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Elias K. Manavathu  
*University of Windsor*

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THE EFFECTS OF CYTOCHALASIN A ON SPORE GERMINATION  
AND GROWTH OF THE WATER MOLD ACHLYA AMBISEXUALIS

BY

ELIAS K. MANAVATHU

A Thesis Submitted to the Faculty of Graduate  
Studies through the Department of Biology in  
Partial Fulfillment of the Requirements  
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at the University of Windsor

WINDSOR, ONTARIO, CANADA

1976

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ABSTRACT

The effect of cytochalasin A (CA), a fungal metabolite, on spore germination, growth, cellulase synthesis and secretion, and respiratory metabolism of the water mold Achlya ambisexualis was investigated.

CA is a potent inhibitor of Achlya ambisexualis spore germination. The effect is rapid, irreversible and concentration dependent. In contrast to the effect on spore germination, the growth of the organism is less sensitive to CA. Both mycelial and spore respiratory metabolisms are impaired by CA. However, mitochondrial respiration is unaffected. The observed inhibition of respiratory metabolism seems to be not mediated by inhibition of sugar uptake.

CA inhibits the synthesis and secretion of cellulase, a secretory enzyme, in Achlya ambisexualis without affecting the general protein synthesis. Lack of accumulation of the enzyme within the mycelia indicates that both synthesis and secretion are affected suggesting that synthesis of certain protein may be controlled at the level of secretion. Thiols such as glutathione (GSH) and cysteine (Cys), added simultaneously, completely inhibits CA responses in Achlya ambisexualis. Among several potential thiol reagents tested only the non-penetrating organomercurial p-chloromercuribenzene sulfonate (pCMBS) mimics the CA response selectively inhibiting cellulase synthesis and secretion suggesting that CA may be acting in Achlya ambisexualis as a thiol reagent.

### ACKNOWLEDGEMENTS

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CHAPTER I  
INTRODUCTION

ACHLYA AMBISEXUALIS

The water mold, Achlya ambisexualis belongs to the fungal class Oomycetes. Members of the genus Achlya are typically aquatic, widely distributed saprobes characterized by the presence of profusely branched coenocytic filamentous mycelium, biflagellate zoospores, cellulose and other glucans in the wall, and the absence of motile gametes. The individual hypha is hyaline with granular cytoplasmic contents in which the nuclei are randomly distributed. Although the somatic mycelium is non-septate, septa are formed at the reproductive phase of the life cycle of the organism to delimit the reproductive structures from the rest of the hypha. The diameter of the hyphae varies considerably with species.

Achlya reproduces asexually by the production of zoospores. Under appropriate conditions the tip of the actively growing hypha is differentiated into a sac-like sporangium which contains an indefinite number of spores. The mature spores after liberation from the sporangium via an apical perforation, encyst in a cluster near the sporangial tip. The biochemical and physiological events that precede the cellular differentiation from somatic hyphae to sporangia are not fully understood. It has been well documented that adverse conditions such as nutrient depletion triggers sporangial differentiation. Even under starved conditions ribonucleic acid (RNA) (Griffin et al., 1969) and protein

(Timberlake et al., 1973) synthesis are essential for the differentiation of sporangia. Both actinomycin D and cycloheximide inhibits the development of sporangia. In 1966 Griffin reported that calcium plays an important role in the sporangial differentiation in Achlya.

Sexual reproduction in Achlya is oogamous.

Morphologically distinct male and female sex organs, called antheridia and oogonia respectively, are produced either on the same thallus (homothallic) or on separate thalli (heterothallic). The antheridium is tubular and multinucleate. The cytoplasmic contents of the spherical oogonium differentiate into one or more globular uninucleate oospheres. Upon mutual contact, one or more antheridia penetrate the oogonium and branch out, sending one branch to each oosphere. These fine hyphal branches connected to oospheres are fertilization tubes. One nucleus of the antheridium now passes through each fertilization tube into each oosphere and fuses with the egg nucleus. Thus fertilization occurs. The fertilized oospheres develop thick walls and are converted into oospores. After a resting period, the oospore germinates by means of a hyphal tube which soon gives rise to a sporangium. Achlya is probably the lowest organism on the evolutionary ladder whose sexual responses are known to be governed by steroid hormones. Raper (1939, 1940, 1942, 1950a, 1950b, 1951) had shown that four distinct hormones called hormones A, B, C and D are involved in the initiation and operation of sexual

process in Achlya. Hormones A and B (otherwise known as antheridiol and oogoniol respectively) have been isolated and structurally characterized (McMorris and Barksdale, 1967; McMorris et al., 1975). In 1967 Thomas and Mullins reported that hormone A, which induces antheridial branching, also elicits a rise in cellulase, a secretory enzyme, which presumably modifies the plasticity of the cell wall.

Although there is no direct economic importance, Achlya has been extensively used by investigators in various disciplines of biology as a research organism. Its non-parasitic mode of life, ameanability to culture in the laboratory under defined conditions, synchronous differentiation of the vegetative cells to reproductive structures and ability to respond to steroid hormones justify its extensive use in cellular and developmental biology.

#### THE CYTOCHALASINS

The cytochalasins (Greek cytos, cell; chalasis, relaxation) are a group of fungal metabolites discovered independently by Carter (1967, 1972), and Rothweiler and Tamm (1966). To date, six members of this class of antibiotics, designated cytochalasin A through F, are available for research purposes. Structurally each of the known cytochalasin is related by having an unsaturated lactone ring in common (Aldridge et al., 1967; Aldridge and Turner, 1969; Aldridge et al., 1972, 1973). In addition to their structural similarity, the cytochalasins share a number of unusual biological activities.



The most commonly studied member of this group of drugs, cytochalasin B (CB), and cytochalasin A (CA) are the metabolites of Helminthosporium dematioideum. Cytochalasins A and B have almost identical structures, the only difference being the former is a ketone and the latter the corresponding secondary alcohol (Figure 1).

In general, the cytochalasins elicit unusual and characteristic effects on many eukaryotic cells. Typically, they are effective at low concentrations ( $2 \times 10^{-6}M$  to  $4 \times 10^{-5}M$ ), rapid in action and readily reversible. Examples of cytochalasin effects include inhibition of the division of the cytoplasm without interfering with nuclear division, inhibition of cell movement, induction of nuclear extrusion (Carter, 1967, 1972; Krishan, 1972; Prescott et al., 1972), inhibition of many transmembrane phenomena such as uptake of sugars (Kletzien et al., 1972; Estensen and Plagemann, 1972; Zigmond and Hirsch, 1972; Taylor and Gagneja, 1976), secretion of macromolecules (Williams and Wolff, 1971; Thoa et al., 1972; Butcher and Goldman, 1974), phagocytosis (Allison et al., 1971; Davis et al., 1971), and inhibition of platelet aggregation and clot retraction (Haslam, 1972; Cerskus, unpublished).

Despite intensive research effort, the mode of action of these drugs at the molecular level is not fully understood. Their rapid and readily reversible effects indicate that their potential target of action may be on the

cell membrane (Carter, 1967, 1972). In support of this assumption, binding studies with isotopically labelled cytochalasins indicate that these drugs have specific binding sites on the mammalian cell membrane (Lin and Spudich, 1974; Mayhew et al., 1974). Other investigators in this field believe that cytochalasins, at least CB, act as antimicro-filament agents. This is resulted from the observation of the inhibition of certain cellular movements by CB accompanied by the disruption or disappearance of microfilaments (Wessells et al., 1971).

Unlike other cytochalasins, CA is found to possess antimicrobial properties. In 1972 Betina et al. reported that CA inhibits both bacterial and fungal growth while CB was found to be ineffective. Although structurally very similar, CA is not as good an inhibitor of glucose uptake as CB (Kletzien, et al., 1972) in mammalian cells. Conversely, CA inhibits sugar uptake in yeast cells (Kuo and Lampen, 1975) whereas CB does not have any effect.

The purpose of this investigation is twofold. First, to study the antifungal properties and mode of action of CA using Achlya ambisexualis as a model system. Second, since cytochalasins are effective inhibitors of secretion, to use them as a research tool to investigate the process of macromolecular secretion in Achlya ambisexualis.

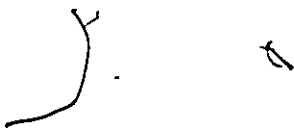
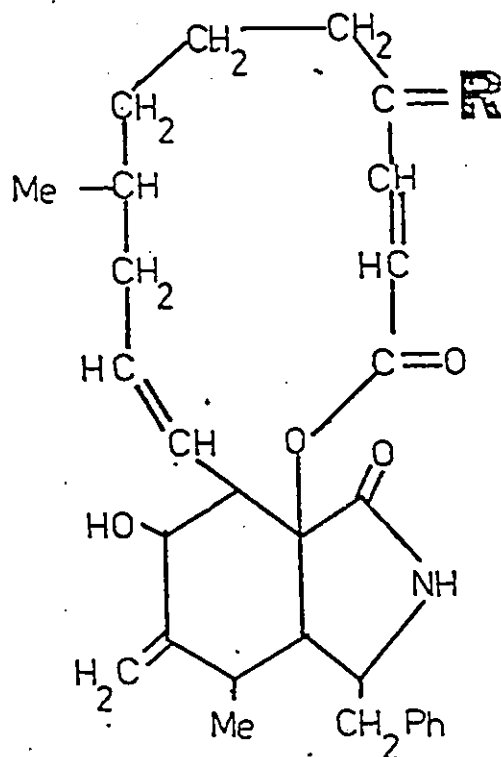


Figure 1. The chemical structure of cytochalasins  
A and B.



CYTOCHALASIN A

$R = O$

CYTOCHALASIN B

$R = H, OH$

CHAPTER II  
EFFECTS OF CYTOCHALASIN A ON SPORE GERMINATION  
AND GROWTH OF ACHLYA AMBISEXUALIS

Most of the available data pertaining to the physiological and biochemical events associated with the differentiation of a resting spore into a vegetative cell are based on the studies conducted with bacterial spores (Keynan and Halvorson, 1965; Gould, 1969; Hansen et al., 1970; Gould and Dring, 1972; Keynan, 1973) and spores of higher fungi (Cochrane, 1956; Cochrane and Cochrane, 1966; Sussman, 1956, 1961; Van Laere and Carlier, 1975; Gottlieb, 1976; Turian, 1976; Van Etten et al., 1976; Lovett, 1976).

Generally speaking, at least three different kinds of sequential events occur in the spore during its differentiation into a vegetative cell. These processes are called activation, germination and outgrowth (Keynan and Halvorson, 1965).

Activation is reversible process which conditions the spores to germinate under appropriate circumstances. The activating agents may either be physical or chemical. Being a reversible process, activation is based on the change of macromolecular structure of the spores and it is not dependent upon the metabolic reactions in the spores. Although spores of Achlya ambisexualis encyst under unfavourable conditions, unlike many fungal spores, they do not require activation to germinate.

The second process in the differentiation of a spore into a vegetative cell is germination. In contrast to activation, germination is an irreversible process during which spore characteristics are lost. During germination extensive macromolecular degradation occurs. Some of the degraded products are excreted into the medium. Evidence available so far indicates that no macromolecular synthesis occurs during germination. This has been further supported by the observation that spores germinate under starved conditions without the supply of exogenous nutrients.

The third and final process in spore differentiation is outgrowth. This is a product of macromolecular synthesis. During this period new kinds of proteins are formed which did not pre-exist in the spore. Since the main feature of outgrowth is macromolecular synthesis many spores do not grow in the absence of exogenous supply of nutrients.

Hyphal tip growth is a highly polarized mode of cell growth by which filamentous fungi, including Oomycetes, expand their somatic thalli preferentially at the apices of growing hyphae. Although the phenomenon of hyphal tip growth in fungi has been described by many authors (Smith, 1923; Castle, 1958; Robertson, 1965; Bracker, 1967), the mechanism of tip extension is not well understood. There is evidence to believe that hyphal tips have structural and cytochemical properties which make them distinct from the rest of the thallus (Robertson, 1958; Zolakar, 1959; Bracker,

1967; Bartnicki-Garcia, 1968; Brenner and Carroll, 1968; McClure, 1968). A series of elegant light microscopic and electron microscopic studies conducted by Grove et al. (1970) shed more light in this regard. Their studies with Pythium ultimum (a member of Oomycetes) using surface markers demonstrated that cell expansion takes place only at the curved portion of the hyphal tip. With respect to internal organization, growing and non-growing regions of the hypha differ considerably. Electron microscopic studies of a growing hypha revealed that its apex consists of three well differentiated regions: (i) apical zone (ii) subapical zone and (iii) zone of vacuolation.

The apical zone is characterized by the abundance of cytoplasmic vesicles. Other cellular organelles such as mitochondria, golgi complex and ribosomes are rarely found. The subapical zone is non-vacuolate and rich in a variety of protoplasmic components. All the synthetic machinery of the cell is found in this region. Farther from the hyphal apex the subapical zone merges into the zone of vacuolation. The degree of vacuolation increases with the age of the hypha and the proportional volume of the cytoplasm is reduced accordingly. Lipid accumulations often appear in this region.

#### METHODS

Preparation of spores: Spores were prepared using a method modified from that of Griffin et al. (1966, 1969). Achlya ambisexualis (male strain E 87) mycelia were grown for 36

hours by inoculating 50 ml of peptone yeast extract glucose (PYG) medium\* in 125 ml Erlenmeyer flasks with  $1 \times 10^3$  spores and incubating at room temperature on a reciprocating shaker (90 cycles/min). After harvesting by filtration mycelia were washed 3 times with 750 ml of  $5 \times 10^{-4}$  M  $\text{CaCl}_2$  solution. The washed mycelia were then transferred to 2800 ml Erlenmeyer flask containing 1 liter of  $5 \times 10^{-4}$  M  $\text{CaCl}_2$  solution and incubated at room temperature with gentle shaking (50 cycles/min). After sporulation, which takes 6 to 8 hours, spores were separated from mycelium by aseptic filtration through nylon mesh. Spores were concentrated by centrifugation at the maximum speed in a clinical centrifuge. Under these conditions 5 to  $6 \times 10^3$  spores/ml were obtained.

Spore germination: To study the effect of CA on Achlya ambisexualis spore germination, washed spores ( $1 \times 10^5$  spores/ml) were resuspended in fresh growth medium and treated with CA ( $0.135 \times 10^{-5}$  M to  $1 \times 10^{-5}$  M)\*\* The treated spores were allowed to germinate at room temperature for 6 hours on a reciprocating shaker (90 cycles/min). Samples were removed at different time intervals, and the percentage of germinated spores\*\*\* determined by microscopic count using hemocytometer.

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\* see appendix

\*\* Cytochalasin A (Aldrich Chemical Co., Milwaukee, Wisconsin) was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of  $2 \times 10^{-3}$  M and stored at  $-20^\circ\text{C}$ .

\*\*\* A spore is considered to be germinated if the length of the germ tube is equal to or greater than the diameter of the spore itself.



As in bacterial colony development, a single fungal colony grows from a single germinated spore, so that plating and colony counting can be used to determine the number of germinated spores. This technique, in contrast to direct microscopic counts, has the advantage that only viable spores are counted. Washed spores ( $1 \times 10^3$  spores/ml) were resuspended in fresh growth medium and treated with CA ( $0.5 \times 10^{-5}$  M to  $4 \times 10^{-5}$  M) at room temperature. After the treatment 0.5 ml samples of the spore suspension were plated on PYG agar. The plates were incubated at room temperature and colonies were counted after 36 to 48 hours of development. To study the reversibility of the response elicited by CA, spores were treated with  $1 \times 10^{-5}$  M CA for 0 to 60 minutes, washed by centrifugation and resuspended in fresh growth medium. Plating and colony counts were done as described above. The control treatments contained a comparable concentration of DMSO.

Germling growth: The effect of CA on the growth of Achlya ambisexualis was investigated as follows: Achlya ambisexualis spores were allowed to germinate and grow for 12 hours in growth medium. Culture vials containing 4 ml of growth medium with CA (final concentration,  $1 \times 10^{-5}$  M) were inoculated with 1 ml of germlings and allowed to grow for different time intervals. At the end of the desired growth period mycelia were harvested by filtration and homogenized in 0.1 N NaOH. After clarifying the homogenate by centrifugation, protein

was precipitated with 10 percent trichloroacetic acid (TCA) and measured according to Lowry et al. (1951).

### RESULTS

Spore germination: Germination of Achlya ambisexualis spore is not synchronous. Experiments dealing with the time course of spore germination indicate that germination begins (in PYG medium at room temperature) after 1 hour of incubation and reaches a maximum within 24 hours (Figure 2). Under these conditions, depending on the spore batch, 50 to 80 percent germination occurs within 6 hours. Therefore, for all experiments dealing with spore germination per se an incubation period of 6 hours has been selected.

Results obtained from the Achlya ambisexualis spore germination study with CA indicate that CA is a potent inhibitor of spore germination. A plot of percent germination as function of time reveals that CA at  $1 \times 10^{-5}$  M concentration significantly reduced spore germination. For example, after 6 hours of incubation, 50 percent of the spores in the control germinated while only 13 percent germination is obtained in the CA treatment (Figure 3). The effect of CA on Achlya ambisexualis spores is rapid, irreversible (Figure 6) and concentration dependent (Figures 4 and 5). Maximal inhibition is obtained within 10 to 15 minutes after the addition of the drug. After this time, prolonged drug treatment appears to have no effect on the 5 to 10 percent of spore population which shows CA resistance.

Figure 2. Time course of Achlya ambisexualis spore germination in PYG medium at room temperature. The vertical bars denote 2X standard deviation.

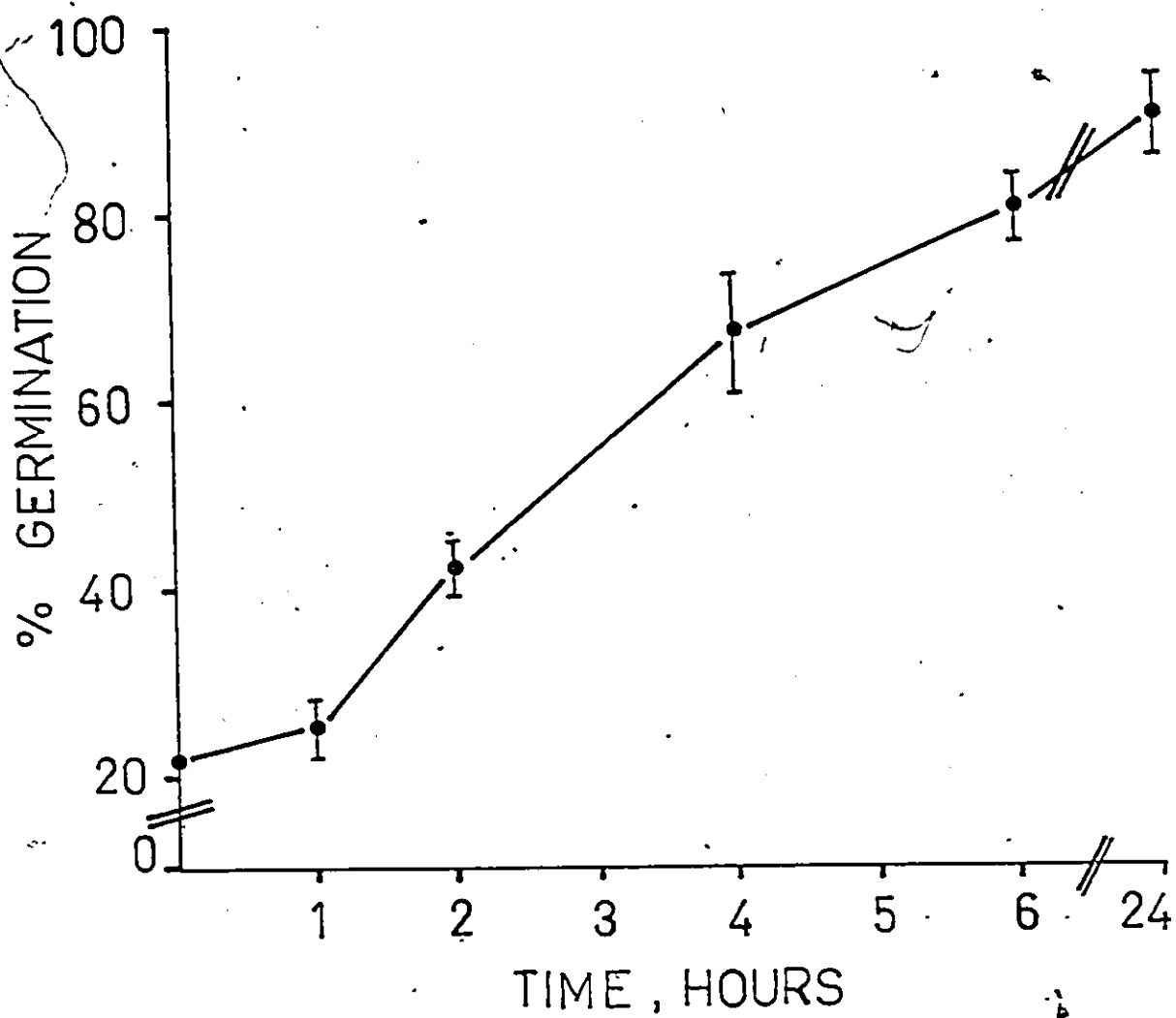


Figure 3. CA inhibition of Achlya ambisexualis spore germination. ●—● Control; ■—■ CA ( $1 \times 10^{-5}$  M). The vertical bars denote 2X standard deviation.

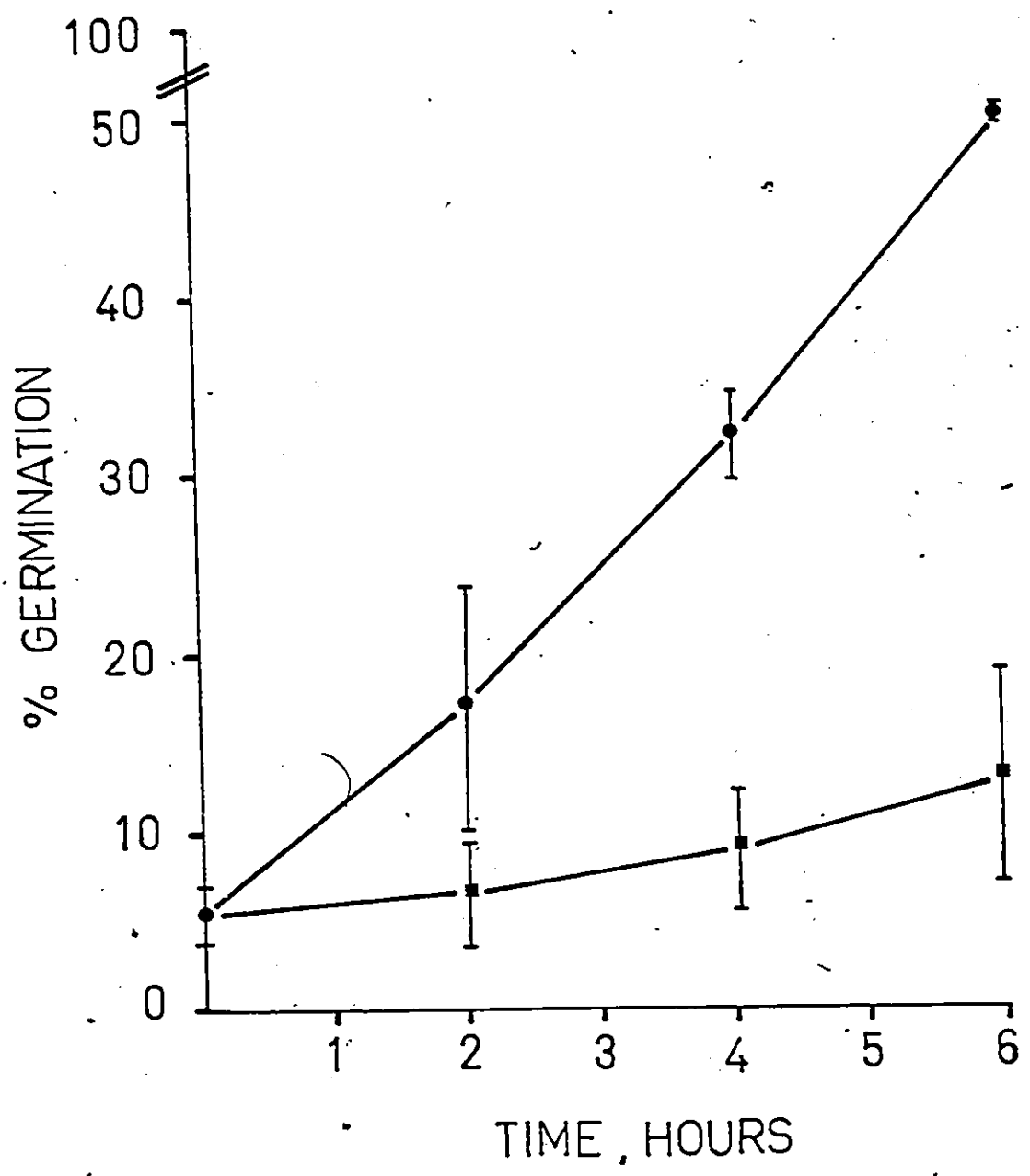


Figure 4. Effect of CA concentration on Achlya  
ambisexualis spore germination. The  
vertical bars denote 2X standard deviation.

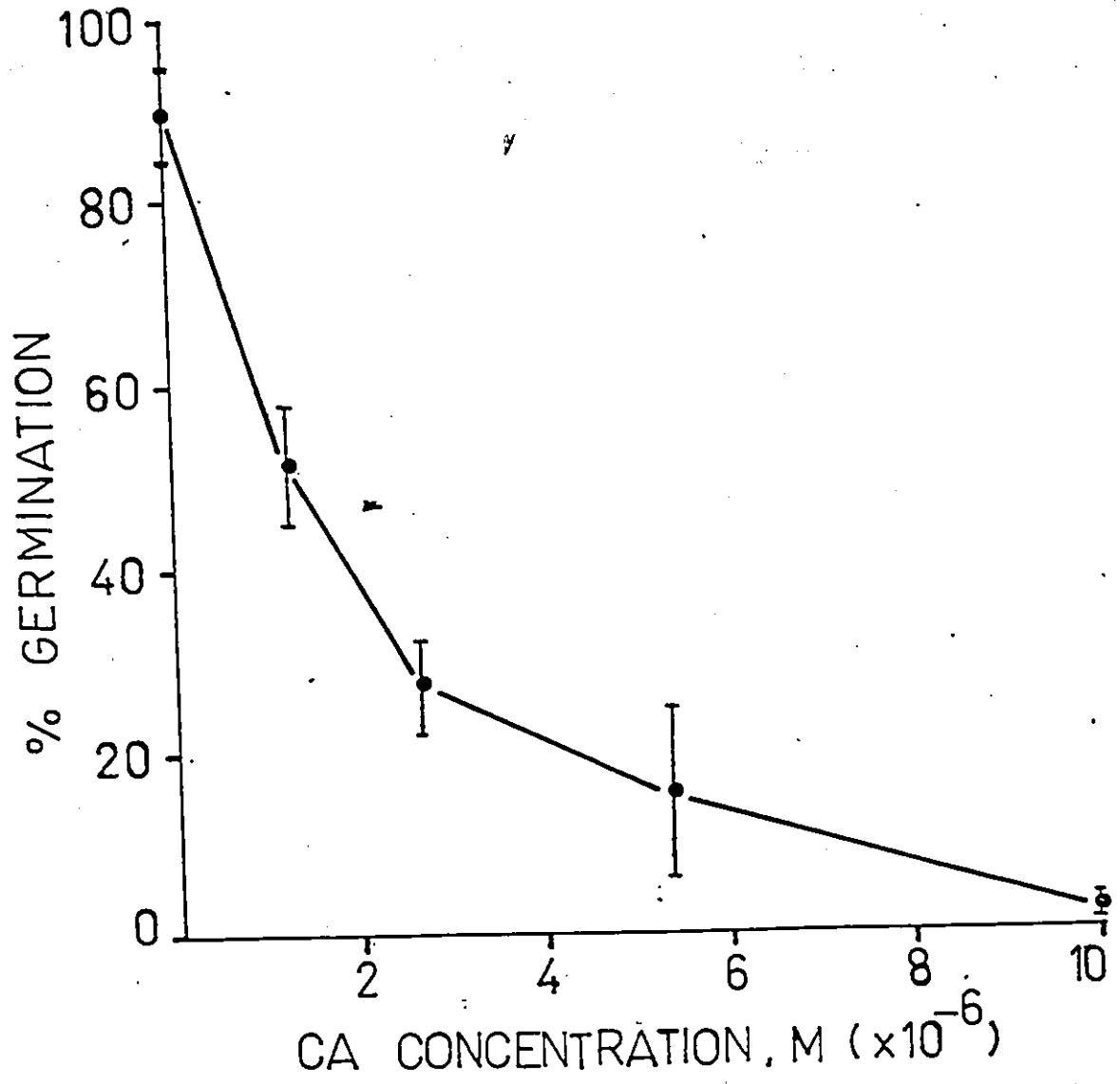




Figure 5. Effect of CA concentration on Achlya  
ambisexualis spore germination and colony  
production. The vertical bars denote 2X  
standard deviation.

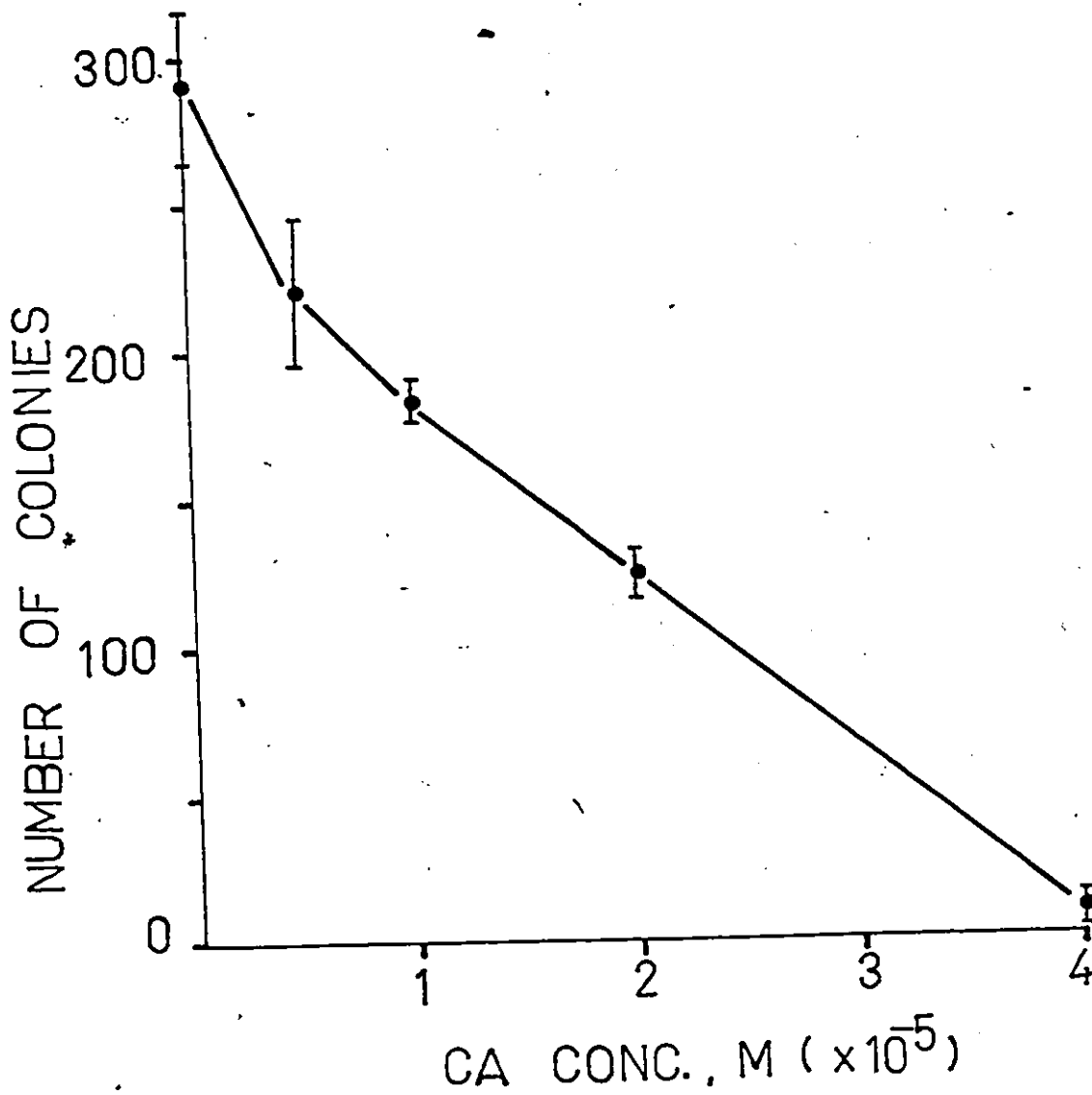


Figure 6. Irreversibility of CA inhibition of Achlya ambisexualis spore germination and colony production following a wash and transfer to fresh medium. The vertical bars denote 2X standard deviation.

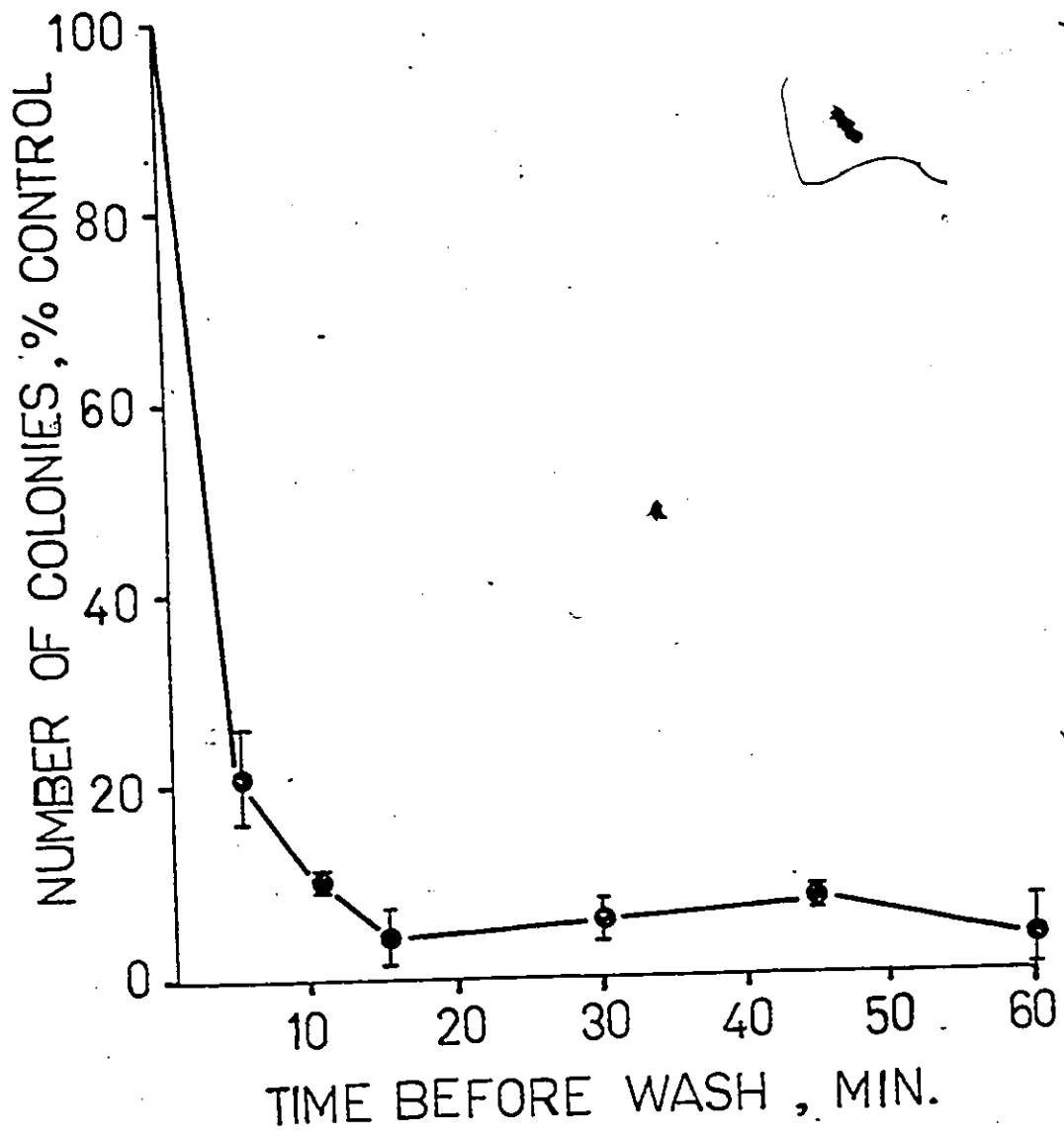


Figure 7. Typical Achlya ambisexualis growth curve, expressed as protein present in mycelial extract of 5 ml culture.

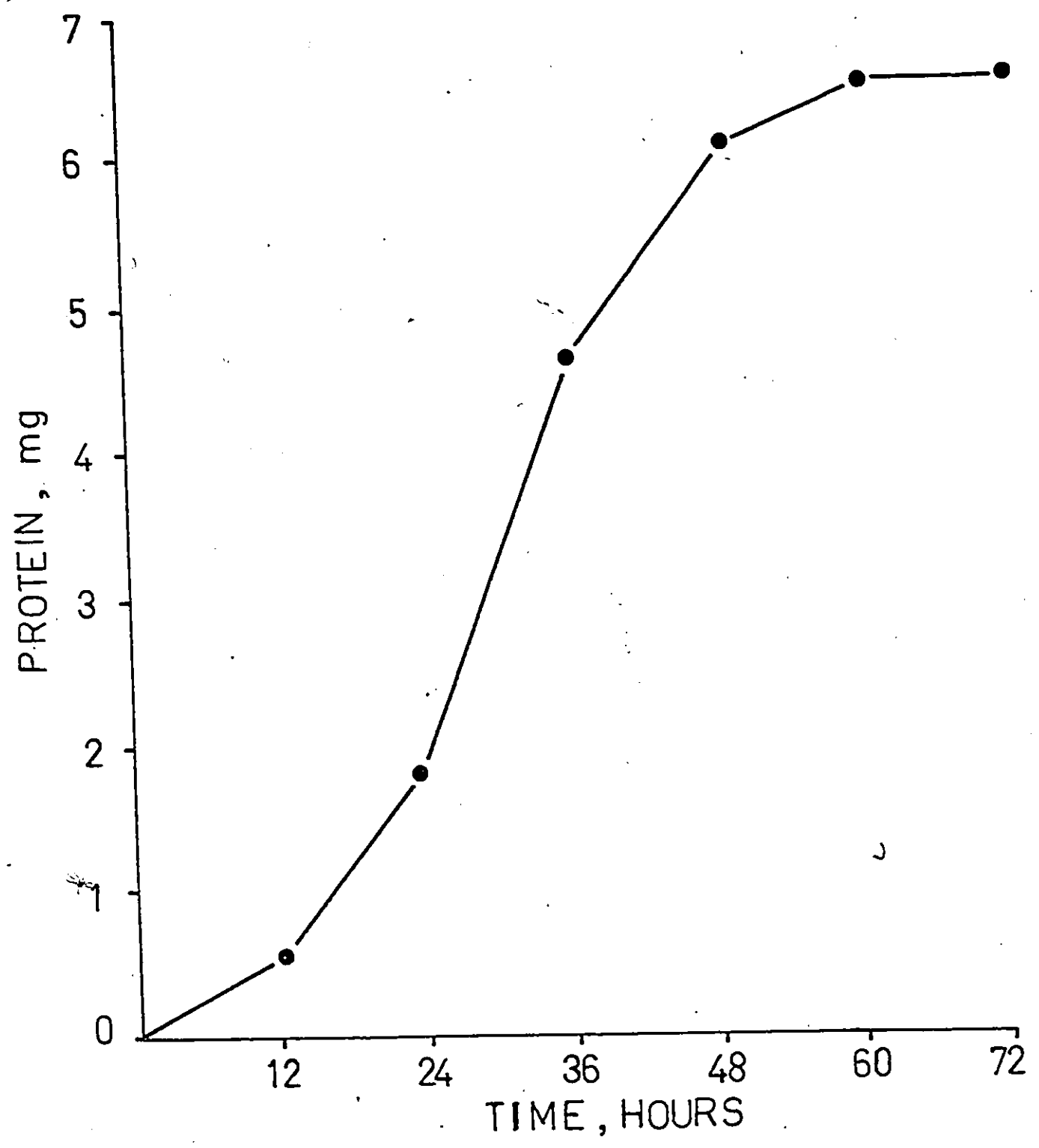
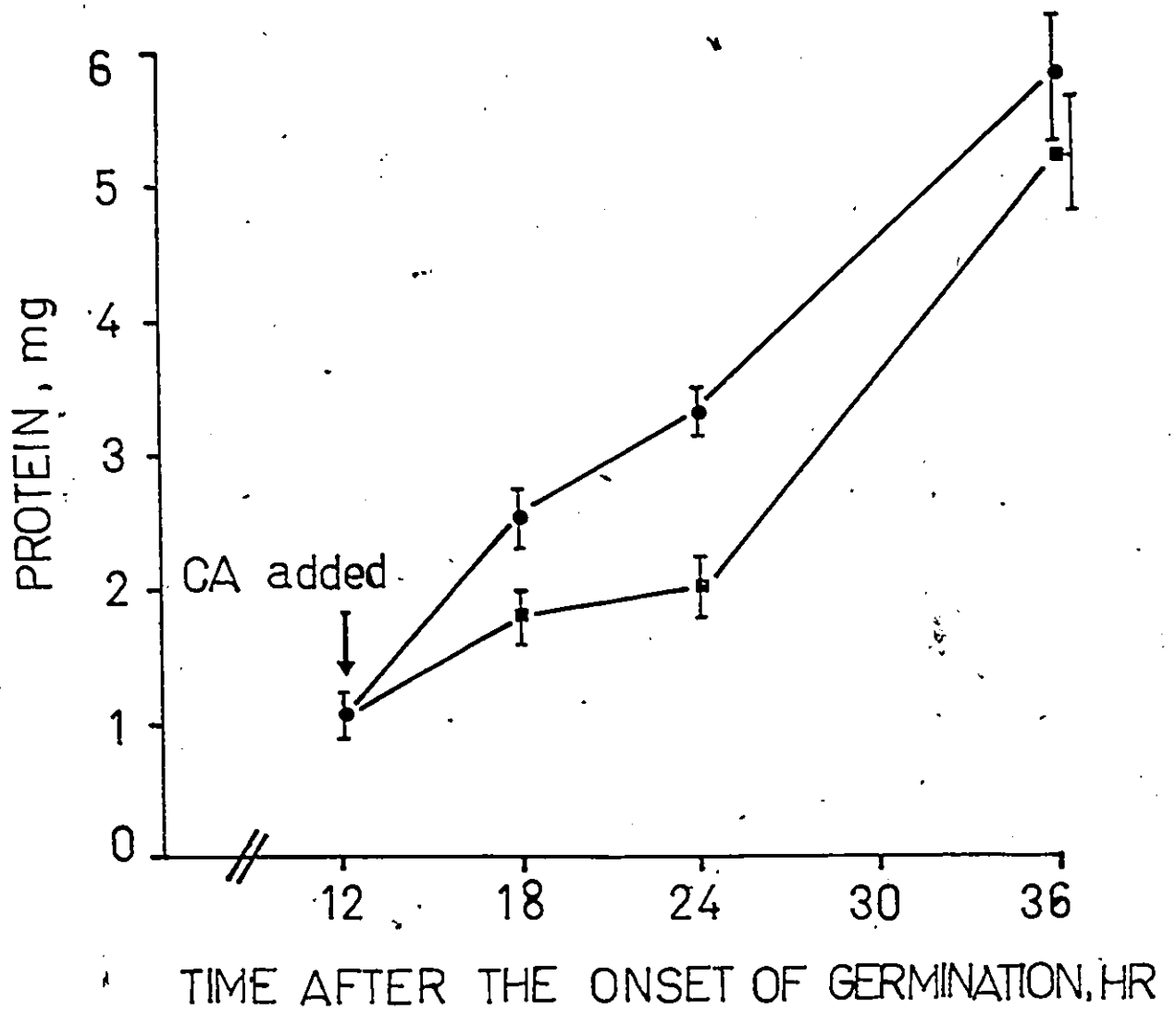


Figure 8. Effect of CA on the growth of Achlya ambisexualis germlings. ●—● Control; ■—■ CA ( $1 \times 10^{-5}$  M). The vertical bars denote 2X standard deviation.





Germling growth: Under the experimental conditions described, the logarithmic phase of Achlya ambisexualis growth begins 12 hours after inoculation and ends after 48 hours (Figure 7). Therefore, any studies on the growth of Achlya ambisexualis should be conducted within this period. Figure 8 shows that CA inhibits the growth of Achlya ambisexualis germlings as measured in terms of TCA-precipitable protein. The magnitude of the inhibition diminishes with time. After 24 hours of incubation growth in the CA treatment is half of that of the control.

#### DISCUSSION

The rapid irreversible CA response observed in Achlya ambisexualis spore germination is uncharacteristic of 'classical' cytochalasin effects, which are readily reversible. Washing and replacement of medium consistently failed to eliminate CA response in Achlya ambisexualis spore germination. These observations are in accord with the findings by Kletzien et al. (1972) that CA inhibition of sugar uptake in chick embryo fibroblasts was not readily reversible. Unlike a large number of fungi, which show greater CA sensitivity with regard to growth than to germination (Imperial Chemical Industries Laboratories, personal communication, 1976), Achlya ambisexualis spore germination is more sensitive to CA effect than the growth of the organism.

The CA inhibition of spore germination is to a certain extent time dependent. Maximum inhibition is observed

within 20 minutes. Longer treatment times are ineffective in inhibiting germination of the 5 to 10 percent of spores that are resistant to CA. Mycelia from these resistant spores produce spores which do not differ in CA sensitivity from the original population. Hence the variability in CA sensitivity of the asexual spores of Achlya ambisexualis appears to be of phenotypic origin.

The mechanism of CA inhibition of Achlya ambisexualis spore germination is not fully understood. Together, the minimal effect of CA on mycelial glucose uptake and the lack of glucose uptake by spores (Chapter IV) exclude the possibility that CA exerts its effect via inhibition of sugar uptake. Since CA inhibits the synthesis and secretion of cellulase (Chapter III), a secretory enzyme which plays a significant role in the morphogenesis of this fungus (Thomas and Mullins, 1967, 1969), the possibility that impaired synthesis and secretion of this enzyme as a possible cause for spore germination was explored. However, cellulase was not detected in the medium during the initial stages of development from spores. Respiratory metabolism of spores, measured by the rate of oxygen uptake, is severely inhibited by CA (Chapter IV). CA at low concentration ( $1 \times 10^{-5}M$ ) inhibits oxygen uptake of spores by approximately 80 percent. CA inhibition of spore germination may therefore be associated with respiratory inhibition.

## CHAPTER III

### EFFECTS OF CYTOCHALASIN A ON THE SYNTHESIS AND SECRETION OF CELLULASE

Cellulases (B-1,4-glucan 4-glucanohydrolase (E.C. 3.2.1.4) are enzymes capable of breaking down cellulose extracellularly for either nutritive or morphogenetic purposes. Their occurrence, in a variety of forms with different substrate specificities, in fungal, in bacterial and in higher plant systems has been reported (King, 1961; Byrne et al., 1975): Depending on their mode of attack on the cellulose molecule, cellulases are divided into exocellulases and endocellulases. Exocellulase cleaves the terminal subunit on the cellulose polymer releasing reducing sugar. On the other hand, the attack of the endocellulase is random and results in the release of subunits with variable lengths.

The role of cellulase in Achlya ambisexualis is morphogenetic rather than nutritive (Mullins, 1973; Thomas and Mullins, 1967). Cellulase secreted by Achlya ambisexualis presumably modifies the plasticity of the cellulosic wall as a prerequisite for the appearance of branch primordia. In support of this argument Thomas and Mullins (1967, 1969) have found that the appearance of branch primordia is associated with the concomitant rise of mycelial cellulase. Under the induction conditions they used, cellulase level starts to rise with the addition of the inducer and reaches

the peak in 2 hours. In the subsequent 24 hours, mycelial cellulase declines, as a result of secretion into the medium, to the level characteristic of non-induced mycelium.

In Achlya ambisexualis, as well as in many other systems, cellulase may exist as a multienzyme system (King, 1961). Degradation of cellulose may be the result of an array of enzymatic reactions, each component of the enzyme system mediating specific reactions. Attempts to characterize cellulases from pea epicotyls support this assumption (Byrne et al., 1975).

This study was conducted to examine the effect of CA on cellulase secretion and synthesis in Achlya ambisexualis.

#### METHODS

Induction and assay of cellulase: In Achlya ambisexualis the synthesis of cellulase can be induced by the addition of the steroid hormone antheridiol, by amino acids, or by mechanical shaking. For experimental reasons mechanical shaking was used to induce cellulase.

Achlya ambisexualis (male strain E 87) mycelia were grown aseptically on a chemically defined medium (M-medium)\*. A 2800 ml Erlenmeyer flask containing 1 liter of growth medium was inoculated with mycelial macerate and aerated with a gentle stream of air. After 5 days of growth at room temperature the mycelia were harvested by filtration and washed with

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\* see appendix

fresh growth medium. Under these conditions 30 to 40 grams (fresh weight) of mycelia were obtained.

To induce the synthesis of cellulase 1 gram fresh weight of mycelium was incubated at room temperature in 10 ml of fresh growth medium without glucose on a reciprocating shaker (120 cycles/min). After 2 hours of shake induction mycelia were harvested by filtration and the filtrate was saved to assay for secreted cellulase. CA was added to the growth medium at the beginning of the induction period. Where appropriate, the controls contained 2 percent (v/v) DMSO.

For mycelial cellulase determinations, mycelia were ground with 1 gram of washed sand in distilled water. The homogenate was centrifuged at maximum speed in a clinical centrifuge for 5 minutes and the supernatant was collected for cellulase assay.

A detailed description of the viscometric assay for cellulase is found elsewhere (King, 1961; Thomas and Mullins, 1967). Briefly, viscometric tubes containing 5 ml of the substrate (carboxy methyl cellulose) were incubated for 10 minutes at 37°C. After equilibration, 1 ml of the enzyme sample was added and mixed well. The enzyme-substrate mixture was incubated for 1 hour at 37°C and the initial and final flow times were recorded. One unit of cellulase was defined as that amount which gave 1 percent reduction in the flow time of the enzyme-substrate mixture during 1-hour incubation at 37°C.

Incorporation of  $^{14}\text{C}$ -leucine into mycelial proteins: The effect of CA on general protein synthesis in Achlya ambisexualis was studied by labelled leucine incorporation into mycelial proteins. Five day old mycelia in 1 gram quantities were incubated at room temperature in 10 ml of fresh growth medium containing DL-leucine- $^{14}\text{C}$  ( $8.5 \times 10^4$  cpm/u mole and  $7.9 \times 10^3$  cpm/u mole) to which CA was added (final concentration,  $4 \times 10^{-5}\text{M}$ ). The controls contained 2 percent (v/v) DMSO. After 1 hour static preincubation, the mycelia were shaken for 3 hours at room temperature on a reciprocating shaker (120 cycles/min). At the end of the incubation period the mycelia were harvested by filtration and washed with 30 ml of medium containing 0.4 mM unlabelled leucine. The washed mycelia were homogenized with 1 gram sand in 0.5 N NaOH containing 10 mM leucine. After clarifying by centrifugation the homogenate was acidified with 2 drops of concentrated HCl. The proteins were precipitated with 10 percent TCA at  $0^\circ\text{C}$  overnight. Pellets were collected by centrifugation at 5000 g for 10 minutes in the cold room and washed twice with 10 percent TCA containing 10 mM leucine and resuspended in 0.5 N NaOH to a final volume of 2.1 ml. Appropriate aliquants were used for liquid scintillation counting and protein determinations. Radioactivity was determined using 15 ml of a solubilising liquid scintillation fluid (Warner and McClean, 1968). Protein determinations were done according to Lowry et al. (1951).

## RESULTS

Figure 9 is typical of the CA response obtained and indicates that a wide range of CA concentrations inhibit both cellulase secretion and synthesis. The response is concentration dependent. The medium and the mycelial cellulase show similar decline as the concentration of CA is increased. Other cytochalasins tested (cytochalasins B and D) failed to inhibit cellulase secretion and synthesis.

The effect of CA on cellulase secretion and synthesis is reversible on removing CA, but never attains the control levels (Table 1). Mycelium preincubated for 1 hour in CA, then transferred to fresh growth medium for 2 hours of shake induction, is capable of cellulase synthesis and secretion. In contrast, mycelium maintained in CA shows little secretion of cellulase and no increase in mycelial enzyme when compared with the levels at the start of preincubation. The possibility that CA acts as a general protein synthesis inhibitor was excluded by the results in Table 2. These results are in agreement with other reports of no cytochalasin inhibition of labelled leucine uptake from the medium, or incorporation into proteins (Zigmond and Hirsch, 1972; Bradley, 1973). Failure of CA to inhibit general protein synthesis in Achlya ambisexualis indicates that the selective inhibition of cellulase synthesis may be a secondary effect, resulting from the inhibition of secretion.

Figure 9. Effect of CA concentration on cellulase secretion and on mycelial cellulase.  
●—●, Medium cellulase; ■—■, Mycelial cellulase. The vertical bars denote 2X standard deviation.



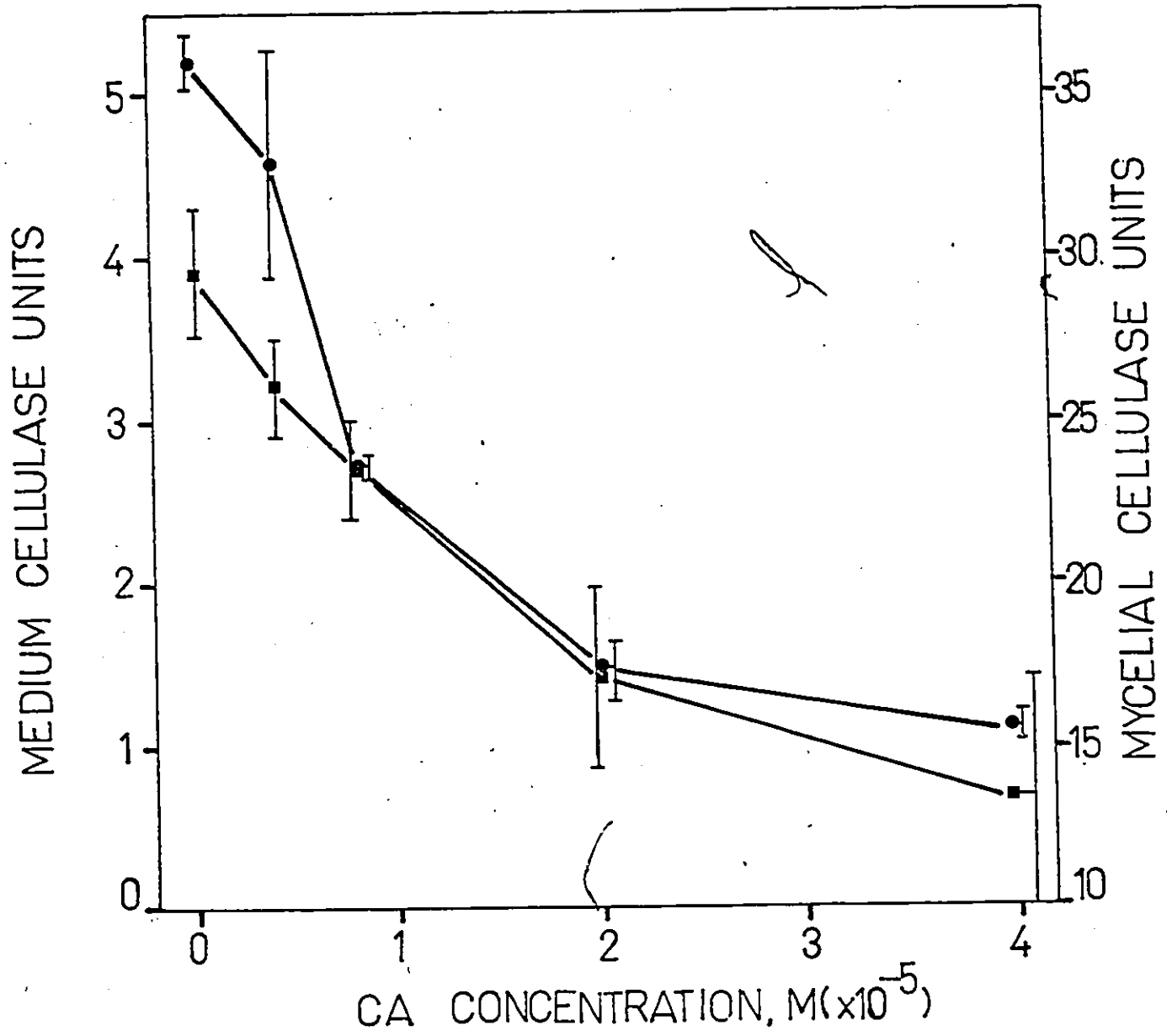


Table 1  
 Recovery of cellulase synthesis and secretion in  
Achlya ambisexualis after CA treatment.

Treatments	CA in medium*	Cellulase activity units	
		Medium**	Mycelium***
1 hour static preincubation	-	2.5 ± 0.14	23.3 ± 2.12
1 hour static preincubation	+	0.0	16.0 ± 0.07
1 hour static preincubation	-,-	25.4 ± 1.41	39.2 ± 0.28
followed by 2 hour shake induction	+,+	1.9 ± 1.41	14.5 ± 0.49
in fresh medium	+,-	11.9 ± 0.42	23.5 ± 4.30

\* Glucose - growth medium, with 2% DMSO, without (-) or with (+) CA at  $4 \times 10^{-5}$  M.

\*\* values represent mean ± standard deviation

\*\*\* Mycelial cellulase levels at the start of preincubation was  $14.9 \pm 0.21$  unit.

Table 2

Effect of CA and cycloheximide on  $^{14}\text{C}$ -leucine incorporation into mycelial protein, and on final radioactivity of medium.

Experiment	Treatments	Total medium activity cpm/ml after 4 hours	Mycelial protein cpm/100 ug after 4 hours
I	Control	2228 $\pm$ 28*	4167 $\pm$ 545*
	CA ( $4 \times 10^{-5}\text{M}$ )	2715 $\pm$ 100	4820 $\pm$ 510 <sup>NS</sup>
	Cycloheximide ( $3.5 \times 10^{-6}\text{M}$ )	2146 $\pm$ 97	230 $\pm$ 21
II	Control	320 $\pm$ 11	177 $\pm$ 4
	CA ( $4 \times 10^{-5}\text{M}$ )	387 $\pm$ 16	247 $\pm$ 73 <sup>NS</sup>
	Cycloheximide ( $3.5 \times 10^{-6}\text{M}$ )	302 $\pm$ 7	62 $\pm$ 3

\* values represent mean  $\pm$  standard deviation

<sup>NS</sup> no significant difference

## DISCUSSION

The effect of CA on cellulase synthesis and secretion of Achlya ambisexualis is partially reversible. This result is in distinct contrast with the irreversible effects of CA on spore germination. The reason for this discrepancy is obscure. Perhaps, the mycelia may be, somehow, more resistant than spores in their sensitivity to CA. Alternatively, CA may have been effectively removed by mycelia via tight binding thereby lowering the effective concentration of CA in the medium.

Although the actual mechanism is not fully understood cytochalasins inhibit secretion of macromolecules. The fact that CA inhibits synthesis and secretion of cellulase, a secretory protein, without affecting general protein synthesis is difficult to explain. If only secretion is affected one would expect higher levels of the enzyme within the mycelia. This is not found. The lack of accumulation of the enzyme within the mycelia indicates that synthesis of certain proteins may be controlled at the level of secretion.

It has been reported that certain transmembrane phenomena such as phagocytosis and pinocytosis show selective sensitivity to certain specific inhibitors (Zanvil and Cohn, 1966, 1970). Phagocytosis is found to be more sensitive to inhibitors of glycolysis. In contrast, pinocytosis is inhibited largely by inhibitors of mitochondrial respiration. Based on such evidence, Zanvil and Cohn (1970) suggested the possible

compartmentalization of ATP derived from glycolysis and mitochondrial respiration. In the light of these findings, one might speculate that CA acts in a similar fashion inhibiting glycolysis (Chapter IV) energy from which would be required for the synthesis and secretion of a secretory protein such as cellulase. On the other hand, general protein synthesis may be primarily dependent for energy on mitochondrial respiration.

CHAPTER IV  
THE EFFECTS OF CYTOCHALASIN A ON THE RESPIRATORY  
METABOLISM OF ACHLYA AMBISexualIS

A great deal is known about the respiratory metabolism of certain fungi, especially the yeasts. Relatively little attention has been directed to members of the Oomycetes, and even the available data are in many respects incomplete. In many fungi investigated, the existence of the Hexose Monophosphate Pathway (HMP), the Embden-Meyerhof Parnas scheme (EMP), the Tricarboxylic acid (TCA) cycle and a terminal cytochrome system were demonstrated (Gottlieb, 1976). Preliminary work by Warren and Mullins (1969) indicates that enzymes representing all the above mentioned schemes are present in Achlya ambisexualis. In addition, glycerol phosphate dehydrogenase and lactic acid dehydrogenase activity were observed, suggesting that the respiratory metabolism of Achlya ambisexualis is essentially similar to that of other fungi. In variance to the general concept (Wessells et al., 1971) that cytochalasins are not respiratory inhibitors, unexpectedly we found that CA inhibits respiratory metabolism of Achlya ambisexualis.

METHODS

Glucose uptake: The effect of CA on glucose uptake of Achlya ambisexualis mycelia was studied as follows. One day old mycelia in 0.2 gram quantities were incubated in 5 ml PYG medium containing CA ( $1 \times 10^{-5} M$ ) and  $5 \times 10^4$  cpm/ml 2-deoxy-D(1-<sup>3</sup>H)glucose (specific activity, 27 Ci/m mol) for 5 minutes

at room temperature. At the end of the incubation time mycelia were harvested by filtration and washed 3 times with 10 mM unlabelled glucose. Washed mycelia were then transferred to 5 ml ethanol and allowed to stand overnight at room temperature. A 2 ml aliquot of the ethanol extract was pipetted into a scintillation vial and, after evaporating to approximately 0.2 ml, radioactivity was determined by liquid scintillation counting as previously described. The controls contained 0.5 percent (v/v) DMSO.

Glucose utilization: The effect of CA on glucose utilization was studied using a procedure modified from that of McElroy et al. (1971). One day old Achlya ambisexualis mycelia were washed and resuspended, in 0.2 gram quantities, in 20 ml culture vials containing 4.5 ml PYG medium. A 4 ml vial containing 0.5 ml of the CO<sub>2</sub> absorbant NCS solubilizer was placed in the bottom of the culture vial. Half ml of PYG medium containing CA (final concentration  $4 \times 10^{-5} M$ ) and  $3.75 \times 10^5$  cpm/ml D-glucose-UL-<sup>14</sup>C (specific activity, 13.42 mCi/ mmol) was pipetted into each vial, immediately capped with a rubber stopper and incubated at room temperature for 1 hour. At the end of the incubation period 0.5 ml 1 M TCA was injected through the rubber stopper into the mycelial suspension and allowed to stand at room temperature for 3 hours. The caps were then removed and the small vials were emptied into clean scintillation vials. Radioactivity was determined by liquid scintillation counting as previously described. The controls contained 2 percent (v/v) DMSO.

Isolation and partial purification of *Achlya ambisexualis*

mitochondria: The isolation and partial purification of *Achlya ambisexualis* mitochondria were done according to C.O. Warren (personal communication, 1976). A schematic outline of the procedure is shown in Figure 10. Briefly, mycelia were grown for 2 days at room temperature, harvested by filtration and washed 3 times with 0.3 M sucrose. After removing excess water from the mycelial mat by pressing, 10 gram fresh weight of mycelia were homogenized with equal weight of washed sand in 30 ml of homogenizing solution (buffer-10 mM TRIS, pH 7.5 and 0.3 M sucrose) for 5 minutes using a mortar and pestle. The homogenate was filtered through a fine nylon mesh and the filtrate was collected. The residue was homogenized two more times reducing the homogenizing solution and grinding time by one half. After pooling, the filtrate was centrifuged at 2000 g for 10 minutes and the supernatant was retained. The low speed supernatant was centrifuged at 12,000 g for 10 minutes and the pellet was resuspended in for 4 ml of 0.3 M sucrose-0.002 M EDTA solution. The volume was adjusted to 10 ml and the centrifugation procedures were repeated twice more. The final pellet was resuspended in EDTA-sucrose solution and stored overnight at 0°C. All operations were done at 0 to 4°C.

Measurement of Oxygen Uptake: The measurement of oxygen uptake by spores, mycelia and mitochondrial suspension were made according to Smith et al. (1975) with a Yellow Spring Instruments (YSI) Oxygen Monitor connected to an oxygen electrode.



The oxygen electrodes were calibrated with respective incubation medium at 27°C.

To study the effect of CA on the oxygen uptake of spores or mycelia (1 day old) were washed and resuspended in fresh growth medium (M-medium) with or without glucose according to the experimental requirements. Four ml samples of the suspension was placed in a YSI reaction chamber in a water bath at 27°C. The suspension was constantly stirred by a magnetic stirrer. The rate of oxygen uptake of the suspension was determined by a YSI oxygen electrode connected to an oxygen monitor. CA was added at the beginning of the experiment.

The measurements of oxygen uptake by mitochondrial suspensions were done similarly. The reaction was initiated by the addition of 0.5 ml 3.3 mM succinate. CA effects were determined by the addition of the drug at the desired concentration after succinate was added. Protein determinations were done with Folin Phenol reagent by the method of Lowry et al. (1951).

## RESULTS

As opposed to other reports of CA inhibition of sugar uptake in yeasts (Kuo and Lampen, 1974, 1975) and chick embryo fibroblasts (Kletzien et al., 1972), Achlya ambisexualis mycelia treated with CA fails to inhibit glucose uptake (Table 3). Spores, on the other hand, do not take up measurable amounts of glucose until 6 hours post-germination (Table 5). This observation is consistent with other reports of the lack

of sugar uptake in many germinating fungal spores (Sussman, 1956, 1961; Gottlieb, 1976). Although CA shows no inhibition of glucose uptake, it significantly diminishes glucose utilization of mycelia (Table 4).

CA at  $1 \times 10^{-5}$  M concentration significantly reduced oxygen uptake by spores as well as mycelia. The inhibition obtained progressed in linear fashion with time. Typically, CA at  $1 \times 10^{-5}$  M diminished oxygen uptake by about 85 percent. Spores and mycelia appear to be equally susceptible to CA inhibition (Figures 11, 12). As with other responses in Achlya ambisexualis, the effect is rapid and irreversible. As soon as the drug is added, oxygen uptake is severely impaired. Similarly, spores treated with CA for 20 minutes with subsequent washing and resuspension in fresh medium had no effect (Figure 13).

Since spores and mycelia are found to be highly sensitive to CA, the effect on isolated mitochondria was studied. As shown in Figure 14 CA ( $1 \times 10^{-5}$  M) has no effect on the uptake of oxygen by isolated mitochondria. Even a four-fold increase in CA concentration does not result in any significant change. Sodium cyanide (NaCN), a classical inhibitor of mitochondrial respiration, significantly inhibits oxygen uptake by the mitochondrial suspension.

The rate of endogenous respiration in many fungi, including Achlya ambisexualis, is known to be very high

Table 3  
Effect of CA on glucose uptake of Achlya  
ambisexualis mycelia

Treatment	Uptake (cpm/0.2 gram mycelia/ 5 min)
Control (DMSO, 0.5%)	652 ± 31*
CA (1 × 10 <sup>-5</sup> M)	575 ± 113 <sup>NS</sup>

\* values represent mean ± standard deviation

<sup>NS</sup> no significant difference

Table 4  
Effect of CA on glucose utilization of Achlya  
ambisexualis mycelia

Treatment	<sup>14</sup> C <sub>0</sub> 2 liberated (cpm/ml)
Control (DMSO, 2%)	2530 ± 315*
CA (4 × 10 <sup>-5</sup> M)	843 ± 32 <sup>a</sup>

\* values represent mean ± standard deviation

<sup>a</sup> significantly different (P < 0.05)

Table 5  
Effect of preincubation time on uptake of glucose  
by Achlya ambisexualis spores

** Preincubation time (hr)	*** Uptake (cpm/2.5 x 10 <sup>3</sup> spores/ 30 min)		
	10	20	30 min)
0	153 ± 39	72 ± 18	139 ± 42*
1	269 ± 50	99 ± 8	75 ± 24
3	146 ± 64	106 ± 15	186 ± 30
6	114 ± 48	52 ± 15	72 ± 13
12	1260 ± 190	1955 ± 82	2679 ± 155
24	3767 ± 89	6813 ± 213	10485 ± 182

\* values represent mean ± standard deviation

\*\* spores were preincubated in PYG medium at room temperature on a reciprocating shaker (90 cycles/min).

\*\*\* absence of significant increase of radioactivity with time is considered to reflect lack of uptake.

Uptake of glucose by spores was measured by incubating 2.5 x 10<sup>3</sup> spores in 1 ml fresh growth medium containing 2.85 x 10<sup>4</sup> cpm/ml 2-deoxy-D(1-<sup>3</sup>H)glucose (specific activity, 27 Ci/ mmol) for 10, 20 or 30 minutes at room temperature on a reciprocating shaker (90 cycles/min). At the end of the incubation period spores were collected on millipore filters (HWAP, 0.45 u), washed 3 times with 10 mM unlabelled glucose. After drying for 1 hour under heat lamp, the filters were transferred to scintillation vials and the radioactivity was determined by liquid scintillation counting.

Table 6

Effect of CA on the uptake of oxygen by Achlya ambisexualis mycelia in the absence of exogenous glucose

Treatment**	O <sub>2</sub> , % saturation after 10 min. incubation
Control (DMSO, 0.5%)	40.8 ± 7.1*
CA (1 x 10 <sup>-5</sup> M)	76.6 ± 5.2 <sup>a</sup>

\* values represent mean ± standard deviation

<sup>a</sup> significantly different (P < 0.01)

\*\* Four ml samples of the mycelial suspension in glucose free M-medium (235 ug protein/ml) were incubated in a YSI reaction chamber at 27°C. CA or DMSO was added at the beginning of the incubation.

Figure 10. A schematic outline of the procedure for isolation and partial purification of Achlya ambisexualis mitochondria.

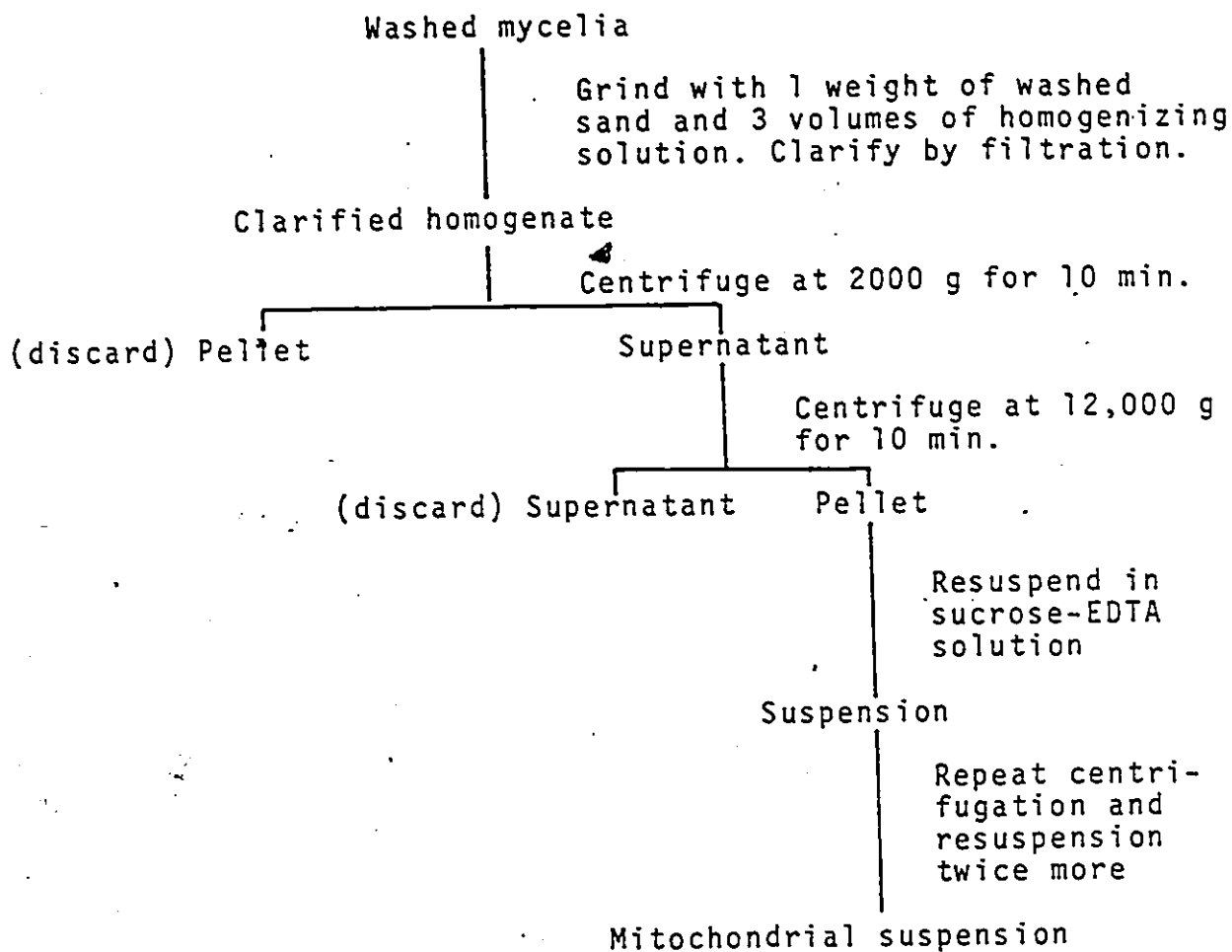


Figure 11. Effect of CA on oxygen uptake of Achlya ambisexualis spores. Four ml samples of the spore suspension ( $8.5 \times 10^5$  spores/ml) were incubated in a YSI reaction chamber at  $27^\circ\text{C}$ . CA ( $1 \times 10^{-5}\text{M}$ ) was added at the beginning of the incubation. Oxygen uptake was measured using a YSI oxygen monitor connected to an oxygen electrode. The controls contained 0.5 percent (v/v) DMSO.

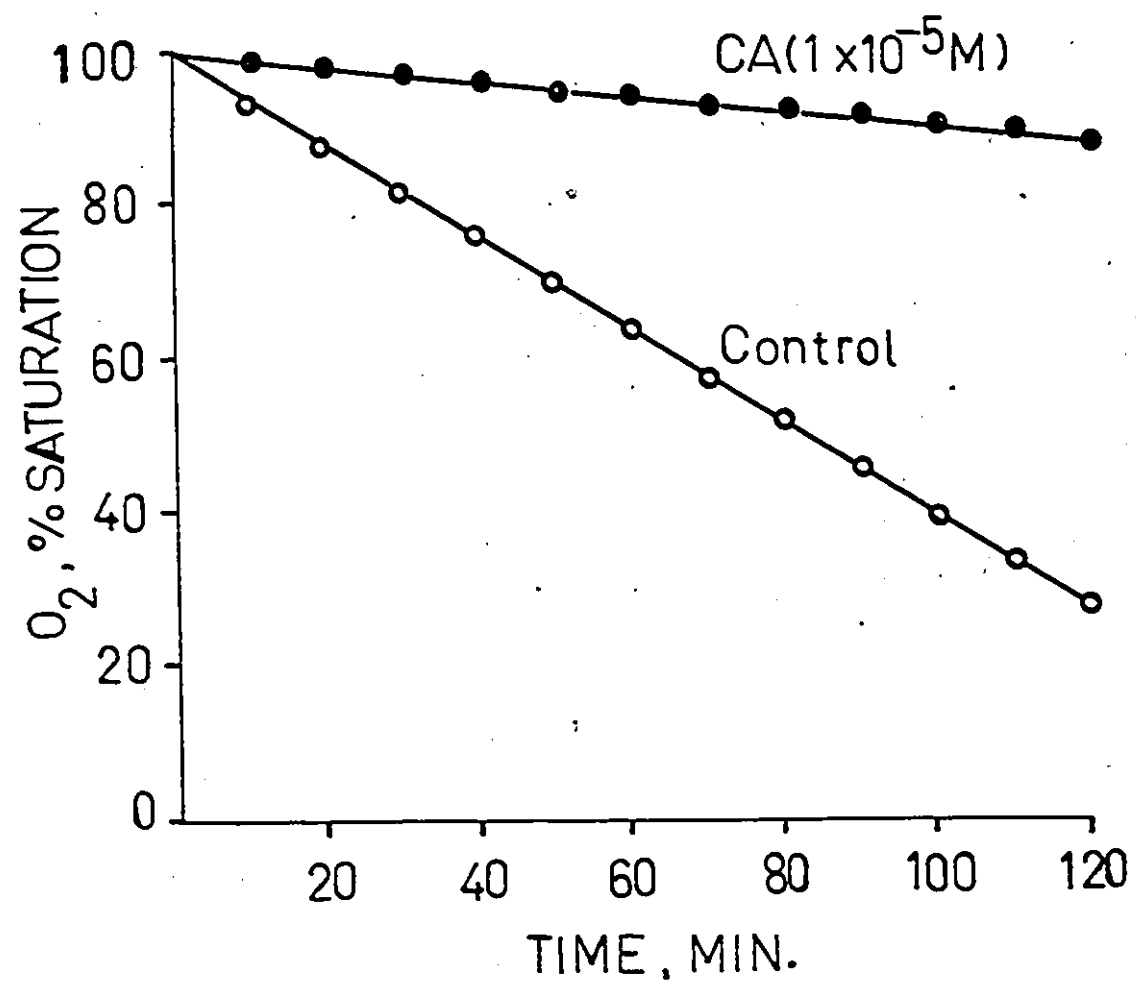




Figure 12. Effect of CA on oxygen uptake of Achlya ambisexualis mycelia in a medium (PYG) containing glucose. Four ml samples of mycelial suspension (135 ug protein/1 ml of culture) were incubated in a YSI reaction chamber at 27°C. CA ( $1 \times 10^{-5}$  M) was added at the beginning of the incubation. Oxygen uptake was measured using a YSI oxygen monitor connected to an oxygen electrode. The controls contained 0.5 percent (v/v) DMSO.

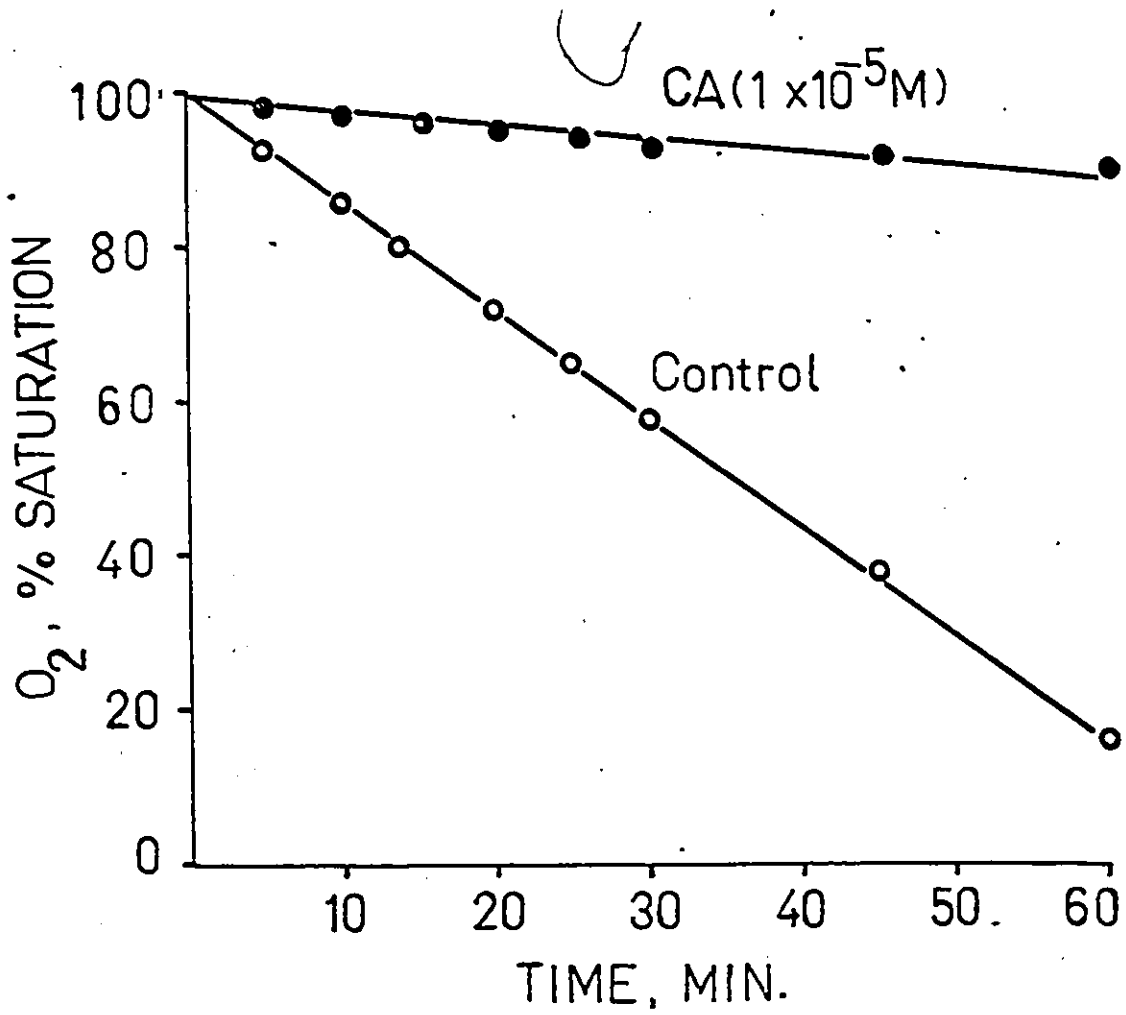


Figure 13. Irreversibility of CA inhibition of oxygen uptake by Achlya ambisexualis spores. Four ml samples of the spore suspension ( $1.7 \times 10^6$  spores/ml) were incubated in a YSI reaction chamber at  $27^{\circ}\text{C}$ . CA ( $1 \times 10^{-5}\text{M}$ ) was applied either during the period of respiratory measurement or as a 20 minute treatment followed by replacement in fresh medium. Oxygen uptake was measured using a YSI oxygen monitor connected to an oxygen electrode. The controls contained 0.5 percent (v/v) DMSO.

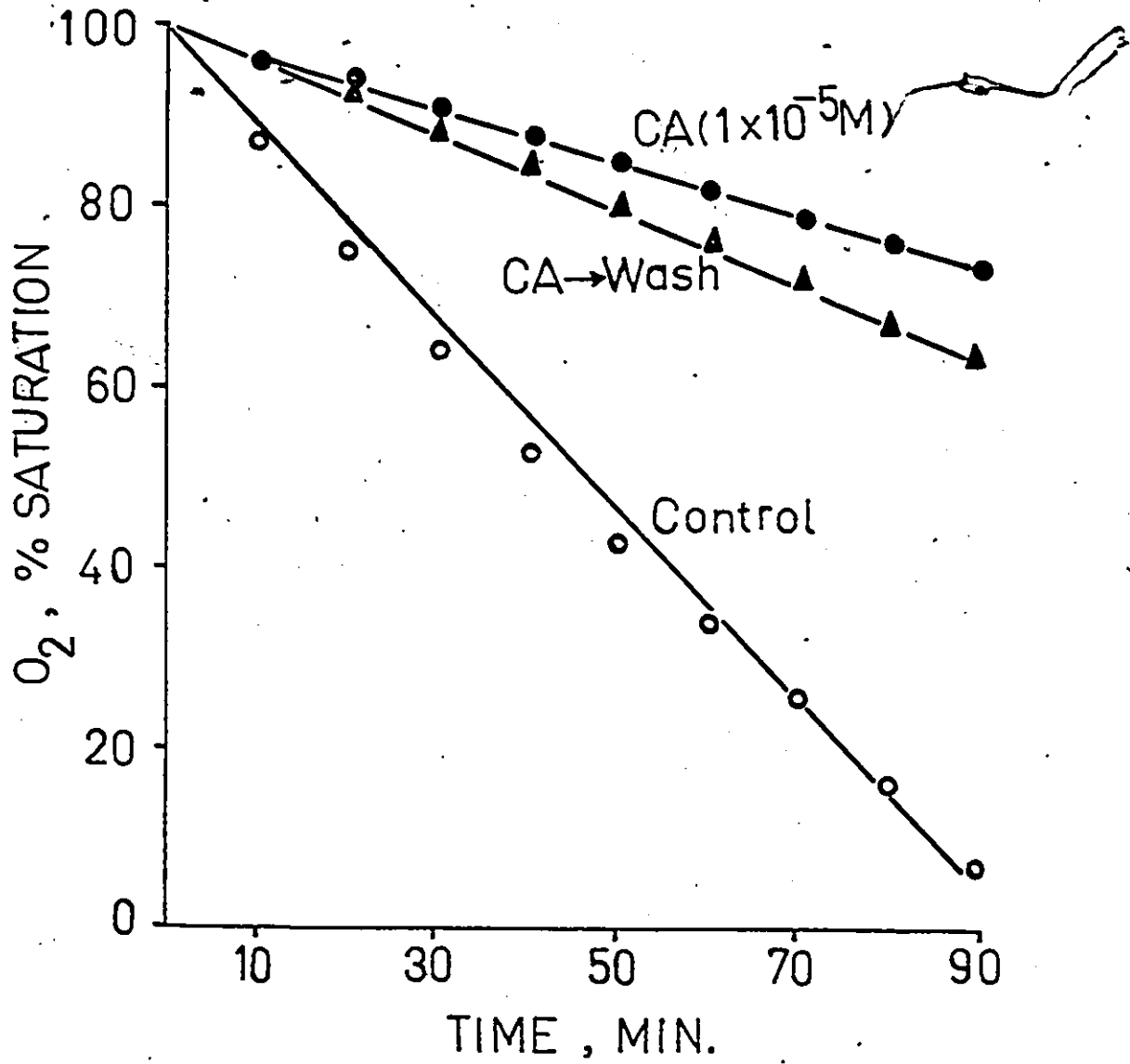
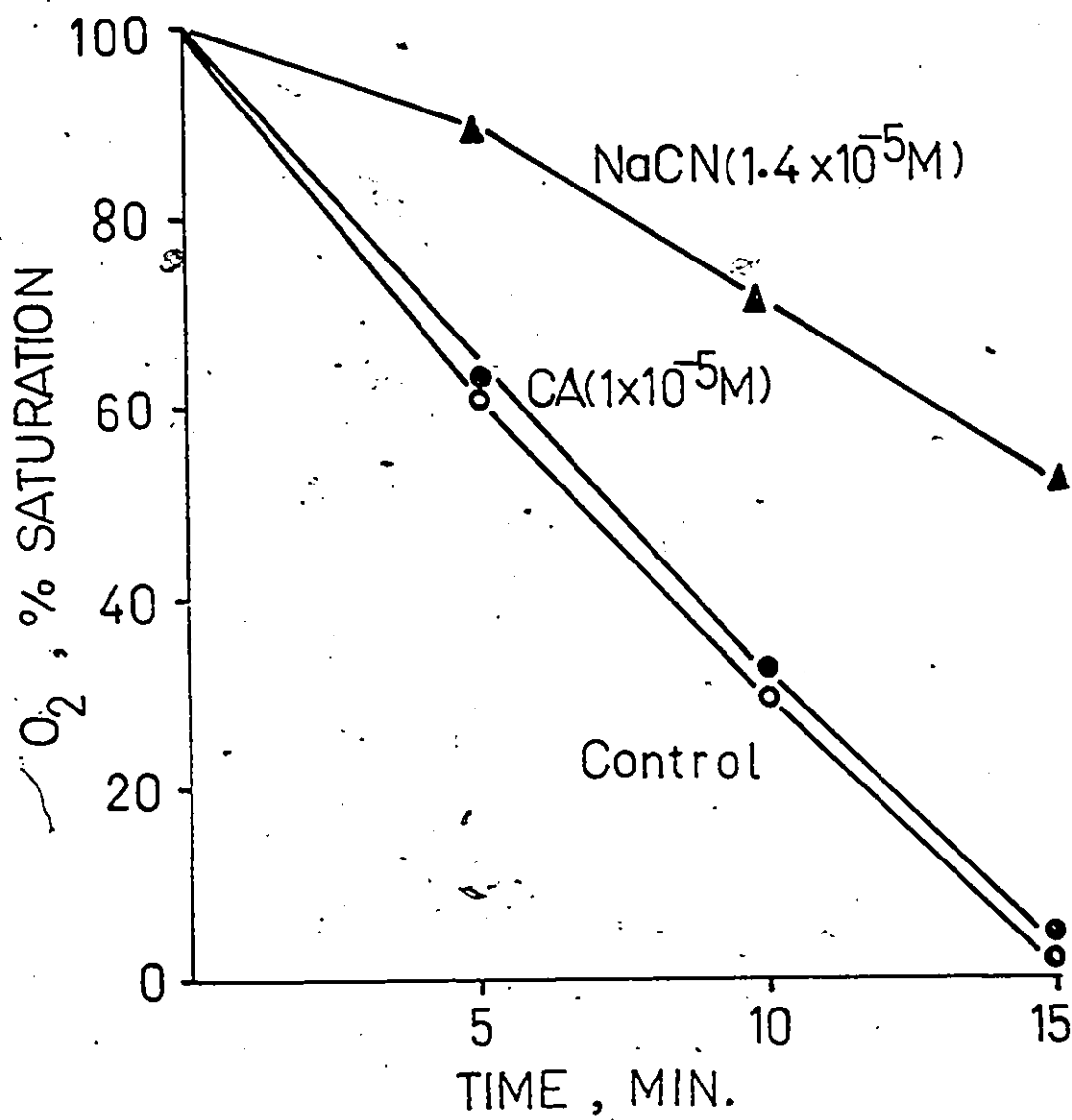


Figure 14. Effect of CA and NaCN on oxygen uptake of Achlya ambisexualis mitochondria. Four ml samples of the mitochondrial suspension (1.2 mg protein/ml) were incubated in a YSI reaction chamber at 27°C. The reaction was initiated by the addition of 0.5 ml 3.3 mM succinate after which CA ( $1 \times 10^{-5}$  M) or NaCN ( $1.4 \times 10^{-5}$  M) was applied. Oxygen uptake was measured using a YSI oxygen monitor connected to an oxygen electrode. The controls contained 0.5 percent (v/v) DMSO.



(Warren and Mullins, 1969; Gottlieb, 1976). Table 6 shows that even in the absence of exogenous supply of glucose CA significantly reduces oxygen uptake by mycelia. Similarly, the fact that spores do not take up measurable amounts of glucose 6 hours post germination suggests that the observed inhibition of oxygen uptake by CA in young spores is not related to inhibition of glucose uptake.

### DISCUSSION

CA is a potent inhibitor of respiratory metabolism in Achlya ambisexualis. At a comparatively low concentration ( $1 \times 10^{-5}M$ ) CA inhibits oxygen uptake by 85 percent. The mechanism of inhibition remains elusive. The lack of effect on sugar uptake and the inhibition of oxygen uptake in the absence of exogenous glucose supply suggest that the inhibitory response is not mediated by the inhibition of sugar uptake. The absence of any effect on isolated mitochondria further indicates that the effect of CA may be on glycolysis. Since CA could act as a potential sulfhydryl reagent (Chapter V) it may well interfere with one or more of the sulfhydryl enzymes of the glycolytic pathway. In any event further work is required to establish the exact site of CA action in the respiratory metabolism of Achlya ambisexualis.

## CHAPTER V

### THE MODE OF ACTION OF CYTOCHALASIN A IN ACHLYA AMBISEXUALIS

Although Carter (1967, 1972) has pointed out that the rapidity and rapid reversibility of cell responses to the cytochalasins are consistent with action on the plasma membrane, evidence on the location of responses is lacking. In contrast, evidence that cytochalasins act, in certain systems, in the manner of thiol reagents (Haslam, 1972; Kuo and Lampen, 1975; Seagull and Thomas, 1976) provides clues to a mode of action for these compounds. It was hypothesized that, if CA acts as thiol reagent, thiol protection might eliminate CA responses in Achlya ambisexualis and, by the same token, other potential thiol reagents such as N-ethyl maleimide (NEM), p-chloromercuribenzenesulfonate (pCMBS), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and N-Iodoacetyl-N'-(5-sulfo-1-naphthyl)-ethylenediamine (1,5-I-AEDANS) might mimic CA responses. The purpose of this investigation was therefore to examine the effect of thiols such as cysteine (Cys), dithiothreitol (DTT) and glutathione (GSH) on CA response and to search for possible mimicry of CA response by other potential thiol reagents.

#### METHODS

Achlya ambisexualis (male strain E 87) mycelia and spores were obtained as previously reported (Chapters II & III). Procedures used for the induction and assay of cellulase, for



spore germination and for the study of  $^{14}\text{C}$ -leucine incorporation into mycelial proteins were similar to those described in chapters II and III.

CA Binding by Mycelia: In the absence of isotopically labelled CA, an indirect measure of CA binding by Achlya ambisexualis, was obtained by a bioassay of a CA containing medium previously exposed to mycelia. To study the effect of time on CA binding 2 day old fresh mycelia in 1 gram quantities were incubated in 5 ml of growth medium containing  $1 \times 10^{-5}\text{M}$  CA for 1 to 20 minutes at  $0^{\circ}\text{C}$ . At the end of the incubation period, mycelia were removed from the medium by filtration and the residual CA in the medium was bioassayed on the basis of its previously determined (Chapter II) ability to inhibit spore germination.

To study the effect of prebinding with a known thiol reagent on the subsequent binding of CA, mycelia in 1 gram quantities were exposed to  $1 \times 10^{-4}\text{M}$  NEM for 5 minutes, washed 3 times with fresh growth medium and incubated in  $1 \times 10^{-5}\text{M}$  CA for 1 hour at  $0^{\circ}\text{C}$ . After separating the mycelia by filtration, the residual CA in the medium was bioassayed as described above. To check the possibility that NEM might interfere with the spore germination bioassay, mycelia identically pretreated with NEM were incubated in fresh growth medium for 1 hour at  $0^{\circ}\text{C}$  and after removing the mycelia by filtration, the ability of the medium to inhibit spore germination was determined.

p-CMBS Binding by Mycelia: Binding of pCMBS by Achlya ambisexualis mycelia was measured using isotopically labelled pCMBS. Fresh mycelia in 0.2 gram quantities were incubated for varying periods of time at room temperature in 5 ml of fresh PYG medium containing 0.08 uCi/ml ( $^{203}\text{Hg}$ )pCMBS (specific activity, 1 mCi/50 mg). Unless otherwise indicated, at the end of the incubation period the mycelia were washed 3 times on fiber glass filters (Whatman) with fresh growth medium. Washing was completed within 3 minutes. Mycelia were homogenized in 3 ml 0.1 N NaOH and a 0.2 ml aliquant of the homogenate was sampled for liquid scintillation counting.

### RESULTS

Thiol inhibition of the CA responses; inhibition of cellulase synthesis and secretion, and inhibition of spore germination, was obtained not only with cysteine (Table 7; Figure 15) but with the relatively non-penetrating (Wallach, 1974) thiol glutathione (Table 8; Figure 16). In testing thiol reagents for CA-like effects on protein synthesis in Achlya ambisexualis, it is found that both the relatively penetrating (Harbury and Schrier, 1974) agent N-ethyl maleimide ( $1 \times 10^{-5}\text{M}$  to  $5 \times 10^{-3}\text{M}$ ) (Figure 17) and the relatively non-permeant (Aledort et al., 1968) thiol reagent p-chloromercuri-benzene sulfonate ( $2.4 \times 10^{-5}\text{M}$  to  $4.8 \times 10^{-4}\text{M}$ ) (Figure 18) inhibited cellulase synthesis and secretion. At  $1 \times 10^{-4}\text{M}$ , cellulase secretion in the presence of NEM or pCMBS was 25 percent and 65 percent of the control values respectively.

Although glutathione eliminated pCMBS inhibition (Table 8), thiol failed to inhibit the NEM response. In contrast to the overall inhibition of protein synthesis elicited by NEM (Table 9), comparable to inhibition by cycloheximide, pCMBS failed to affect overall protein synthesis, as determined by labelled leucine incorporation into mycelial protein (Table 9). Thus, pCMBS resembles CA in selectively inhibiting cellulase without affecting general protein synthesis.

Incubation of mycelia in fresh growth medium containing ( $^{203}\text{Hg}$ )pCMBS resulted in rapid binding of radioactivity which attained a maximum level within 1 hour (Figure 19). Further incubation upto 3 hours, was not accompanied by any substantial increase in bound radioactivity. Labelled mycelium, washed and subsequently incubated in fresh growth medium shows no loss of bound radioactivity (Figure 20). A similar treatment with a normally inhibitory concentration of pCMBS, followed by a wash and replacement in fresh medium has no significant inhibitory effect on cellulase induction and secretion, indicating that pCMBS inhibition is not mediated by tightly bound reagent (Table 10). From Figure 20 it is clear that about 70 percent of the bound radioactivity of ( $^{203}\text{Hg}$ )pCMBS is lost within 5 minutes of treatment with the thiol DTT or with unlabelled pCMBS, indicating that pCMBS acts in Achlya ambisexualis, as in other systems (Aledort et al., 1968) as a relatively non-penetrating reagent.

Table 7  
Cysteine (Cys) inhibition of CA response in Achlya  
ambisexualis

Treatments**	Cellulase activity units	
	Medium	Mycelial
Control	70.4 ± 0.71	45.5 ± 1.63*
CA (4 × 10 <sup>-5</sup> M)	33.3 ± 1.77	31.0 ± 1.13 <sup>a</sup>
CA (4 × 10 <sup>-5</sup> M) + (Cys (1×10 <sup>-3</sup> M)	70.9 ± 0.64	43.1 ± 0.85 <sup>NS</sup>

\* values represent mean ± standard deviation

<sup>a</sup> significantly different (P < 0.01)

<sup>NS</sup> no significant difference

\*\* All treatments contained 0.5 gram fresh weight of mycelium in 5 ml PYG medium. Cysteine and CA were added to the growth medium simultaneously at the start of the experiment.

Table 8  
 Glutathione (GSH) inhibition of CA and pCMBS responses  
 in Achlya ambisexualis

Experiment	Treatments**	Cellulase activity units
I	Control	27.4 ± 3.55*
	CA ( $4 \times 10^{-5}$ M)	7.5 ± 0.45
	CA ( $4 \times 10^{-5}$ M) + GSH ( $3.25 \times 10^{-4}$ M)	30.0 ± 2.05
II	Control	18.2 ± 0.70
	pCMBS ( $1.92 \times 10^{-4}$ M)	13.1 ± 0.35
	pCMBS ( $1.92 \times 10^{-4}$ M) + GSH ( $3.25 \times 10^{-4}$ M)	19.4 ± 0.65

\* values represent mean ± standard deviation

\*\* All treatments contained 0.5 gram fresh weight of mycelium in 5 ml PYG medium. Glutathione (GSH) and CA or pCMBS were added to the growth medium simultaneously at the start of the experiment. CA and pCMBS produced cellulase levels different from controls ( $P < 0.01$ ).

Table 9

Effects of a penetrating (NEM) and non-penetrating (pCMBS) thiol reagent, and cycloheximide on  $^{14}\text{C}$ -leucine incorporation into mycelial protein

Experiment	Treatments**	Mycelial protein cpm/ mg after 3 hours
I	Control	2604 $\pm$ 133*
	pCMBS ( $2.4 \times 10^{-4}\text{M}$ )	2660 $\pm$ 15 <sup>NS</sup>
	Cycloheximide( $7 \times 10^{-5}\text{M}$ )	720 $\pm$ 68
II	Control	755 $\pm$ 50
	NEM ( $4 \times 10^{-4}\text{M}$ )	447 $\pm$ 22 <sup>a</sup>
	Cycloheximide( $7 \times 10^{-5}\text{M}$ )	407 $\pm$ 19

\* values represent  $\pm$  standard deviation

<sup>a</sup> significantly different ( $P < 0.05$ )

<sup>NS</sup> no significant difference

\*\* All treatments contained 0.5 gram fresh weight of mycelium in 5 ml of PYG medium containing 0.5 mM leucine. The specific activities of the L-U- $^{14}\text{C}$ -leucine used in these experiments were  $8.7 \times 10^3$  cpm/u mol and  $1.3 \times 10^3$  cpm/u mol respectively. Radioactivity was determined by liquid scintillation counting as reported previously. In Achlya ambisexualis, cycloheximide is a known inhibitor of protein, including cellulase, synthesis (Kane et al., 1973).

Table 10

Lack of effect of bound pCMBS on the synthesis and secretion of cellulase (Cx) in Achlya ambisexualis. pCMBS was applied either during a 30 minutes pre-incubation or during a 3 hour Cx induction.

Preincubation	Treatment medium		Cx activity units
		Cx induction period	
FGM	FGM	(Control)*	26.8 ± 2.47 <sup>****</sup>
FGM	pCMBS <sup>**</sup>		13.3 ± 0.07 <sup>a</sup>
pCMBS	FGM <sup>***</sup>		24.1 ± 2.26 <sup>NS</sup>

\* Mycelia, 0.5 g, were preincubated in 5 ml FGM (fresh growth medium) for 30 min, washed 3 times with FGM and resuspended in 5 ml FGM for cellulase (3 hour) induction.

\*\* Cx induction medium contained pCMBS ( $2.4 \times 10^{-4}$ M).

\*\*\* Mycelia, 0.5 g, were preincubated in 5 ml FGM containing pCMBS ( $2.4 \times 10^{-4}$ M) for 30 minutes, washed 3 times with FGM and resuspended in 5 ml FGM for Cx (3 hour) induction.

Cx was induced with shaking on a reciprocating shaker (120 cycles/min) at room temperature.

\*\*\*\* values represent mean ± standard deviation

<sup>a</sup> significantly different ( $P < 0.01$ )

<sup>NS</sup> no significant difference

Table 11

Effect of time on CA binding to Achlya ambisexualis mycelia at 0°C. Residual CA after treatment with mycelia was bioassayed by spore germination.

Binding time (min)	Percent germination**
1	75.5 ± 3.4*
10	67.9 ± 5.4
20	68.8 ± 3.1

\* values represent mean ± standard deviation

\*\* Control (DMSO, 0.5%) and CA  $1 \times 10^{-5}$  M gave 75.8 ± 4.9 and 15.5 ± 7.2 values respectively.



Table 12

Effect of NEM pretreatment on the subsequent binding of CA by Achlya ambisexualis mycelia. Binding is shown by an increased percentage in the spore germination bioassay.

Spore germination medium	Percent germination	
	Expt. I	Expt. II
Control	93.4 ± 6.4	64.0 ± 5.7*
CA ( $1 \times 10^{-5}$ M)	9.4 ± 7.0	6.7 ± 4.7
CA ( $1 \times 10^{-5}$ M) treated with fresh mycelia	97.3 ± 1.3	58.3 ± 4.6
CA ( $1 \times 10^{-5}$ M) treated with NEM ( $1 \times 10^{-4}$ M) pretreated fresh mycelia	42.3 ± 12.0	35.4 ± 5.7
Fresh growth medium treated with NEM pretreated fresh mycelia	95.5 ± 4.5	62.1 ± 6.5

\* values represent mean ± standard deviation

Figure 15. Varying effectiveness of cysteine additions at different times during CA treatment. Vertical bars denote 2X standard deviation.

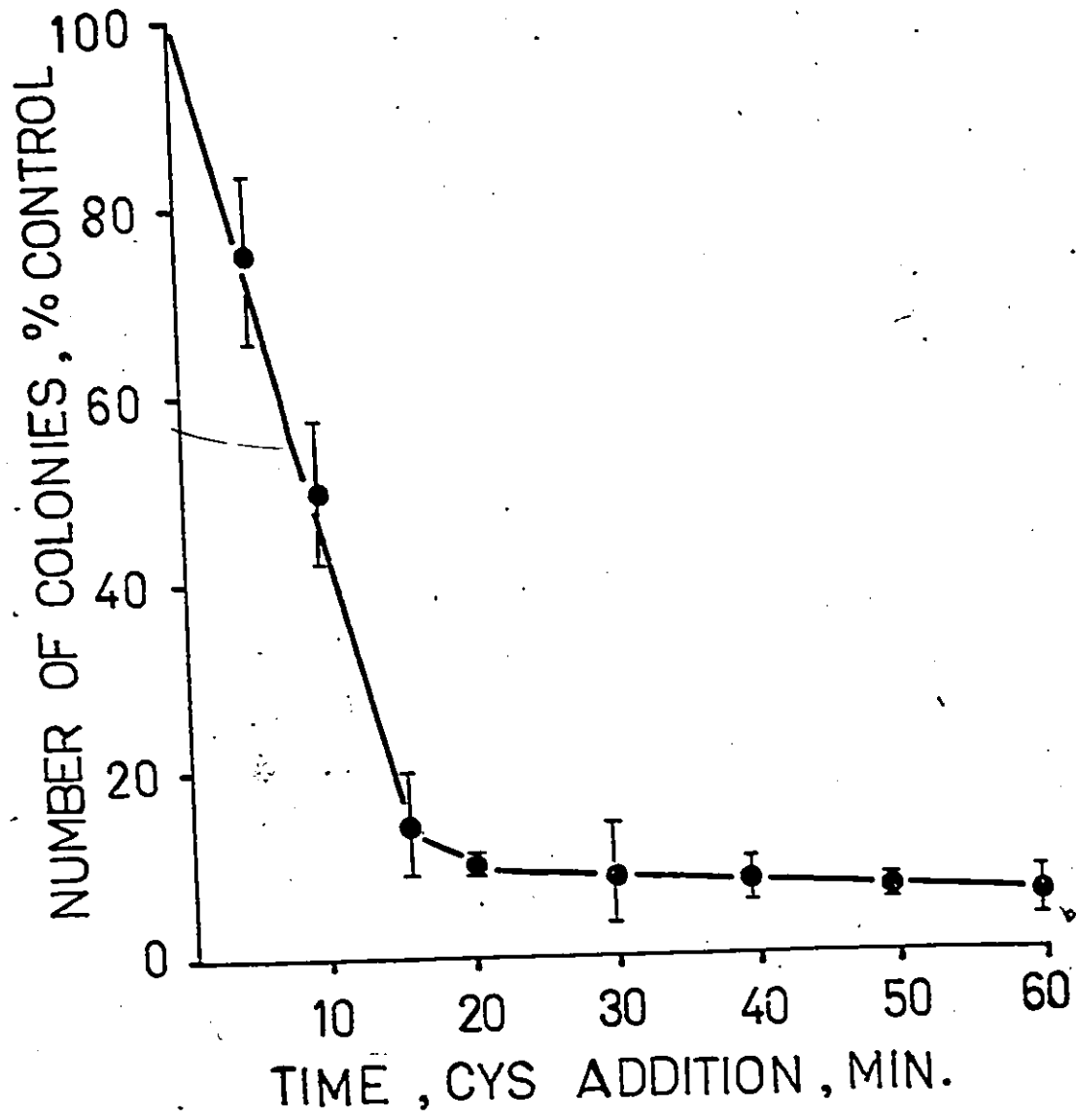


Figure 16. Varying effectiveness of GSH additions at different times during CA treatment. Vertical Bars denote 2X standard deviation.

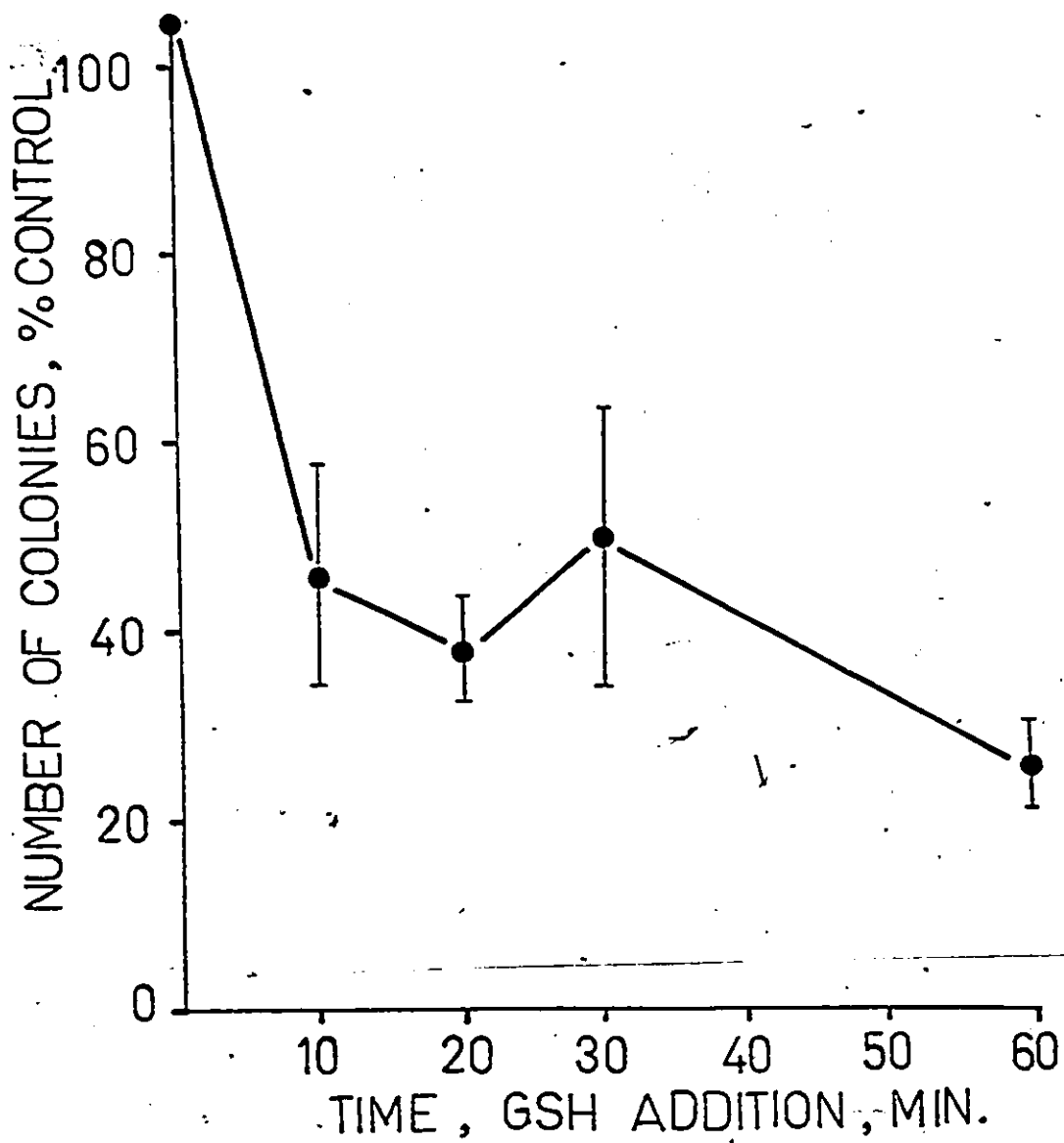


Figure 17. Effect of NEM concentration on cellulase synthesis and secretion. Vertical bars denote 2X standard deviation.

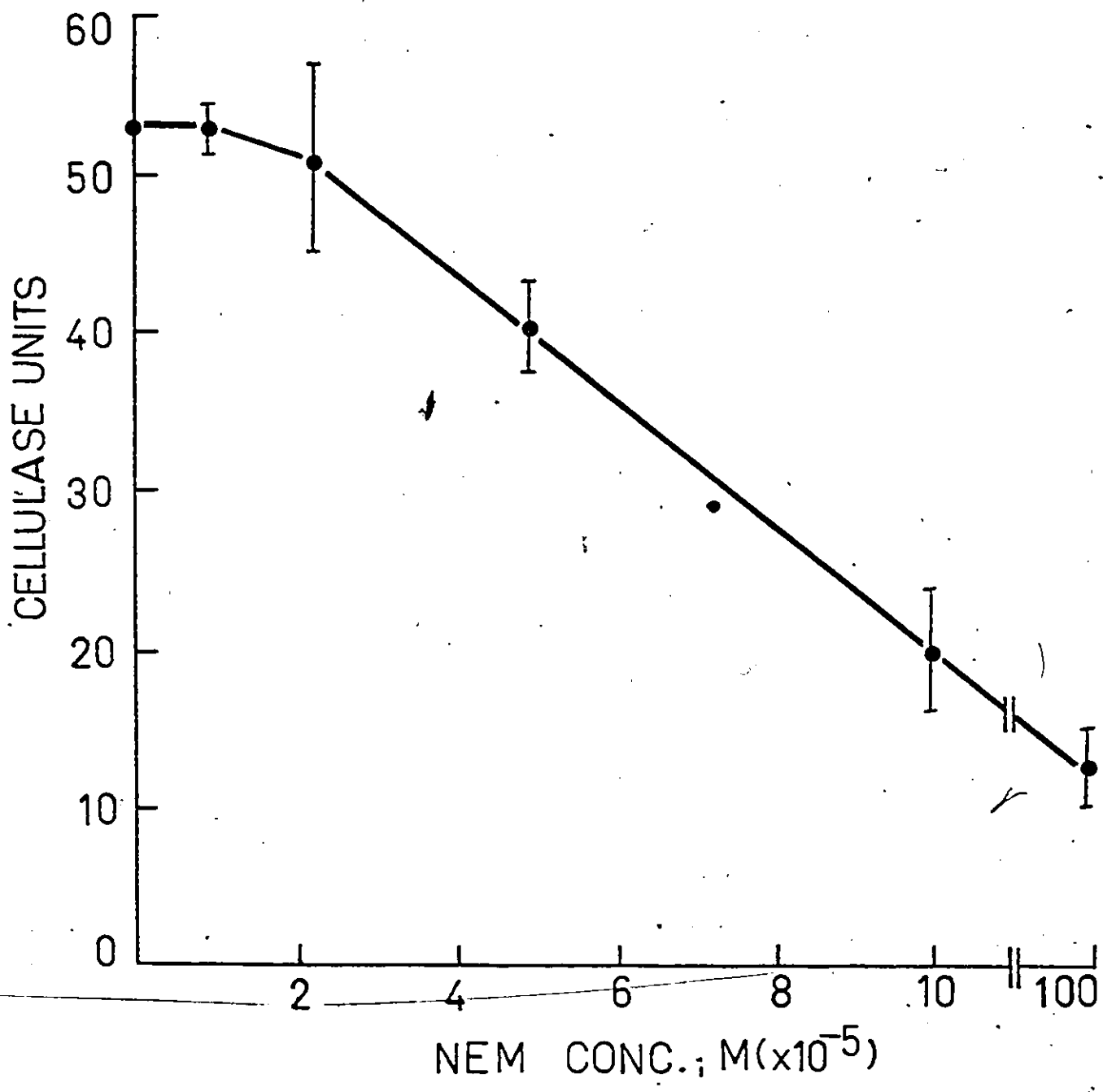


Figure 18. Effect of pCMBS concentration on cellulase synthesis and secretion. Vertical bars denote 2X standard deviation.



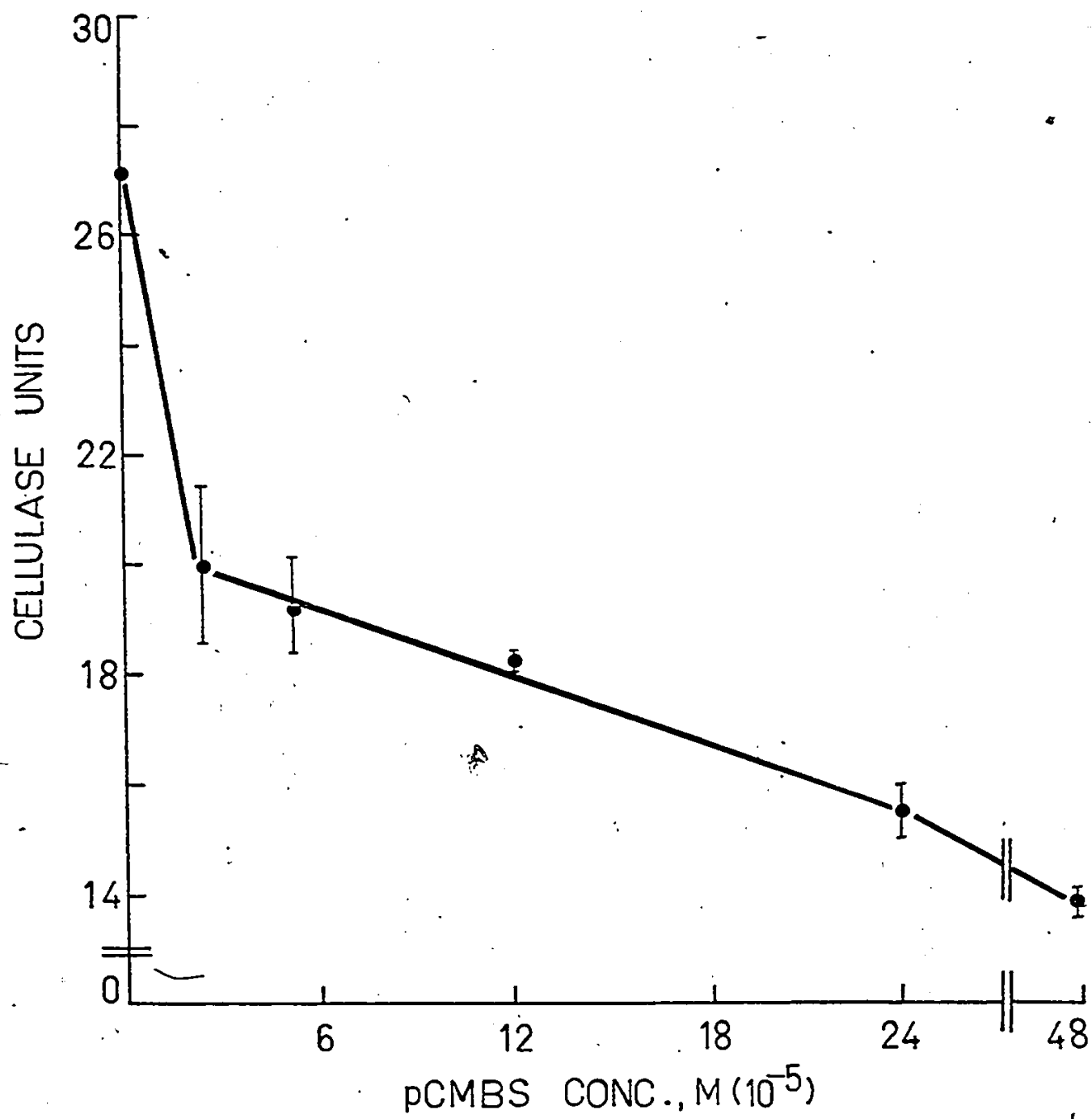


Figure 19. Binding of pCMBS by Achlya ambisexualis mycelia. Fresh mycelia in 0.2 gram quantities were incubated in duplicate for varying periods of time at room temperature in 5 ml of fresh growth medium containing 0.08  $\mu$  Ci/ml ( $^{203}\text{Hg}$ )pCMBS (1 mCi/50 mg). After incubation, the mycelia were washed 3 times with fresh growth medium and the associated radioactivity was determined as previously described. The vertical bars denote 2X standard deviation.

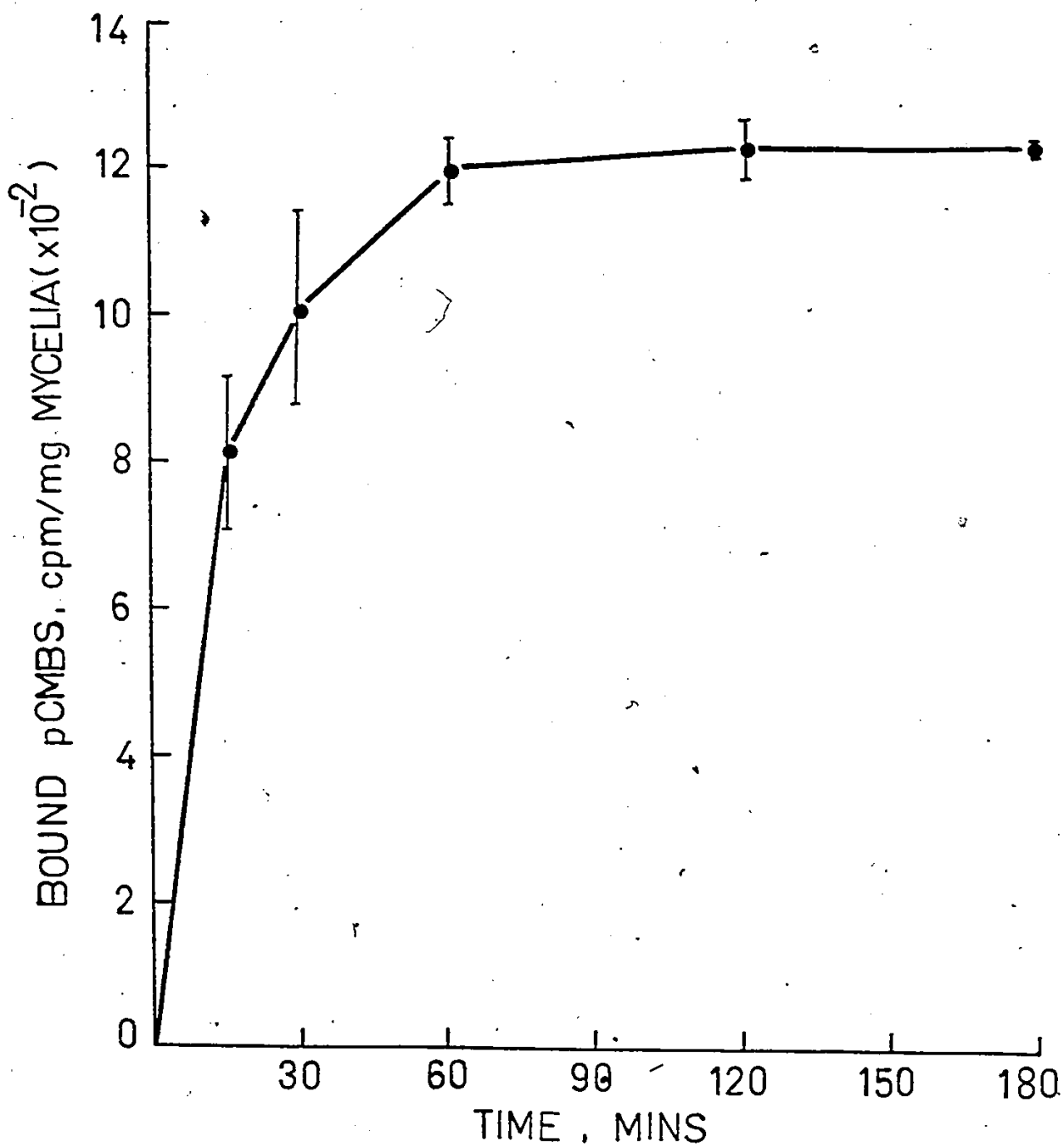
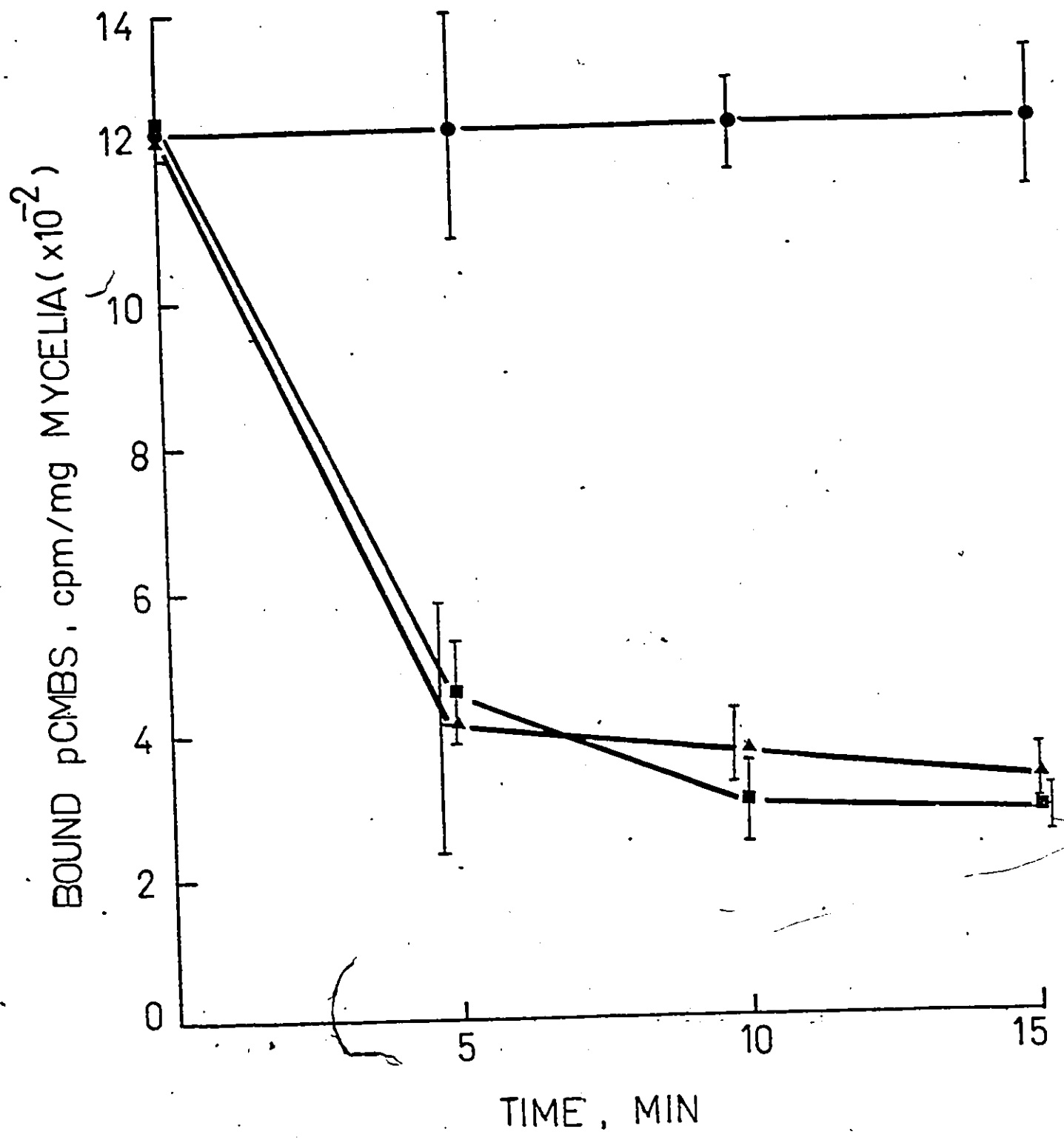


Figure 20. Elution of pCMBS from Achlya ambisexualis mycelia. Fresh mycelia in 0.2 gram quantities were preincubated in fresh growth medium containing 0.08 uCi/ml ( $^{203}\text{Hg}$ )pCMBS (1 mCi/50 mg) at room temperature for 1 hour and then washed 3 times with fresh growth medium to remove unbound pCMBS. The washed mycelia were then transferred to fresh growth medium (●—●), or fresh growth medium with 1 mM unlabelled pCMBS (■—■), or with 1 mM dithiothreitol (DTT) (▲—▲). After incubation periods of varying times, mycelia were harvested by filtration and the associated radioactivity was determined as previously described. Vertical bars denote 2X standard deviation.



The binding of CA by Achlya ambisexualis mycelia is rapid and reaches a maximum within minutes. Longer incubation does not result into any significant changes (Table 11). No significant difference in binding is observed at 0°C and room temperature. Therefore, all subsequent experiments were conducted at 0°C to minimize possible metabolic breakdown of the compound. In order to gain some insights into the target of CA binding mycelia were pretreated with a known thiol reagent, NEM, for 5 minutes and used for subsequent CA binding. As shown in Table 12, NEM pretreatment significantly reduced subsequent CA binding.

#### DISCUSSION

As in other systems, simultaneous addition of thiols such as cysteine and glutathione completely inhibits CA responses in Achlya ambisexualis. If the thiol addition is delayed until 20 to 30 minutes after CA addition, no protection is obtained. The lack of effect of thiols after 20 to 30 minutes post CA addition suggests that the effect can be prevented but cannot be reversed. These observations, consistent with other reports (Haslam, 1972; Kuo and Lampen, 1975), indicate that the effect of CA in Achlya ambisexualis is not readily reversible.

Among several potential thiol reagents such as NEM, pCMBS, DTNB and 1,5-I-AEDANS tested, only the relatively non-permeant pCMBS mimics the CA response, selectively inhibiting cellulase synthesis and secretion. Although NEM severely

inhibits cellulase synthesis and secretion in Achlya ambisexualis, this effect is attributable to its ability to inhibit general protein synthesis. Rapid binding and rapid elution of ( $^{203}\text{Hg}$ )pCMBS by mycelia indicate that, as in other systems, pCMBS acts as a non-penetrating reagent in Achlya ambisexualis. Although indirect, evidence which suggests CA binding by Achlya ambisexualis mycelia has been presented. The fact that a known thiol reagent, NEM, interferes with the binding of CA indicates that at least one of the binding sites for CA in Achlya ambisexualis may be sulfhydryl groups. Together, the facts that the CA responses in Achlya ambisexualis are eliminated by glutathione, a non-penetrating thiol, that CA fails to inhibit a thiol reagent sensitive system such as overall mycelial protein synthesis, and that the relatively non-penetrating thiol reagent pCMBS mimics the CA response, selectively inhibiting secretory protein synthesis without affecting general protein synthesis, indicate that CA may be acting in Achlya ambisexualis as non-penetrating thiol reagent, and that inhibition of cellulase synthesis may be exerted via relatively superficial thiol groups in the plasma membrane.

## CHAPTER VI

### GENERAL CONCLUSIONS

1. Cytochalasin A (CA) inhibits both spore germination and growth of Achlya ambisexualis. Unlike a large number of fungi, which show greater CA sensitivity with regard to growth than to germination, Achlya ambisexualis spore germination is more sensitive to CA effect than the growth of the organism. In contrast to the general concept that cytochalasins are poor inhibitors of respiratory metabolism, CA is a potent inhibitor of respiratory metabolism of Achlya ambisexualis. Lack of CA responses on sugar uptake and on isolated mitochondrial respiration suggest that the target of action may be glycolysis, and this inhibitory effect on respiratory metabolism may be associated with the CA inhibition of spore germination and growth of Achlya ambisexualis.

2. CA selectively inhibits the synthesis and secretion of cellulase, a secretory enzyme, without affecting general protein synthesis of Achlya ambisexualis. The lack of accumulation of the enzyme within the mycelia suggests that both synthesis and secretion are affected indicating that the synthesis of certain protein may be controlled at the level of secretion.

3. CA acts as a thiol reagent in Achlya ambisexualis. Simultaneous addition of thiols completely prevents CA responses in Achlya ambisexualis. The fact that neither delayed thiol addition nor washing and replacement of medium can reverse CA responses in Achlya ambisexualis suggests that, as in certain



other systems, the effect is irreversible. Among several potential thiol reagents tested, only the non-penetrating thiol reagent p-chloromercuribenzenesulfonate (pCMBS) mimics the CA response selectively inhibiting synthesis and secretion of cellulase in Achlya ambisexualis suggesting that CA may be acting in a similar manner interacting with superficial thiol groups.

4. Among several cytochalasins such as CA, CB, CD and CE tested, only CA, in accordance with certain other reports, is found to possess antifungal properties and its inhibitory effects in Achlya ambisexualis may be attributable to its ability to act as a potential thiol reagent.

## CHAPTER VII

### APPENDICES

#### 1. Composition of Growth Media

##### (a) Peptone yeast extract glucose (PYG) medium

Component	gram/liter of distilled water
peptone (Difco)	1.2
yeast extract (Difco)	1.2
glucose	3.2
pH	7.2

##### (b) Mating (M) medium

Component	gram/liter of distilled water
monosodium glutamate	0.40
glucose	2.80
l-methionine	0.0017
KCl	0.0165
MgSO <sub>4</sub>	0.0137
CaCl <sub>2</sub>	0.0061
EDTA (disodium)	0.0022
KH <sub>2</sub> PO <sub>4</sub>	0.0227
metal mix (#4)	0.0200
pH	6.9

## 2. Preparation of substrate for cellulase assay

Carboxymethyl cellulose (Hercules) was dissolved in NaOH-citric acid buffer (2 gram NaOH, 5.3 gram citric acid/1500 ml distilled water, pH 7.2) to obtain a 1.2 percent (w/v) as follows. After warming-up the buffer to 50 to 60°C required amount of carboxymethyl cellulose was added slowly and gently blended for 10 minutes. The solution was filtered through miracloth and after cooling down to room temperature thimersol (0.05%) and toluene (0.3%) were added. The solution was stored in at 4°C.

## 3. Chemicals used and suppliers

Unless otherwise indicated, all the chemicals used were reagent grade and purchased either from Sigma Chemicals, St. Louis, Missouri or from Fisher Scientific Co., Don Mills, Ontario.

Radiochemicals and related items were purchased from Amersham/Searle, Arlington Heights, Illinois.

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Zigmond, S.H. and Hirsch, J.G. Cytochalasin B: Inhibition of D-2-deoxyglucose transport into leukocytes and fibroblasts. *Science*, 176, 1432-1434 (1972).

VITA AUCTORIS

- July 19, 1943 - Born, Kerala, S. India.
- May 1963 - Granted Secondary School Leaving Certificate.
- July 1967 - Granted B.Sc., University of Kerala, S.India.
- January 1968 to July 1970 - Employed as teacher at the Baptist English High School, Kohima, India.
- September 1971 - Commenced studies at the University of Windsor, Windsor, Ontario.
- October 1974 - Granted B.Sc., University of Windsor, Windsor, Ontario.