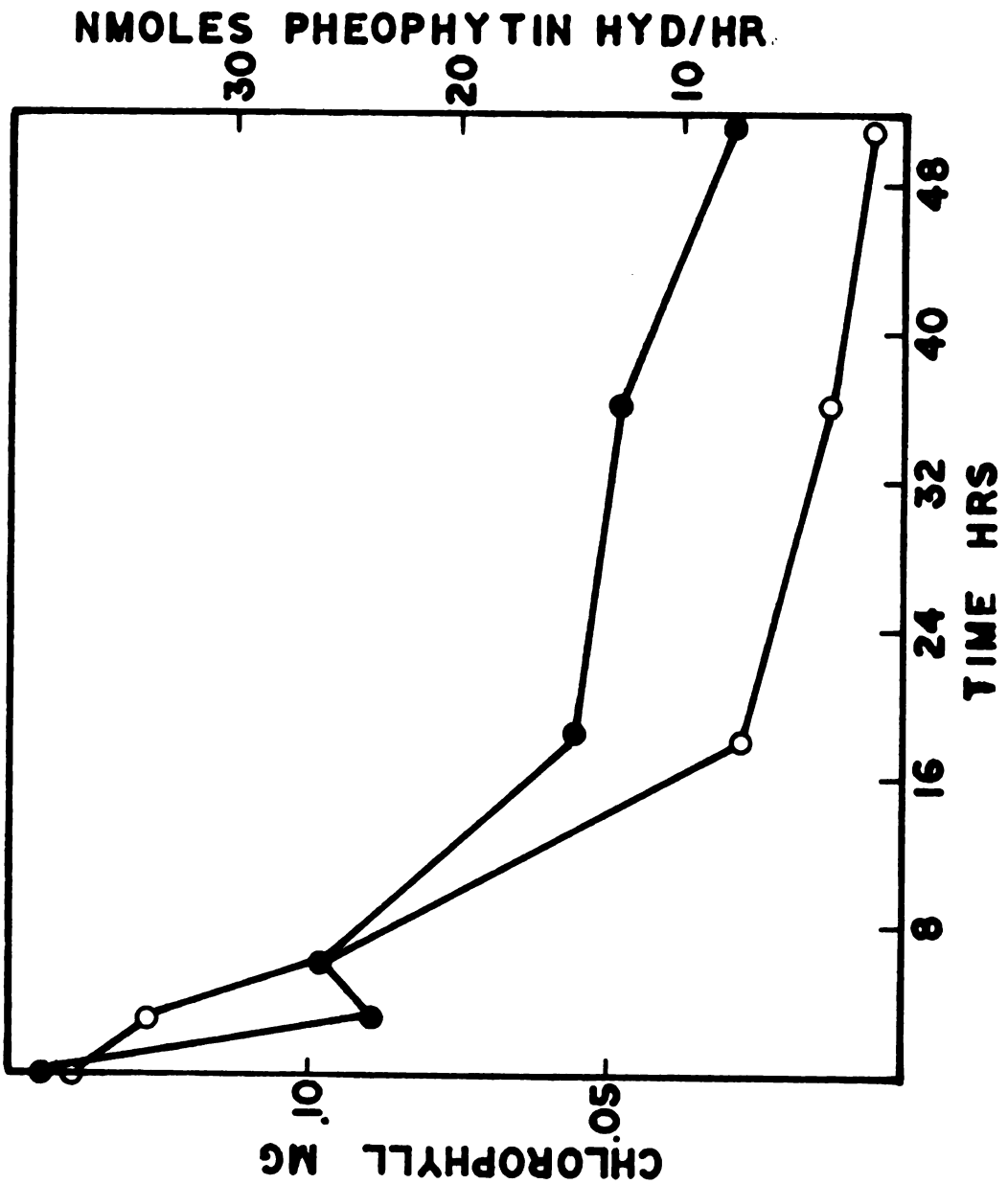

NMOLES PHEOPHYTIN HYD/HR/L
300
200
100
8
16
24
32
40
48
TIME HRS
.10
.05
CHLOROPHYLL MG/L

Figure 4.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a bleaching culture of C. protothecoides. Conditions used were the same as in Figure 3.

O———O chlorophyll, ●———● chlorophyllase activity


NMOLES PHEOPHYTIN HYD/HR
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48
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32
24
16
8
TIME HRS
.10
.05
CHLOROPHYLL MG

bleaching period. Therefore, expression of the results on a per cell basis makes it appear that there is a parallel loss of both chlorophyll and chlorophyllase activity during bleaching. There is approximately a three-fold excess of chlorophyllase to account for the rate of chlorophyll degradation. Assuming the *in vivo* rate of hydrolysis is approximately equal to the rate measured *in vitro*. Experiments were terminated when cells became pale yellow in color. At this point, extraction of intracellular material was made difficult by the high lipid content of the cells. Also, the readings of chlorophyll concentration were affected because lipids would precipitate on the sides of the cuvettes. It should be noted that chlorophyllase does not disappear totally even though cells may be bleached for an extended period of time.

Inhibitor Experiments

Figure 5 shows the effect of cycloheximide (5.3×10^{-5}M) on the greening of cells, the antibiotic was in contact with the cells 15-16 hours before addition of urea and exposure to light. A control under the same conditions was also run. Results are expressed on a per liter basis.

As may be observed, cycloheximide completely inhibits the formation of chlorophyll. Chlorophyllase

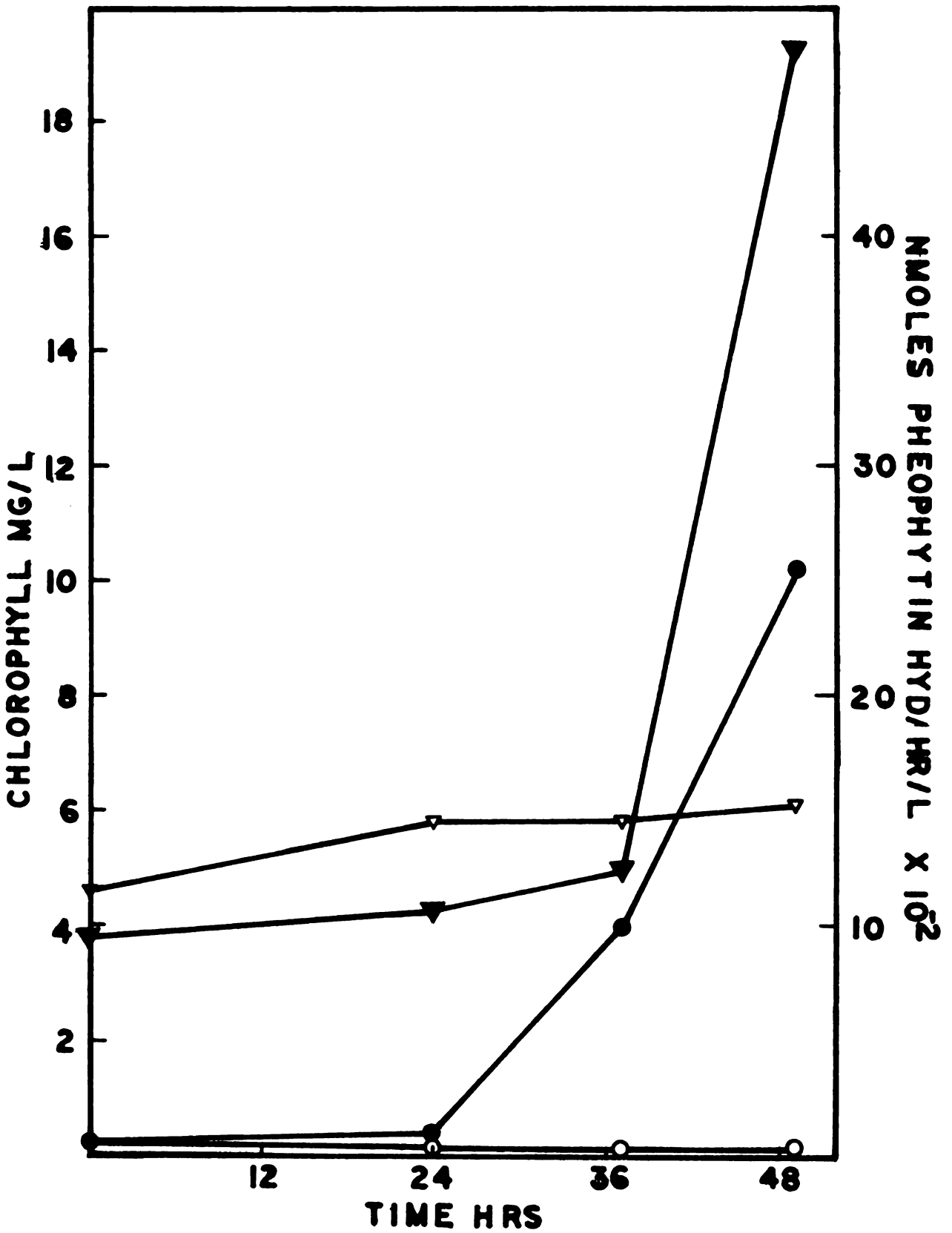
Figure 5.--Chlorophyll and chlorophyllase activity changes per liter of culture in greening cells of C. protothecoides with 5.3 x 10^{-5}M cycloheximide added to the greening medium. Cycloheximide was added to bleached cells in basal medium 16 hours prior to addition of urea. Changes in a control culture carried out at the same time and under the same conditions are also shown.

●———● chlorophyll in control culture

O———O chlorophyll in cycloheximide treated cells

▼———▼ chlorophyllase activity of the control culture

▽———▽ chlorophyllase activity of the cycloheximide treated cells


CHLOROPHYLL MG/L
NMOLES PHEOPHYTIN HYD/HR/L X 10²
TIME HRS
18
16
14
12
10
8
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12
24
36
48

activity does not change significantly during the course of the experiment. Figure 6 shows the same results on the basis of 10^9 cells. The situation does not appear markedly different than when expressed on a per liter of culture basis. The control culture shows a typical lag between chlorophyll synthesis and initial rise in chlorophyllase activity. It should be noted that although practically no chlorophyll exists, there is a measurable level of chlorophyllase activity in the experimental culture.

Figure 7 shows the effect of cycloheximide on the bleaching of cells. Treatment was analogous to that of greening cells, but 1% glucose was added to the culture at the end of the 16-hour pre-incubation period and the cells were maintained in darkness. The results are expressed on the basis of a liter of culture. Cycloheximide stops bleaching after 24 hours completely.

Figure 8 shows the same results on the basis of 10^9 cells, it seems to indicate a close relationship between chlorophyll degradation and loss of enzyme activity as indicated by Chiba et al. (1967). The higher chlorophyll content in the treated cells, at time zero, may be due to partial inhibition of bleaching by cycloheximide even in the dark.

Figure 9 shows the effect of chloramphenicol (2×10^{-2}M) on greening cells. The inhibitor was

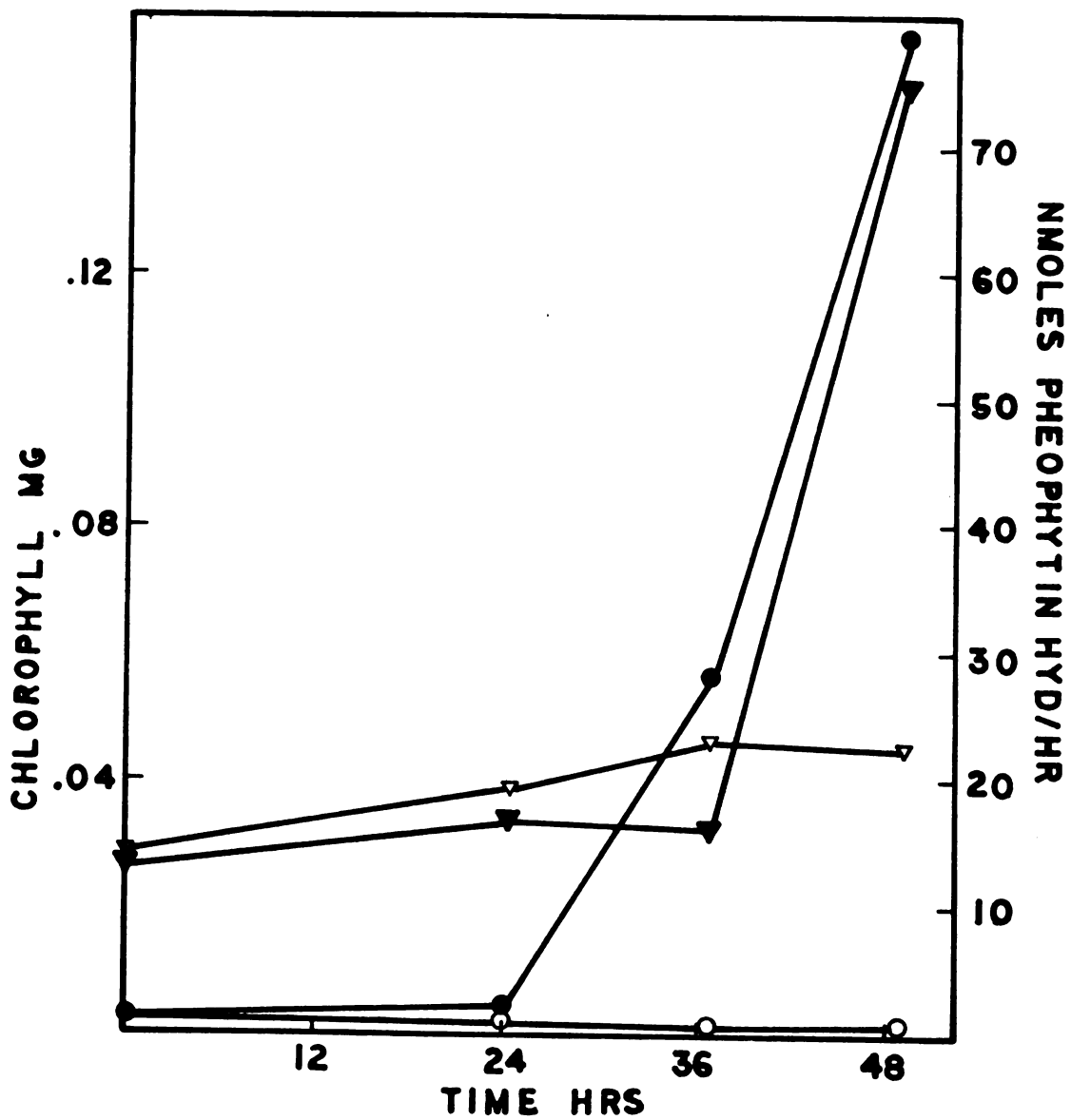
Figure 6.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a greening culture of C. prototheocoides with 5.3×10^{-5}M cycloheximide added to the greening medium. Conditions are the same as those described in Figure 5. Control culture results are also shown.

●——● chlorophyll in the control culture

○——○ chlorophyll in the cycloheximide treated cells

▼——▼ chlorophyllase activity of control culture

▽——▽ chlorophyllase activity of cycloheximide treated cells


CHLOROPHYLL MG
.12
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NMOLES PHEOPHYTIN HYD/HR
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TIME HRS

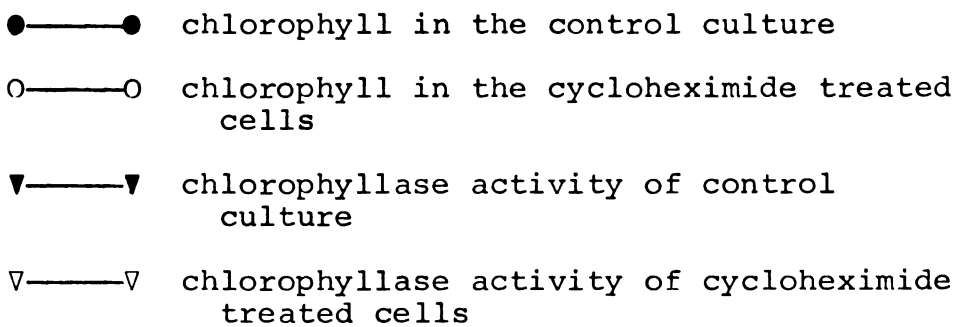

Figure 7.--Chlorophyll and chlorophyllase activity changes per liter of culture in bleaching cells of C. protothecoides with 5.3 x 10^{-5}M cycloheximide added to the bleaching medium. Cycloheximide was added to green cells in basal medium 16 hours prior to the addition of glucose. Changes in a control culture carried out at the same time are also shown.

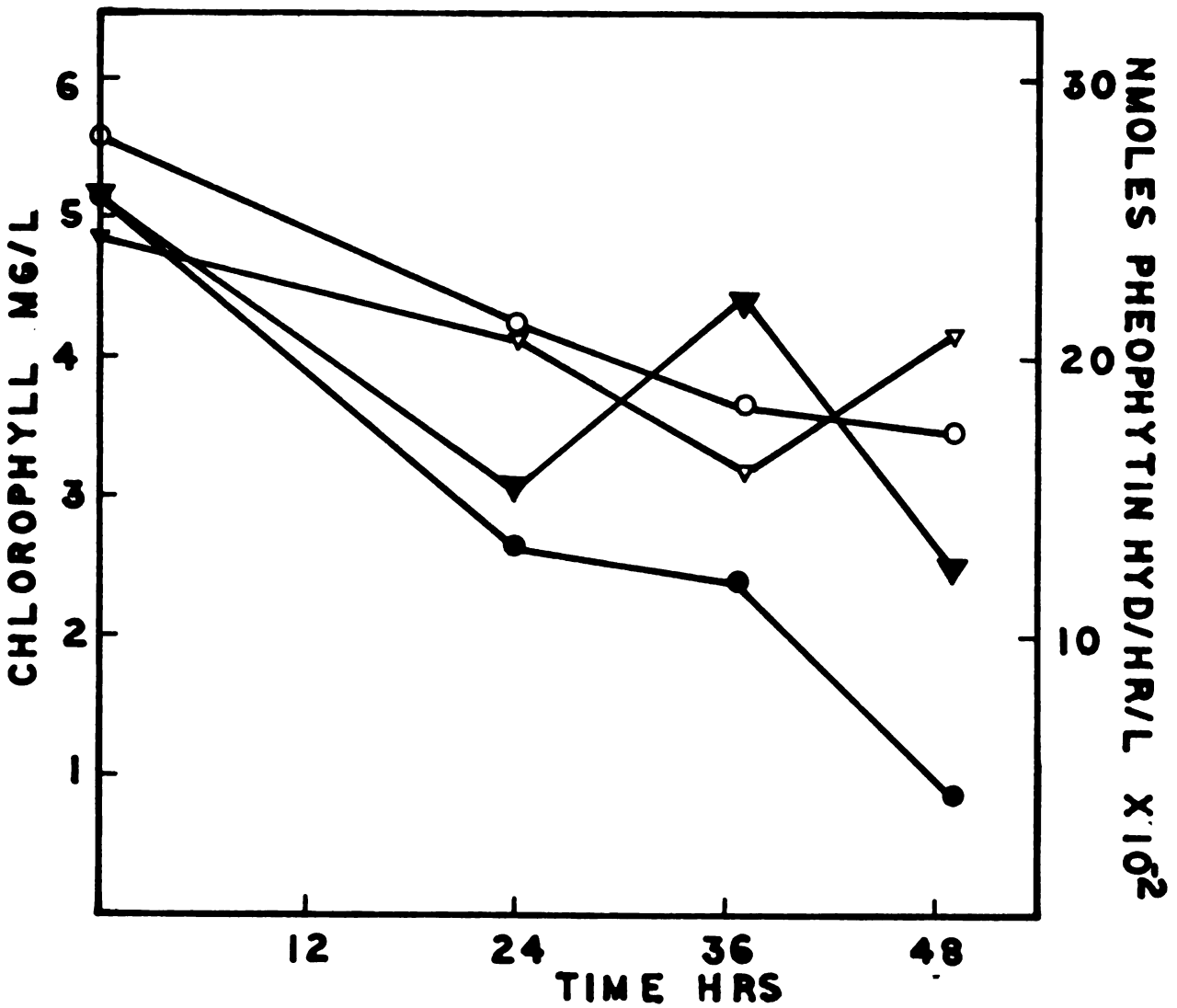
CHLOROPHYLL MG/L
NMOLES PHEOPHYTIN HYD/HR/L X 10²
TIME HRS
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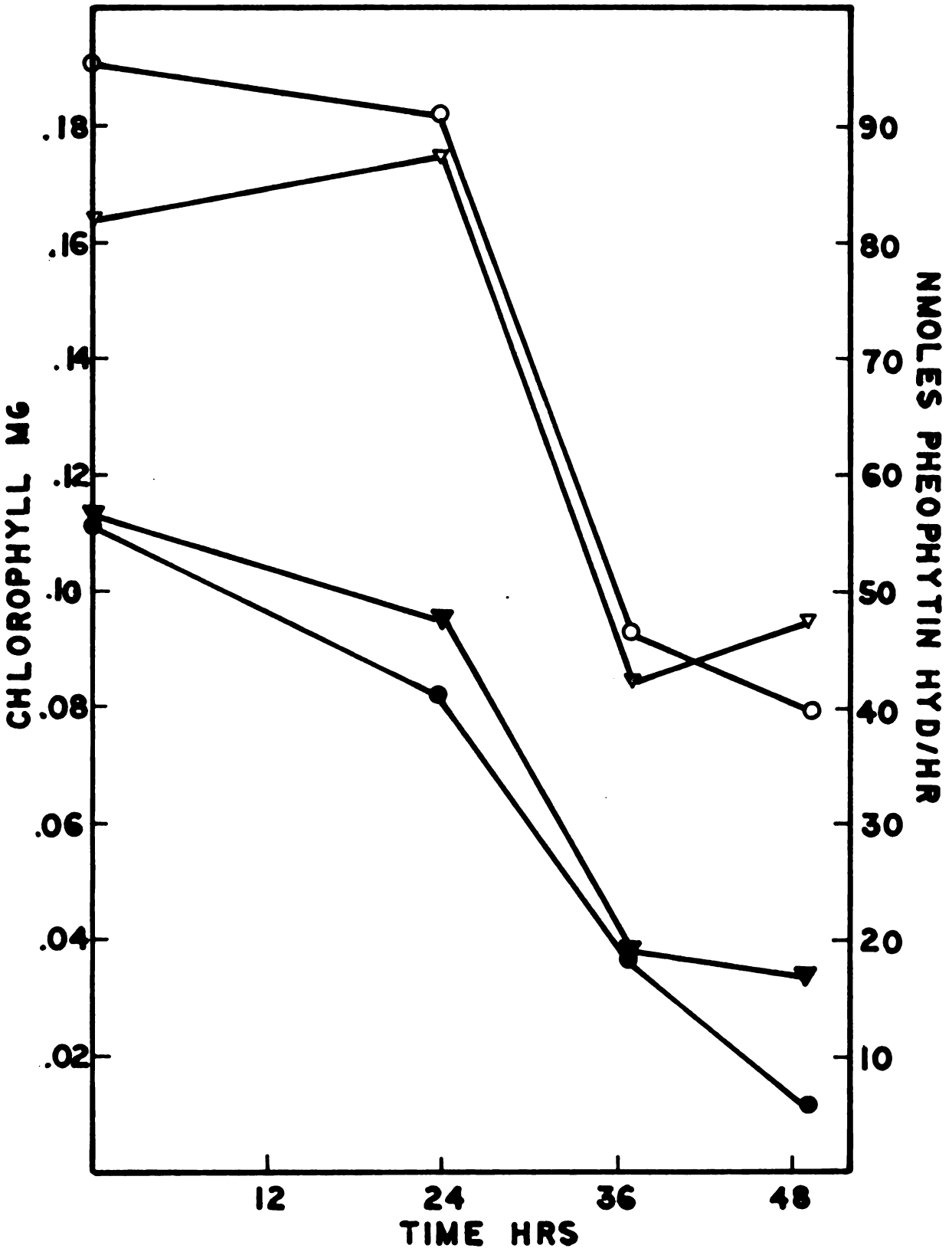
Figure 8.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a bleaching culture of C. prototheocoides with 5.3×10^{-5}M cycloheximide added to the bleaching medium. Conditions are the same as those described for Figure 7. Control culture results are also shown.

●———● chlorophyll in the control culture

O———O chlorophyll in the cycloheximide treated cells

▼———▼ chlorophyllase activity in control culture

▽———▽ chlorophyllase activity of cycloheximide treated cells


CHLOROPHYLL MG
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.06
.04
.02
NMOLES PHEOPHYTIN HYD/HR
90
80
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30
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TIME HRS

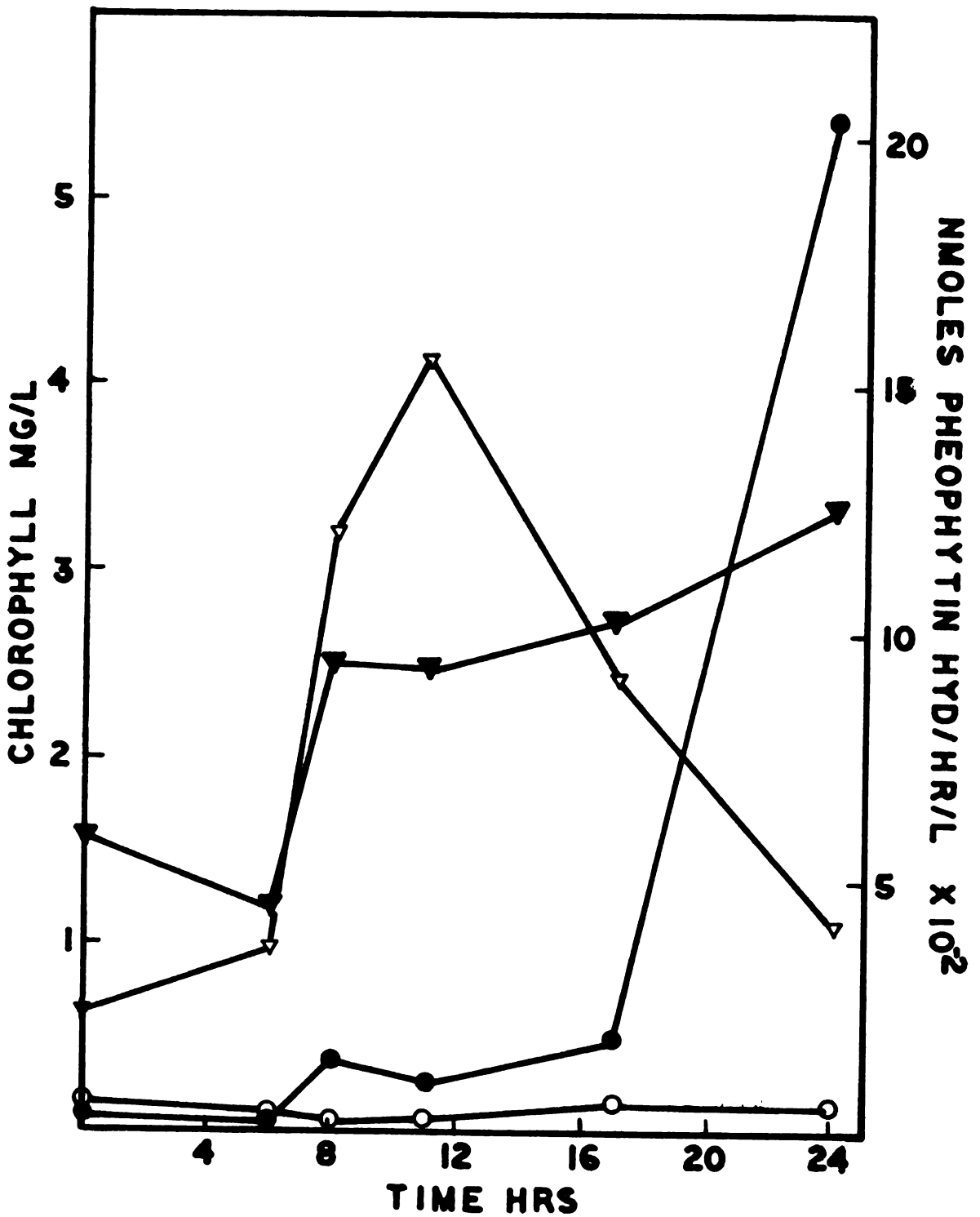
Figure 9.--Chlorophyll and chlorophyllase activity changes per liter of culture in greening cells of C. prototheocoides with 2 x 10^{-2}M chloramphenicol added to the greening medium. Chloramphenicol was added to bleached cells in basal medium 16 hours before addition of urea. Changes in a control culture carried out at the same time are also shown.

●——● chlorophyll in the control culture

O——O chlorophyll in the chloramphenicol treated cells

▼——▼ chlorophyllase activity in control culture

▽——▽ chlorophyllase activity of chloramphenicol treated cells


CHLOROPHYLL MG/L
NMOLES PHEOPHYTIN HYD/HR/L X 10²
TIME HRS
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added 15-16 hours prior to addition of urea and exposure to the light. The treated cells show an increase in chlorophyllase activity while no synthesis of chlorophyll occurs.

Figure 10 shows the effect of chloramphenicol on the bleaching of cells. Concentration of chloramphenicol was the same as that used for greening cells. Addition of chloramphenicol to the cells already in the dark, was 15 to 16 hours prior to adding glucose. Cells were kept in the dark during the experiments. Results are expressed on the basis of a liter of culture.

Figure 11 shows the effect of chloramphenicol on the bleaching of cells, this time expressed on the basis of 10^9 cells. No significant differences between the treated cells and the control are noticeable.

Figure 12 shows, on the basis of a liter of culture, the effect of methyl pyropheophorbide a on the chlorophyll content and chlorophyllase activity during the greening of cells. Methyl pyropheophorbide a was added after 24 hours of incubation in the dark. The compound was dissolved in a 2% Triton X-100 solution. The final concentration of Triton X-100 in the medium was 0.02%. The final concentration of methyl pyropheophorbide a in the medium was 500 nmoles/ml. Control cells were also grown in a medium containing 0.02% Triton X-100.

Figure 10.--Chlorophyll and chlorophyllase activity changes per liter of culture in bleaching cells of C. protothecoides with 2 x 10^{-2}M chloramphenicol added to the bleaching medium. Chloramphenicol was added to green cells in basal medium 16 hours before addition of glucose. Changes in a control culture carried out at the same time are also shown.

●———● chlorophyll in the control culture

O———O chlorophyll in the chloramphenicol treated cells

▼———▼ chlorophyllase activity in control culture

▽———▽ chlorophyllase activity in chloramphenicol treated cells

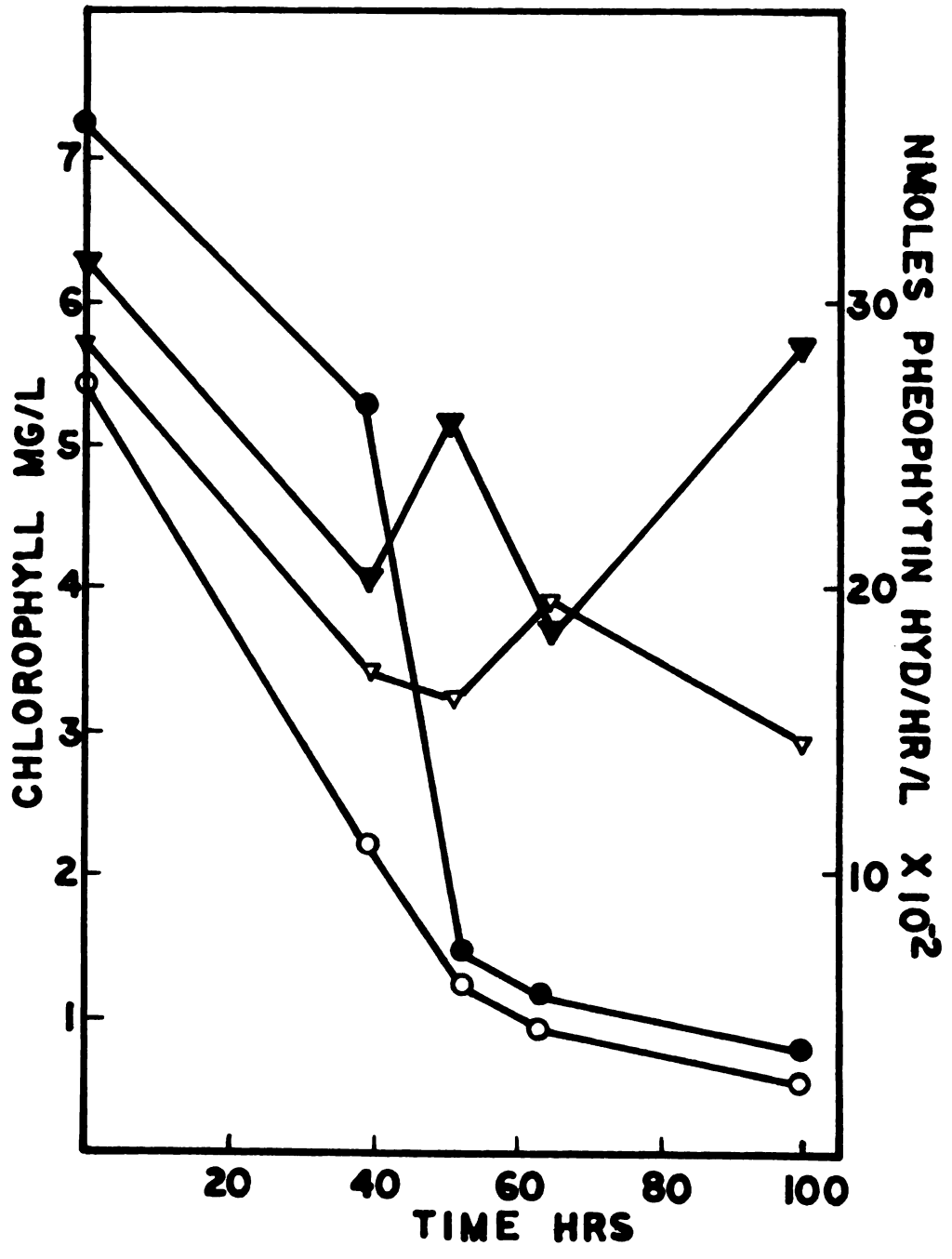
CHLOROPHYLL MG/L
NMOLES PHEOPHYTIN HYD/HR/L X 10²
TIME HRS
1
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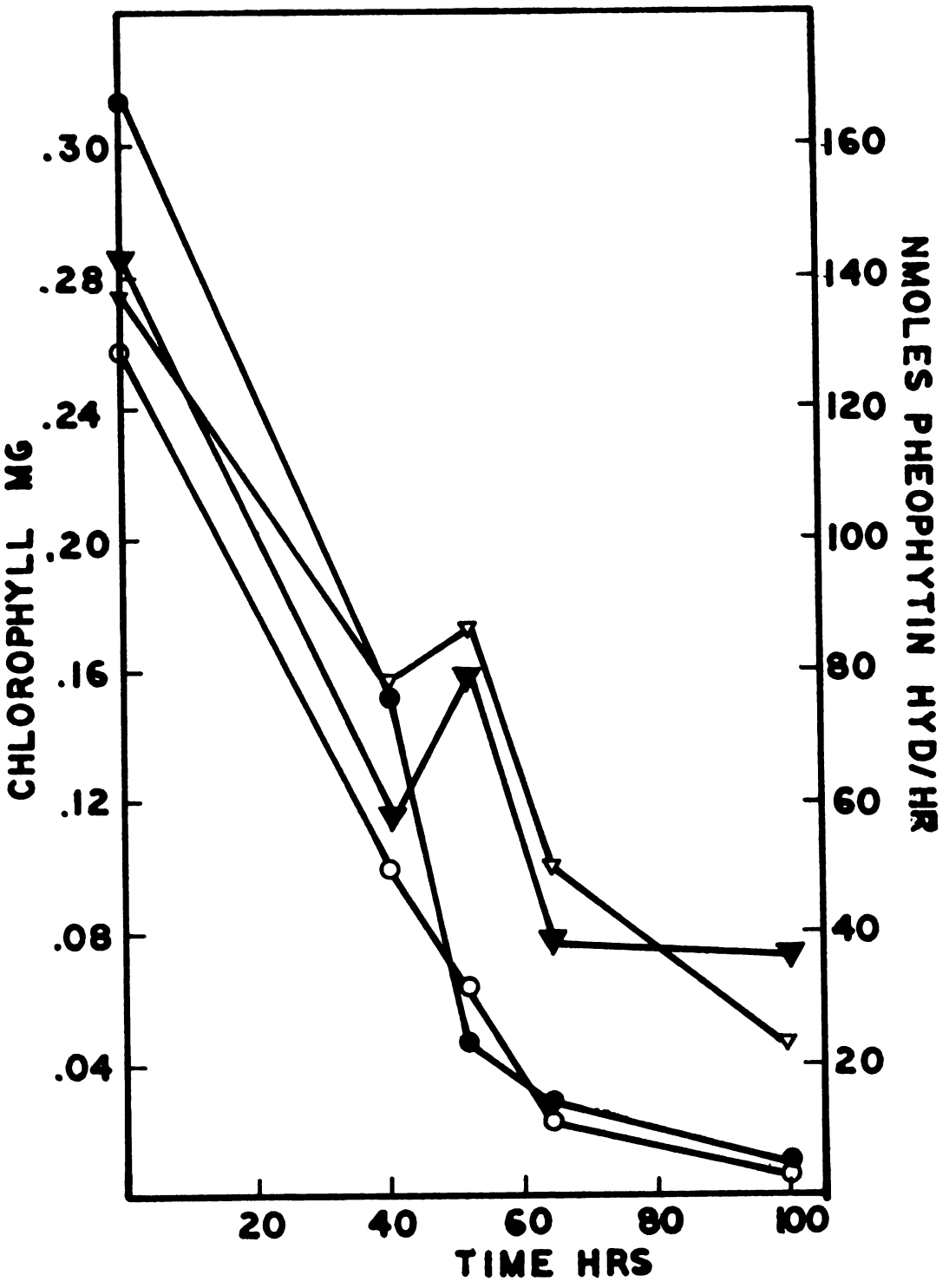
Figure 11.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a bleaching culture of C. prototheocoides with 2 x 10^{-2}M chloramphenicol added to the bleaching medium. Conditions are the same as those described for Figure 10. Control culture results are also shown.

●———● chlorophyll in the control culture

O———O chlorophyll in the chloramphenicol treated cells

▼———▼ chlorophyllase activity in the control culture

▽———▽ chlorophyllase activity in the chloramphenicol treated cells


CHLOROPHYLL MG
.30
.28
.24
.20
.16
.12
.08
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NMOLES PHEOPHYTIN HYD/HR
160
140
120
100
80
60
40
20
20
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60
80
100
TIME HRS

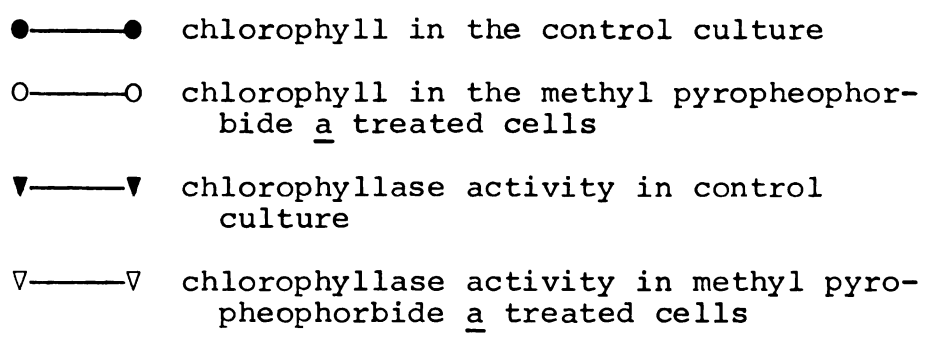
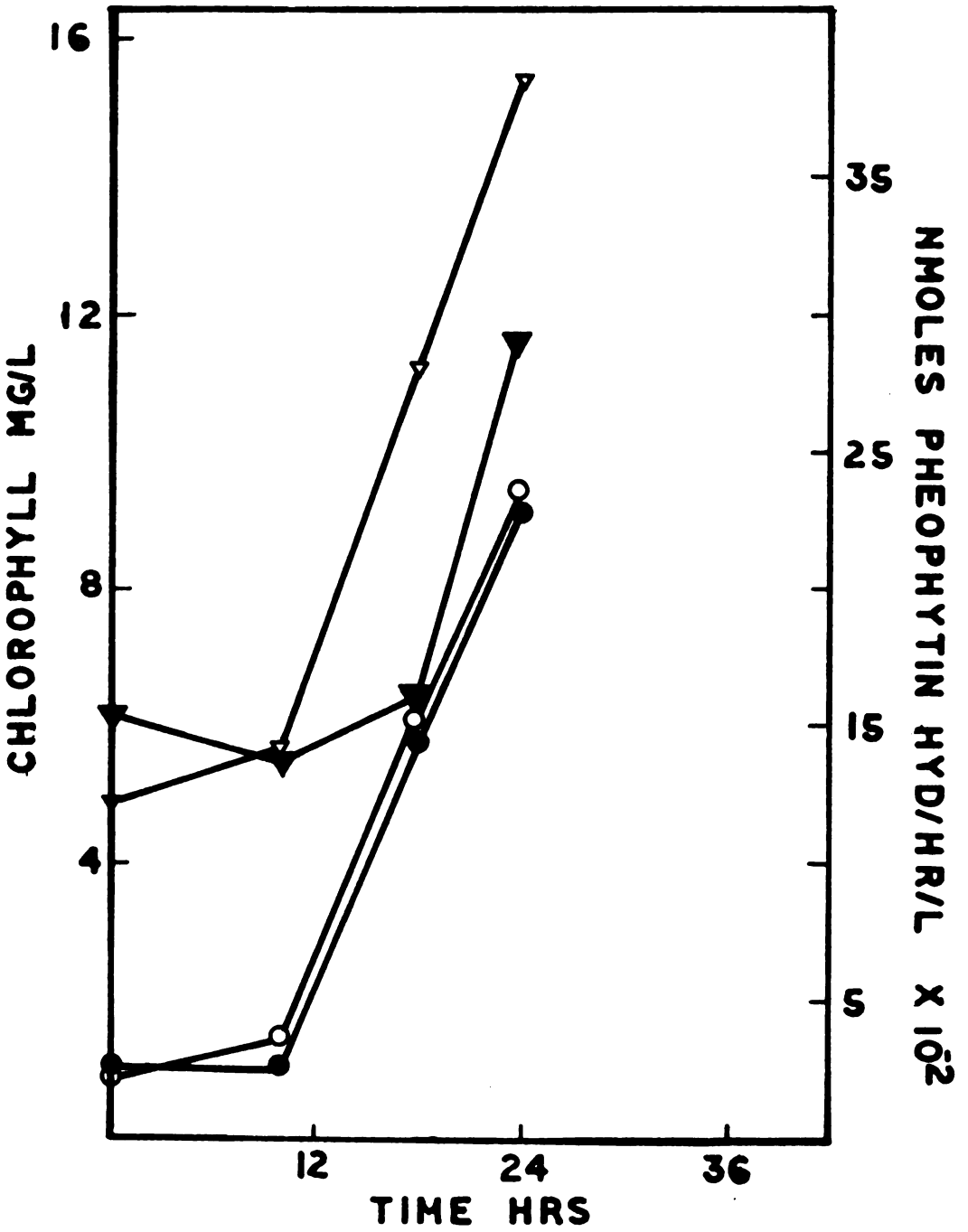


Figure 12.--Chlorophyll and chlorophyllase activity changes per liter of culture in greening cells of C. protothecoides with 500 nmoles of methyl pyropheophorbide a per milliliter of culture medium added. Methyl pyropheophorbide a was added to white cell in basal medium at the same time urea was added. Changes in a control culture carried out at the same time are also shown.


16
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CHLOROPHYLL MG/L
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NMOLES PHEOPHYTIN HYD/HR/L X 10²
12
24
36
TIME HRS

The chlorophyll content of the treated cells behaves much like the control culture, but chlorophyllase activity of treated cells rises sharply 6 hours earlier than the control. Figure 13 shows the results expressed on the basis of 10^9 cells. Basically the same pattern is observed. The significance of this early rise in chlorophyllase activity is not clear at this time.

Figure 14 shows the effect of methyl pyropheophorbide a on the chlorophyll content and chlorophyllase activity of bleaching cells on the basis of a liter of culture. One observes that chlorophyll degradation occurs at about the same rate in both cultures. Chlorophyllase activity, however, is noticeably different. While a consistent decline of enzyme activity cells occurs, activity increases in the treated cells.

Figure 15 shows that on the basis of 10^9 cells the results are similar to those in Figure 14. Low initial activity of the enzyme in treated cells may be due to the effect of residual ether-acetone at the time of adding the compound to the culture. The compound was dissolved in ether-acetone (80:20) and then suspended in a 2% Triton X-100 solution. The ether-acetone was evacuated at low pressure. Large losses of the compound were unavoidable unless some of the ether-acetone remained in the Triton X-100 solution.

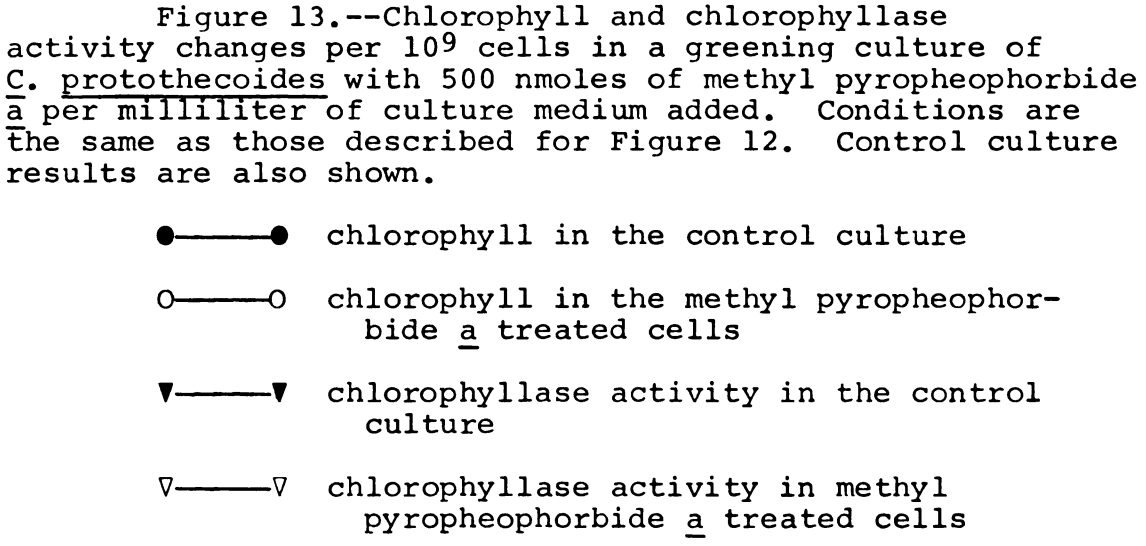


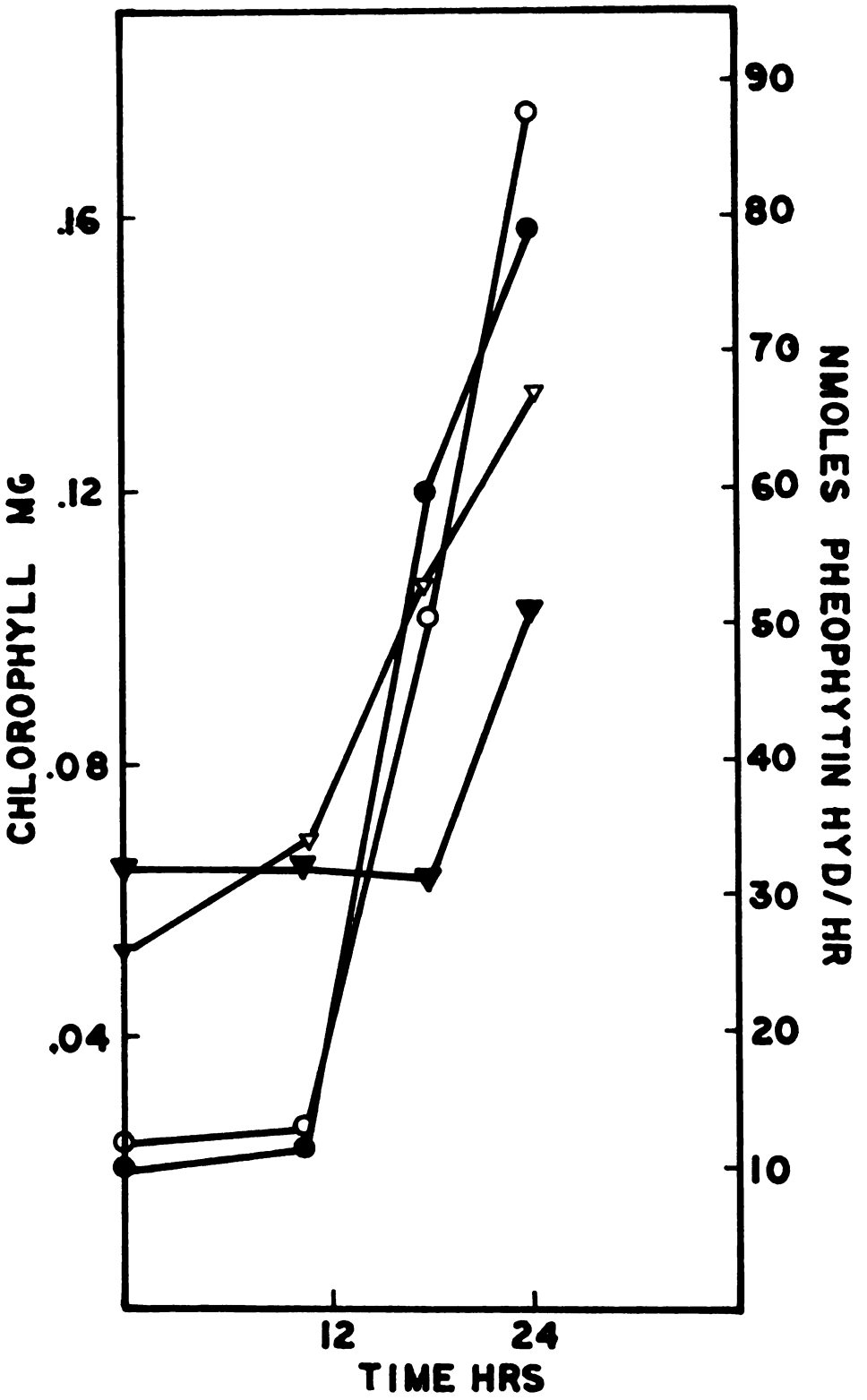
Figure 13.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a greening culture of C. protothecoides with 500 nmoles of methyl pyropheophorbide a per milliliter of culture medium added. Conditions are the same as those described for Figure 12. Control culture results are also shown.

●——● chlorophyll in the control culture

○——○ chlorophyll in the methyl pyropheophorbide a treated cells

▼——▼ chlorophyllase activity in the control culture

▽——▽ chlorophyllase activity in methyl pyropheophorbide a treated cells


CHLOROPHYLL MG
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NMOLES PHEOPHYTIN HYD/HR
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TIME HRS

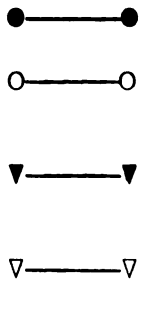


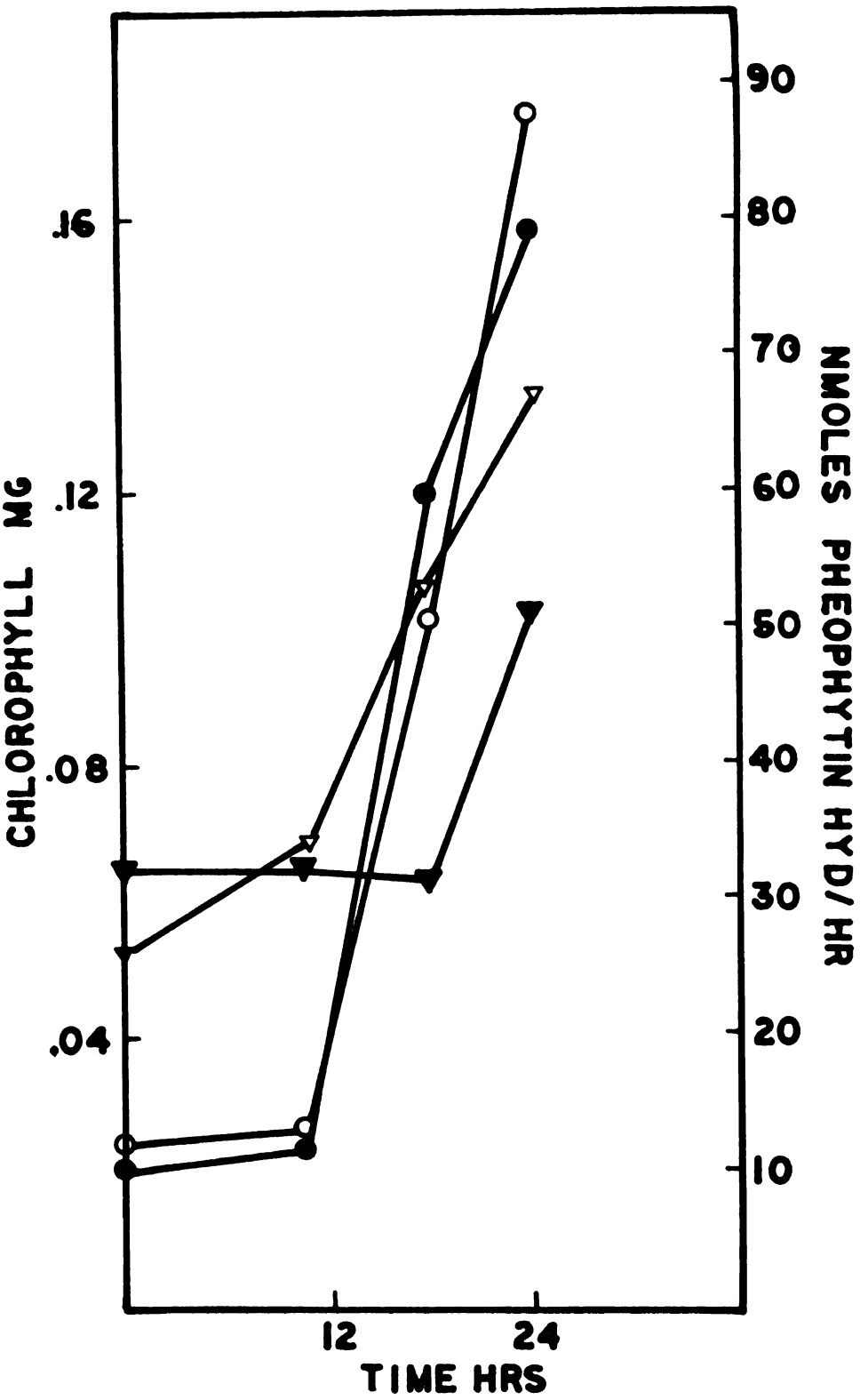
Figure 13.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a greening culture of C. prototheccoides with 500 nmoles of methyl pyropheophorbide a per milliliter of culture medium added. Conditions are the same as those described for Figure 12. Control culture results are also shown.

●——● chlorophyll in the control culture

O——O chlorophyll in the methyl pyropheophorbide a treated cells

▼——▼ chlorophyllase activity in the control culture

▽——▽ chlorophyllase activity in methyl pyropheophorbide a treated cells


CHLOROPHYLL MG
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NMOLES PHEOPHYTIN HYD/HR
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TIME HRS

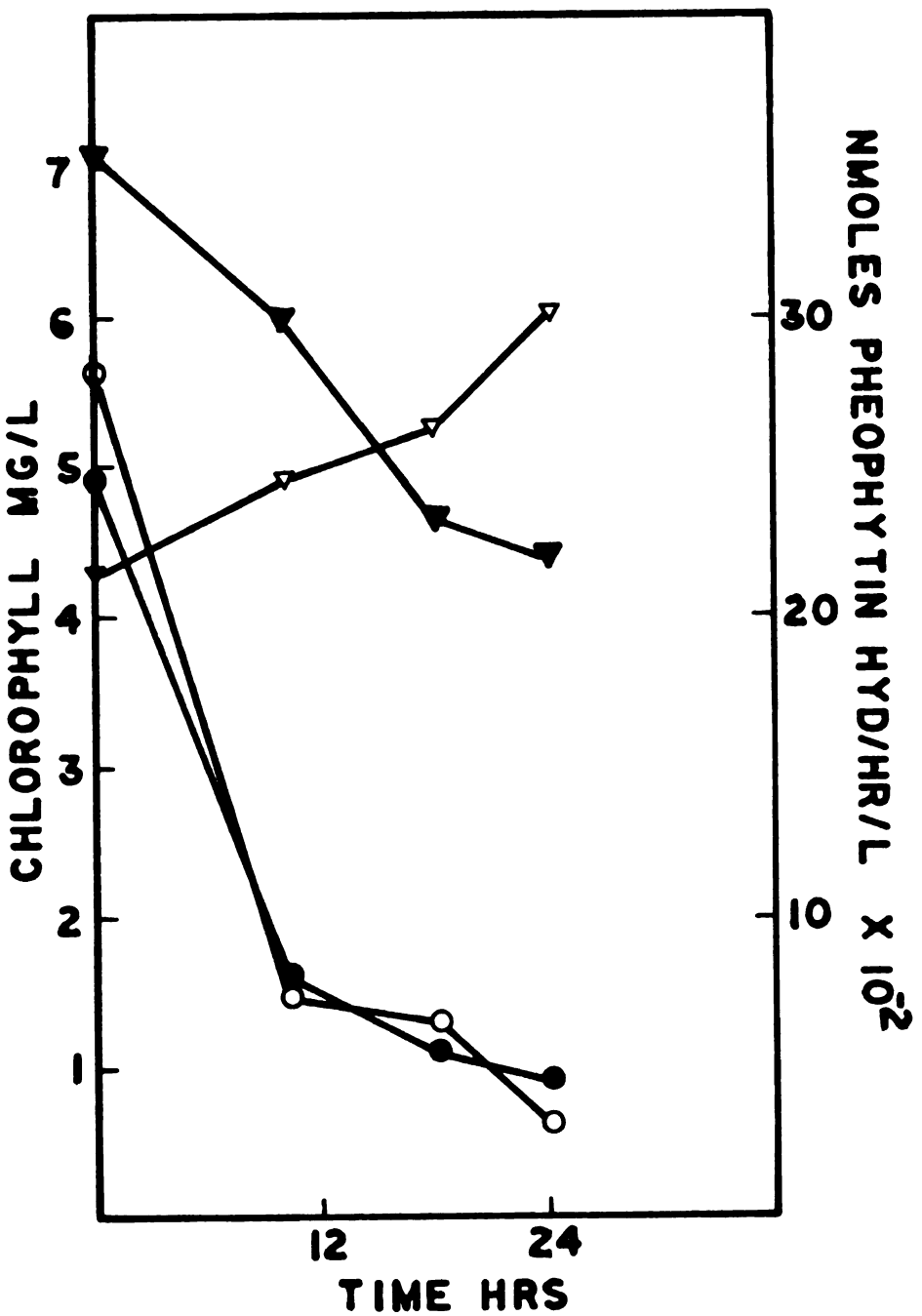
Figure 14.--Chlorophyll and chlorophyllase activity changes per liter of culture in bleaching cells of C. protothecoides with 500 nmoles per milliliter of medium of methyl pyrophephorbide a added. Methyl pyrophephorbide a was added to green cells in basal medium at the same time as glucose. Changes in a control culture carried out at the same time are also shown.

●———● chlorophyll in the control culture

O———O chlorophyll in the methyl pyropheophorbide a treated cells

▼———▼ chlorophyllase activity in control culture

▽———▽ chlorophyllase activity of methyl pyropheophorbide a treated cells


CHLOROPHYLL MG/L
NMOLES PHEOPHYTIN HYD/HR/L X 10^2
TIME HRS
1
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12
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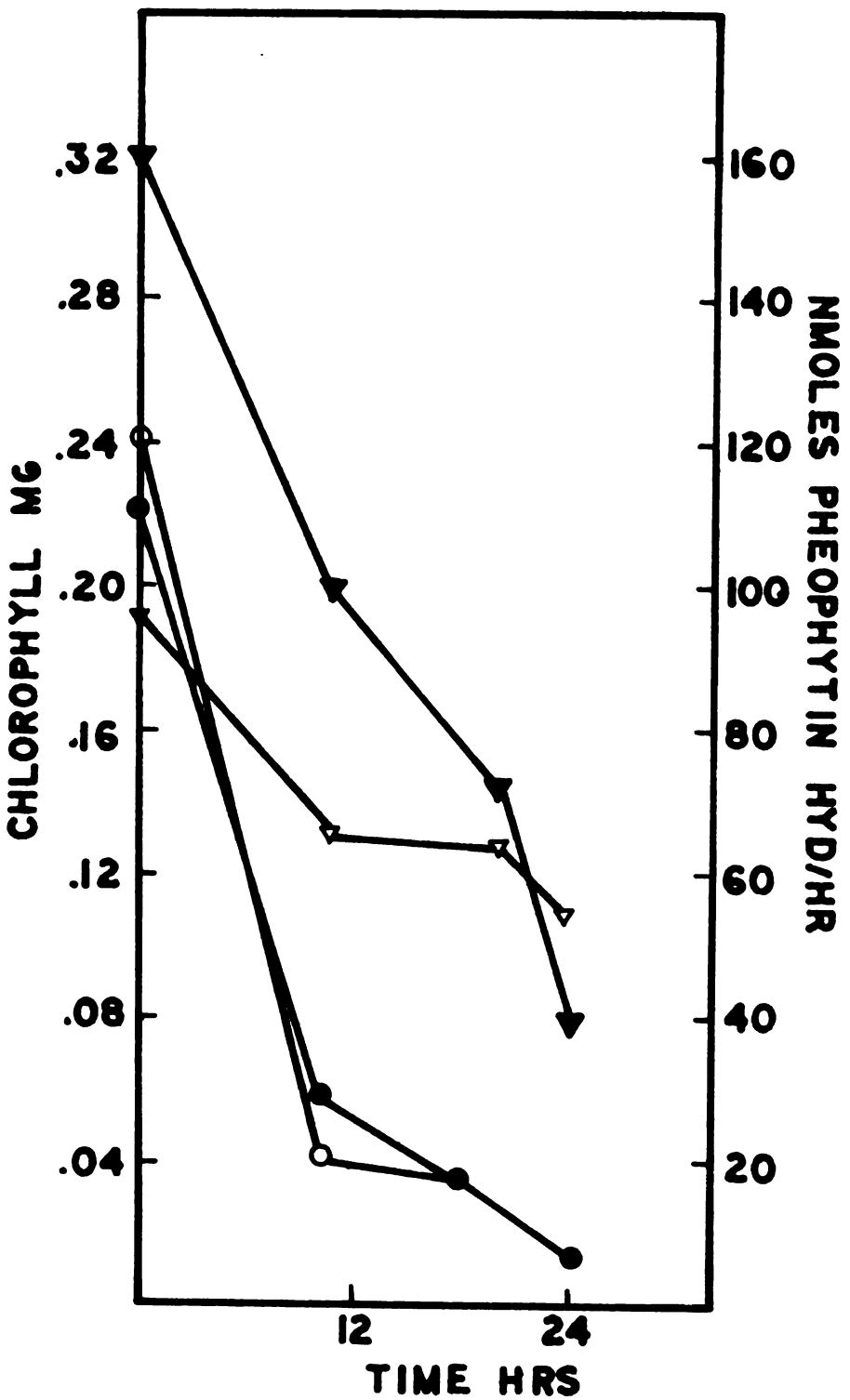
Figure 15.--Chlorophyll and chlorophyllase activity changes per 10^9 cells in a bleaching culture of C. protothecoides with 500 nmoles of methyl pyropheophorbide a added per milliliter of culture medium. Conditions are the same as those described for Figure 14. Control culture results are also shown.

●——● chlorophyll in the control culture

O——O chlorophyll in the methyl pyropheophorbide a treated cells

▼——▼ chlorophyllase activity in control culture

▽——▽ chlorophyllase activity of methyl pyropheophorbide a treated cells


.32
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CHLOROPHYLL MG
160
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NMOLES PHEOPHYTIN HYD/HR
12
24
TIME HRS

Data on the incorporation of methyl pyropheophorbide a into the cells are given in Table 1.

TABLE 1.--Incorporation of methyl pyropheophorbide a into C. protothecoides

Time (hr)	Bleaching Culture	Greening Culture
	μmoles per 10^9 cells*	
0	0.161	0.067
11	0.142	0.045
18	0.145	0.054
24	0.145	0.062

*Cells were washed twice with acetone to remove all loosely bound methyl pyropheophorbide a. The washings were discarded before grinding the cells.

Concentration of methyl pyropheophorbide a inside each cell may be calculated if one assumes spherical and uniformly distributed cells. The average diameter for a cell was 10 microns. For the greening cells at 11 hours the calculated methyl pyropheophorbide a concentration was 1.5×10^{-4}M.

A K_i for methyl pyropheophorbide a has not been determined. However, the K_m for methyl pheophorbide a is approximately 1.5×10^{-6}M (McFeeters, unpublished observation). Since the K_i of pyropheophytin a is similar to the K_m of pheophytin a (McFeeters et al., 1971), by analogy, it is estimated that the K_i for methyl pyropheophorbide a is near 10^{-6}M. Since the

concentration of the compound in the cells is approximately 100 fold greater, it appears that a reasonable level of methyl pyropheophorbide a was achieved.

CHAPTER V

DISCUSSION

Culture Maintenance

A great deal of effort was required in solving some of the particular problems of culturing the cells of Chlorella protothecoides. Because this area constitutes an important aspect of this research, some of the problems will be mentioned. Due to the relatively slow growth of algae as compared to other microorganisms, purity of the culture must be maintained throughout the many transfers that take place in the course of an experiment. To circumvent this problem, it was necessary to start new cultures from the mother culture for each experiment. Each container and all equipment in direct or indirect contact with the cells was sterilized either by heat or by sterile filtration. This insured that pure cultures were used for the experiments. During sampling contamination was kept to a minimum by using sterile equipment. If under microscopic examination, contamination was detected, the culture was discarded and the experiment repeated.

Cultures kept under aereation for more than 3-4 days would invariably become contaminated. It was found that after a short period of use, the sterilized Millipore filters would develop cracks which accounted for the contamination. For this reason, sterile filters were regularly replaced.

Liquid cultures kept in the refrigerator would lose their viability after 1-2 weeks unless kept on an agar slant. Mother cultures were kept on agar slants at 4°C. Cells should be transferred to fresh agar slants every three months to maintain viability of the mother culture. Cells from agar slants which had been used over 10 times for the initiation of liquid cultures showed considerably slower growth and were more prone to contamination.

Data Presentation

As a general point, data are reported both on a per liter of culture basis and on a per 10^9 cells basis. By expressing results on the basis of 10^9 cells, it is possible to compare the present data with that of Chiba *et al.* (1967). Use of the per liter expression provides a constant base to evaluate changes. For example, the number of cells in an experiment may increase, but no change in chlorophyll may occur. Expression of the results on the constant base of a

liter of culture shows that no change in chlorophyll content occurs. If the results are computed on the basis of a fixed number of cells, there appears to have been chlorophyll degradation because the chlorophyll per cell has decreased.

Greening

The greening experiments indicate that there is a 2-4 hour lag between the time rapid chlorophyll synthesis begins and a rapid increase in chlorophyllase activity is noticed. This delay was not observed in the control culture of the chloramphenicol experiments and in the cells treated with methyl pyropheophorbide a. In no case was an increase in chlorophyllase activity noticed prior to the increase in chlorophyll. This implies that chlorophyllase is not needed for synthesis of chlorophyll. However, there is a low level of chlorophyllase activity even in completely bleached cells. This could be sufficient to account for the initial chlorophyll synthesis. Chiba et al. (1967) reported a parallel synthesis of chlorophyll and chlorophyllase. It appears that they did not sample frequently enough to see a lag in chlorophyllase synthesis.

The experiments with chloramphenicol where chlorophyllase activity increases without synthesis of chlorophyll may prove to be useful for other studies.

This study was able to separate the processes of chlorophyll synthesis and increase of chlorophyllase activity. This has never been shown in the literature.

The experiments with cycloheximide indicate that chlorophyllase activity does not increase when cytoplasmic protein synthesis is inhibited. When chloramphenicol is used, chlorophyllase activity increase is not inhibited. These experiments suggest that chlorophyllase synthesis occurs in the cytoplasm of the cell.

Bleaching

The bleaching experiments show that while chlorophyll decreases sharply, chlorophyllase activity remains at approximately the same high levels throughout the experiments. It was difficult to follow the pattern of chlorophyllase activity for longer periods because of contamination. It may, however, be inferred that chlorophyllase activity decreases to a constant low level after an extended period of bleaching since chlorophyllase activity in completely bleached cells is very low.

The high chlorophyllase activity during bleaching does not agree with the conclusion of Chiba *et al.* (1967) that chlorophyll and chlorophyllase decline in a parallel fashion. This is very likely a problem of data presentation. Cell division occurs during bleaching. When the results are expressed on the basis of a constant cell number, there is a parallel decrease (see Figure 4).

If the per liter expression is used, it may be clearly seen that chlorophyll degrades while chlorophyllase remains relatively constant.

The high levels of chlorophyllase activity during bleaching of the cells tend to indicate hydrolytic activity *in vivo*. However, even though the enzyme is present, it is not possible to say with certainty that it is active during the bleaching process.

Chloramphenicol fails to inhibit chlorophyll degradation. Cycloheximide partially inhibits chlorophyll degradation at the time of addition and stops bleaching completely after 24 hours.

Methyl pyropheophorbide a

Methyl pyropheophorbide *a* was added to the cultures with the intent of using it as a specific chlorophyllase inhibitor. At this point, it is not certain whether the compound enters the chloroplast. However, there is evidence that the compound was at least associated with the cells. In addition there is a definite effect on the chlorophyllase activity of cultures treated with the compound. The greening cells show an increase in the chlorophyllase activity earlier than the control cells. This stimulation of earlier enzyme synthesis had no effect on chlorophyll synthesis. Since other inhibitor experiments indicate cytoplasmic

synthesis of chlorophyllase, this implies the methyl pyropheophorbide a at least reached the cytoplasm.

The bleaching cultures show an increase in chlorophyllase activity although chlorophyll is being degraded at the same rate as in the control culture. The significance of these effects on chlorophyllase activity is not clearly understood at this time.

Since no effect on chlorophyll metabolism occurred, this experiment does not help to determine the role of chlorophyllase. However, studies with other competitive inhibitors of the enzyme or combination of two or more metabolic inhibitors may help give further insight into the role of chlorophyllase in chlorophyll metabolism.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The chlorophyll and chlorophyllase activity changes during greening and bleaching of the alga Chlorella prothotecoides were investigated in this study. The following conclusions were derived:

A. The relationship between chlorophyllase activity and chlorophyll synthesis during greening of the cells does not suggest a synthetic role for the enzyme. Contrary to the results obtained by Chiba et al. (1967), the data indicate that increase in chlorophyllase activity occurs after synthesis of chlorophyll has begun.

B. The high chlorophyllase activity during bleaching of the cells suggests that chlorophyllase may be involved in chlorophyll degradation. Chiba's assertion that a parallel loss of chlorophyllase activity and chlorophyll occur during bleaching is an artifact of the way in which the data were presented. The data in this study indicate that little chlorophyllase activity is lost during bleaching.

C. Chloramphenicol (2×10^{-2}M) allows for the separation of the processes of chlorophyll synthesis and chlorophyllase activity increase. Chloroplast ribosome inhibitors do not inhibit chlorophyllase synthesis.

D. Cycloheximide (5.3×10^{-5}M) inhibits both chlorophyll synthesis and degradation. Chlorophyllase activity increase was inhibited under greening conditions. This indicates that chlorophyllase is synthesized in the cytoplasm.

E. Experiments with methyl pyropheophorbide a, competitive inhibitor of the enzyme, indicate that some effect on chlorophyllase activity occurs. However, no effect on chlorophyll synthesis or degradation was observed.

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LITERATURE CITED

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