SERINE PROTEASES, THEIR INHIBITORS, AND CHITIN SYNTHETASE IN *PHYCOMYCES*

Thesis by

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to my mother

Acknowledgments

With this thesis four years filled with exciting new experiences are coming to an end. There are many people who helped to make this time unforgettable for my family and me.

I thank Max Delbrück for his great example as a scientist and even more as a person. This quotation from a speech he gave in 1976 will illustrate my point:

> "There is great happiness in doing research, coming in in the morning, burning to see what answer Nature has given to your experiment."

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ABSTRACT

Phycomyces shows a positive growth response to blue light. Mutants are available which exhibit abnormal phototropism. They have been analysed genetically by complementation analysis. Seven complementation groups have been shown to be involved in the light information channel. They are designated mad A, -, G. Four groups are connected with the output end.

These findings encourage biochemical investigations. We make the hypothesis that light directly regulates the activity of chitin synthetase. It is investigated whether limited proteolysis represents one mode of such regulation.

Three serine proteases are isolated and characterized. The molecular weights are determined to be 18.000, 22.000, and 60.000 daltons. The proteases are solubilized by detergent or salt treatment. There are two specific soluble inhibitors present, which are also isolated and characterized. Both these proteins have a MW of 10.000 daltons. Each protease forms a 1:1 complex with its inhibitor. The inhibitors are present in excess in the cells. An acid protease is able to take the inhibitor off a serine protease-inhibitor complex. This protease has been partially purified.

The proteases and inhibitors of three mutant

strains have been partially purified and compared with wild type. These mutants are disturbed at the output end of the light information channel. Mutant specific changes are detected but a connection to the changed behavioral responses has not been demonstrated.

Chitin synthetase activity is detected in three different fractions. One form appears to be soluble; the two particulate forms can be separated by density gradient centrifugation. The high density material is probably a plasma membrane fraction.

The serine proteases are able to activate all three forms of chitin synthetase.

A hypothetical cascade for chitin synthetase activation is discussed.

In an appendix the amino acid composition of cytochrome c of Phycomyces is presented.

TABLE OF CONTENTS

SERINE PROTEASES, THEIR INHIBITORS, AND CHITIN SYNTHETASE IN *PHYCOMYCES*

Abbreviations

MW	Molecular Weight
CS	Chitin Synthetase
OD	Optical Density
STP	Sensory Transduction Pathway
spph	Sporangiophore of Phycomyces
WT	Wild Type
ATP	Adenosine triphosphate
BTNA	N-Benzoyl-L-tyrosine p-nitroanilide
cAMP	Adenosine 3',5'-cyclic monophosphate
CM-	Carboxymethyl-
DEAE-	Diethylaminoethyl-
DFP	Diisopropylfluorophosphate
EDTA	Ethylene diaminetetraacetic acid
HAP	Hydroxylapatite
Hepes	N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
Metrizamide	2-(3-acetamido-5-N-methylacetamide 2,4,6-triiodobenzamido)-2-deoxy-D-glucose
NADPH	Nicotinamide adenine dinucleotide phosphate
NAG	N-acetyl-D-glucosamine = 2-acetamido-2-deoxy-D-glucose
NSAN	N-succinyl-(L-ala)$_3$-p-Nitroanilide
PEG	Polyethylene glycol
PHMB	p-hydroxymercuribenzoate
PMSF	Phenylmethylsulfonylfluoride
PPO	2,5-Diphenyloxazole
POPOP	p-bis[2-(5-phenyloxazole)]-benzene
SDS	Dodecyl sodium sulfate
SP-	Sulphopropyl-
TCA	Trichloroacetic acid
Triton X-100	Octylphenoxylpolyethoxy methanol
TPCM	Tosyl-L-phenylalanylchloromethane
TLCM	N-Tosyl-L-lysylchloromethane
UDP-NAG	Uridine-5'-diphospho-NAG

For amino acids the three-letter abbreviations are used; they are listed in (58).

Chapter A

Background and Program

BACKGROUND AND PROGRAM

1) Introduction to *Phycomyces*

1.1) The Organism

Phycomyces is a unicellular polynucleate microorganism. The species *Phycomyces blakesleeanus* belongs within the lower fungi to the class zygomycetes. In common with many (but not all) other fungi it has a cell wall whose principal structural element is a system of fibrils composed of chitin (poly-N-acetylglucosamine) rather than cellulose (poly-glucose). In the vegetative life cycle after spore germination and mycelial growth sporangiophore formation is initiated. The sporangiophore is a gigantic, single-celled, cylindrical, aerial hypha, which is sensitive to at least five distinct stimuli: light, gravity, mechanical stretch, wind, and some unknown stimulus by which it avoids solid objects. The sporangiophore is ideally suited (through its size (several cm) and reaction time (minutes)) for physiological experimenting. It was therefore selected by Delbrück for the study of sensory transduction processes (1). A review of *Phycomyces* research is provided by Bergman *et al.* (2).

1.2) Photophysiology and Mutants

The light growth response of *Phycomyces* has been investigated in the hope to provide a paradigm for

the understanding of signal transduction; the underlying question is to see if the reductionist's approach to biology can be pushed far enough to give a complete understanding of the sensory processes in a microorganism."Light growth response" describes the observation that the growth velocity of a symmetrically illuminated sporangiophore shows transient changes in response to variations in light intensity. This effect has been characterized under a broad range of conditions using an automated tracking machine (3,4).

A number of behavioral mutant strains have been isolated which show characteristic physiological differences (5). The genetic analysis is described in part B of this thesis; the results indicate that only a small number of genes is involved in the sensory transduction process. This gives hope that one might be able to describe a behavioral mutation on the molecular (biochemical) level. Evidence had been presented that the output end of the light transduction channel can be identified with the enzyme UDP-N-Acetylglucosamine: chitin-N-Acetylglucosaminyl transferase (chitin synthetase) (6). It appears reasonable to start a biochemical approach with the investigation of the regulation of this enzyme.

2) Biochemical Background

2.1) Cell Wall Composition and Synthesis

Variation of the rate of elongation of the sporangiophore implies a change in the rate of cell wall synthesis, which could be achieved by regulating the enzyme that catalyzes it. The sporangiophore cell wall is mainly composed of polyaminosaccharides (7). Chitin and chitosan (a deacetylated form of chitin) account for about 62% and 13% of the dry weight of wall material (2, 6). Regulation of cell wall synthesis could occur directly by activating chitin synthetase and/or chitosan synthetase or indirectly by controlling the activity of a cell wall loosening enzyme. In the work described here we test the idea of an immediate regulation of chitin synthetase. The basic properties of this enzyme in Phycomyces and its cellular localization are described by Jan (6). This enzyme activity has been investigated also in other organism, like Saccharomyces cerevisiae and Mucor rouxii; the most influential work stems from Cabib (8,9) who proposed a scheme for its regulation in yeast, where chitin occurs only during part of the cell cycle synthesizing the primary septum. Cabib shows that chitin synthetase is synthesized as a zymogen which is converted to the active enzyme by a protease which in turn is usually inhibited by an inhibitor protein. Chitin synthetase in

yeast was chosen by Cabib because it represents a suitable system for the study of molecular aspects of morphogenesis (9).Cabib's hypothesis is illustrated in Fig. A1.

2.2)Proteases and Inhibitors

Proteases are known to be involved in a number of regulatory processes (20) and the zymogen activation in case of digestive enzymes and clotting factors is textbook knowledge (58).As far as microorganisms are concerned a great deal of information has been collected starting from the pioneering work of Hata (12).Lenney(47) identified the first specific protease inhibitor for yeast.The properties of intracellular proteases in microorganisms (like yeast,Neurospora crassa) have been extensively reviewed (38).

Three types of proteolytic activity could be distinguished;they are designated A,B and C.The A proteases have a pH optimum around three (" acid protease"); the B proteases show their main activity at slightly alkaline pH and turn out to be serine proteases;the C proteases are carboxypeptidases.For each enzyme specific inhibitors could be identified.

This background made the investigation of the involvement of limited proteolysis in the regulation of cell wall synthesis look like a reasonable approach.

3) Program and Outline of Results

The program that was undertaken in this thesis was derived from the three following observations,which summarize this introduction:

a) The chitin synthetase from WT can be activated by light,whereas the enzyme of a photomutant from the output end (see section B) of the light information channel is not (6);this finding points to chitin synthetase being involved in the light growth response.

b) The number of complementation groups that are involved in the light information channel is small (seven groups are established at the present,April 1977); see section B for details.

c) A mechanism for the regulation of chitin synthetase in yeast has been proposed (See Fig. A1) and the participation of proteases has been established by Cabib (8).

The following program was developed: Find out whether in Phycomyces corresponding proteases and inhibitors can be detected. Purify and characterize these proteins.Find out whether they are able to activate or inhibit any activation of chitin synthetase. Try to characterize this enzyme within the framework of these experiments. Compare the situation in WT and in appropriate mutants to see whether mutant specific changes point to the involvement of limited proteolysis in physiological

responses.

The results can be summarized as follows: There are three serine proteases in Phycomyces, which can be distinguished with respect to molecular weight and charge. All three are inhibited by two proteins,which differ in charge but not in MW. An acid protease is detected, which can digest these inhibitors.

Different forms of chitin synthetase are found, which react differently if exposed to the serine proteases.In most cases,however,increase in chitin synthetase is observed;the activation is inhibited by the inhibitor proteins.

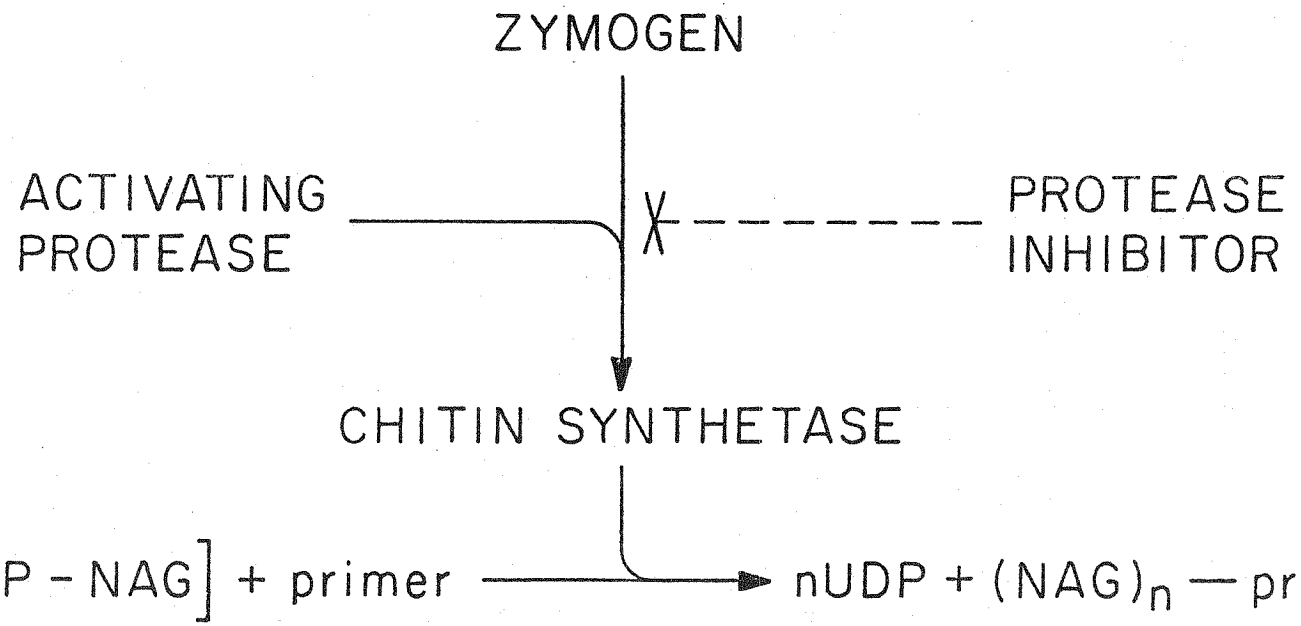
Analysis of the mutant strains reveals one mutant specific change,but no conclusions can be presented about the physiological relevance.

The investigation of serine proteases and their relation to biological control processes in microorganisms is only at the beginning and will provide an interesting area for future research.

Fig. A1: The Chitin Synthetase System

Hypothetical scheme for the regulation of chitin synthetase by limited proteolysis.Chitin synthetase is converted from its zymogen form to the active enzyme by a protease; this step is blocked by an inhibitory protein.

THE CHITIN SYNTHETASE ACTIVATING SYSTEM



NAG: *N* - acetylglucosamine

(Adapted from E. Cabib in Curr. Topics in Cell. Reg., Vol. 8
Academic Press (1974) p. 2-30)

Chapter B

Analysis of Mutants of *Phycomyces* with Abnormal Phototropism

1) Complementation Analysis

1.1) Identification of Five Complementation Groups: *mad* A, - , E

Molec. gen. Genet. 131, 233—246 (1974)

Complementation between Mutants of *Phycomyces* with Abnormal Phototropism

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Summary. Fifteen mutants of *Phycomyces blakesleeanus* with abnormal phototropism (phenotype *mad*), obtained by mutagenesis with nitrosoguanidine, were tested for genetic complementation using heterokaryons produced by grafting stage I sporangiophores. Five complementation groups were found, defining five genes, designated *madA* to *madE*. This grouping of the mutants is completely consistent with the phenotypic classification scheme of Bergman *et al.* (1973), based on sensory responses of the sporangiophore other than those to light (geotropism and avoidance), and two responses of the mycelium to light. At two points the complementation grouping refines this scheme. The groups *A*, *B* and *C* coincide with the class 1 *mad* mutants of Bergman *et al.* which have a high threshold for the photoresponses but show normal geotropism and avoidance. Class 1 can be subdivided further into 1.1 (groups *A* and *B*) and 1.2 (group *C*), depending on whether the mutants do not or do show normal mycelial responses. The groups *D* and *E* coincide with class 2 which is defective on the output side of the sporangiophore growth response.

1. Introduction

Phycomyces does not naturally anastomose, but an efficient method to obtain heterokaryons in *Phycomyces* has been developed by grafting stage I sporangiophores (Ootaki, 1973), and was applied to complementation tests for mutants deficient in carotenogenesis (*car* mutants, Ootaki *et al.*, 1973). Three genes, *carA*, *carB* and *carR*, were detected in these color mutants; the *carA* mutant is white and contains a very low level of carotenoids, the *carB* mutant is also white but accumulates phytoene, and the *carR* mutant is red because it accumulates lycopene. In the present experiments, we applied the above method for heterokaryon formation to do complementation tests of mutants with defects in sporangiophore phototropism (phenotype *mad*).

Bergman *et al.* (1973) characterized the *mad* mutants and classified them into three groups based on other responses to stimuli such as geotropism, autochemotropism (avoidance) and mycelial responses to light. As illustrated in Fig. 1, class 1.1 mutants show normal geotropism and autochemotropism, but no mycelial responses to light, class 1.2 mutants are abnormal only in the photoresponses of the sporangiophores, and class 2 mutants with abnormal autochemotropism and geotropism, but normal mycelial responses to light. These authors presumed that the class 1.1 mutants had some faults in the photoreceptor system or in the early transducers present in both the mycelium and the growing zone of the

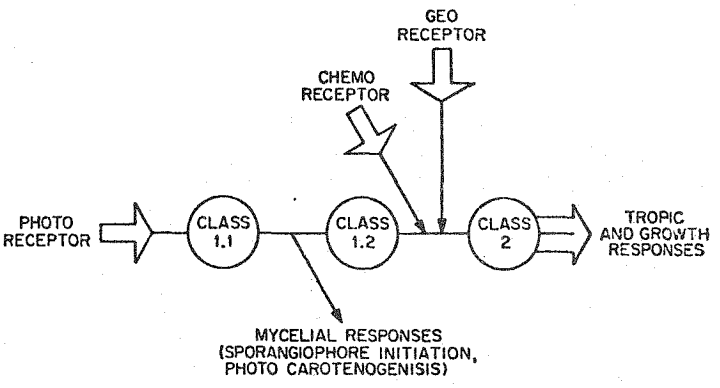

Fig. 1. Network of stimulus-response system of *Phycomyces*. Adapted from Bergman *et al.* (1973)

sporangiophores. Class 1.2 mutants appear to be affected in steps unique to phototropism, and class 2 mutants in the growth system controlling all the tropic reactions. To assess how many different genes are involved in this information channel it was desirable to perform complementation tests by formation of heterokaryons among *mad* mutants selected from each class.

2. Materials and Methods

a) Strains. The standard wild types of *Phycomyces blakesleeanus* used in this work were NRRL1555(−) obtained from Northern Regional Research Laboratory, Peoria, Ill. and UBC24(−) isolated by Dr. R. J. Bandoni, Botany Department, University of British Columbia, Canada. The mutant strains used, derived from these wild types by mutagenesis with nitrosoguanidine, are listed in Table 1. The procedure of mutagenization and mutant isolation has been described in detail by Bergman *et al.* (1973). The C strains were obtained from the culture collection at the California Institute of Technology and the S strains from that of Drs. A. P. Eslava and E. Cerdá-Olmedo at the University of Sevilla. By definition, any mutant carrying the genotypic designation *mad* is abnormal for phototropism. Although large numbers of *mad* mutants were available for the present study, only fifteen strains, all of the same (−) mating type, were selected. These mutants show no phototropic response at all to light of intensity below $\log_2 I = -13$ (see below) and their behavior other than phototropism, such as geo- and autochemotropism and photocontrol for sporangiophore initiation, had previously been characterized (Bergman *et al.*, 1973). Perhaps fortuitously, each of the mutants of class 1.2 carries in addition a mutation at the *carA* locus (Bergman *et al.*, 1973; Ootaki *et al.*, 1973). There are class 1.2 mutants available which do not carry a *car* mutation, but all of these happen to be of the (+) mating type.

b) Culture Conditions. To obtain sturdy stage I sporangiophores, best suited for grafting, *Phycomyces* was cultured on potato-dextrose agar (PDA) of double strength (80 g Potato Dextrose Agar, dehydrated, Difco Lab., Detroit, in 1 l H_2O) or enriched with potato extract (40 g Potato Dextrose Agar in 1 l potato extract prepared according to Gruen, 1959). The methods for the culture have been detailed by Ootaki (1973).

c) Light Intensity. Intensities of light (tungsten light source, Corning 5-61 blue filter), measured by the method of Bergman *et al.* (1973), are represented on logarithmic scale, to the base 2: $\log_2 I = 0$ is equivalent to 10 μwatts/cm^2. This scale is convenient to express the wide intensity range (Delbrück and Reichardt, 1956).

d) Threshold Measurement. The thresholds of all *Phycomyces* strains, wild types and mutants, used in this work were measured in the threshold box described by Bergman *et al.* (1973). It consists of ten chambers, each illuminated by horizontal light with a different

Table 1. Strains of *Phycomyces blakesleeanus* used in this work

Class	Strain	Genotype[a]	Color[b]	Growth rate (mm/hr)	Origin[c]
1.1	C21	*madA7*(−)	yellow	2.1 ± 0.1	NRRL1555
	C47	*madA35*(−)	yellow	1.7 ± 0.1	NRRL1555
	C109	*madB101*(−)	yellow	1.4 ± 0.1	NRRL1555
	C111	*madB103*(−)	yellow	1.5 ± 0.1	NRRL1555
	C112	*madB104*(−)	yellow	1.5 ± 0.1	NRRL1555
	C114	*madB106*(−)	yellow	1.8 ± 0.2	NRRL1555
1.2	C141	*carA5 madC51*(−)	white	2.8 ± 0.2	C59 × C2
	C148	*carA5 madC119*(−)	white	1.7 ± 0.1	C2
	S5	*carA51 madC202*(−)	white	3.6 ± 0.1	UBC24
	S14	*carA53 madC205*(−)	white	3.1 ± 0.1	UBC24
	S18	*carA57 madC209*(−)	white	2.9 ± 0.1	UBC24
2	C68	*madD59*(−)	yellow	2.5 ± 0.2	NRRL1555
	C107	*madD99*(−)	yellow	2.3 ± 0.1	NRRL1555
	C110	*madE102*(−)	yellow	2.3 ± 0.2	NRRL1555
	C149	*madD120*(−)	yellow	2.4 ± 0.1	NRRL1555

[a] Based on phenotype. *mad* indicates a mutant with abnormal phototropism. *car* indicates a mutant with abnormal carotene complement. The numbers refer to independent isolates. The *mad* genes involved (*A* to *E*) as inferred from the present work. The *car* genes involved as inferred from Ootaki *et al.* (1973).

[b] Both wild types, NRRL1555(−) and UBC24(−), are yellow because of biosynthesis of β-carotene. *carA* mutants lack colored carotenes and are white.

[c] C2 is a white mutant carrying the genotype *carA5*(−). The phototropism is normal. C59, genotype *mad-51*(+), is yellow and abnormal for phototropism.

intensity, from $\log_2 I = 0$ to $\log_2 I = -26$. To reduce the light intensity, the beam from the light source (750 W Westinghouse CWA lamp) is successively passed through ten mirrors coated with titanium oxide for 50% transmission (Keim Optical Co., Glendale, Ca., U.S.A.) or through ten Borosilicate Crown Glass mirrors, front surface inconel coating and back surface SL/AR coating, with 13–21% transmission (Systems and Industries Optical, Monrovia, Ca.). Several shell vials, 1 cm diameter, 3 cm high, with solid culture medium, with young stage IV sporangiophores, are placed in each experimental chamber of the box for 6 to 9 hr to be tested for phototropic response.

e) Complementation Tests. Fig. 2 shows the procedure for complementation tests between *mad* mutants. Heterokaryons between two strains are made according to Ootaki (1973). Sturdy stage I sporangiophores (1–2 cm long) from each strain are removed from mycelia, placed on plain agar blocks, tips facing each other, and grafted by inserting a little bit of the decapitated tip of one sporangiophore into the other. For each pair of mutants to be tested at least thirty grafts were made. About 24 hr after grafting, the grafts are tested for success under a dissecting microscope by observing the formation of new cell wall connecting the two sporangiophore portions and the fusion of cell contents in the graft region. Spores were collected only from the grafts showing a single sporangiophore regenerated at the graft union of a successful graft. Such a sporangiophore is almost always heterokaryotic (Ootaki, 1973). The spores, uninucleate or multinucleate, and homo or heterokaryotic, resulting from segregation within the sporangium of the regenerate (Heisenberg and Cerdá-Olmedo, 1968), were suspended in a shell vial containing 1 ml sterile distilled water (one sporangium per vial). After heat-activation at 48°C for 20 min the spores were diluted and inoculated in shell vials containing solid PDA (one spore per vial). Alternatively, the heat-activated spores were streaked on acid glucose-asparagine-yeast medium (GAY acid, Cerdá-Olmedo and Reau, 1970). A piece of mycelium from each small colony which appeared on the 2-day-old plate was transferred

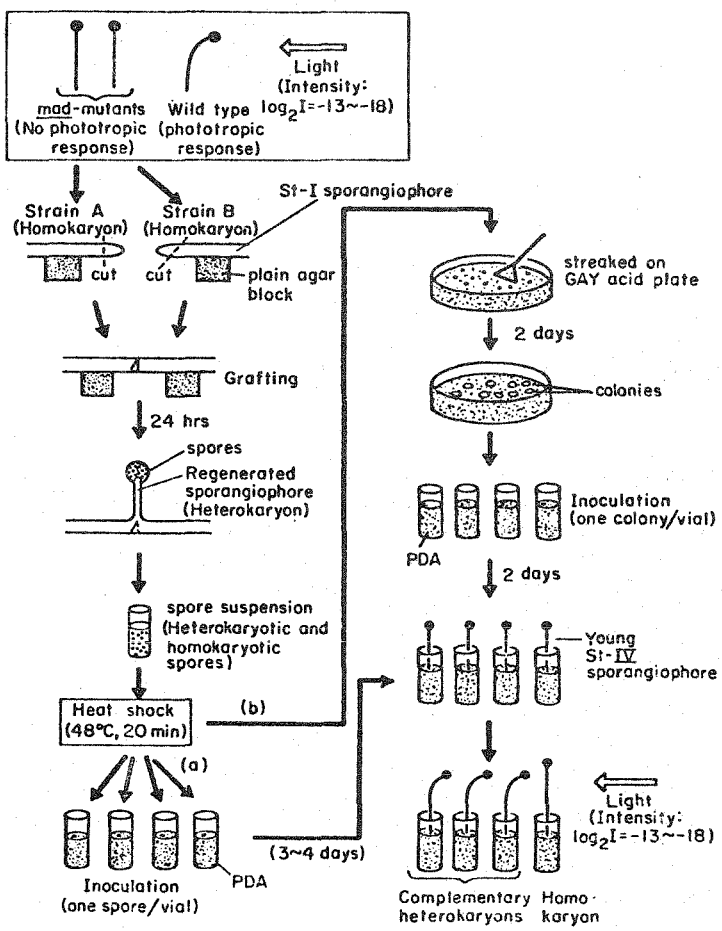

Fig. 2. Procedure for complementation tests between *mad*-mutants of *Phycomyces*. For explanation see text

into a vial containing PDA (one colony per vial). The vial with either a spore or a piece of mycelium was placed into a deep Petri dish (9 cm in diameter, 5 cm in height) with a lid and kept at 21 to 23°C under continuous overhead diffuse white light with intensity of about $\log_2 I=0$. When the sporangiophores appeared, the Petri dish was uncovered. Fuzzy first crop sporangiophores were removed. Only one straight young stage IV sporangiophore, about 3 cm long, from the second or third crop was left in each vial. The vials were placed in the threshold box or a test box for 6 to 9 hr to test the phototropic responses. In the threshold box sporangiophores were placed only in the chambers with light intensities between $\log_2 I=-13$ and -18, i.e. strong enough to cause phototropism for the wild types but not for *mad* mutants. Alternatively, a test box was used (20 × 14 × 13 cm) with a window on one side to admit horizontal light. The light was obtained from a fluorescent lamp (General Electric F30 Cool White or Toshiba FL4W ND White) and screened through layers of white paper sandwiched between colorless opaque lucite plates to provide a diffuse light of appropriate intensity. Intensities between $\log_2 I=-13$ and -18 were chosen. At these intensities the wild-type NRRL1555(−) clearly bent, and the mutant C47, the least blind among the mutants used in this work, did not. Whenever in these test systems some of the heterokaryotic sporangiophores bent clearly towards the light, the mutants constituting the

heterokaryon were scored as a complementing pair. If all the sporangiophores grew straight, even after repeating the experiment using another graft, we judged that the mutations failed to complement each other. The bending angle was recorded, and the length of each sporangiophore was measured at the end of each experiment to check whether the sporangiophores had grown at a normal rate during the experiment.

3. Results

First, the thresholds for phototropism of the sporangiophores of the wild types (NRRL1555 and UBC24) and of all the *mad* mutants used in this work were measured. Figs. 3a and b represent the bending angles of sporangiophores of each strain as a function of light intensity. For light intensities above $\log_2 I = -21$, the bending angles of NRRL1555 are about 70° from the vertical at phototropic and geotropic equilibrium, at lower intensities the angle gradually decreases and reaches zero at $\log_2 I = -26$. Phototropism of UBC24 sporangiophores saturates at intensities above $\log_2 I = -18$ and disappears at $\log_2 I = -26$. Compared with these wild types, all *mad* mutants were obviously blind at low intensities (night-blind). The sporangiophores of the class 1.1 mutants C109, C111, C112 and C114 did not respond at all at light intensities below $\log_2 I = -7$ or -10, while the threshold of C21 and C47 was around $\log_2 I = -12$. The five white mutants carrying the genotype *carA* were also quite blind; C141 and C148 showed no phototropism up to $\log_2 I = -9$ and -3 respectively, and three S-strains were blind up to $\log_2 I = -7$. The four yellow class 2 mutants, C68, C107, C110 and C149, showed little bending response even at $\log_2 I = 0$. These results are in good agreement with the measurements by Bergman *et al.* (1973) and show that light intensities between $\log_2 I = -13$ and -18 are strong enough to give saturating responses for the wild types and none for the *mad* mutants. This range, therefore, can be used for the complementation tests.

The first series of complementation tests was made among six class 1.1 mutants with all possible combinations. The tests gave clear-cut results indicating two types of pairs, i.e. complementing pairs in which some of the sporangiophores showed clear phototropism, and noncomplementing pairs in which none of the sporangiophores responded to light. In the complementing pairs the bending angles of *heterokaryotic* sporangiophores varied. The sporangiophores that showed no phototropic responses were judged to be either homokaryons or very unbalanced heterokaryons. In the pairs in which none of the sporangiophores respond it is difficult to prove that the sporangiophores are actually heterokaryotic, because these strains do not carry other genetic markers such as color or auxotrophy. Therefore, we repeated the experiment using another successful graft regenerating a *single* sporangiophore at the graft union. The results shown in a matrix (Fig. 4) indicate that the six class 1.1 mutants fall into two complementation groups: C21, C47, hereafter referred to as the locus *madA*, and C109, C111, C112, C114, to be called *madB*.

To assess the phototropic phenotype of complementing strains, the threshold of the sporangiophores of each complementing pair of class 1.1 mutants was measured. To do so, a piece of mycelium was picked from the vial which produced sporangiophores responding with maximum bending angle in the above complementation test, and was transferred to a Petri dish containing PDA. Two days

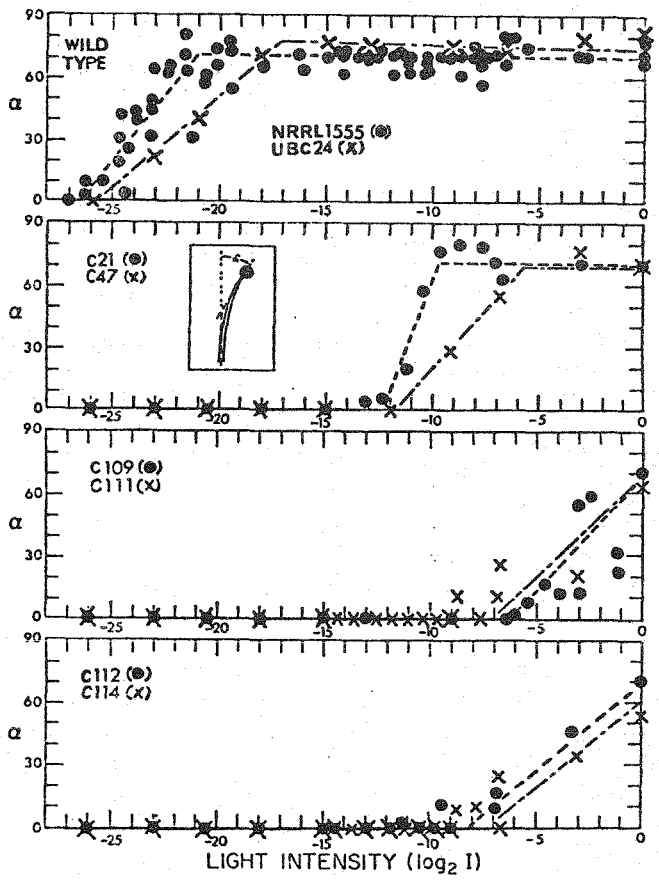

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Fig. 3. a Phototropic responses of the wild types (NRRL1555 and UBC24) and six class 1.1 mutants of *Phycomyces* in the threshold box against different intensities of light (abscissa, logarithmic, base 2). Each point represents an average of at least three sporangiophores grown in vials containing potatodextrose agar and exposed to light for 6 to 9 hr. $\log_2 I=0$ is equivalent to 10 μwatts/cm². α is the angle between the sporangiophore and the vertical. The phototropism of these strains, which we retested and confirmed here, has been described previously by Bergman *et al.* (1973). b Phototropic responses of nine class 1.2 (upper half) and class 2 (lower half) *Phycomyces* mutants in the threshold box against different intensities of light (abscissa) illuminated horizontally. See legend of Fig. 3a for details

later pieces of mycelia grown on the Petri dish were transferred to 80 to 120 shell vials containing PDA and were cultured until young stage IV sporangiophores suitable for testing in the threshold box developed. The results are represented in Fig. 5. The wild-type NRRL1555 curve from Fig. 3a is drawn for comparison (dashed line). The heterokaryotic sporangiophores of each complementing pair showed normal phototropism at intensities above $\log_2 I=-18$. It is noteworthy, however, that the sensitivity of these heterokaryons is slightly less than that of the wild type.

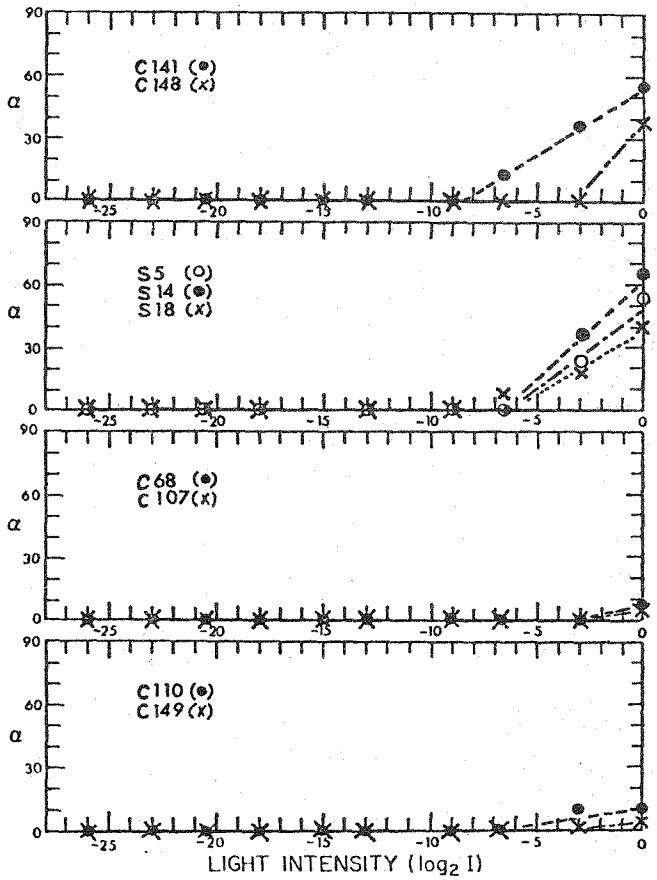

Fig. 3b

White class 1.2 mutants were tested in a second series of complementation against C47, genotype *madA35*(−), as representative of the genotype *madA* and C111, genotype *madB103*(−), as representative of the genotype *madB*. This series includes a recombinant resulting from a genetic cross (C141), one two-step mutant (C148), and the three S strains, S5, S14 and S18, derived from a different wild type. All five strains were classified into class 1.2, since they gave rim forests of sporangiophores by daily exposure to 1 hr of light at $\log_2 I = -3$, indicating the normal photoinitiation (M. Delbrück, personal communication). All five of these mutants carry a mutation at the *carA* locus. In this series of experiments, spores collected from the successful graft were streaked on GAY acid plates. When both white and yellow colonies of various shades appeared on the plates, the mycelium of each pale yellow colony was transferred into a shell vial (one colony per vial). Segregation of white and yellow colonies from *one* sporangium proves that the regenerated sporangiophore of the graft is heterokaryotic (Heisenberg and Cerdá-Olmedo, 1968). The pale yellow colonies are expected to be heterokaryotic with a high degree of certainty. The young stage IV sporangiophores grown in the shell

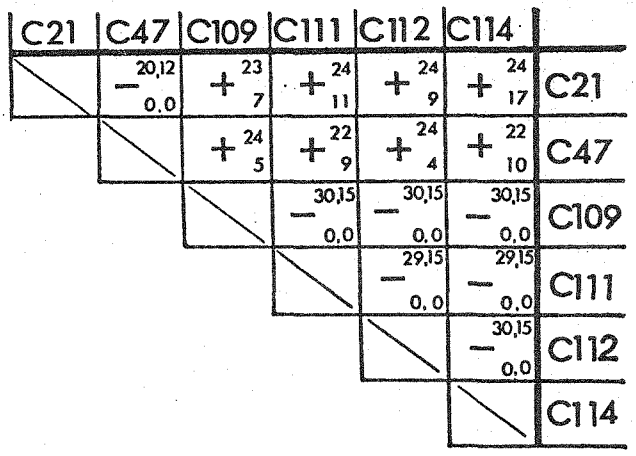

Fig. 4. Complementation tests among class 1.1 *mad*-mutants. For each pair, 30 to 60 grafts were made of which 30% to 87% (average 58%) were successful. Only the graft which regenerated a single sporangiophore at the graft union was used for collection of heterokaryotic spores. Spores were inoculated into vials containing PDA (after dilution, one spore/vial), and sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism. + Complementation (phototropic response). — Failure to complement (no phototropic response). Upper number: Number of sporangiophores inspected for phototropism in the threshold box. Lower number: Number of sporangiophores which showed more or less phototropism as a result of complementation. When no complementation occurred, the experiment was repeated by the use of another graft for each pair

Table 2. Complementation tests of *albino*, class 1 *mad*-mutants with C47(*madA*) and C111 (*madB*)

	C47(*madA*)	C111(*madB*)
C141	+	+
C148	+	+
S5	+	+
S14	+	+
S18	+	+

For each pair, 30 grafts were made. Spores were collected from the sporangium derived from a singly regenerating sporangiophore at the graft union and streaked on GAY acid plate (one sporangium/plate). The plate was inspected for segregation of yellow, pale-yellow and white colonies 2 to 4 days after streaking. Small pieces of mycelia from different pale-yellow colonies were inoculated into vials (one colony/vial). Young stage IV sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism in the threshold box. + Complementation. — Failure to complement.

vials were inspected for phototropism: each of the albinos complemented with both C47 and C111, indicating that the mutation in these white strains occurred at a locus different from *madA* and *madB* (Table 2).

The next complementation test was done to find out how many new genes are involved in these albinos. Pairs were made in all possible combinations. None of the pairs complemented (Fig. 6) showing that the five *mad* mutations occurred

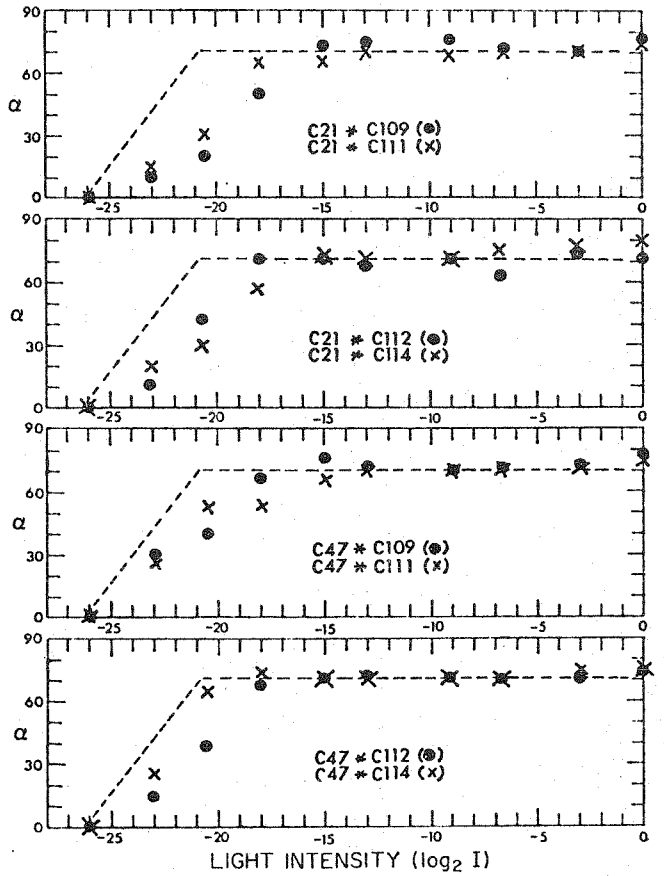

Fig. 5. Phototropic responses of eight pairs of class 1.1 mutants, which are complementing, in the threshold box against different intensities of light (abscissa, logarithmic, base 2) illuminated horizontally. Each point represents an average of at least three sporangiophores grown in vials containing potato-dextrose agar and exposed to light for 6 to 9 hr. $\log_2 I=0$ is equivalent to 10 μwatts/cm². α is the angle between the sporangiophore and the vertical. The wild-type NRRL1555 from Fig. 3a is included for comparison (dashed line)

in the same locus to which we assign *madC*. These strains carry the *carA* mutations representing abnormal carotene biosynthesis (Ootaki *et al.*, 1973), in addition to *madC*.

These facts raise the possibility that in some of the class 1.1 mutants we had tested (Fig. 4) the *madC* locus might be mutagenized in addition to *madA* or *madB*. C47 and C111 cannot be mutated at the *madC* locus because they complement with C148 carrying *madC* (Table 2), but other might be. Heterokaryons of these other class 1.1 mutants (C21, C109, C112, C114) with C148 as representative of the genotype *madC* were made and tested. They responded normally, proving that none of the class 1.1 strains were mutated at the *madC* locus (Table 3).

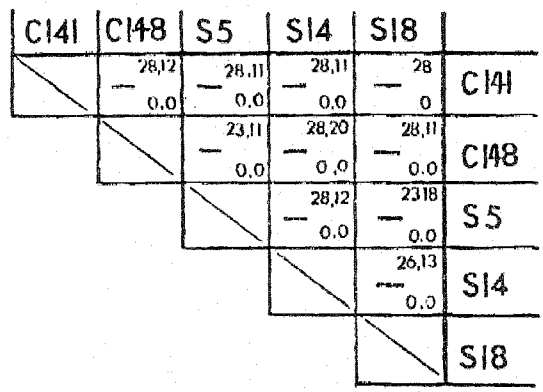

Fig. 6. Complementation tests among *albino* class 1.2 *mad*-mutants. For each pair, 30 to 60 grafts were made. Spores were collected from the sporangium derived from a singly regenerated sporangiophore at the graft union and inoculated in vials containing PDA after dilution (one spore/vial). Sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism. + Complementation. — Failure to complement. Upper number: Number of sporangiophores inspected for phototropism in the threshold box. Lower number: Number of sporangiophores which showed more or less phototropism as a result of complementation. When no complementation occurred, the experiment was repeated by the use of another graft for each pair

Table 3. Complementation tests of class 1.1 *mad*-mutants with C148(*madC*)

	C148(*madC*)
C21	+
C47	+[a]
C109	+
C111	+[a]
C112	+
C114	+

For each pair, 30 grafts were made. Spores were collected from sporangium derived from a singly regenerated sporangiophore at the graft union and inoculated into vials containing PDA (after dilution, one spore/vial). Sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism in the threshold box. + Complementation. — Failure to complement.

[a] Cited from the data shown in Table 2.

Similarly, the four yellow class 2 mutants were each paired with a representative (C47, C111 and C148) of each of the genotypes *madA*, *madB* and *madC*, with the results shown in Table 4. Normal phototropism was found for the heterokaryotic sporangiophores in all combinations, showing that one or more loci other than *A*, *B*, *C* were mutagenized in the class 2 strains. To detect how many new genes were involved, the class 2 mutants were paired among themselves and the results are shown in a matrix (Fig. 7) which brings out the fact that complementation only occurred in the heterokaryons between C110 and all the others. Hence, two

Table 4. Complementation tests of class 2 *mad*-mutants with C47(*madA*), C111(*madB*) and C148(*madC*)

	C47(*madA*)	C111(*madB*)	C148(*madC*)
C68	+	+	+
C107	+	+	+
C110	+	+	+
C149	+	+	+

For each pair, 30 to 90 grafts were made. Spores were collected from the sporangium derived from a singly regenerated sporangiophore at the graft union and inoculated in vials containing PDA (after dilution, one spore/vial). Fifteen to sixty-eight sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism. + Complementation. – Failure to complement.

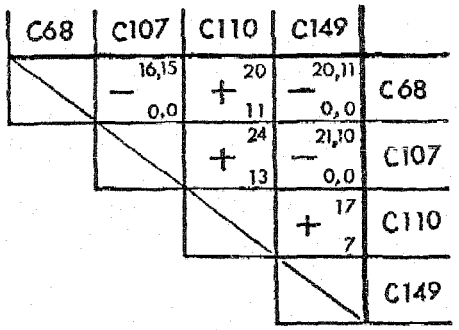

Fig. 7. Complementation tests among class 2 *mad*-mutants. For each pair, 30 grafts were made. Spores were collected from the sporangium derived from a singly regenerated sporangiophore at the graft union and inoculated in vials containing PDA (after dilution, one spore/vial). Sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism. + Complementation. — Failure to complement. Upper number: Number of sporangiophores inspected for phototropism in the threshold box. Lower number: Number of sporangiophores which showed more or less phototropism resulting from complementation. When no complementation occurred, the experiment was repeated by the use of another graft

complementation groups were revealed, i.e. (C68, C107, C149) and (C110) for which the genotypes *madD* and *madE* were assigned respectively.

A last series of experiments tested whether or not some of the class 1.1 and 1.2 mutants (including the albinos) were mutagenized also in the *madD* and/or *madE* locus in addition to *madA*, *madB* and *madC*. Table 5 shows that all class 1 mutants could complement with both C68 (*madD*) and C110 (*madE*) indicating no evidence of a second or third mutation in these strains.

4. Discussion

Bergman *et al.* (1973) classified the phototropic mutants into three groups: class 1, which was subdivided into two groups according to whether the mycelia responded to light normally (class 1.2) or not (class 1.1), and class 2. In a model

Table 5. Complementation tests of class 1 *mad*-mutants with C68(*madD*) and C110(*madE*)

	C68(*madD*)	C110(*madE*)
C21	+	+
C47	+[a]	+[a]
C109	+	+
C111	+[a]	+[a]
C112	+	+
C114	+	+
C141	+	+
C148	+[a]	+[a]
S5	+	+
S14	+	+
S18	+	+

For each pair, 30 to 60 grafts were made. Spores were collected from the sporangium derived from a singly regenerated sporangiophore at the graft union. For yellow mutants, spores collected were inoculated in vials containing PDA (after dilution, one spore/vial). For white mutants, spores collected were streaked on GAY acid plate (one sporangium/plate) and segregation of yellow, pale-yellow and white colonies was checked. Small pieces of mycelia from different pale-yellow colonies were inoculated into vials (one colony/vial). Sporangiophores grown in vials (one sporangiophore/vial) were inspected for phototropism in the threshold box. + Complementation. — Failure to complement.

[a] Cited from the data shown in Table 4.

of the information channel, they assumed that class 1 mutants were defective on the input side (photoreceptor system) and normal on the output side (response), since these mutants could respond to other stimuli, while class 2 mutants were defective on the output side since they showed no tropic response to any stimuli. To assess and refine this model complementation was tested in heterokaryons among fifteen *mad* mutant strains selected from each of the phenotypic groups: six class 1.1 mutants, five white class 1.2 mutants carrying the genotype *carA* besides *mad*, and four class 2 mutants. Our results reveal the existence of five genes neatly consistent with the phenotypic grouping, i.e. *madA* and *madB* in class 1.1, *madC* in class 1.2, and *madD* and *madE* in class 2. The functional difference between *madA* and *madB* mutants of class 1.1 is unknown, but a phenotypic difference between them is that *madA* strains (C21, C47) have a somewhat lower threshold than *madB* mutants (C109, C112, C114) (Fig. 3a). Foster and Lipson (1973) have reported a phenotypic difference even between the two *madA* mutants C21 and C47, by analysis of the growth responses to light pulses using a tracking machine which automatically records the growth response of *Phycomyces*. Above the threshold C21 has an abnormally delayed maximum, while C47 shows a completely normal growth response. The phenotype of C47 might be interpreted by assuming a reduced concentration of photoreceptor pigment. Our complementation tests, however, fail to provide a genetic basis for the different behavior of C21 and C47. In class 2 mutants we also found two kinds of mutants, *madD* mutants (C68, C107, C149) and *madE* mutant (C110) (Fig. 7). A possible phenotypic difference between them is that in the light the *madD* produce slightly less β-carotene than the *madE*. The latter's production is equal to that of wild type (Bergman *et al.*, 1973). Thus, the complementation results obtained in this work

completely concord with, and at two points refine, the network model proposed for the sensory responses of *Phycomyces*. It is interesting and encouraging that all these strains are single locus mutants with respect to the known *mad* genes and that relatively very few genes are involved in each phenotypic group.

The heterokaryotic sporangiophores which complement respond to light with bending angles ranging from the maximum (70 to 80°) to hardly recognizable angles. It is likely that the bending angle is greatly affected by the difference of the proportion of nuclei derived from the parental strains and that the sporangiophores with a balanced proportion of nuclei give the maximum bending angle, closest to the wild phenotype. This is the case for the complementing heterokaryons among *car* mutants deficient with respect to β-carotene; the mycelia and sporangiophores of heterokaryons give various shades of color according to the nuclear proportion (Heisenberg and Cerdá-Olmedo, 1968; De La Guardia *et al.*, 1971). Even the complementing heterokaryons with the best balanced nuclear ratio, however, are not functionally and phenotypically equal to the wild type. This may be a reason why the thresholds of the complementing heterokaryons between class 1.1 mutants, shown in Fig. 5, are always higher than those of wild type. This is true also in the balanced heterokaryons of *car* mutants: the production of total carotenes or β-carotene is cut down and the relative proportion of intermediate carotenes is altered (De La Guardia *et al.*, 1971; Ootaki *et al.*, 1973).

The clear-cut results of the complementation tests and the finding of relatively few genes involved in phototropism opens the way to further genetic analysis using many other behavioral mutants and also indicates that the mechanism and pathway might be simple enough to understand. Our results fit together with the information obtained by biophysical analysis (Foster and Lipson, 1973) and by biochemical analysis, such as enzymatic study of chitin synthetase which is believed to play an important role in the behavioral output (Jan, 1974).

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1.2) *mad F*

The complementation analysis was continued to include the strain C6 (listed in part C 2.5 of this thesis); the physiology of this mutant is described by Bergman *et al.* (5). It seemed important to include C6 because it was reported by Leighton in the 1973 Cold Spring Harbor Workshop that while *mad D* and *mad E* mutants are abnormal in chitin synthetase function (e.g. in their reaction to stimulation by ATP) the enzyme of C6 cannot be distinguished from the one in WT; in addition the threshold curve of C6 is characteristically different from that of the other stiff mutants.

Complementation analysis was performed as described and the results are listed in table B1. The results from table B1 demonstrate the existence of another complementation group, which is designated *mad F*.

To confirm these results threshold measurements were made as described in the previous section. The results are shown in Fig. B1.

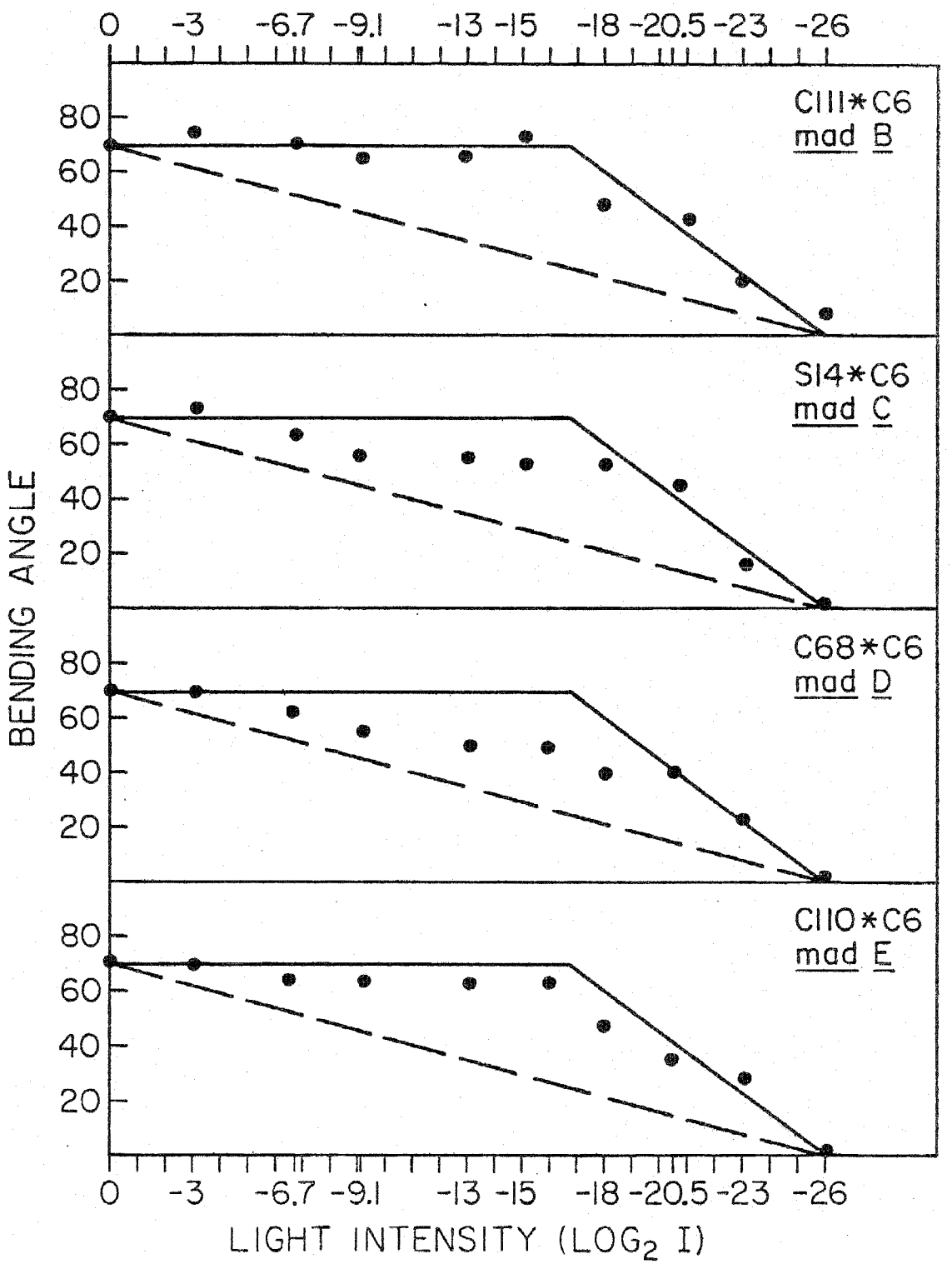
Complementation group	A	B	C	D	E
Representative strain	-	C 111	S 14	C 68	C 110
C6	Not performed	+ 12/11	+ 23/2o	+ 40/31	+ 42/33

Table B1: Complementation tests between C6 and representative strains from established complementation groups.The + sign indicates phototropic response; the upper number gives the number of sporangiophores inspected for phototropism,the lower number gives the number of sporangiophores which showed a phototropic response as a result of complementation.

Fig. B1: Threshold curves

Threshold curves for the heterokaryons of representative mad strains with C6; the solid (dashed) line represents the behavior of WT (C6); both were taken together with the measurements of the heterokaryons. Each point is the average of five samples.

For details,see the legend to Fig.3 in the enclosed publication in the previous section.


0 -3 -6.7 -9.1 -13 -15 -18 -20.5 -23 -26
C111*C6
mad B
S14*C6
mad C
C68*C6
mad D
C110*C6
mad E
80
60
40
20
BENDING ANGLE
LIGHT INTENSITY (LOG2 I)

2) Linkage Analysis and Summary

The results of complementation analysis and physiological classification are illustrated in Fig.B2 (designed by T.Ootaki).

To determine the linkage between these mad genes sexual crosses were studied between the corresponding mutants (11). These experiments were made possible through establishing conditions for high and reproducible germinations of zygospores by A.P.Eslava (11).Clear evidence was found that a standard meiotic process is operating in the generation of recombinants. No evidence was found for linkage, supporting the notion that here as in other eukaryotic organisms functionally related genes are not clustered on the same linkage group but are dispersed over the genome.

During these recombination tests one mad D strain (C 107) was found to be a double mutant.A new gene, tentatively designated mad G, was segregated from a cross involving that strain (11). This finding suggests that a few more genes can be expected to be involved in the sensory transduction processes that have been described so far; nevertheless the number appears to be small. This fact encourages biochemical investigations especially of the regulatory processes at the output end; evidence has been presented earlier for Phycomyces (6,7) that chitin synthetase

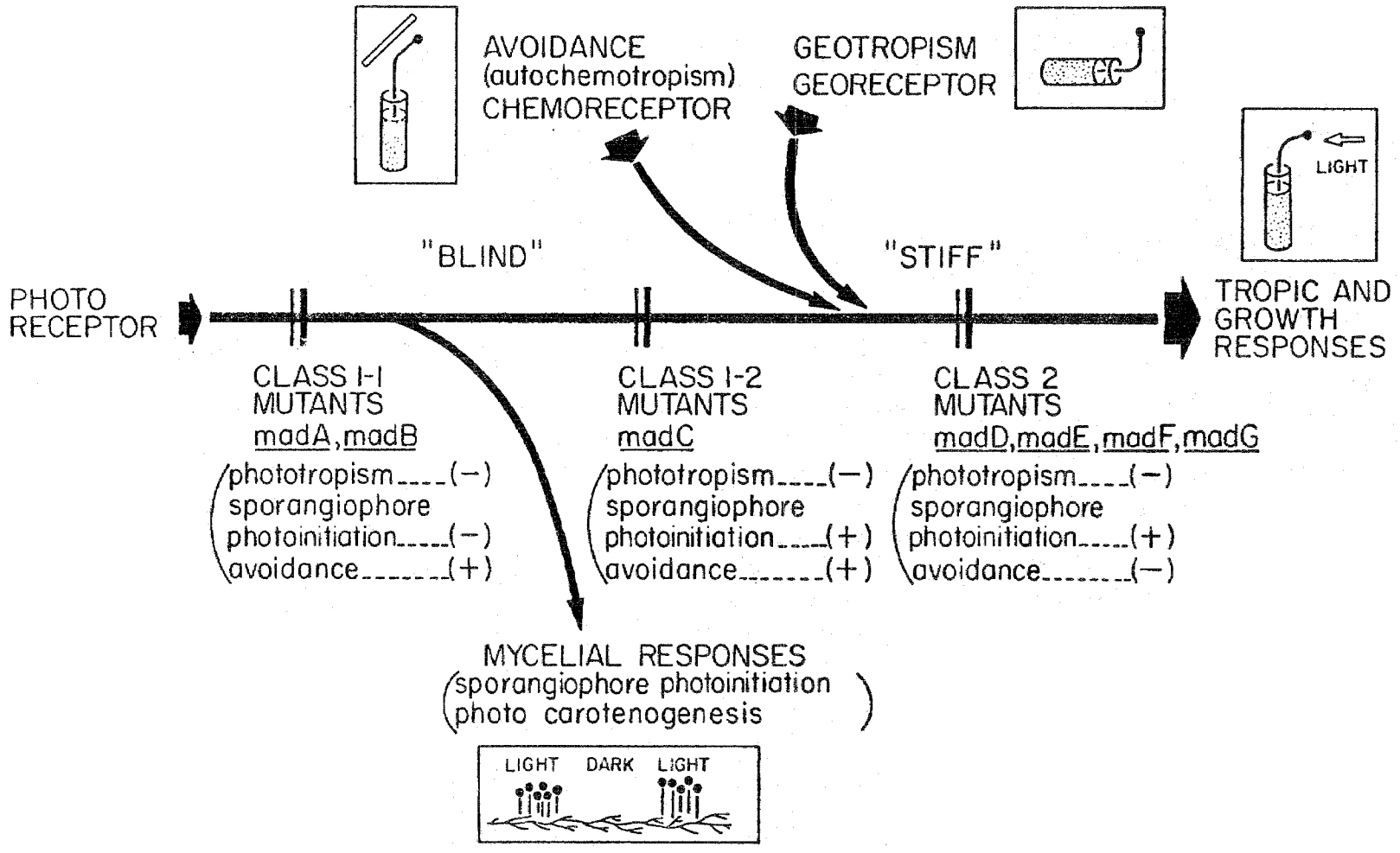
can be activated by light in WT but not in some of the mutant strains.

The following part is the description of the studies of the question whether limited proteolysis is a physiologically relevant mode of regulating cell wall synthesis.

Fig. B2 : The light information channel

Complementation groups and physiological classes form a stimulus-response network in Phycomyces.

For details, see text and Bergman et al. (5).


AVOIDANCE
(autochemotropism)
CHEMORECEPTOR
GEOTROPISM
GEORECEPTOR
LIGHT
"BLIND"
"STIFF"
PHOTO
RECEPTOR
TROPIC AND
GROWTH
RESPONSES
CLASS I-1
MUTANTS
madA, madB
phototropism ---- (−)
sporangiophore
photoinitiation ---- (−)
avoidance ---- (+)
CLASS I-2
MUTANTS
madC
phototropism ---- (−)
sporangiophore
photoinitiation ---- (+)
avoidance ---- (+)
CLASS 2
MUTANTS
madD, madE, madF, madG
phototropism ---- (−)
sporangiophore
photoinitiation ---- (+)
avoidance ---- (−)
MYCELIAL RESPONSES
sporangiophore photoinitiation
photo carotenogenesis
LIGHT
DARK
LIGHT

Chapter C

Serine Proteases and Their Inhibitors in *Phycomyces*

SERINE PROTEASES AND THEIR INHIBITORS IN PHYCOMYCES

Our goal is to understand the molecular events controlling the behavioral output (i.e. the change of the rate of cell wall elongation); we explore the hypothesis that this variation involves the regulation of an enzyme that catalyzes cell wall synthesis.

1) Materials and Methods

For all biochemical studies the strain C2 was used; it is derived from WT NRRL 1555 by nitrosoguanidine mutagenesis and carries the genotype car A5(-) . The growth and tropic responses are identical to WT (4); it has the advantage of producing very little β-carotene.

Instead of using spphs as material for biochemical studies, cells which have been grown for either 24 or 48 hours are homogenized. In these germlings the activity of the enzymes under consideration per mg of material (and also per mg protein) is much greater compared to the spphs, because most of the cell wall synthesizing activity is found in only a very small part of the spph (namely the growing zone), as demonstrated by Jan (6).

1.1)Materials

UDP-N-acetyl-D-1-[^{14}C]-glucosamine was purchased from Amersham/Searle Corporation,Illinois;TPCM,TLCM,NSAN, trypsin,chymotrypsin,azocoll were purchased from Calbiochem,

California; imidazole,Triton X-100,cholic acid,PPO,POPOP were purchased from Sigma Chem. Comp.,Missouri; acrylamide, Bis,Temed,ammonium persulfate are products of Biorad,Cal.; [^{3}H]-DFP and Aquasol were purchased from New England Nuclear in Mass.; DEAE-cellulose DE 52 and DE 23 are products of Whatman,Kent,England; CM-Sephadex,SP-Sephadex,G 75,G 100 are products of Pharmacia Fine Chem.,Sweden; Metrizamide and spheroidal hydroxylapatite were purchased from Gallard-Schlesinger,New York; ampholytes for electrofocusing gels were purchased from LKB Comp.,Sweden; calibration proteins are taken from Combitheks I and II from Boehringer/Mannheim,West-Germany.

1.2)Culture Methods

Spores were heatshocked (100 ml with 10^7 spores/ml were put for 60 min in a heat block which was set at 48^{o}C) and inoculated in 9 liter GAY (glucose,asparagine plus yeast) medium in a container of total volume 15 liter. GAY contains 30g glucose,2g L-asparagine-H_2o, 0.5g $MgSO_4 \cdot 7H_2O$, 1.5g KH_2PO_4, 1g yeast extract(Difco Co,Michigan), 0.25mg thiamine-HCl in 1 liter of distilled water. The culture was kept at 22^{o}C ($\pm$ 1^{o}C) with overhead diffuse white light of intensity 10 μwatts/cm^2 on a Fisher jumbo magnetic stirrer set at half the maximal speed;vigorous aeration was applied.The formation of foam was prevented

by applying Antifoam A (Dow Chemicals). This procedure gives about 500g of cells (wet weight) after 48 hours.

1.3)Assays of Proteases

1.3.1)Protease A is assayed according to the method of Hata et al.(12) by incubating 5oo µl of 1.2% acid denatured hemoglobin (at pH 3) with up to 100 µl of enzyme solution at 30°C. The reaction is stopped by adding 5oo µl 10% TCA; after 20 min the samples are centrifuged and the supernatant is processed according to a variation of the Lowry procedure as described by Mc Donald et al. (14); the OD 635 is recorded.

The preparation of the hemoglobin is crucial: 2.4 g Hb are stirred with 5o ml H_2O for 20 min,the pH is lowered to 1 with 1 N HCl; after 15 min 2 ml lactic acid (85%) are added;the pH is then adjusted to 3 with 1 N NaOH; water is added to bring the volume to 200 ml. If stored in the refrigerator,this solution can be used for 3 weeks.

1.3.2)Protease B is assayed according to the method of Saheki et al.(10) by incubating 5oo µl 1.2% azocoll suspension (in 0.1 M phosphate buffer,pH 7.0) with up to 100 µl enzyme solution under vigorous shaking at 3o°C; the reaction is stopped with 500 µl 10% TCA, the samples are centrifuged and the OD 52o of the super-

natant is recorded.

1.3.3)Protease C is assayed according to the method of Aibara et al.(13) by adding 0.2 ml of a 3 μM BTNA (in dimethylformamide) to 1 ml of a mixture pre-heated to 30°C of enzyme solution with 0.1 M phosphate buffer,pH 7.0.The OD 410 is recorded.

1.3.4)Inhibitory activity (of B type proteases) is determined by adding various amounts of inhibitory protein solution to a standardized crude protease preparation.

1.3.5)One unit of proteolytic activity is defined as the amount of enzyme that causes a reading of one OD unit at the corresponding wavelength in one hour incubation time. One unit of inhibitory activity inhibits one unit of proteolytic activity.

1.4)Preparation of Column Material

The column material (DE23,DE52,CM-Sephadex) was prepared according to the manufacturer's recommendations. (16). Spheroidal hydroxylapatite was suspended in 0.2 N NaOH and washed with distilled water till pH was 5; the column was poured and equilibrated with 20 volumes buffer.

1.5)Purification of Inhibitor Protein

48 hours old germlings are harvested by filtration through miracloth; the buffer is 25 mM imidazole-acetate,pH 7.2 (buffer II). Cells are homogenized

as descibed above.Alternatively the germlings are homogenized in a Gaulin Laboratory Homogenizer (J. H. Vossler Co.,California) at 9,000 p.s.i.; in this case the material can be concentrated considerably.

In a typical preparation 400 g cells (wet weight) in 1600 ml are used.The material is heated to 90°C ($\pm$3°C) for 15 min and cooled in ice. The following steps are all performed at room temperature (22°C) after addition of 0.02% NaN_3 .

The material is centrifuged at 2000 x g x 15 min (Sorvall RC-2B centrifuge,GSA rotor) and the pellet is discarded.The pH of the supernatant is adjusted to 7.2 with 1 N NaOH. TCA is added to a final concentration of 15% (w/v). After two hours of constant stirring the material is centrifuged at 2000 x g x 30 min and the pellet is resuspended in buffer II and dialyzed for 2 x 3 hours against 50-fold excess of buffer. The sample is then loaded on a DEAE- cellulose column (DE52 brand, 3 x 25 cm); at a flow rate of 120 ml/h,12 ml fractions are collected; the column is washed with 2oo ml buffer II,followed by application of a linear NaCl gradient (O-O.4 N, 250/250 ml). Two peaks of activity can be separated; both are dialyzed against 4 l 0.2 M NH_4HCO_3 for 3 h and lyophilized. The powder is resuspended in 50 mM acetate buffer,pH 5.5 and

filtered on Sephadex G75 (apply 3 ml to a 2.5 x 90 cm column,collect 4 ml fractions, 150 cm H_2O pressure); the active fractions are run through an SP-Sephadex column (1 x 5 cm,flow rate 120 ml/h); the passthrough is lyophilized as described above and then dissolved in buffer II.

Alternatively the supernatant of the boiled homogenate can be directly loaded onto a DEAE-cellulose column or it can be concentrated in a rotatory evaporator. All column chromatography is done in Biorad columns,the OD 280 is monitored with the Isco U5A absorbance monitor, the fractions are collected with the Isco Golden Retriever.

1.6)Purification of Proteases of Type B

Harvesting and homogenization of cells as described in section 1.5; the buffer is the same as buffer II, only the pH is shifted to 6.8 (buffer III).

In a typical preparation 1 kg of germlings (wet weight) is suspended in 1 l buffer III; the homogenized cells are centrifuged at 1000 x g x 15 min; the pH of the supernatant is lowered to 5 with glacial acetic acid; 0.1% of an antibiotic mix (50 mg streptomycin, 4o x 10^3 I.U. Penicillium G,10 x 10^3 I.U. Nystatin (Lilly Co.) per ml) and 2 ml/l of 10% NaN_3 is added and the material is incubated for 20 h at 28°C.The pH is then adjusted to

6.8 with 50% NaOH and 1% Triton X-loo (w/v) is added; the sample is stirred in ice for 60 min.

All the following steps are done at 4°C (± 2°C) unless otherwise stated.The material is centrifuged at 100.000 x g x 60 min.; the pellet is discarded. The supernatant is a crude protease preparation; it is used for the assays of protease inhibitor described above.

The purification of the proteolytic enzymes is achieved in the following way (see Fig. C2) : The crude preparation is loaded onto a DEAE-cellulose column (DE 23 brand, 5x30 cm, flow rate 2oo ml/h, 20 ml fractions are collected). The activity is monitored by azocoll hydrolysis.

The passthrough contains part of the activity; it is cooled to -5°C, acetone (precooled to -10°C) is added in a ratio 2:1; the mixture is stirred for 15 min. and then centrifuged at 0°C with 3000 x g x 30 min.; the pellet is resuspended in buffer III (with 3% Triton X-100 (w/v) to prevent aggregation of the material) and dialyzed overnight against 100-fold volume of buffer III.

The material that was retained on the DE 23 column is eluted with a linear salt gradient (0 - 0.5 N NaCl, 500/500 ml); two peaks of activity can be separated. (Note: It does not make any difference whether this column is run with or without detergent; usually Triton X-100 is kept until after the gel filtration.)

All three separated fractions with protease B type activity are run on a G 75 Sephadex column (6 x 120 cm, 150 cm H_2O pressure, 20 ml fractions collected), which is equilibrated with buffer III and 1% Triton X-100. The detergent is removed in the next step by passing the active fractions over either a CM-Sephadex column (the acetone precipitated material) or a DEAE-cellulose (DE 52) column (all columns measure 1 x 10 cm, 5 ml fractions are collected at a flow rate of 100 ml/h). The columns are washed till all detergent is removed (detected by monitoring OD 280). All activity is retained and eluted then with a linear NaCl gradient (0 - 0.4 N NaCl, 50/50 ml). The active fractions are dialyzed (2 x 3 hs, 50 volumes buffer III) and loaded (each separately) on a spheroidal hydroxylapatite column (1 x 5 cm, 120 ml/h flow rate, 4 ml fractions). All activity is absorbed; the retained proteins are eluted by a linear phosphate gradient (0 - 0.3 M phosphate buffer, pH 6.8) and dialyzed (3 h, 50 volumes buffer III). The different fractions are designated as indicated in Fig. C2.

1.7) SDS Polyacrylamide Gel Electrophoresis

SDS gel electrophoresis is performed as described by Laemmli (57) at pH 8.8 and 10% or 15% acrylamide. The gels are stained for 60 min. in .25% Coomassie Brilliant Blue (in 10% acetic acid, 25% isopropylalcohol) and de-

stained in 10% acetic acid.

1.8) Isoelectric Focusing

Isoelectric focusing was performed as described in the LKB application book (18); ampholines were selected for a pH range from 3.5 to 10; the sample was lyophilized and dissolved in 6 M urea; 20 µl of a solution with 1 mg protein/ml was applied to a Whatman #1 strip (5x10 mm) and placed on the gel.

The pI was determined by cutting the gel in strips and mixing these slices with de-aerated distilled water; alternatively linear interpolation with the following standards was used (pI as given in (68)): myoglobin (6.99), hemoglobin (7.23), cytochrome c (10.1), serum albumin (4.7), insulin (5.35).

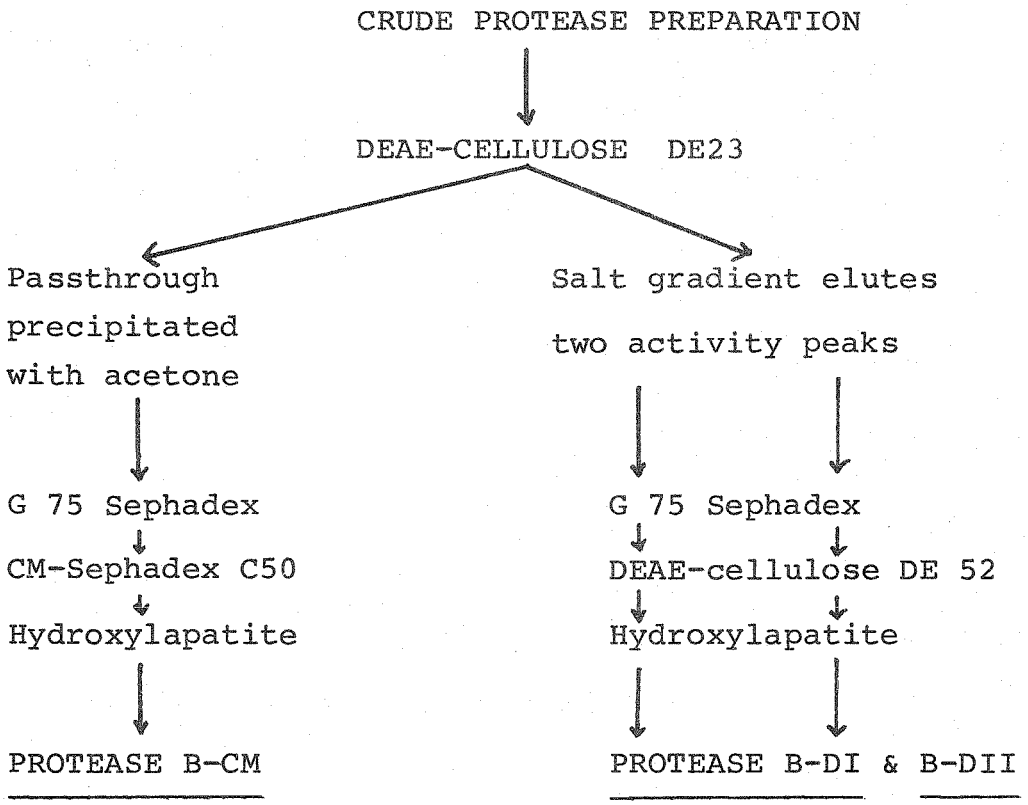


Fig.C2 : Purification scheme for the three B type proteases that can be found in Phycomyces.For details, see see section 1.6 of methods.

1.9)Labeling with [1-^{3}H(N)]-DFP

The radioactive material is supplied in propylene glycol solution with 0.051 mg/.25 ml;the specific activity is o.9 Ci/mmole; 10 µl are added to 2 ml of protein solution (0.15 mg/ml) and stirred for 5 min (all steps - with obvious exceptions - are performed under a hood (velocity ≃ 100 ft/min)).

The sample is dialyzed overnight against 4 l H_2o and concentrated 50-fold by lyophilization. It is submitted to SDS polyacrylamide gel electrophoresis. The gel is sliced into 20 slices (0.50 cm each),mixed with 1 ml 30% hydrogen peroxide and heated for 6 h at 75°C. Aquasol (5 ml) is added and the samples are counted in a liquid scintillation counter.

1.10)Protein Determination

The protein was determined by the Lowry procedure (19) or with fluorescamine (Pierce Company) in the following way: Sample and H_2O were mixed to give a total of 500 µl; if a TCA precipitation is done first enough 1 N NaOH is added to neutralize the pH; 1 ml of 0.2 M borate (pH 9) was added.

Fluorescamine (=Fluram) is dissolved in acetone (3 mg/10 ml) and 500 µl of this solution is added and vortexed immediately for at least two seconds.

The sample is excited with 390 nm and the emission

at 480 nm is recorded (Perkin-Elmer MPF Fluorescence-spectrometer).This procedure is applied if the protein concentration is low ($\leq$ 50 µg/ml).

2)Results

2.1)Purification of Two Inhibitors

The inhibitor proteins are heat-stable,TCA-resistant and nondialyzable; this stability suggested the purification procedure outlined in Methods,section 1.5. The results are summarized in Table C1. Two forms of the inhibitors can be distinguished; the two forms carry different charges but apparently have identical molecular weights (Fig. C2, Table C2).

A similar situation is found in yeast (41); the appearance of two inhibitors is not a boiling artifact: two strains of yeast could be identified which carry only one or the other form, whereas baker's yeast appears as a hybrid with both proteins (H. Holzer, personal communication). No difference between the two inhibitors other than the charge has been detected. The data from Table C1 indicate that the inhibitors make up about 1% of the protein in the boiled extract or about 0.3 % in the original homogenate. All inhibitor protein is soluble. The relevance of these observations will be discussed in the next section. After the purification steps listed in Table C1 the sample appears homogeneous with respect to molecular weight as illustrated in Fig. C3, and quantitative analysis (59) gives a molecular weight of 10.000

daltons (Fig C5). This result is supported by a similar determination on G75 Sephadex performed according to (32); see Fig. C18 for details.

In order to see if the visible protein band represents the activity of interest the gels were sliced and kept overnight in phosphate-buffer. As is shown in Fig. C4 the extracted activity corresponds to the stained band.

The Lineweaver-Burk plots of inhibited azocoll hydrolysis (Fig. C6) show a shift of the apparent type of inhibition from competitive (crude inhibitor) to non-competitive (purified inhibitor) inhibition. This shift may have a trivial explanation: on one hand the substrate is a suspension of a red powder (cowhide with an azo-dye attached) and as such may not disturb the purified enzyme-inhibitor equilibrium making the Lineweaver-Burk plot indicate noncompetitive inhibition.On the other hand, the crude inhibitor (=boiled extract) contains other factors that interact with the protease and/or the inhibitor protein.

Table C2 summarizes some properties of the two proteins.

Table C 1 Purification of two protease B inhibitors from *Phycomyces*

Fraction	Volume (ml)	Units ml	Total units	Protein mg/ml	Units mg prot.	Yield %	Purification
Boiled extract	1600	3.2	5202	1.2	2.7	100	1
TCA precitation	70	48.2	3380	3.2	15.3	65	6
DE 52							
# I	140	3.7	512	0.07	54.2	10	20
# II	220	5.9	1300	0.05	125.0	25	46
G 75 and lyophiliz.							
# I	5	41.6	208	o.30	138.2	4	51
# II	5	124.8	624	0.43	287.3	12	106
SP-Sephadex							
# I	5	20.8	104	0.14	143.1	2	53
# II	5	72.8	364	0.18	411.5	7	152

Table C1

	Molecular weight (daltons)		Isoelectric point
	from G 75	from SDS gel	(± 0.15)
Inhibitor I	9.500	10.000	4.50
Inhibitor II	9.000	10.000	4.95

Table C2: Molecular weights and isoelectric points of two protease inhibitors from Phycomyces

Since no difference in specificity was detected we will talk about the inhibitor in the following sections. All reported experiments were done with the protein designated inhibitor II in Table C2.

The next part deals with the purification of the corresponding protease; the enzyme is monitored by azocoll hydrolysis. This is a general proteolytic substrate and more than one protease might exhibit activity in the assay. We intended to isolate proteases that hydrolyze azocoll and can be inhibited by the inhibitor proteins whose isolation was described in this section.

Figure C 12:

Chromatography of inhibitor protein on DEAE-cellulose. Elution of two peaks of inhibitory activity from a DEAE-cellulose column; the percent of inhibition of azocoll hydrolysis (-●-) is plotted together with the OD 280 (-▲-) against the number of fractions; the straight line indicates the NaCl gradient.

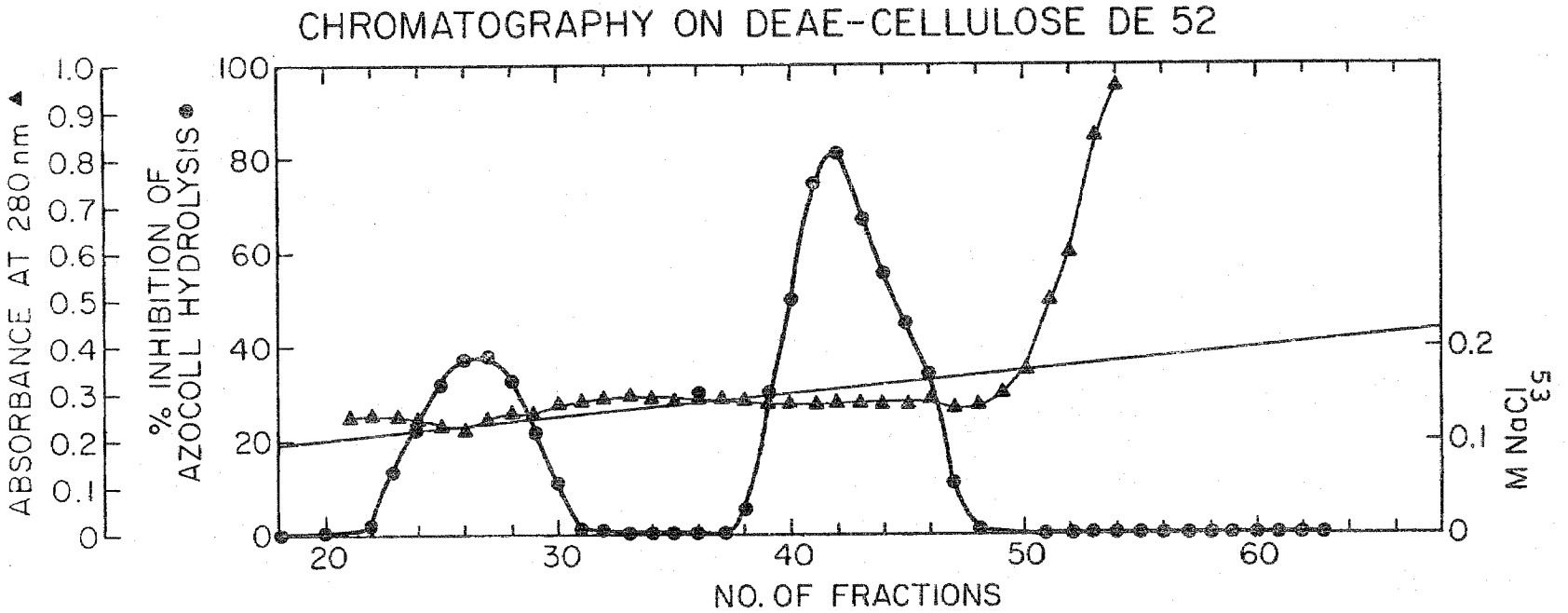

CHROMATOGRAPHY ON DEAE-CELLULOSE DE 52
ABSORBANCE AT 280 nm ▲
% INHIBITION OF AZOCOLL HYDROLYSIS ●
M NaCl
NO. OF FRACTIONS
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
100
80
60
40
20
0
0.2
0.1
0
20
30
40
50
60

Figure C3: SDS polyacrylamide gel electrophoresis

1) and 6) Crude inhibitor (boiled extract)

2) Partially purified inhibitor (after two column steps)

3) and 4) The two forms of the inhibitor; the molecular weight appears to be identical

5) Two standard proteins: cytochrome c (MW 12.500) and BSA (MW 68.000)

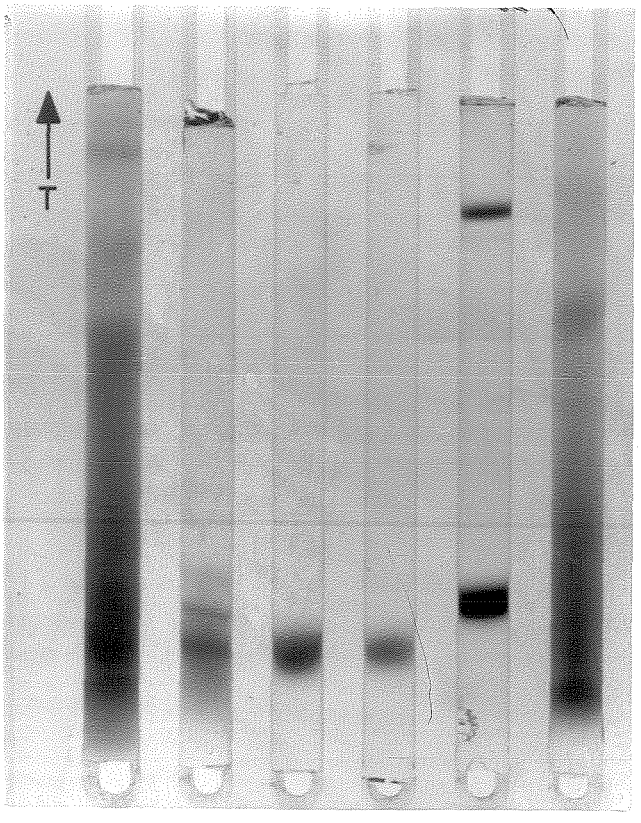


Figure C 4: Extraction of inhibitory activity from SDS polyacrylamide gel, one gel is stained with Coomassie Brilliant Blue; one gel is sliced and the slices are ground in phosphate buffer; aliquots from each sample are incubated with a crude protease preparation.

EXTRACTION OF INHIBITORY ACTIVITY FROM POLYACRYLAMIDE GEL

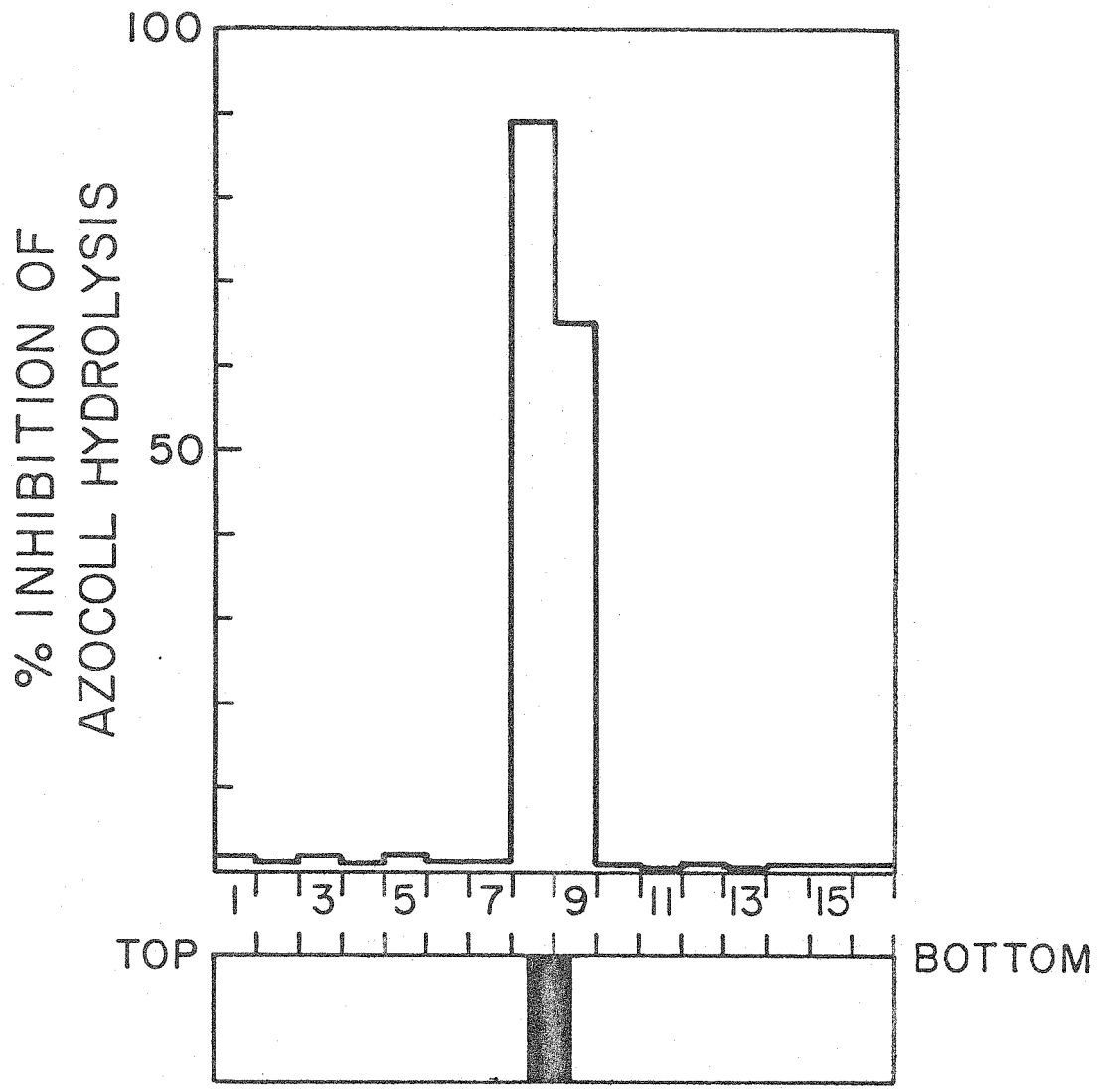


Figure C 5: Determination of molecular weight of inhibitor protein from Phycomyces by SDS gel electrophoresis. The mobility is calculated as described by Weber and Osborn (59).

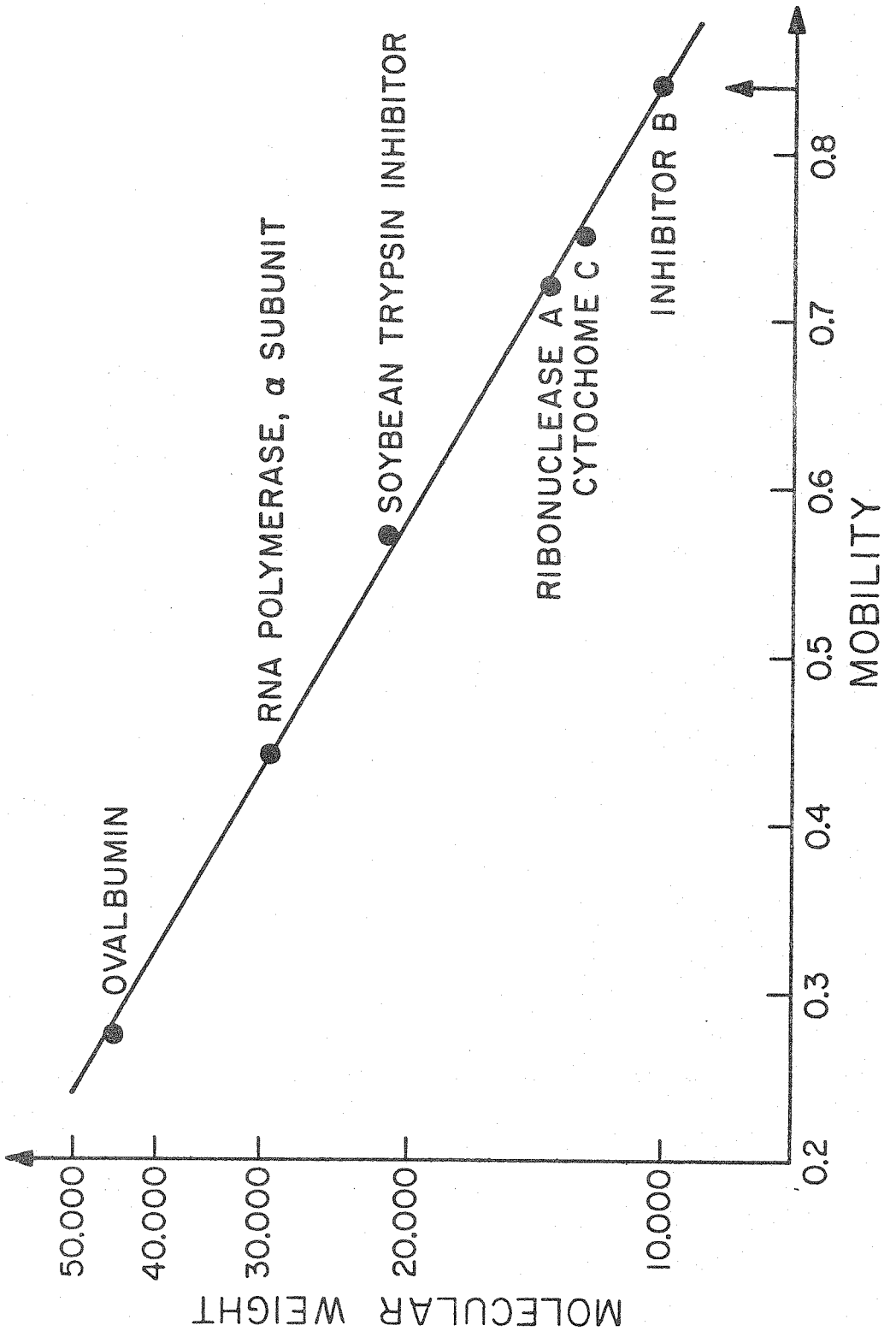
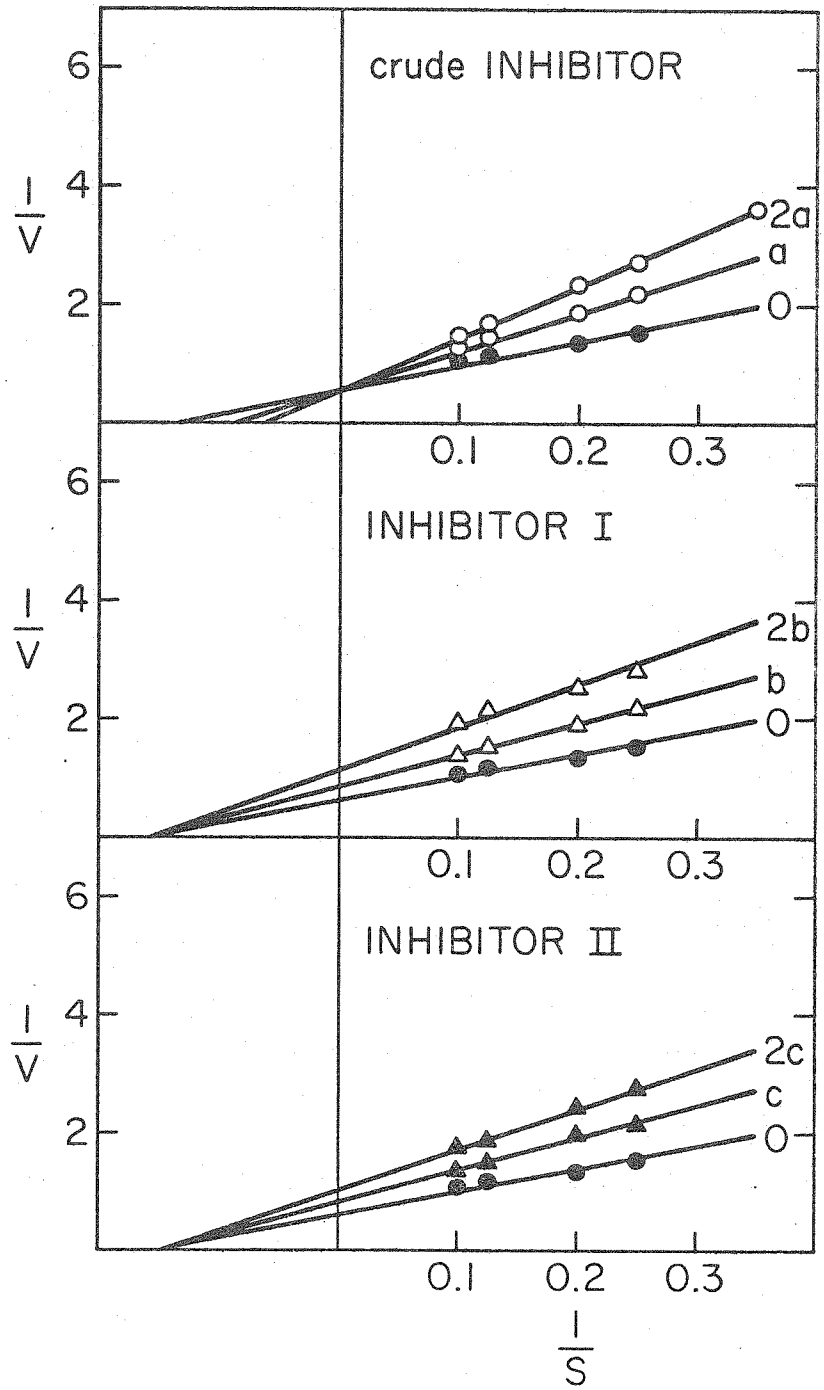

MOLECULAR WEIGHT
50,000
40,000
30,000
20,000
10,000
0.2
0.3
0.4
0.5
0.6
0.7
0.8
MOBILITY
OVALBUMIN
RNA POLYMERASE, α SUBUNIT
SOYBEAN TRYPSIN INHIBITOR
RIBONUCLEASE A
CYTOCHOME C
INHIBITOR B

Figure C 6: Lineweaver-Burk plots of azocoll hydrolysis by a crude protease from Phycomyces inhibited with crude inhibitor and purified inhibitors I and II.

LINEWEAVER-BURK PLOTS OF AZOCOLL HYDROLYSIS BY PROTEASE WITH CRUDE INHIBITOR, INHIBITOR I, AND INHIBITOR II



2.2) Purification of Three Serine Proteases

We use the terms B type protease and serine protease interchangeably in this thesis.

As was described in section 2.1 there is a great amount of inhibitor protein in the homogenate; to detect any proteolytic activity of type B this inhibitor has to be removed. This removal is achieved by incubation at pH 5.0 at room temperature. Figure C 7 illustrates the emergence of proteases and the disappearance of inhibitor.

The incubation can also be done at pH 7.0 with similar results. Surprisingly this does not hold for pH 6.0. The protease A (an acid protease with a pH optimum at around pH 3.0) seems to be responsible for the activation at low pH; (further evidence for this conjecture is presented in part 2.4). The identification of the inactive precursor of protease B as an inhibitor-enzyme complex was made in yeast some time ago (47); the results shown in Fig. C validate this idea for Phycomyces.

Only a small part of the activated protease B is soluble without special treatment and even sonication has only negligible effects on the solubilization. Salt and detergent (Triton X-100) prove more helpful as illustrated in Fig. C 8. These results indicate that part of the proteolytic activity is membrane-bound in the way

it is described for peripheral membrane proteins (60). Similar effects could be achieved by cholic acid, Tween 80, Brij 36 and other detergents.

Two effects of detergent were observed: Protease B was solubilized and soluble protease was activated. This finding confirms reports (50) about the stabilizing effect of detergents on proteolytic activities in yeast.

In the further purification steps the total protease B activity splits into three subfractions.The whole preparation is summarized in Table C3.Without detergent treatment only one type of protease B is solubilized, namely, the one referred to as B-DI; this finding is in agreement with the observation that protease B-CM aggregates if concentrated too much.Another helpful effect of the detergent can be noticed during gel filtration: without Triton X-100 recovery from e.g. G75 Sephadex would be less than 20%.This recovery is not influenced by variations of the salt concentration.

The results indicate that the protease type B accounts for 0.04% of the protein in the original homogenate.Thus (compared with 0.3% inhibitor protein) there is a nearly 10-fold excess of inhibitor over protease. This excess makes the regulatory scheme (Fig. A1) rather unlikely.Instead,one may conceive the inhibitor as a

security force, immediately inactivating any protease that manages to escape from its compartment into the cytoplasm. A similar excess of inhibitor has been reported for yeast (23).

All three B type proteases are inhibited by DFP and thus can be identified as serine proteases.The influence of other inhibitors is summarized in Table C4. It shows that all three serine proteases have a critical sulfhydryl group (cysteine). The specific inhibitors for trypsin (trypsin soybean inhibitor, TLCM) and for chymotrypsin (TPCM) have no effect on the Phycomyces serine proteases. This indicates that their specificity is wider than the ones for the pancreatic proteases.

NSAN is a substrate specifically designed for elastase. Trypsin e.g. does not hydrolize it. Of the three Phycomyces serine proteases only the high molecular weight form (B-DII) is active against NSAN and thus can be assigned elastase specificity.

The endogeneous inhibitor on the other hand has no influence on mammalian serine proteases; on the contrary, trypsin destroys it.

So far it appears there is no difference between two of the three B proteases, but differences become obvious when looking at some steps of the purification

procedure. Fig. C9 shows the elution profile of the DE 23 column; if both peaks are mixed and filtered on G 75 Sephadex, the different molecular weights become apparent (Fig. C 10). If the passthrough of the DEAE-cellulose column is run through a CM-Sephadex column a single peak of activity is eluted (Fig. C 11); the final purification step is done with a small hydroxylapatite column (Fig. C 12); apparently the activity elutes as a homogeneous sample.

The purity is further checked by SDS gel electrophoresis; the results are shown in Fig. C 13. In order to assure that the bands in the gel, which are visible after staining, represent serine proteases, the enzymes were labelled with ^{3}H- DFP as outlined under Methods. In Fig. C 14 the results of staining and counting of SDS gels are presented. They indicate that the assumption is safe, that the major component of the isolated fractions is a serine protease in case of B-DI and B-CM; in case of B-DII no true peak was separated from the background due to the low counting efficiency with tritiated material. The experiment was not repeated.

Figure C7: Effect of incubation at pH 5 on protease B (-●-), protease A (--▲--), inhibitor of B (--o--) and total protein (-Δ-); no inhibition of A type activity could be detected; no protease C type activity was found.

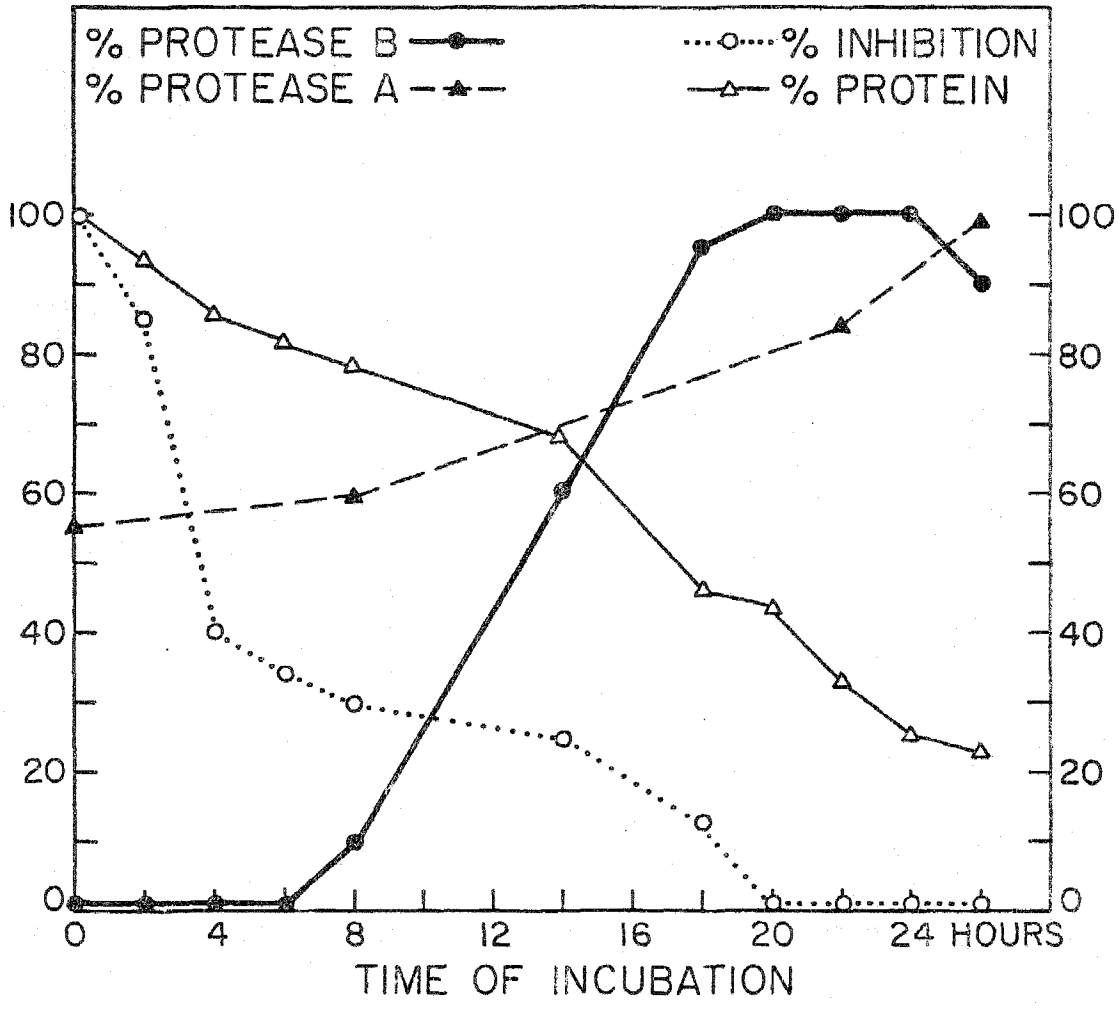

% PROTEASE B
% PROTEASE A
% INHIBITION
% PROTEIN
100
80
60
40
20
0
0
4
8
12
16
20
24 HOURS
TIME OF INCUBATION

Figure C8: Solubilization of acid (A) and serine (B) proteases from an activated homogenate of Phycomyces by treatment with salt (NaCl) or detergent (Triton X-100).
The salt or detergent is added to the homogenate, which is subsequently centrifuged at 100.000 x g for 60 min. The activity of the resulting supernatant is determined and plotted in % of the activity of the homogenate.

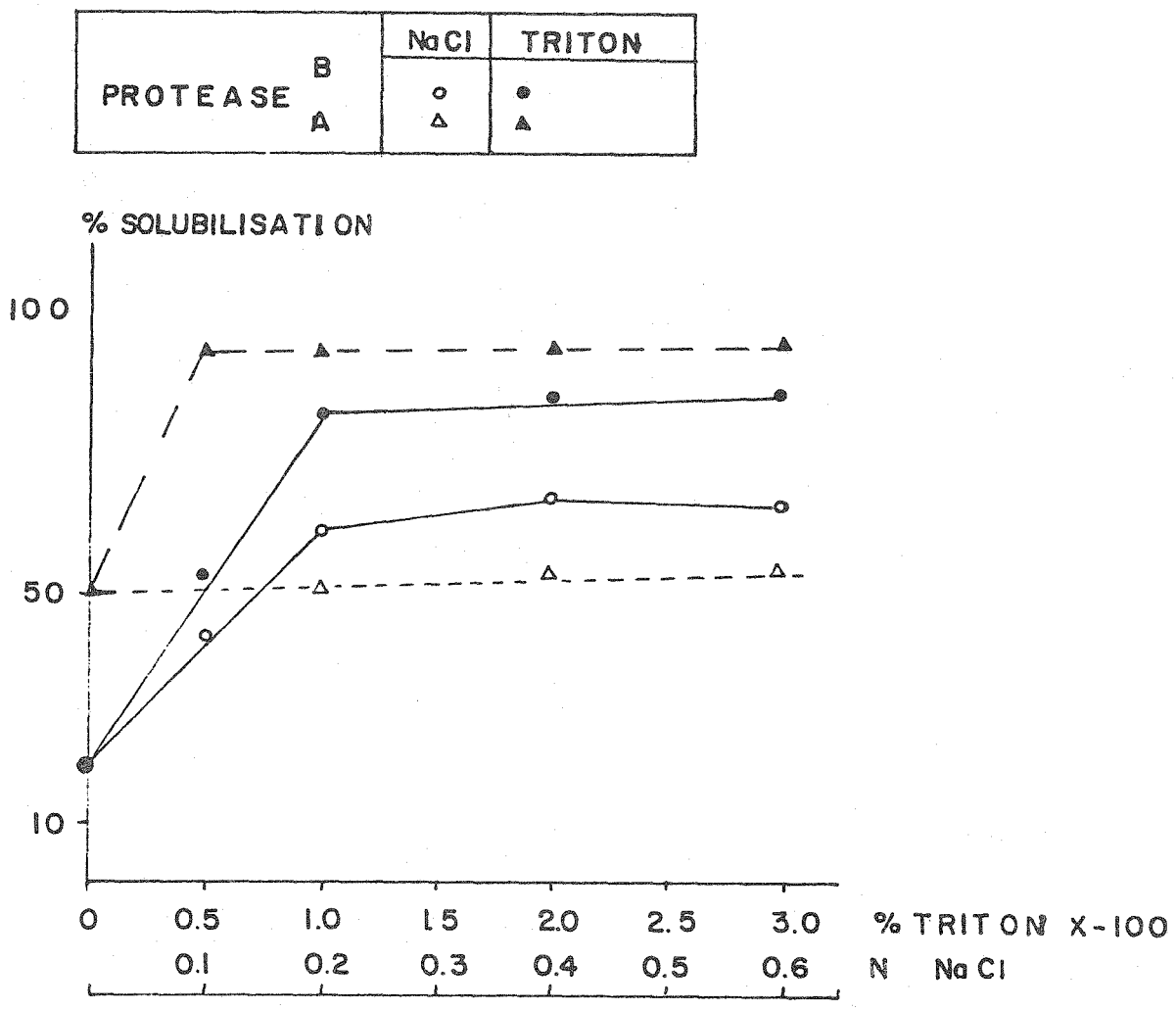

NaCl
TRITON
PROTEASE
B
A
% SOLUBILISATION
100
50
10
0
0.5
1.0
1.5
2.0
2.5
3.0
% TRITON X-100
0.1
0.2
0.3
0.4
0.5
0.6
N NaCl

Table C3: Purification table of three serine proteases of *Phycomyces* ; for details see text.

Fraction	Vol. (ml)	Units / ml	Total units	Protein mg/ml	Units / mg prot	Yield %	Purification
Homogenate	5430	14,8	80.434	14,3o	1,0	100	1
Supernatant	5420	12,0	65.151	8,75	1,4	81	1
Passthrough	5520	3,6	19.867	3,52	1,o	24,7	1
Acetone prec.	182	56,2	10.215	4.80	11,7	12,7	11
G 75 - CM	405	19,2	7.754	1,13	17,0	9,6	16
CM-Sephadex	37	94,0	3.475	0,46	204,2	4,3	196
HAP-CM	21	88,1	1.850	0,12	734,2	2.3	706
DEAE-Cell. I	370	14,4	5.309	6,62	2,2	6,6	2
G 75 - I	208	14,7	3.056	2,10	7,0	3,8	7
DE 52 - I	44	53,0	2.332	0,54	98,1	2,9	94
HAP - DI	25	54,7	1.367	o,13	420,6	1,7	404
DEAE-Cell. II	510	16,6	8.445	4,14	4,0	10.5	4
G 75 - II	35o	13.6	4.746	3,22	4,2	5,9	4
DE 52 - II	49	52,5	2.574	0,31	169,4	3,2	162
HAP - DII	22	69,4	1.528	0.14	469,1	1,9	477

Figure C9: Elution of two B type proteases of Phycomyces on DEAE-cellulose. Protease A is completely separated in this step.

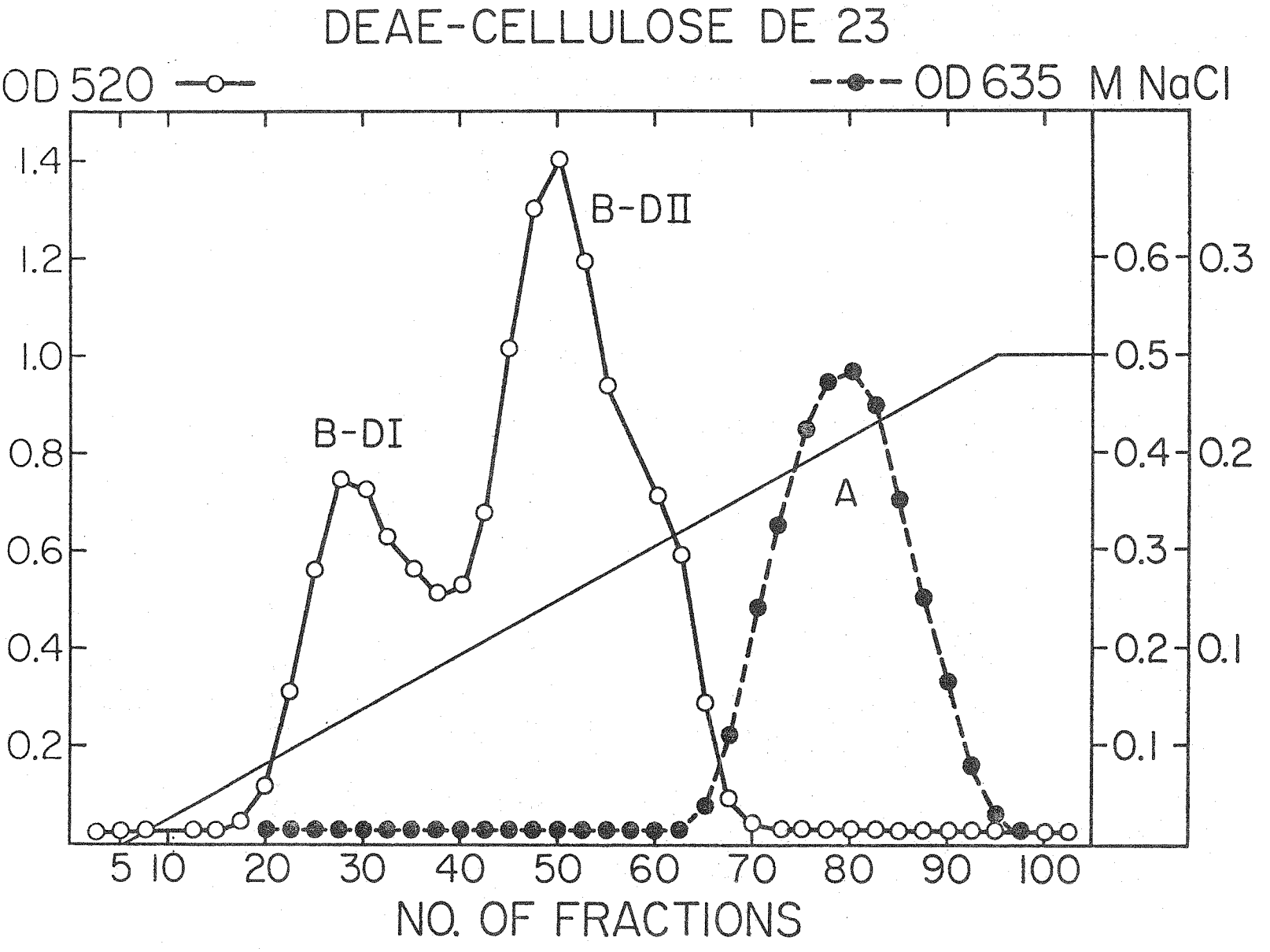

DEAE-CELLULOSE DE 23
OD 520
OD 635
M NaCl
B-DI
B-DII
A
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.6
0.5
0.4
0.3
0.2
0.1
0.3
0.2
0.1
5 10 20 30 40 50 60 70 80 90 100
NO. OF FRACTIONS

Figure C10: Gel filtration with G75 Sephadex of both B type activities which are retained on a DEAE-cellulose column (see Fig. C9); the position of marker proteins of known molecular weight is indicated by the arrows.

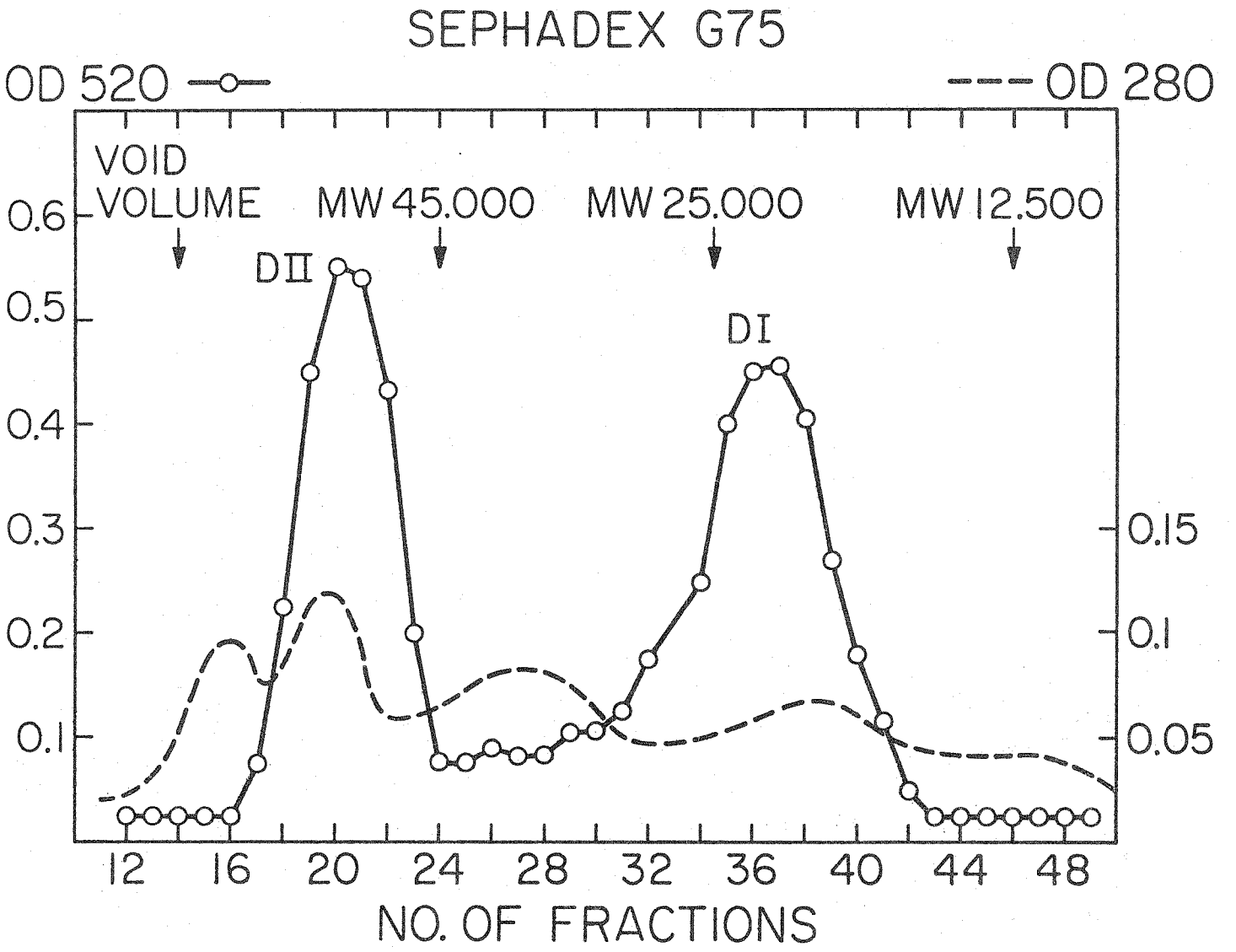

SEPHADEX G75
OD 520
OD 280
VOID VOLUME
MW 45.000
MW 25.000
MW 12.500
DII
DI
0.6
0.5
0.4
0.3
0.2
0.1
0.15
0.1
0.05
12
16
20
24
28
32
36
40
44
48
NO. OF FRACTIONS

Figure C11: Elution of a single peak of B type proteolytic activity from a CM-Sephadex column (-o-).

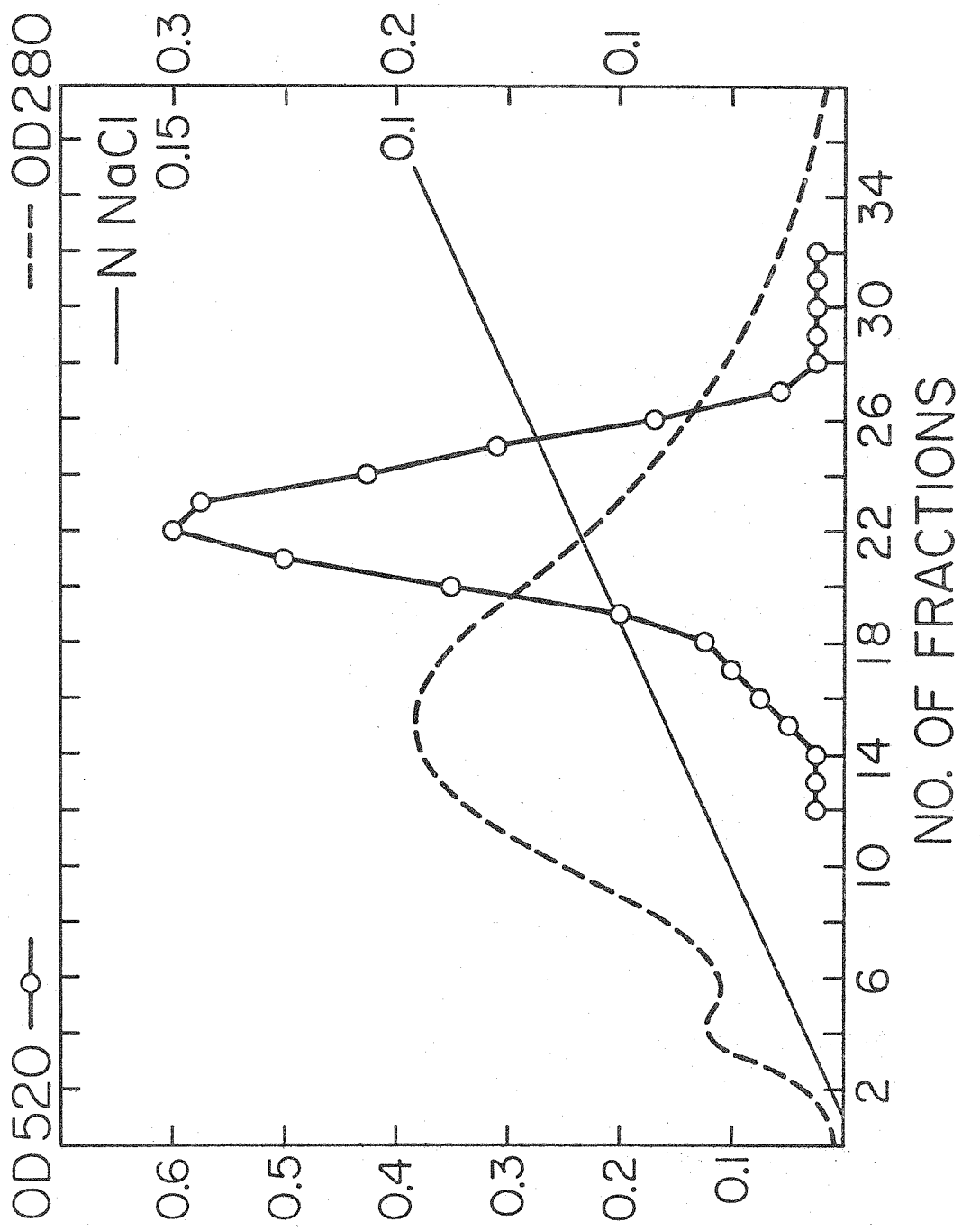

CM SEPHADEX C50
OD520
OD280
N NaCl
0.6
0.5
0.4
0.3
0.2
0.1
0.15
0.3
0.1
0.2
0.1
2
6
10
14
18
22
26
30
34
NO. OF FRACTIONS

Figure C12: Final purification step of Protease B-CM on HAP; the activity is eluted as a single protein peak as indicated by calculating the specific activity of each fraction (-x-).

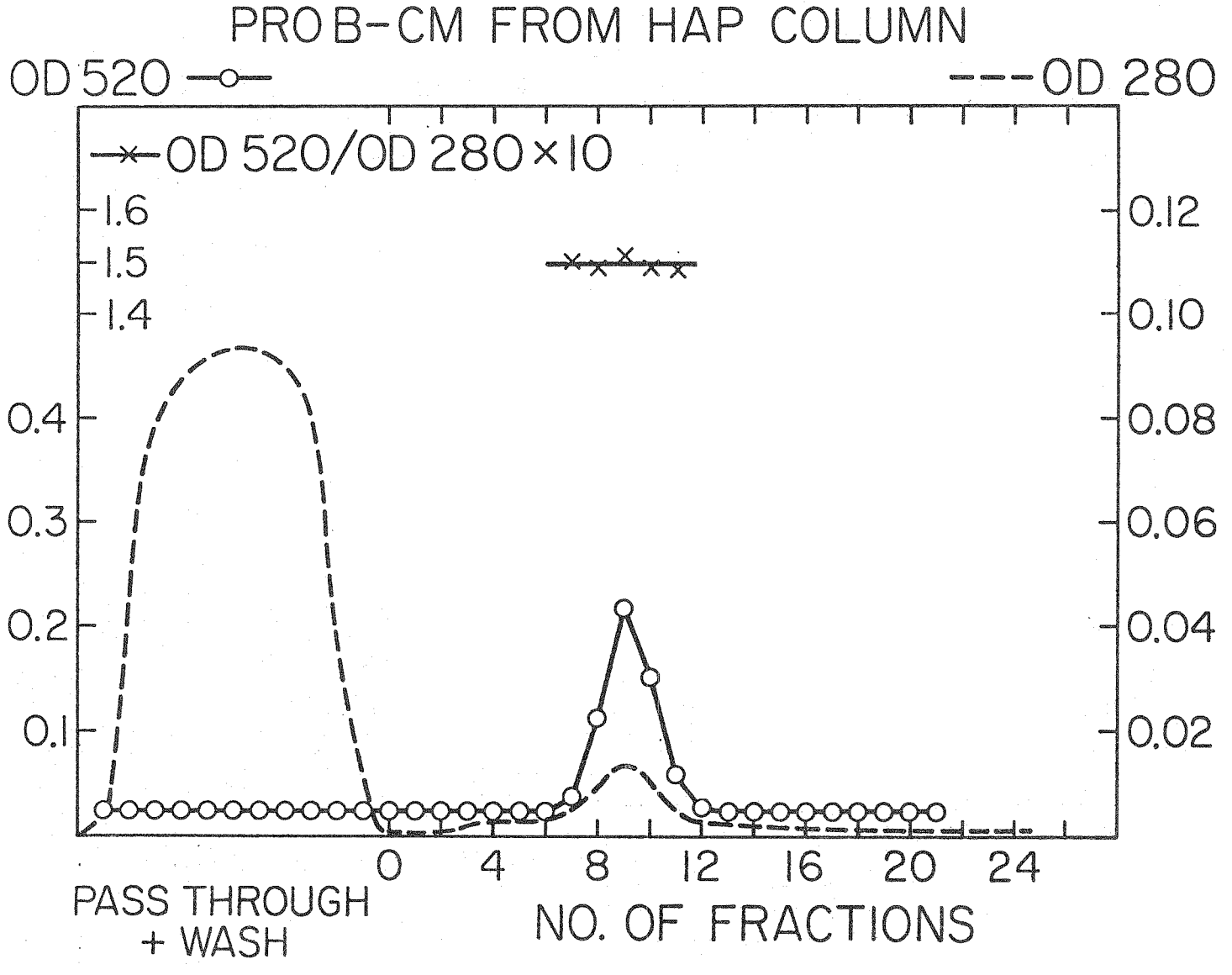
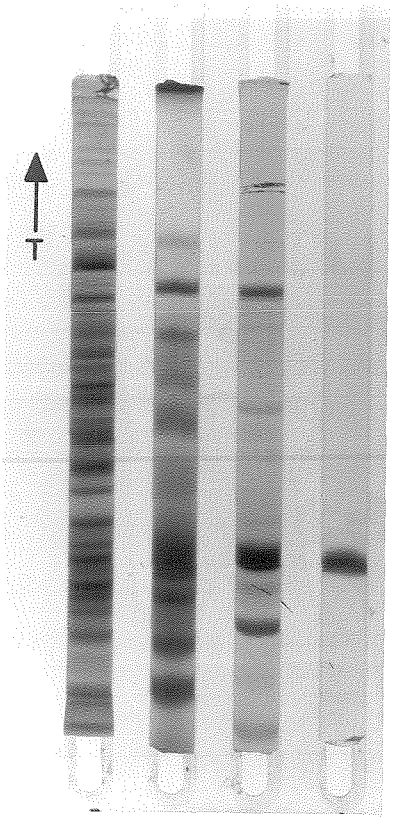

PROB-CM FROM HAP COLUMN
OD 520
OD 280
OD 520/OD 280 × 10
1.6
1.5
1.4
0.4
0.3
0.2
0.1
0.12
0.10
0.08
0.06
0.04
0.02
0
4
8
12
16
20
24
PASS THROUGH + WASH
NO. OF FRACTIONS

Figure C13: SDS gel electrophoresis of serine proteases from Phycomyces

1) Homogenate
2) Supernatant of incubated homogenate
3) Acetone precipitated material
4) Protease B-CM
5) Protease B-DII
6) Protease B-DI



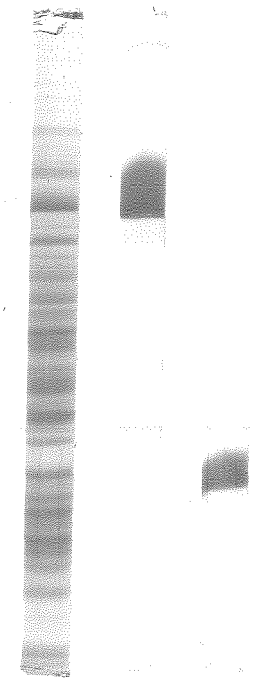


Figure C14: Labelling of Serine proteases B-DI (—●—) and B-CM (....o....) from Phycomyces with ^{3}H-DFP. The drawings at the bottom indicate the result of corresponding gels stained with Coomassie Brilliant Blue.

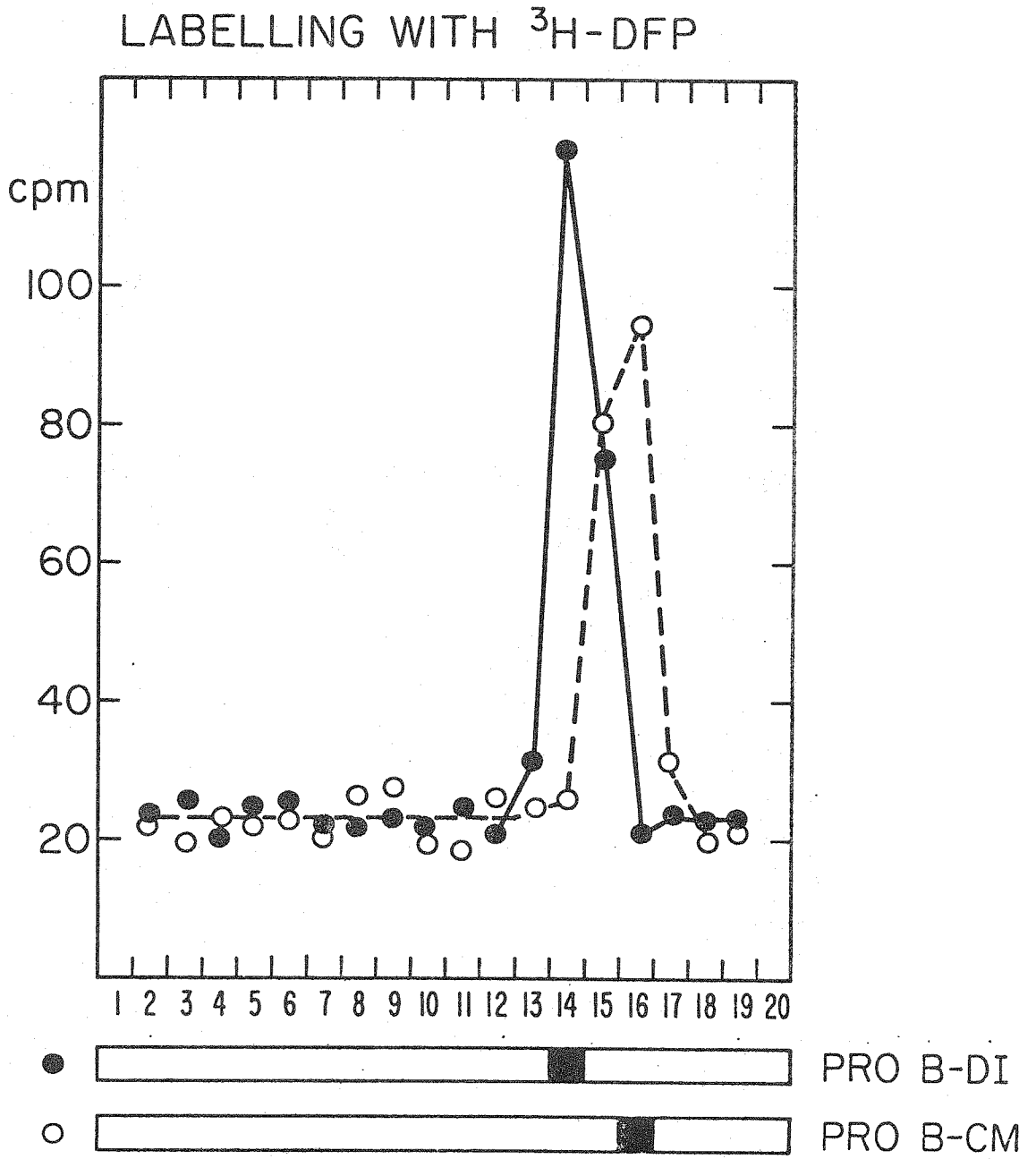

LABELLING WITH ^{3}H-DFP
cpm
100
80
60
40
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
PRO B-DI
PRO B-CM

Table C4: Effect of various inhibitors on Phycomyces proteases.

+ : Inhibition - : No inhibition

Agent	Protease A	B-CM	B-DI	B-DII
EDTA	-	-	-	-
PMSF	-	+	+	+
PHMB	-	+	+	+
DFP	-	+	+	+
NaN_3	-	-	-	-
TPCM	-	-	-	-
TLCM	-	-	-	-
Soybean Trypsin inh.	-	-	-	-
Inhibitor B from Phycomyces	-	+	+	+
Pepstatin A	+	-	-	-

2.3)Some Properties of Proteases and Inhibitors.

From section 2.1 it is known that the Lineweaver-Burk plot establishes noncompetitive inhibition for purified inhibitor when interacting with a crude protease preparation. This finding is confirmed for each of the separated forms in Fig. C15; these curves indicate similar Michaelis constants for the B-CM and B-DI proteases and a smaller value for B-DII. A similar conclusion can be drawn from Fig. C16, which is a graph of the dependence of the reaction rate on substrate concentration. All proteases follow the standard saturation curve.

The interaction of proteases and inhibitors deserves further attention in the following direction: A typical inhibition curve is Fig. C17. A conspicuous feature is the slight activation at low concentrations of inhibitor.This observation might indicate allosteric interactions,but the increase (1%) is insignificant. The 95% confidence range is around 3%.

From the point in Fig.17 in which 50% activity is left a value for the association constant can be estimated. The inhibitor is used at .1 mg/ml protein concentration; we thus find $K_{ass} \simeq 10^7$ [M^{-1}]. Using the Dixon-plot (66) gives a value of the same order of magnitude.

This value can be considered high (31), which in turn leads to all the problems that are involved in determining K_{ass} and which are discussed in (67).

The pH optimum of the three proteases is determined as shown in Fig. C 18; the ionic strength neither influences the proteolytic activity nor affects the interaction with the inhibitor protein in the range between .025 and .25 M. The molecular weights of all proteases are determined by SDS gel electrophoresis and gel filtration. The results are shown in Fig. C 19 and C 20 and summarized in Table C 4. The molecular weight of the protease-inhibitor complex is estimated using gel filtration; the results demonstrate the formation of a 1:1 complex.

The experiment was done by combining protease and inhibitor to give 60% inhibition of the proteolytic activity and running the mixture on the filtration column. The OD 280 is recorded and compared with the profile of a run that featured either the protease or the inhibitor alone.

The tightly associated complex is not broken in high salt or at low pH. The inhibitor is released by 3 M urea at pH 5, but this denatures the protease. One way to regain active protease (this procedure would help to use affinity chromatography) could be a short exposure only to urea at low pH followed by immediate dilution. Some small

scale preliminary tests have been performed successfully.

The purified proteases are rather unstable as shown in Fig. C 21. The observation that BSA can prevent the rapid loss of activity supports the notion that the proteases digest themselves. It is also observed that partially purified protease is rather stable.

All three isolated proteases lose activity if exposed to pH 5; they are completely inactivated (denatured) if exposed to pH 4 or less.

As can be seen in Table C 5 the MW estimated by SDS gel electrophoresis (in two cases) comes out higher than the one determined by gel filtration; this underlines the hydrophobic nature of these enzymes, for, as was pointed out by Reynolds and Tanford (61), the cooperativity of the SDS binding is disturbed in case of hydrophobic proteins.

Protease	Molecular weight (daltons)		Isoel. point	pH optimum
	SDS gel elctroph.	gel filtr.	(± .15)	
CM	20.000	18.000*	7.6	7.5
DI	23.500	22.000*	5.1	7.5
DII	60.000	60.000**	4.4	7.0

*) G 75 Sephadex

**) G 100 Sephadex

Table C5: Some properties of three serine proteases isolated from Phycomyces; see text for nomenclature.

Figure C15: Lineweaver-Burk plots of the inhibition of azocoll hydrolysis of three purified serine proteases of Phycomyces by purified inhibitor protein.

The reciprocal of the substrate concentration (% azocoll) is plotted against the reciprocal of the OD 520 (incubation time was 20 min.).

The lowest curve in each case is a plot of the data taken in the absence of inhibitor; the upper two curves illustrate measurements of protease activity in the presence of 1.5 and 3 µg inhibitor.

LINEWEAVER-BURK PLOTS

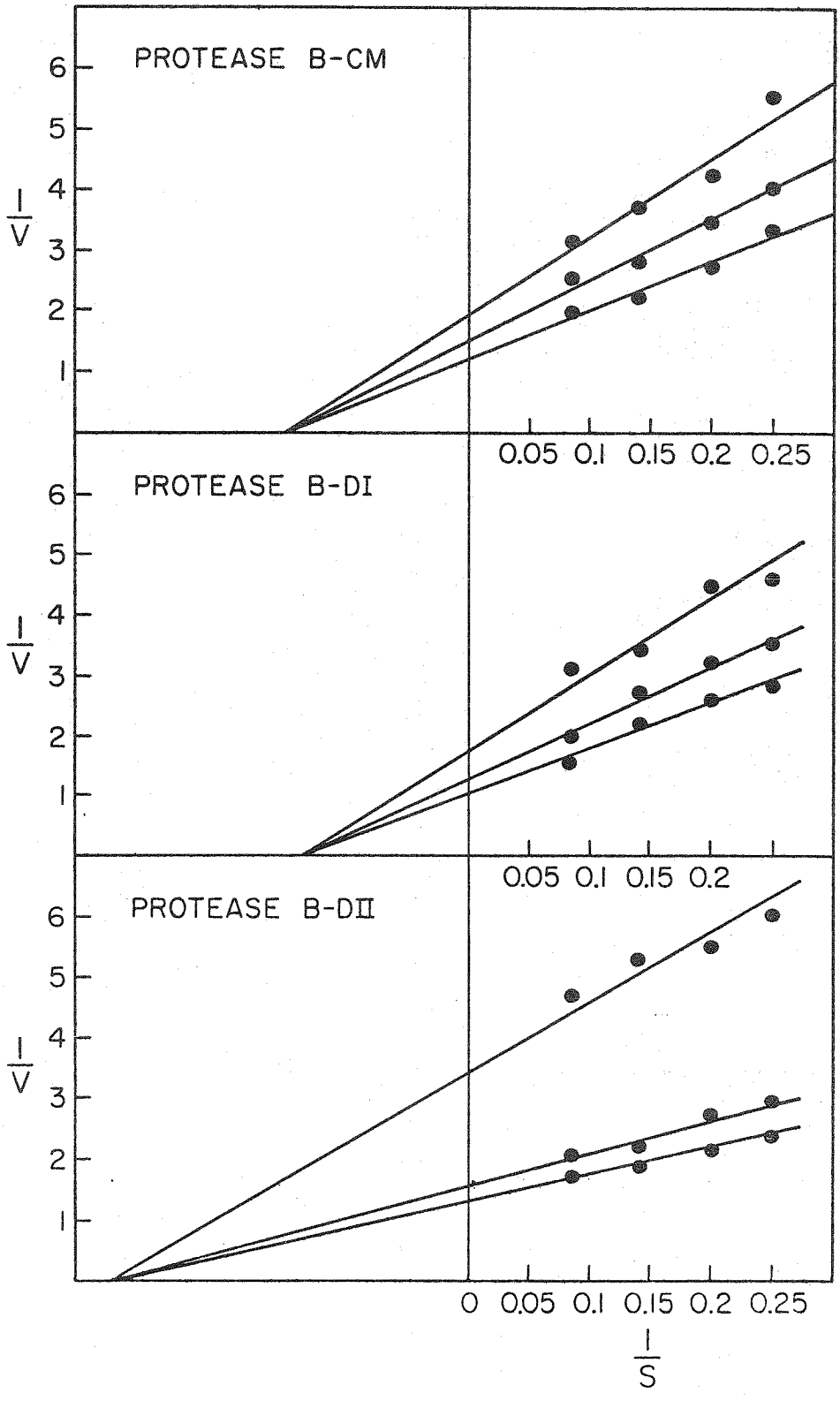


Figure C 16: Dependence of activity of the three purified serine proteases of Phycomyces on substrate concentration; the arrows indicate the substate concentration at which half the maximal activity is achieved.

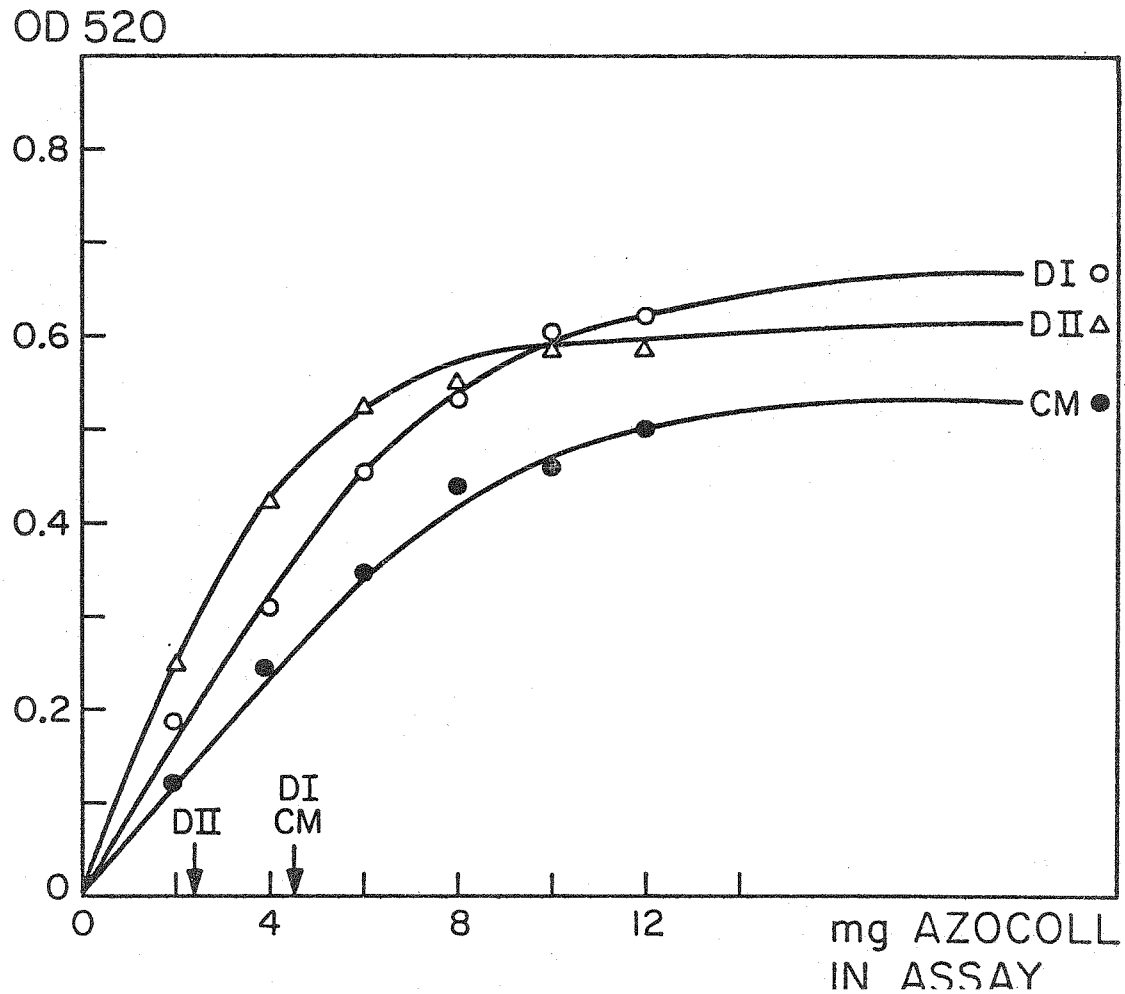

OD 520
0.8
0.6
0.4
0.2
0
DI
DII
CM
DII
DI
CM
0
4
8
12
mg AZOCOLL
IN ASSAY

Figure C 17: Inhibition of azocoll hydrolysis of serine protease DI of Phycomyces; note a slight activation for small amounts of inhibitor (—●—). At 50% inhibition 2 µg of inhibitor are needed in 600 µl assay volume; this allows an estimation of the dissociation constant of about 3×10^{-7} M.

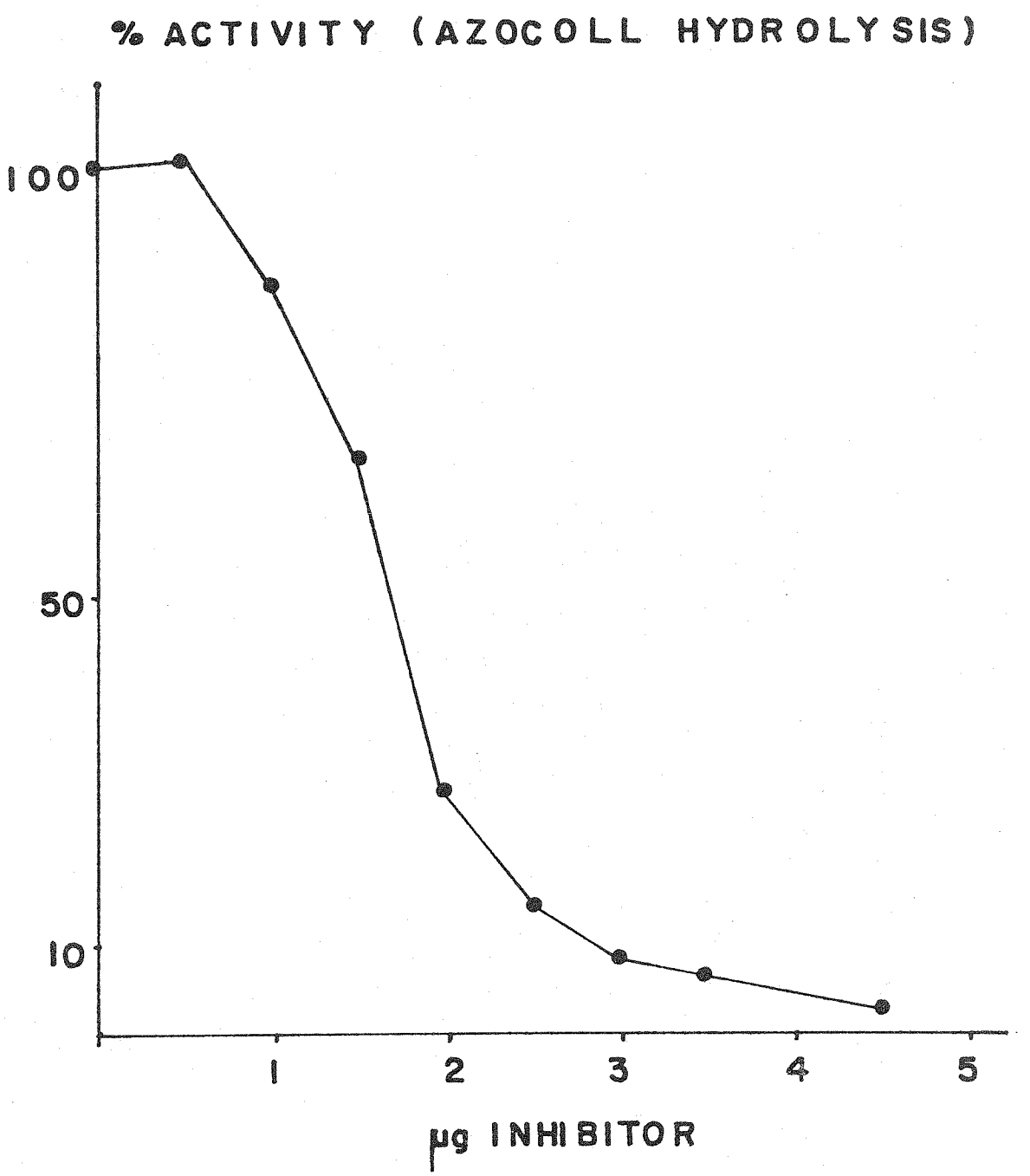

% ACTIVITY (AZOCOLL HYDROLYSIS)
100
50
10
1
2
3
4
5
µg INHIBITOR

Figure C18: The activity of three serine proteases of *Phycomyces* as a function of pH.

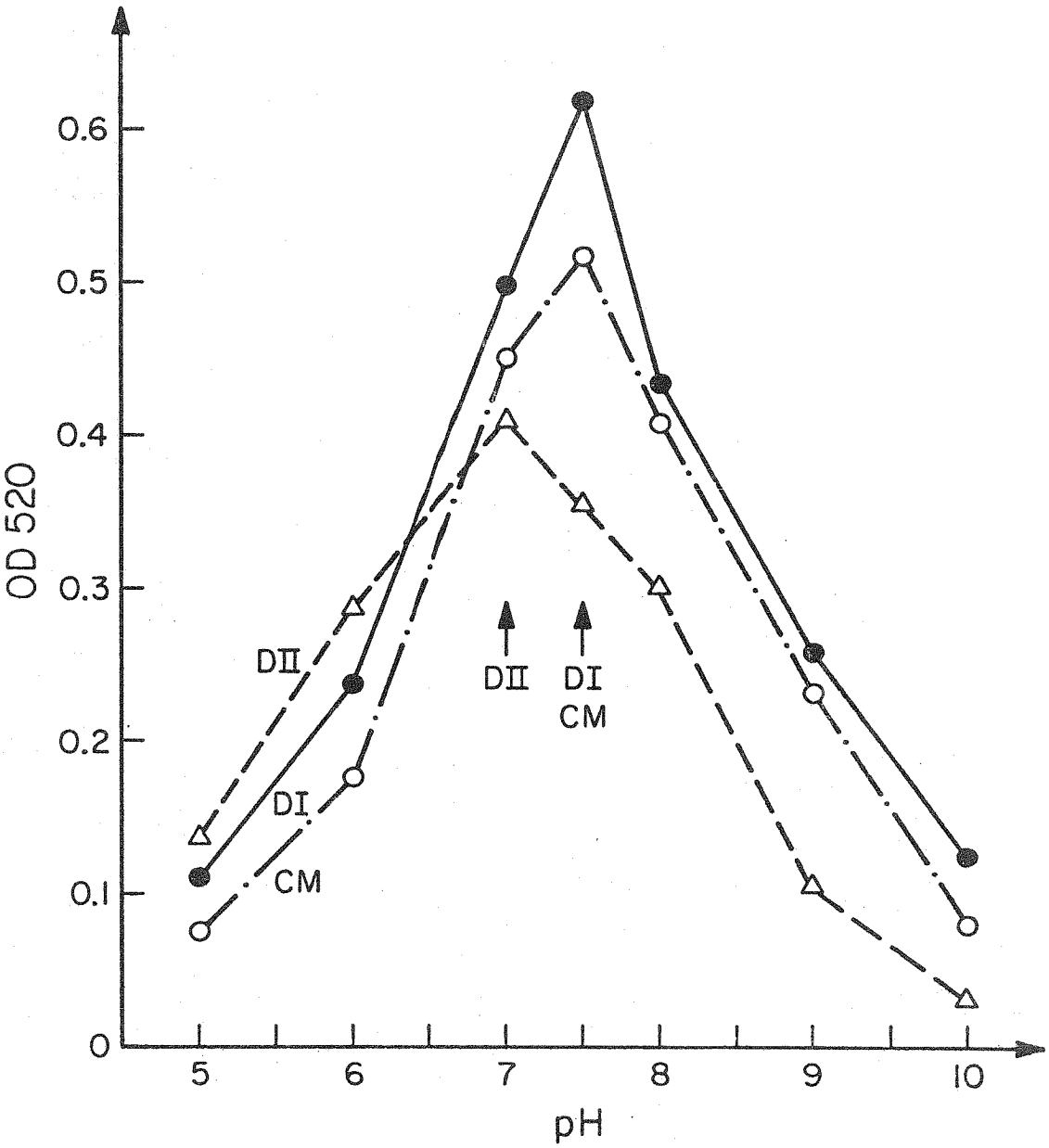

OD 520
0.6
0.5
0.4
0.3
0.2
0.1
0
5
6
7
8
9
10
pH
DII
DI
CM
DII
DI
CM

Figure C 19: Determination of the molecular weight of three serine proteases from Phycomyces on SDS gel electrophoresis. The proteases are designated B-CM, B-DI, B-DII as explained in Fig. C2. The mobility is calculated as described by Weber and Osborn (59).

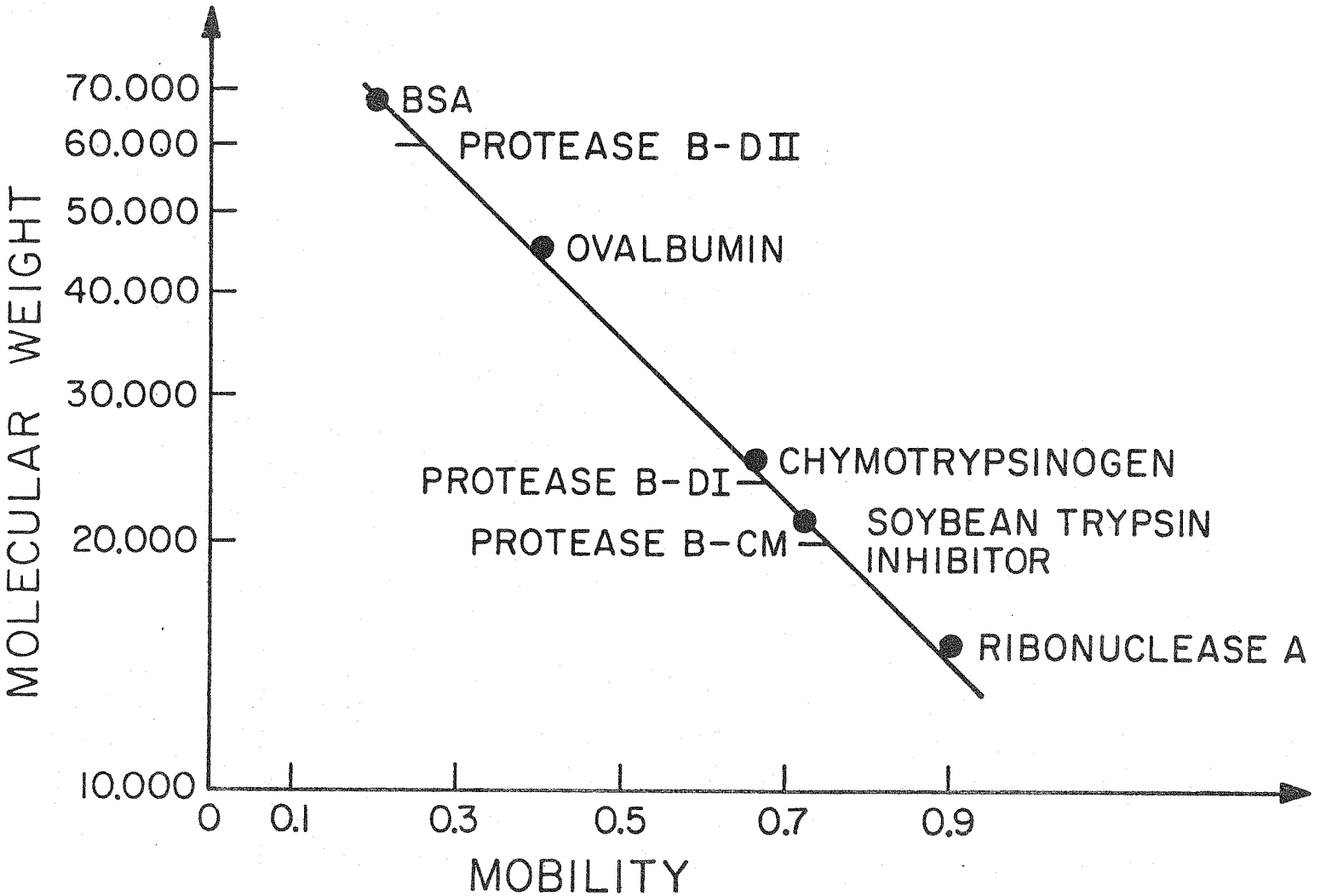

MOLECULAR WEIGHT
70.000
60.000
50.000
40.000
30.000
20.000
10.000
BSA
PROTEASE B-DII
OVALBUMIN
CHYMOTRYPSINOGEN
PROTEASE B-DI
SOYBEAN TRYPSIN INHIBITOR
PROTEASE B-CM
RIBONUCLEASE A
0
0.1
0.3
0.5
0.7
0.9
MOBILITY

Figure C 20: Determination of the molecular weight of serine proteases, their inhibitors, and the protease-inhibitor complex (designated as INH/B...) on G 75 Sephadex. Similar results for protease B-DII and the corresponding complex are achieved on G 100 Sephadex.

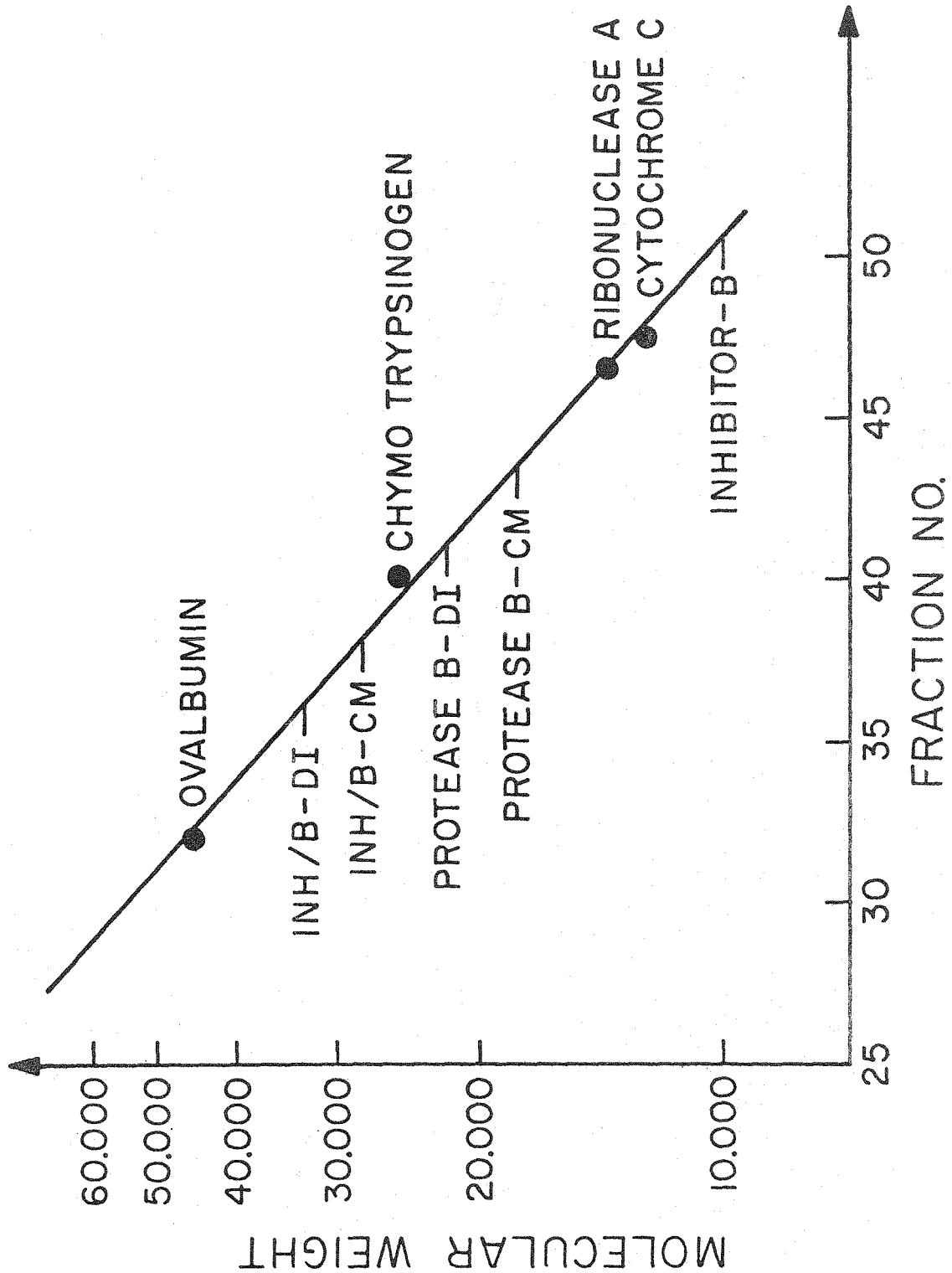
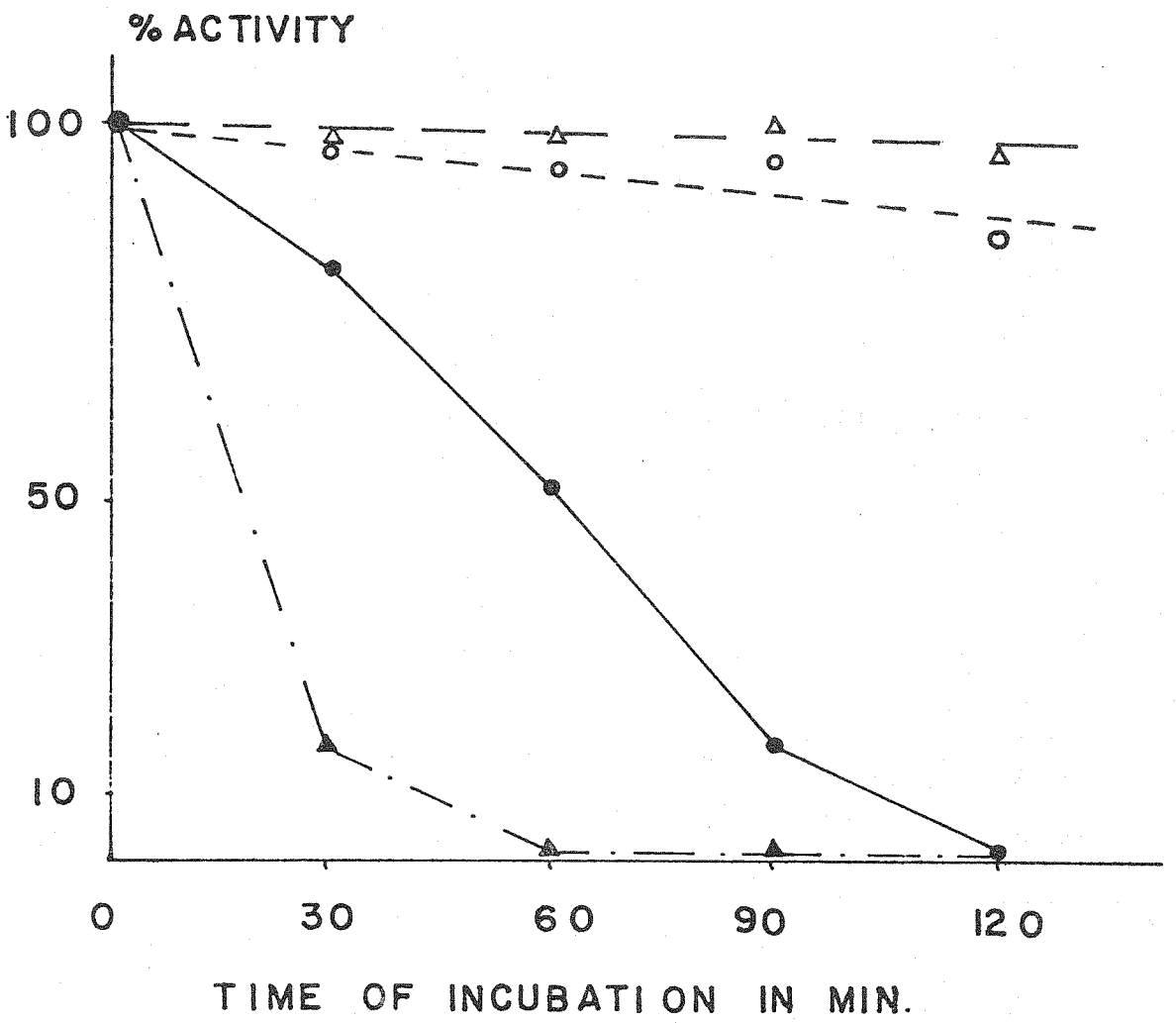

MOLECULAR WEIGHT
60.000
50.000
40.000
30.000
20.000
10.000
25
30
35
40
45
50
FRACTION NO.
OVALBUMIN
INH/B-DI
INH/B-CM
CHYMO TRYPSINOGEN
PROTEASE B-DI
PROTEASE B-CM
RIBONUCLEASE A
CYTOCHROME C
INHIBITOR-B

Figure C21: Loss of azocoll hydrolysis activity of purified protease B-CM (●) by incubation at 30°C compared to a sample to which 10-fold excess of BSA was added (○); protease B-DI (▲) is destroyed even more rapidly; control B-DI in ice (△).


% ACTIVITY
100
50
10
0
30
60
90
120
TIME OF INCUBATION IN MIN.

2.4) An Acid Protease in Phycomyces

To detect any proteolytic activity of type B the inhibitor has to be destroyed. As shown in Fig. C7 there is always a protease A activity present; this activity also increases during the incubation of the homogenate at low pH, but no inhibitor was detected in a reproducible way. The activation might just be the expression of the loss of total protein.

To investigate the question whether protease A might cause the emergence of protease B type activity a partially purified sample of protease A was prepared. Protease A can be separated from any serine protease as shown in Fig. C11; only one peak of activity is detected. The material is then passed through a Sephadex G100 column and concentrated with PEG.

By the gel filtration on a calibrated column an apparent molecular weight of 24.000 daltons is established. The effect of various inhibitors on this sample (purification is about 40-fold) is listed in Table C4; the formula of pepstatin is given by Subramanian et al. (62); it is a general inhibitor of acid proteases.

By incubating a mixture of protease B and its inhibitor (80% of the B activity inhibited) with the partially purified protease A at pH 5 the inhibition

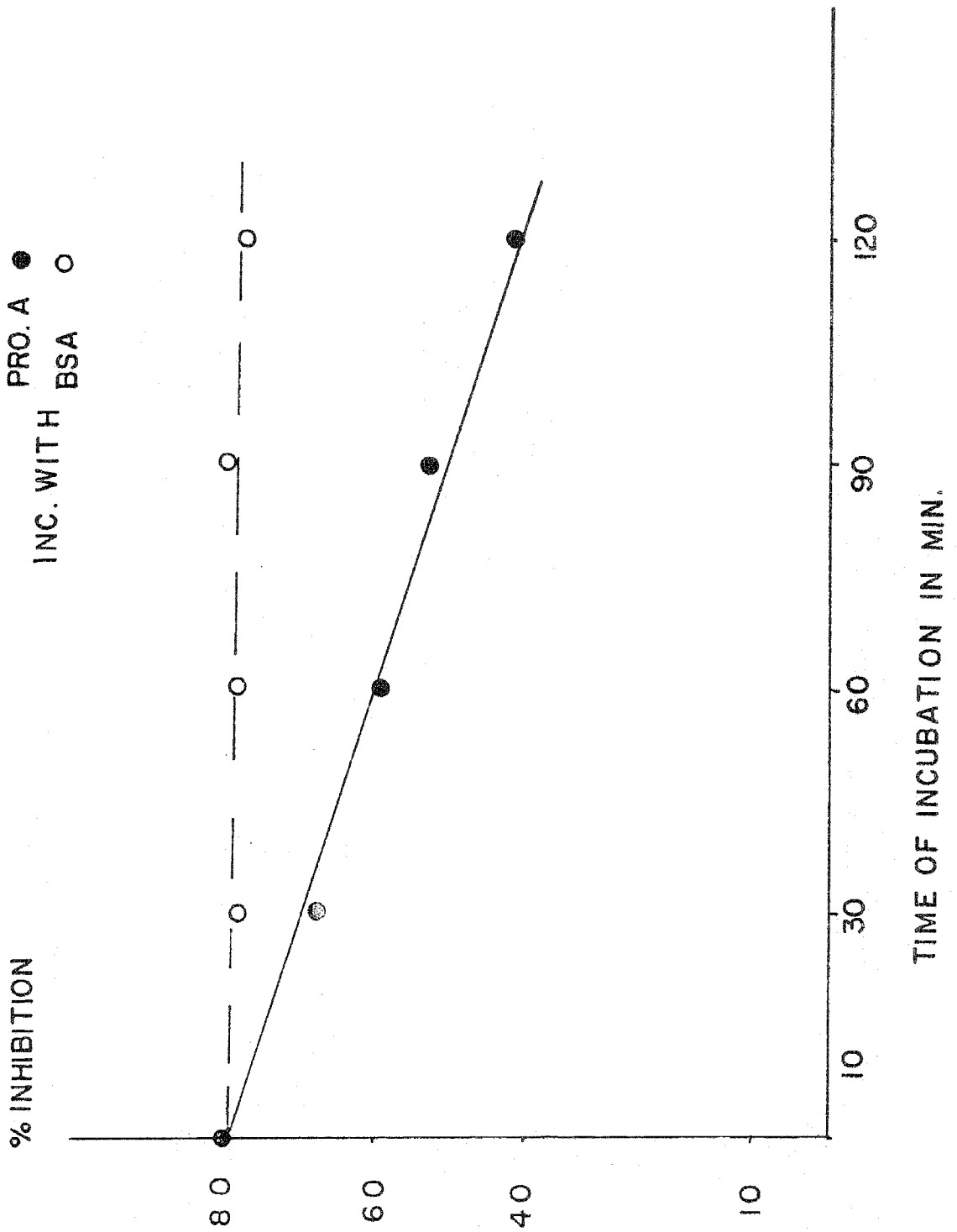
is removed (Fig.C22). The acid protease destroys the inhibitor also if incubated with this protein alone.

These findings suggest that the activation of protease B is due to the acid protease.

A similar conclusion can be drawn from the fact that adding pepstatin A to a homogenate cuts down the emergence of protease B by about 80 %.

Two other observations, though, do not support this suggestion. First, activation of serine proteases is possible by incubation at neutral pH. Second, three mutants (see Fig. C23) have very high protease A activity, but the serine proteases do not exhibit activity earlier than in WT.

Figure C22: The effect of incubating protease B-DI - inhibitor complex with partially purified acid protease from Phycomyces (●); the control is done by adding BSA instead (o). The incubation is done at 30°C and pH 5.


% INHIBITION
INC. WITH PRO. A ●
BSA O
80
60
40
10
10
30
60
90
120
TIME OF INCUBATION IN MIN.

2.5) Analysis of Three Mutant Strains

As was outlined in part B several behavioral mutants of Phycomyces are available which can be arranged in a physiological scheme that has an underlying genetic structure. Only a limited number of complementation groups is detected. In the present context the mutations of interest are the ones near the output end of the light information channel (Fig.B2). These mutants show a fairly good light growth response (3) but they no longer phototrope, they are"stiff".They share the characterictic of a prolonged growth response with normal latency. Their behavior can be explained by assuming a response saturation at a small increase in growth rate. These strains are listed in Table C6.

The proteins used in these experiments were taken through two column steps. The inhibitors were partially purified with DEAE-cellulose and Sephadex G 75; the proteases were run through DEAE-cellulose and Sephadex G 75 resp. CM-Sephadex column material.

Mutant strain	Genotype	Origin	Mutagen
C 2	carA5(-)	NRRL 1555	Nitroso-guanidine
C 6	carA12carR27madF48(-)		
C 68	madD59(-)		
C 110	madE102(-)		

Table C6: Mutant strains used in biochemical analysis

The results can be described as follows:

1) The mutant strains contain both forms of the inhibitor in approximately the same amounts per mg protein as wild type. The inhibitors elute at the same salt concentrations from ion exchange columns and behave indistinguishably on gel filtration columns.

2) The mutant strains contain all three forms of the serine protease and they all have protease A type activity. However, the amounts vary considerably both with respect to the total activity and with respect to the distribution of protease B activity among the three forms. The last statement is demonstrated in Fig. C23. There is much higher acid protease activity in the mutant strains (especially in C68 (mad D) and C110 (mad E)) and less B type activity, though among the mutants the ones with high A activity also show the greater B activity. The incubation illustrated in Fig. C23 was done starting with identical total protein amount in each case.

3) The serine proteases behave similarly to WT in ion exchange chromatography, with the exception of the DII form of C6; this enzyme elutes at a lower salt concentration than the protease from C2 (WT) does (0.11 N as compared to 0.14 N). Thus protease B-DII of C6 (mad F) may be abnormal.

4) The mutant inhibitors inactivate all proteases

of the mutant strains with a possible exception: The C6 inhibitor consistently has little effect against the CM form of the serine protease from the other three strains (Fig. C24). The abnormality again singles out C6 (mad F): either its protease B-CM or its inhibitor is abnormal.

5) The protease B-CM from mutant strain C110 appeared very unstable even in the practically purified form in ice. It lost about 30% of its activity between the calibration experiment and the actual test (about 120 min.).

6) The effect of the DI and DII forms of the protease B on membrane chitin synthetase was tested without finding any significant variation from WT.

The analysis of the mutant enzymes is being continued (April 1977).

The results at present indicate some mutant specific changes (especially for C6), but conclusions about the relevance with respect to photophysiology would be premature, since e.g. C6 is a known triple mutant (Table C6), and the other strains may also harbor adventitious genetic alterations.

Figure C23: Change of protease A and B type activity during incubation at pH 5 (28°C) in the wild type C2 and in three *mad* mutant strains of *Phycomyces*. For details, see text.

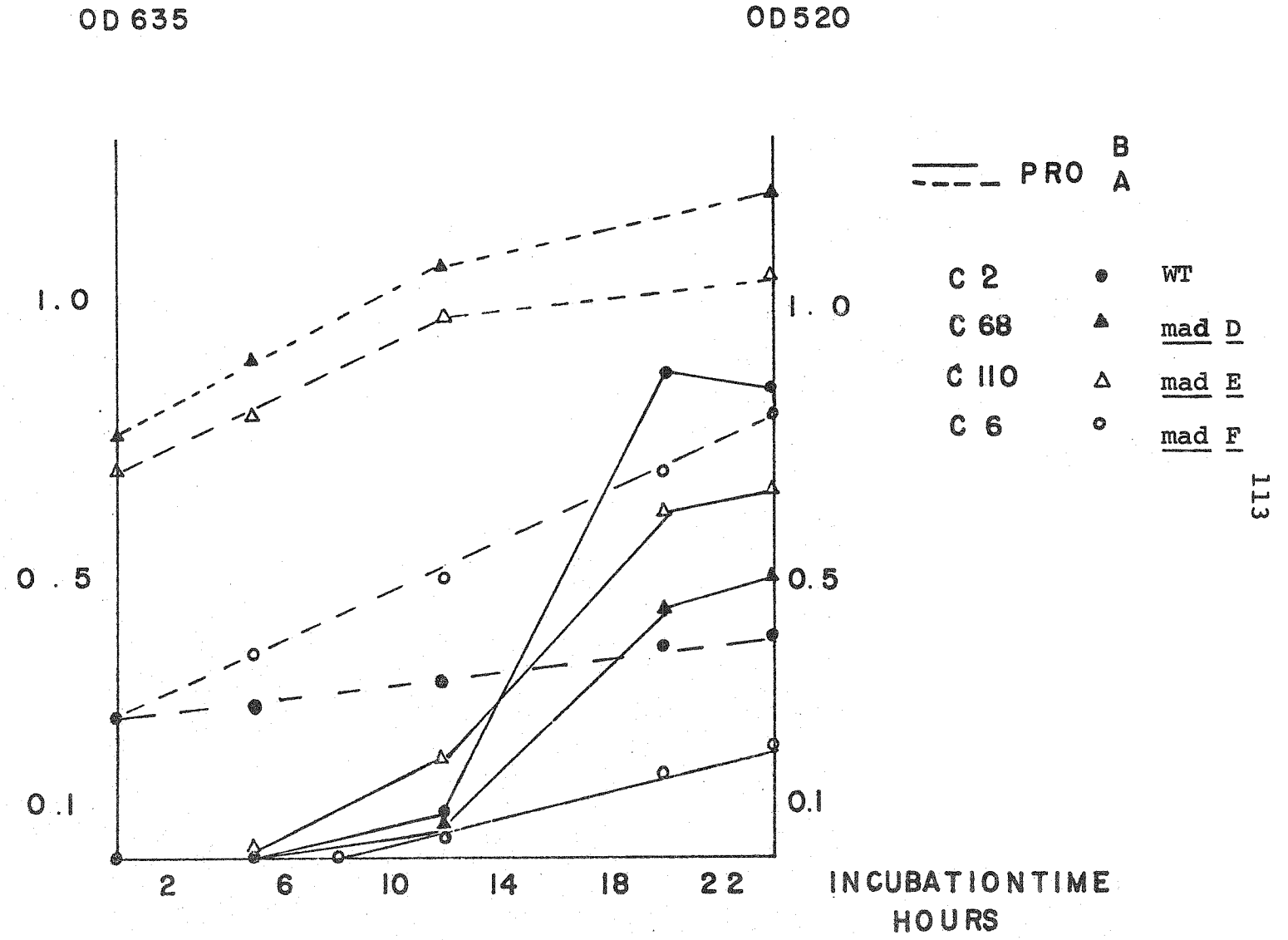
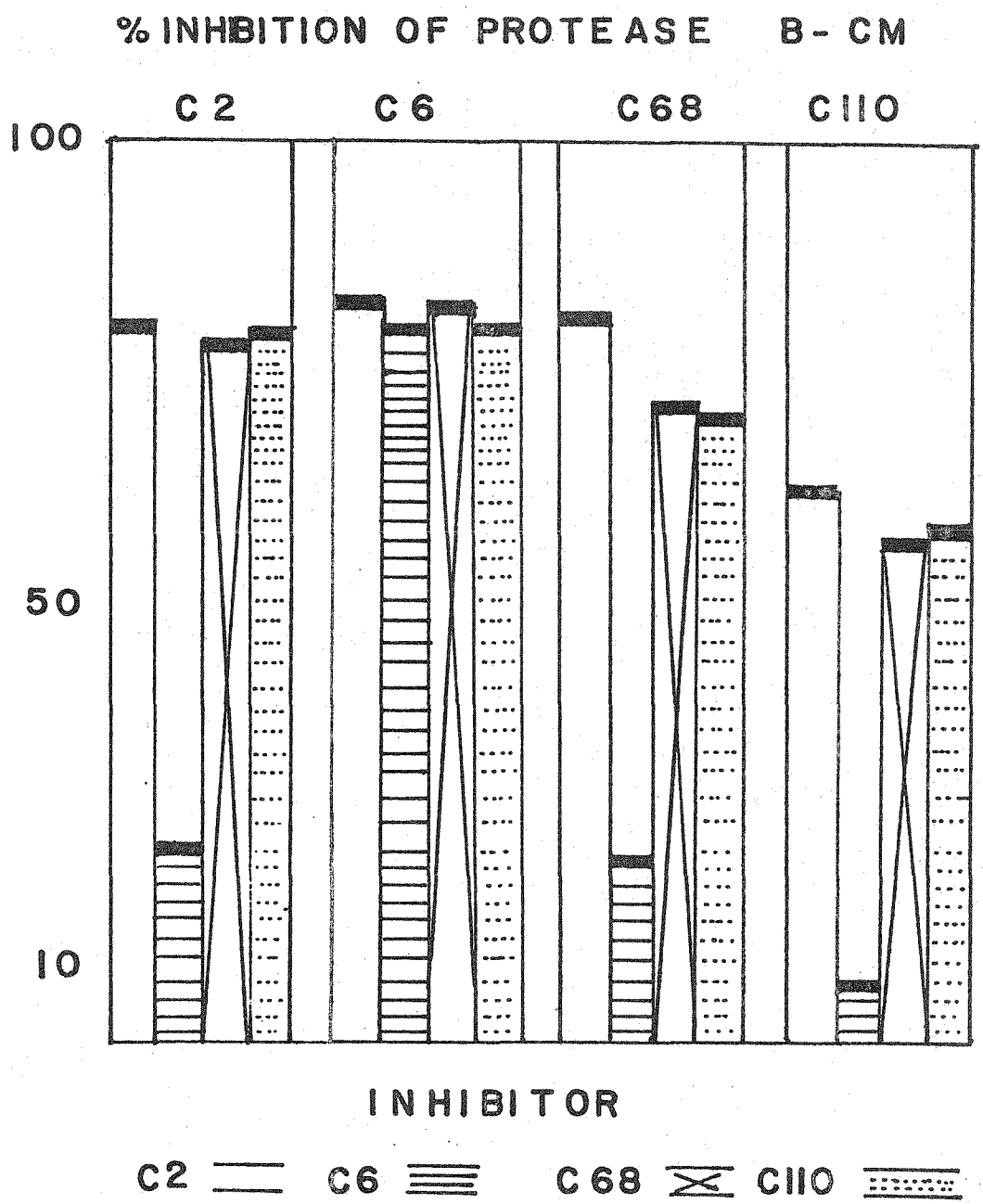

OD 635
OD 520
1.0
0.5
0.1
1.0
0.5
0.1
2
6
10
14
18
22
INCUBATION TIME
HOURS
PRO B
PRO A
C 2 WT
C 68 mad D
C 110 mad E
C 6 mad F

Figure C 24: The effect of inhibitors from various mutants on CM type serine proteases from the same strains; for details, see text.


% INHIBITION OF PROTEASE B-CM
C 2
C 6
C 68
C 110
100
50
10
INHIBITOR
C2
C6
C 68
C110

Chapter D

Chitin Synthetase Activities in *Phycomyces*

CHITIN SYNTHETASE ACTIVITIES IN PHYCOMYCES

1) Methods and Materials

1.1) General Methods

The materials are listed in section C 1.1, the culture conditions are described in C 1.2.

1.2) Chitin Synthetase Assay

The reaction mixture contains a total of 100 µl; it is made up of 5 µl radioactive solution (0.33 mM UDP-NAG plus 0.125 µCi UDP-[1-^{14}C]-NAG with a specific activity 0.28 mCi/mM), 25 µl chitin synthetase buffer (60 mM Hepes, pH 7.2, 40 mM $MgCl_2$, 50 mM NAG) and up to 70 µl of enzyme solution; it is a modified version of the method described by Jan (6).

This mixture is incubated for 90 min. at 28 ^{0}C with overhead diffuse white light of intensity 10 µwatts/cm. The reaction is stopped with 1 ml cold 5 % TCA; the samples are then filtered through Whatman GF/C filter and washed with 5 % TCA and ethanol. The filter paper is dried and mixed with 5 ml scintillation fluid, consisting of 0.4 % PPO and 0.02 % POPOP in toluene. The samples are counted in a liquid scintillation counter.

1.3) Chitin Synthetase Preparation

Germlings grown for 24 hours were harvested from

the liquid culture by filtration through Miracloth (Chicapee Mills, Inc.); this allows spores to pass through. The cells are washed extensively with water and resuspended in homogenization buffer I: 250 mM sucrose, 25 mM imidazole-acetate, pH 6.8, 10 mM $MgCl_2$, 0.05% (V/V) β-mercaptoethanol.

Cells are homogenized by shaking 25 g cell material (wet weight) in 100 ml buffer I together with 200 g glass beads (Ø 5 mm) in 500 ml flasks on a New Brunswick Scientific shaker at 300 rpm in icewater for 60 min. This disrupts about 95% of the germlings according to inspection with a light microscope.

The homogenate is centrifuged at 1000 x g for 10 min. (or alternatively again filtered through Miracloth); both the supernatant and the pellet show chitin synthetase activity. The two fractions are processed differently.

a) The pH of the supernatant (typically now at about pH 6) is readjusted to pH 7 with 1 N NaOH; the material is centrifuged at 50.000 x g for 45 min. in the Beckman L5-50 ultracentrifuge; again chitin synthetase activity is found in both the supernatant and the pellet (see Fig. D1); the pellet is resuspended in buffer I (without sucrose) and centrifuged (along with the active supernatant) at 100.000

x g for 60 min.. This time all activity is pelleted; the material is resuspended in buffer I (without sucrose and $MgCl_2$) and designated granular chitin synthetase.

b) The first pellet (see Fig. D1) contains cell wall material; according to Nurminen et al.(15) the plasma membrane is still attached to it. This fraction is resuspended in buffer I and homogenized with a Teflon Pestle Tissue grinder in ice; five strokes are sufficient; the material is filtered through Miracloth and washed with buffer I (without sucrose and $MgCl_2$) until the filtrate appears clear. The sample ("cell wall fraction") is resuspended in the washing buffer (using 10 ml per 100 ml starting material) and sonicated 3 x 20 sec. with the Branson J-22 sonifier at 45 watts; it is then centrifuged at 1,000 x g x 10 min. (Sorvall RC/2B,SS-34 Rotor); the pellet is discarded, the supernatant is a crude plasma membrane fraction; this material can be concentrated by centrifugation (100,000 x g x 60 min.) if necessary for preparative work or electron microscopy.

c) Particulate chitin synthetase and the crude plasma membrane fraction are further processed by density gradient centrifugation with metrizamide (22). A 1 ml sample is layered on 11 ml gradient either 10 - 40% or 10 - 58%

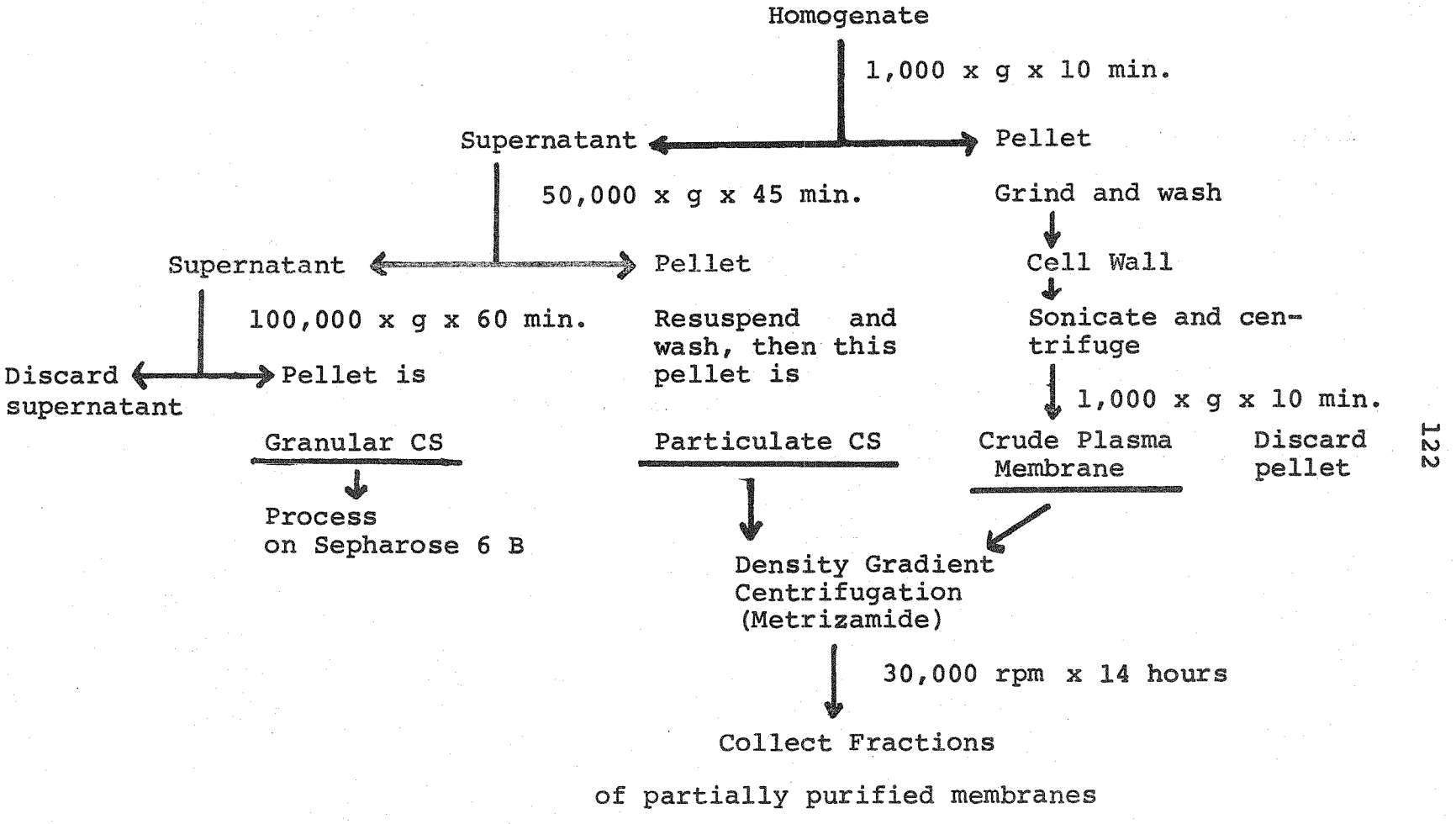
metrizamide and centrifuged at 30,000 rpm for 14 hours in a SW 41 rotor using cellulose nitrate tubes. Fractions of 500 µl are collected.

The density of the material is calculated according to (22) from the equation

$$\rho_{4^{\circ}C} = 3.453 \; \eta_{20^{\circ}C} - 3.601$$

where $\eta_{20^{\circ}C}$ is the refractive index at 20°C.

Figure D 1: Preparation of chitin synthetase fractions; for a detailed description of the procedure, see under methods. For a description of differences among these activities, see results.
CS stands for chitin synthetase.
The crude plasma membrane fraction can be concentrated by ultracentrifugation (if necessary for preparative work) as described in the text.


Homogenate
1,000 x g x 10 min.
Supernatant
Pellet
50,000 x g x 45 min.
Grind and wash
Supernatant
Pellet
Cell Wall
100,000 x g x 60 min.
Resuspend and wash, then this pellet is
Sonicate and centrifuge
Discard supernatant
Pellet is
1,000 x g x 10 min.
Granular CS
Particulate CS
Crude Plasma Membrane
Discard pellet
Process on Sepharose 6 B
Density Gradient Centrifugation (Metrizamide)
30,000 rpm x 14 hours
Collect Fractions
of partially purified membranes

1.4) Electron Microscopy

Electron microscopical inspection of various chitin synthetase preparations were performed by Dr. William Van der Woude, UC Riverside. The methods are described by Van der Woude (20); as a special feature the Roland Stain technique (21) was used. This stain is specific for plasma membranes of plants.

2) Results

2.1) Different Forms of Chitin Synthetase.

According to the scheme outlined in Fig. D1 total chitin synthetase activity can be fractionated into three different forms. They are called granular, particulate, and plasma membrane chitin synthetase. The names are explained as follows:

The supernatant of the medium speed centrifugation (Fig. D1) was shown in Mucor rouxii (20) to consist of granules with a diameter of around 400 A; the number of the granules can be increased by incubating the homogenate with substrate (UDP-NAG) and activator (NAG) of chitin synthetase ("solubilization"). This material can be passed through Sepharose 6B. The last two observations are also true in case of Phycomyces; this fraction contains about 10% of the activity of the crude homogenate. We have not tried to purify this fraction any further. One reason for this omission is the belief that the plasma membrane bound chitin synthetase is the physiologically relevant one. Evidence for this assumption is given by Jan (6). Also a regulation of chitin synthesis through transport of granules would be more complicated than through activating a membrane bound enzyme.

The name particulate chitin synthetase is assigned to the activity in the medium speed pellet; as will be shown later this activity is different from the one produced by sonication of a cell wall preparation. The latter fraction yields the plasma membrane chitin synthetase , for by adding Mg^{++} to the homogenization buffer the plasma membrane remains attached to the wall, as first pointed out by Nurminen et al. (15). The cell wall material was monitored by electron microscopy and the result is shown in Fig. D 2. It presents evidence for a plasma membrane rich fraction through the use of the Roland stain. A typical result for a chitin synthetase preparation is given in table D 1 (the specific activity is given as nmoles UDP-NAG incorporated/ min. x mg protein).

Fraction	Total Activity %	Specific Activity
Homogenate	loo	3.1
Granular CS	7	1.9
Particulate CS	58	11.2
Crude Plasma Membrane	11	8.4

Table D 1: Chitin synthetase preparation

This distribution differs from the one given by Jan (6) for the chitin synthetase of spphs. The specific activities are considerably larger.

Each measurement of chitin synthetase activity is accompanied by a control which includes the substrate analog Polyoxin D in the incubation mixture. Polyoxin D is a powerful competitive inhibitor of chitin synthetase; its action in Phycomyces is described by Jan (6,7).

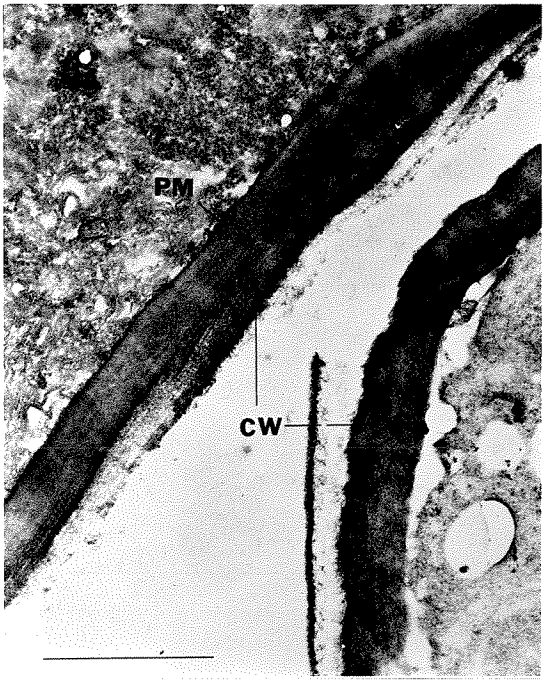
Figure D 2 a - d: Electron micrographs of cell wall material prepared from Phycomyces; the bar in each picture represents 1 µm. The Roland staining technique was applied. This technique stains the plasma membrane black, while other membranous vesicles appear gray. The pictures were taken by Dr. William Van der Woude.

a. The cell wall with plasma membrane visible as a dark line (Roland stain).

b. The cell wall fraction.

c. The effect of sonication; (plasma) membrane vesicles form along the cell wall, indicating that they were shaken loose from the wall.

d. Crude plasma membrane fraction after centrifugation of sonicated wall fraction; note that most of the visible vesicles stain black, which indicates they are plasma membrane material (21).

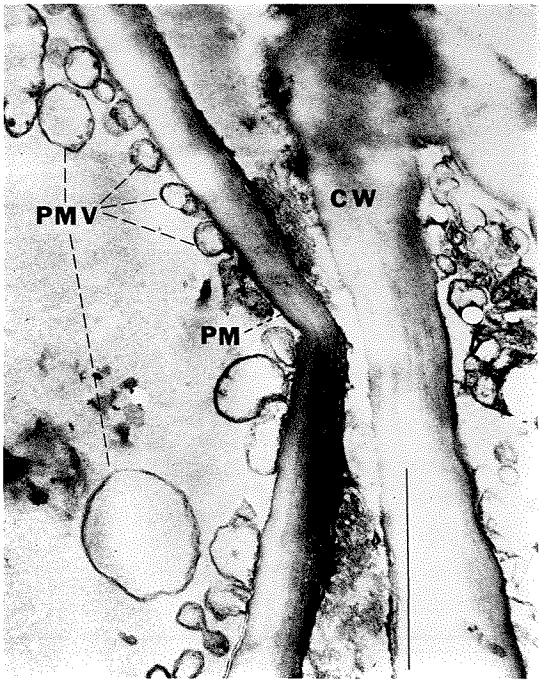


a

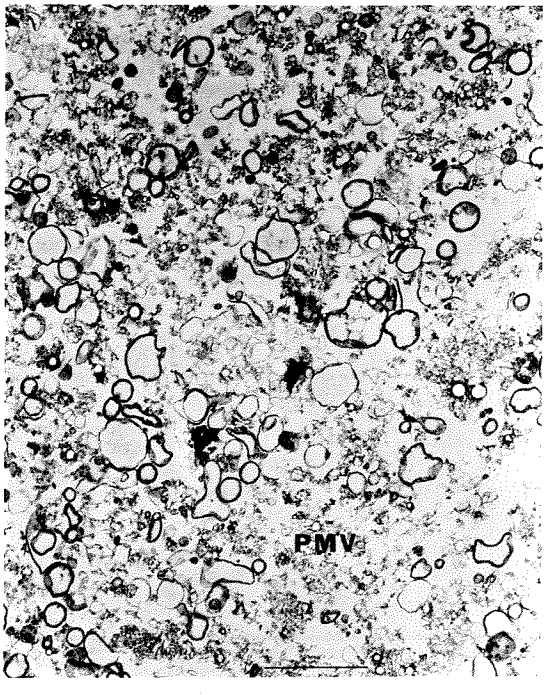
b

Fig. D2

c



d



2.2) Density Gradient Centrifugation

Both the particulate and the membrane chitin synthetase are further processed on metrizamide density gradients as described by Rickwood et al.(22); metrizamide is a tri-iodinated benzamido-derivative of glucose; there are no ionizable groups.

Both forms of the chitin synthetase are separated on such a gradient. The membrane chitin synthetase bands at a density of ρ = 1.19 ($\pm$ 0.01) g/cm^3; the particulate enzyme bands at ρ = 1.165 ($\pm$ 0.01) g/cm^3.

The results are illustrated in Fig. D 3. The nonsoluble protease B activity is associated with another membrane type as indicated in Fig. D 4; particulate protease B bands at ρ = 1.14 ($\pm$ 0.01) g/cm^3. These results are in agreement with the literature. Membranes are known to band between 1.14 and 1.23 g/cm^3 in metrizamide gradients (22). Both the A and B proteases are connected with the same membranous material, as shown in Fig. D 5.

Solubilized protease B does not enter the gradient. This holds only for the particular conditions of centrifugation (14 h runs). In runs of 60 hours soluble proteins band at their corresponding density .

We thus find the enzymes of interest in different subcellular fractions. Any model that suggests an

interaction between these proteins has to take this compartmentalization into account.

Figure D 3: Separation of two forms of chitin synthetase from Phycomyces; the gradient extended from 10 - 58 % metrizamide; the material that presumably is plasma membrane bound (—o—) bands at higher density than the particulate activity (--●--). 70 µl of each fraction were assayed as described above. Metrizamide interferes slightly with the activity.
The banding positions of the activities under investigation are indicated by arrows.
Metrizamide may be removed from the samples by diluting the fractions tenfold, centrifuging them at 100.000 x g x 60 min., and resuspending each pellet.

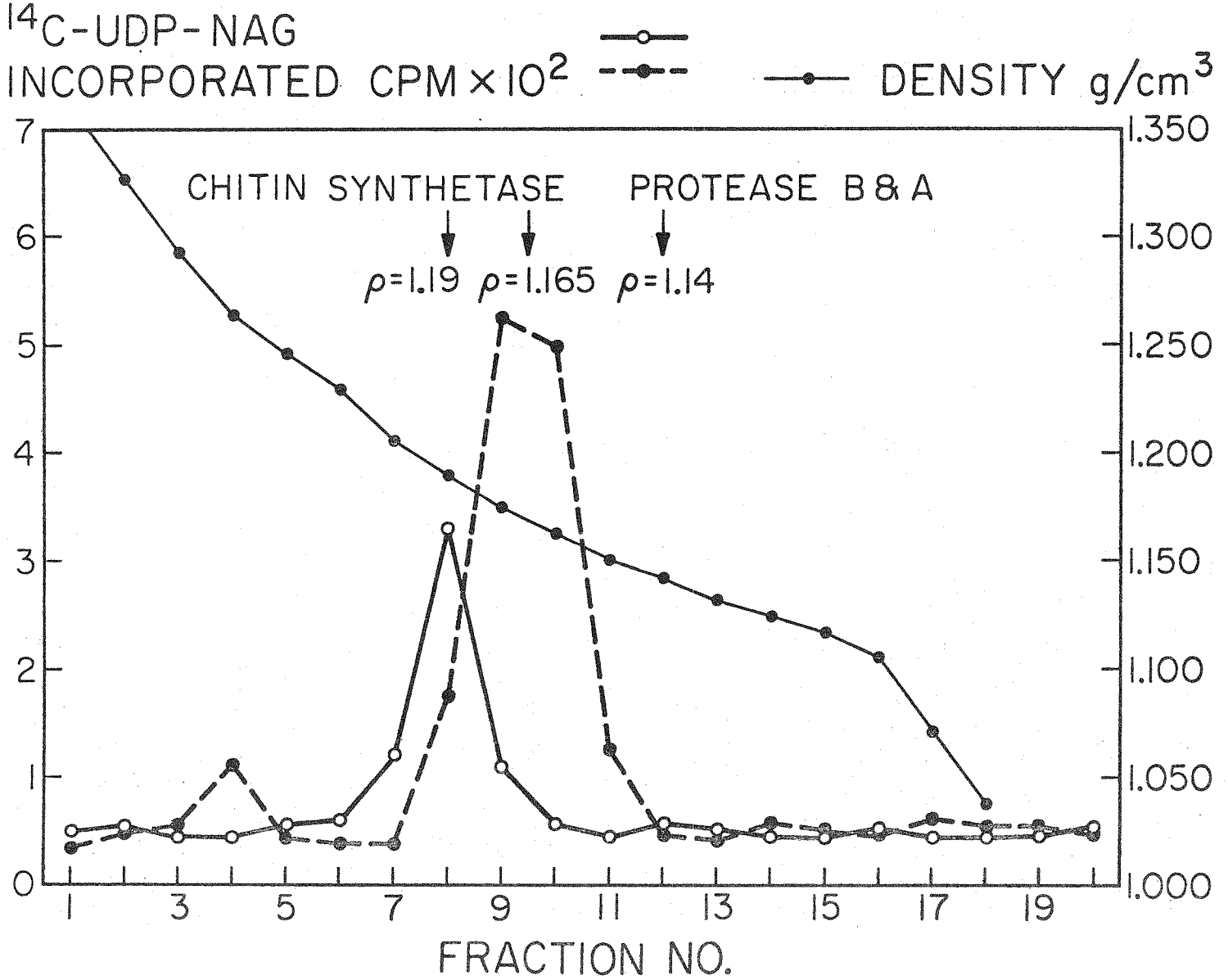

^{14}C-UDP-NAG
INCORPORATED CPM × 10^2
DENSITY g/cm^3
CHITIN SYNTHETASE
PROTEASE B & A
ρ=1.19
ρ=1.165
ρ=1.14
0
1
2
3
4
5
6
7
1.000
1.050
1.100
1.150
1.200
1.250
1.300
1.350
1
3
5
7
9
11
13
15
17
19
FRACTION NO.

Figure D 4: Separation of presumably plasma membrane bound chitin synthetase and nonsolubilized protease B (very likely a vacuolar fraction according to Lenney et al.(48)) on a 10-40 % metrizamide gradient. 70 µl of each fraction is assayed as described above; metrizamide interferes slightly with the chitin synthetase assay.

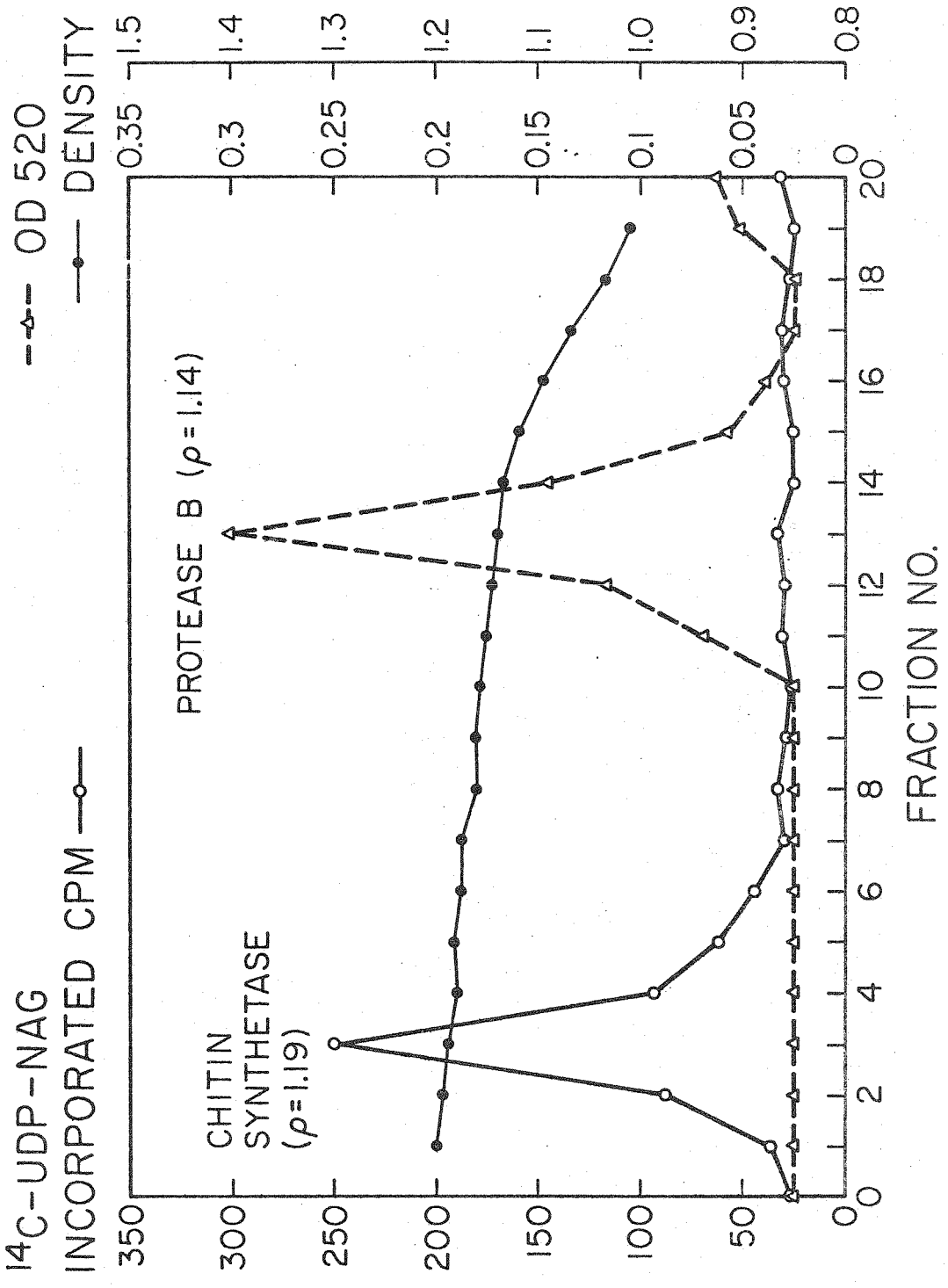
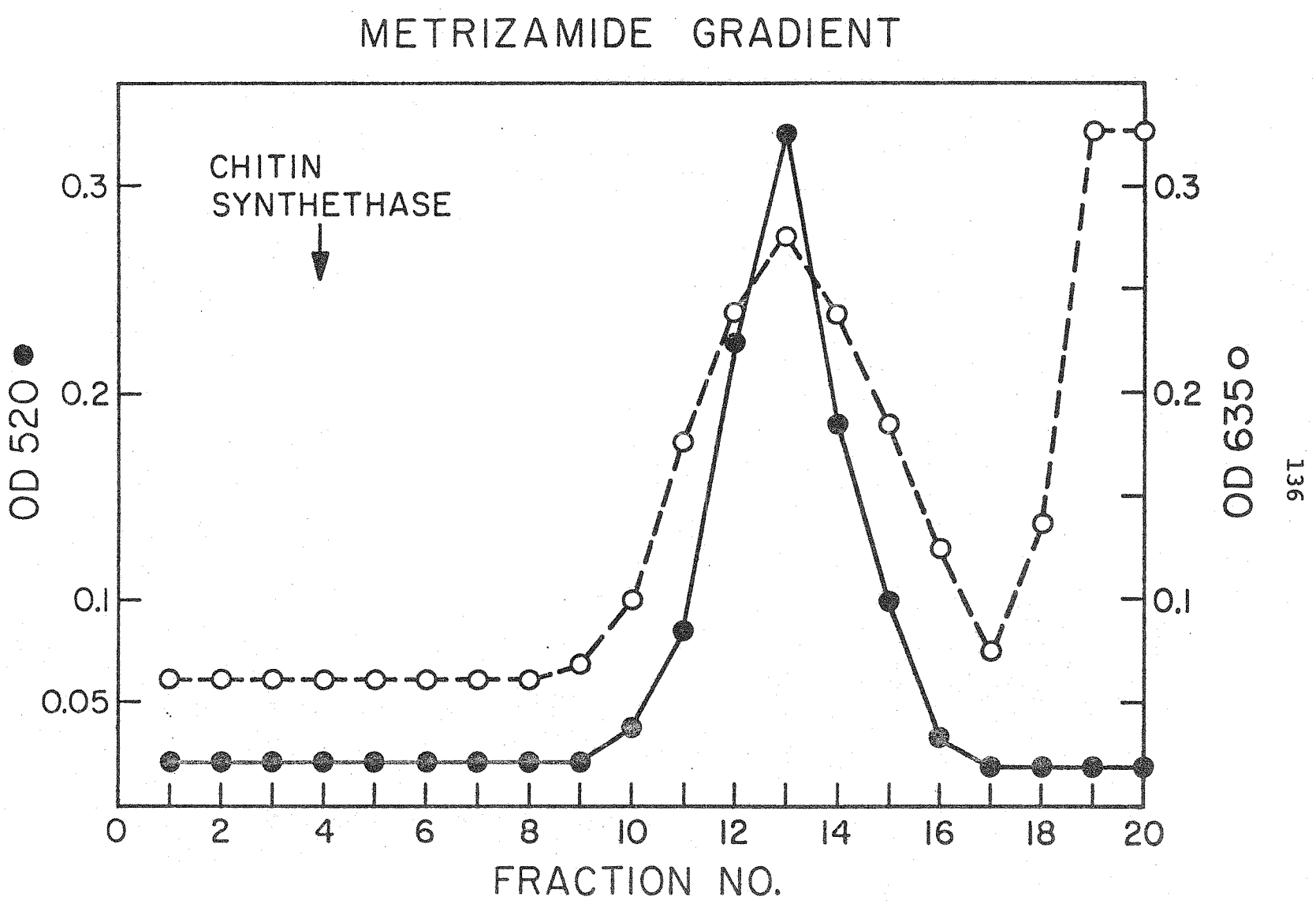

METRIZAMIDE GRADIENT
^{14}C-UDP-NAG INCORPORATED CPM
OD 520
DENSITY
CHITIN SYNTHETASE (ρ=1.19)
PROTEASE B (ρ=1.14)
FRACTION NO.

Figure D 5: Particulate proteases A and B of Phycomyces band at the same density on a 10 - 40 % metrizamide gradient, indicating they are connected with the same membrane fraction. 100 µl of each fraction are assayed as described in section C 1.3.


METRIZAMIDE GRADIENT
CHITIN
SYNTHETHASE
OD 520 ●
OD 635 ○
0.3
0.2
0.1
0.05
0
2
4
6
8
10
12
14
16
18
20
FRACTION NO.

2.3) The Effect of Serine Protease on Chitin Synthetase Activity.

In neither of the separated forms of chitin synthetase activity was protease B type activity detected; nevertheless adding purified inhibitor slightly reduces chitin synthesis. In the case of the particulate enzyme the decrease is about 10 %; in the case of the plasma membrane material the effect is less than 5 %. This observation presents difficulties for the hypothetical regulatory scheme (Fig. A 1).

The effect of the serine protease is studied in the following way: Protease B solutions are made, which are equally effective in azocoll hydrolysis, and added to chitin synthetase preparations standardized with respect to protein content. The mixture is incubated at 30 ^{o}C. In the control buffer III is added to the chitin synthetase fractions. At various times aliquots are removed and mixed with enough inhibitor to stop all protease B type activity (determined by azocoll hydrolysis in the presence of the chitin synthetase samples) and kept in ice for the remaining incubation time. The zero time point is done by mixing the protease with the inhibitor first.

The results of these experiments show clear

differences between the chitin synthetase preparations: Figs. D6, D7, D8.

The "granular" fraction (Fig.D6) shows a strong activation after a delay of thirty minutes and a possible slight initial drop in response to protease B-DI.

The plasma membrane bound chitin synthetase (Fig.D7) is destroyed faster than in the control or at best stabilized (by protease B-DII).

The activity in the particulate fraction (Fig.D8) is clearly stimulated by incubation with serine proteases. In this fraction the synthesis rate of chitin increases slightly by itself.

The effect of incubation with serine proteases on the protein population was monitored by SDS gel electrophoresis in case of the B-DI form of the enzyme acting on the crude plasma membrane fraction. No specific changes (e.g. the dissapearance of one band) could be detected. On the contrary, the protease proved to be rather wild in changing the gel banding pattern at least at 15 positions.

We note the particulate chitin synthetase can be activated by protease B. This finding can be interpreted within the framework of the model. Newly synthesized chitin synthetase (the zymogen) would not be in

the plasma membrane but stored in membrane vesicles which are on their way to be incorporated into the plasma membrane. This may be the nature of the particulate fraction. This material can be activated.

The chitin synthetase that is already in the plasma membrane is expected to be fully active; then exposure to proteolytic action should decrease the overall activity.

This simple explanation unfortunately cannot be maintained for explaining the experiments illustrated in Figs.D9 and D10. The first graph (Fig.D9) demonstrates the capability of one of the three serine proteases (B-DI) to bind to membranous material (among others to the plasma membrane). In this situation (Fig.D10) the chitin synthetase assay includes the corresponding amount of bound protease B and the result is an increase in chitin synthesis. Adding the protease after the gradient is run gives a similar though somewhat reduced activation.

We thus see a crude plasma membrane preparation contains components which can direct the serine proteases. These components are probably removed in the purified plasma membrane fraction and the activity of chitin synthetase is enhanced.

The regulatory role of the serine proteases is

is emphasized by the observation that any incubation of a chitin synthetase preparation with a crude protease fraction results in a rapid decrease of chitin synthesis.

In summary the regulation of chitin synthesis in Phycomyces presents itself as a problem which involves more than the interaction of three proteins and thus can be expected to need at least better characterization of various subcellular fractions and their proteins before a definite picture may emerge.

Figure D6: Effect of incubation of granular chitin synthetase fraction with serine protease DI from Phycomyces. The control shows a decay in activity (--O--). Note the strong activation after a slight initial loss of activity by incubation with protease (—●—).

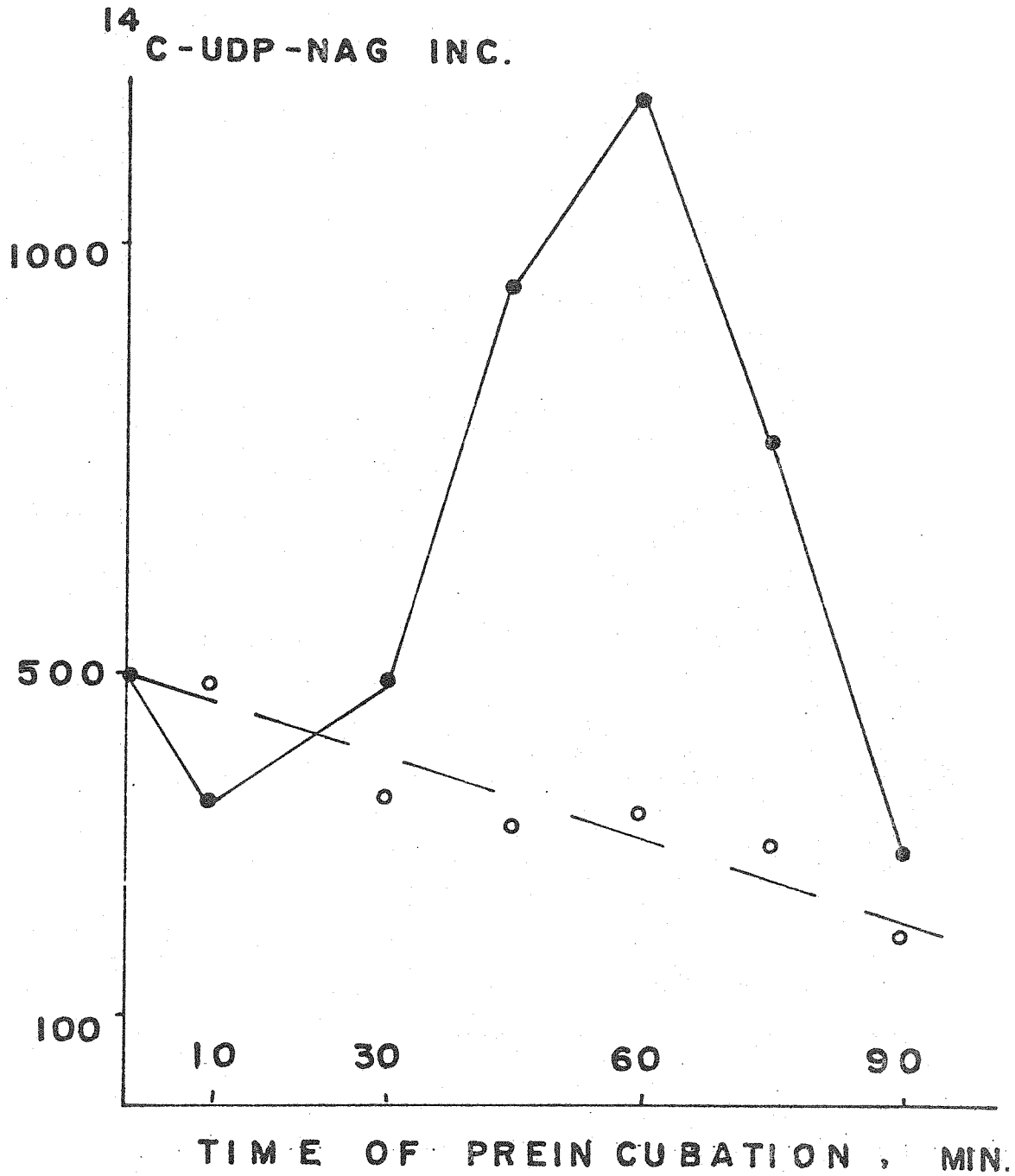

14 C-UDP-NAG INC.
1000
500
100
10
30
60
90
TIME OF PREINCUBATION, MIN.

Figure D7: Effect of incubation of a crude plasma membrane fraction with three serine proteases from Phycomyces on chitin synthetase activity. The control (▲) shows a decay of activity. This decay is accelerated by two of the enzymes (B-CM ○ and B-DI ●). It is reduced by the high molecular weight protease (B-DII △).

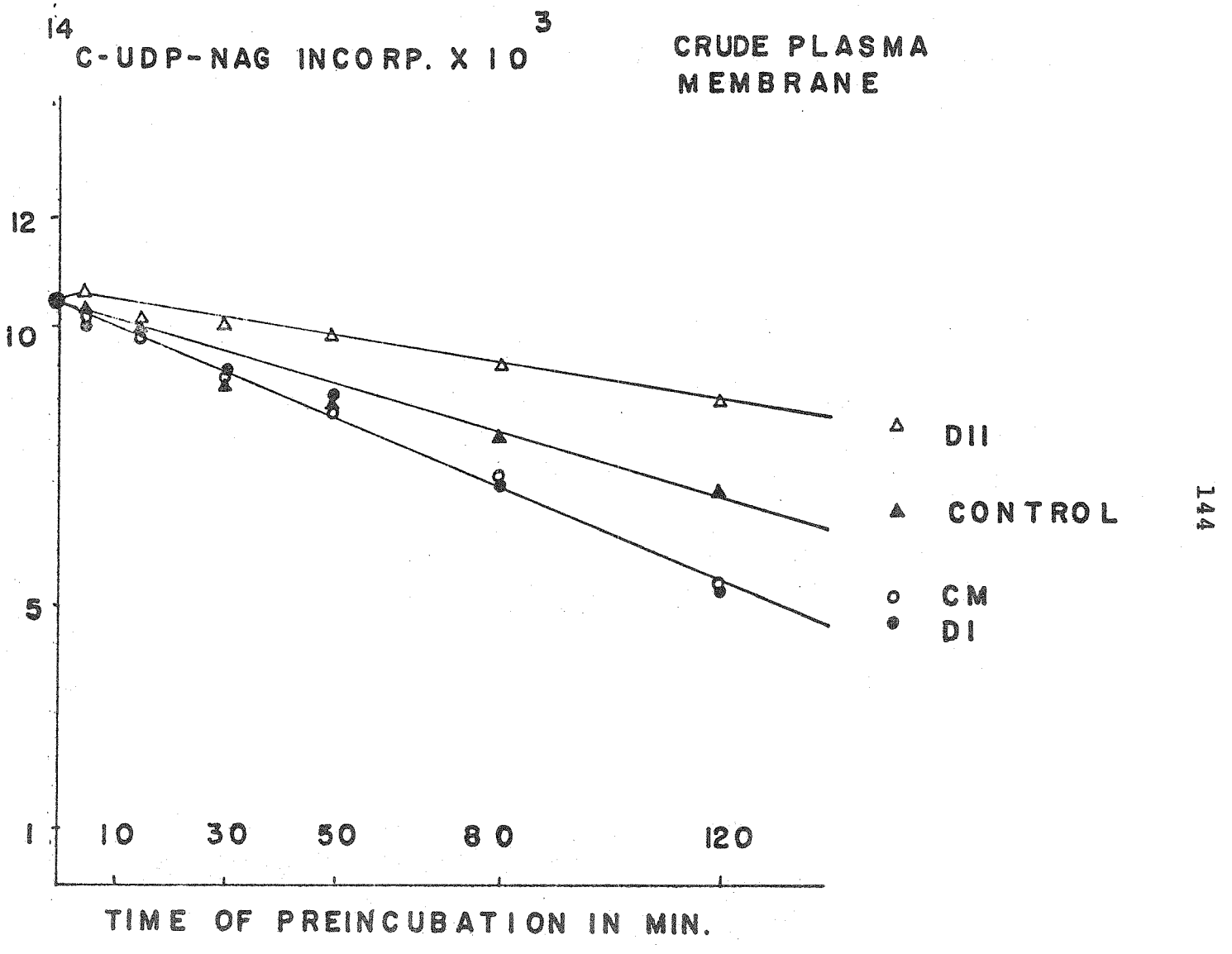
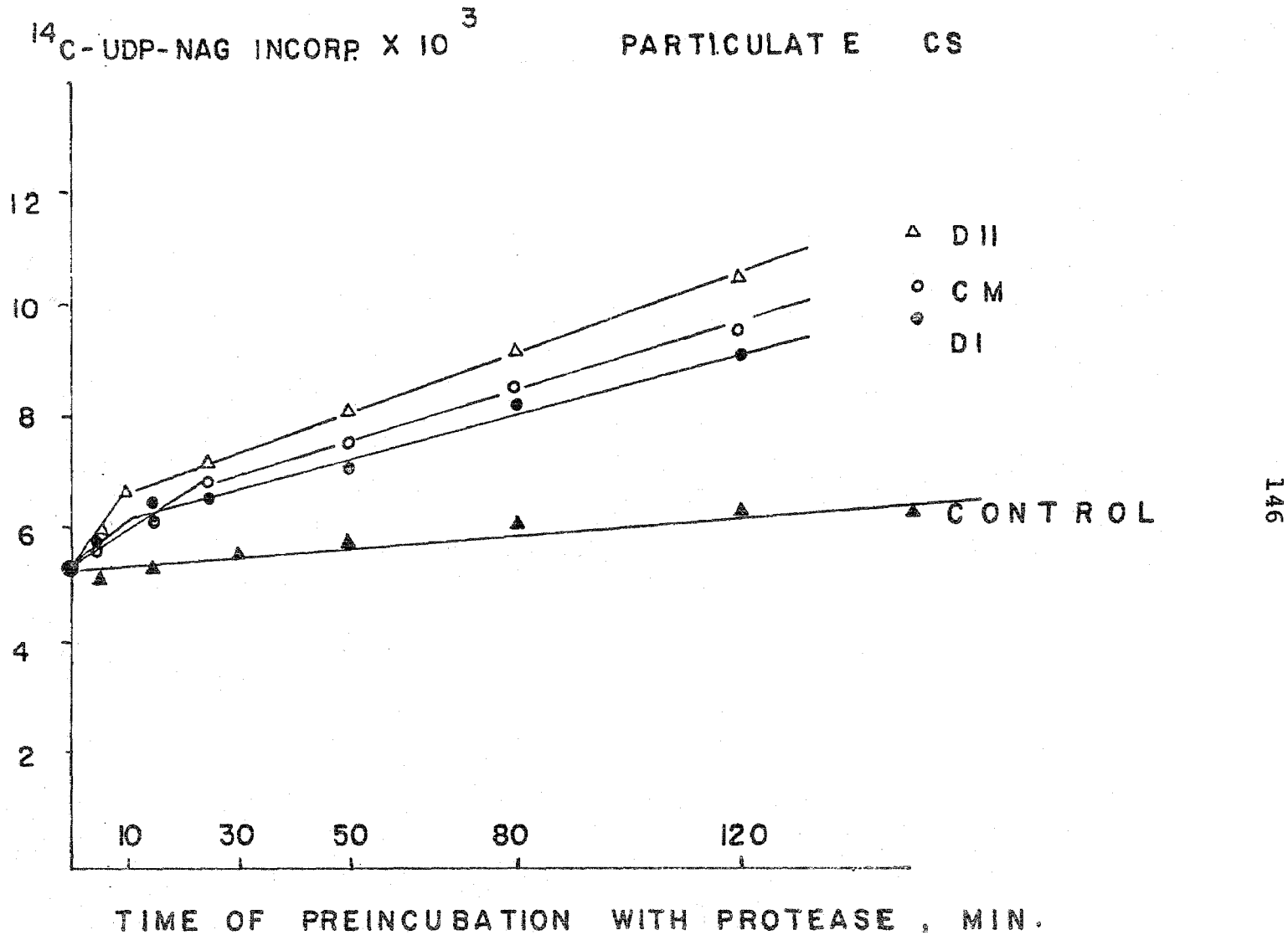

^{14}C-UDP-NAG INCORP. X 10^{3}
CRUDE PLASMA MEMBRANE
12
10
5
1
10
30
50
80
120
TIME OF PREINCUBATION IN MIN.
DII
CONTROL
CM
DI

Figure D 8: Effect of incubation of particulate chitin synthetase with three serine proteases from Phycomyces. All proteases (B-CM ○ , B-DI ● , B-DII △) increase the activity with the high molecular weight enzyme leading the way. Note that the fraction (▲ control) shows slight self-activating properties.


^{14}C-UDP-NAG INCORP. X 10^{3}
PARTICULATE CS
12
10
8
6
4
2
D II
C M
D I
CONTROL
10
30
50
80
120
TIME OF PREINCUBATION WITH PROTEASE, MIN.

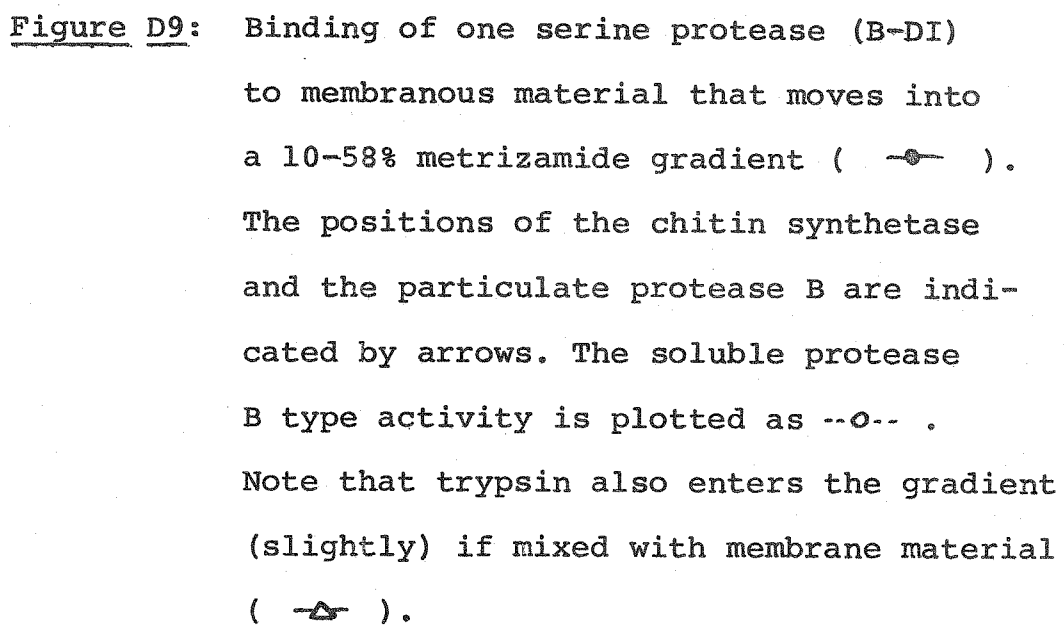


Figure D9: Binding of one serine protease (B-DI) to membranous material that moves into a 10-58% metrizamide gradient (—●—). The positions of the chitin synthetase and the particulate protease B are indicated by arrows. The soluble protease B type activity is plotted as --o-- . Note that trypsin also enters the gradient (slightly) if mixed with membrane material (—△—).

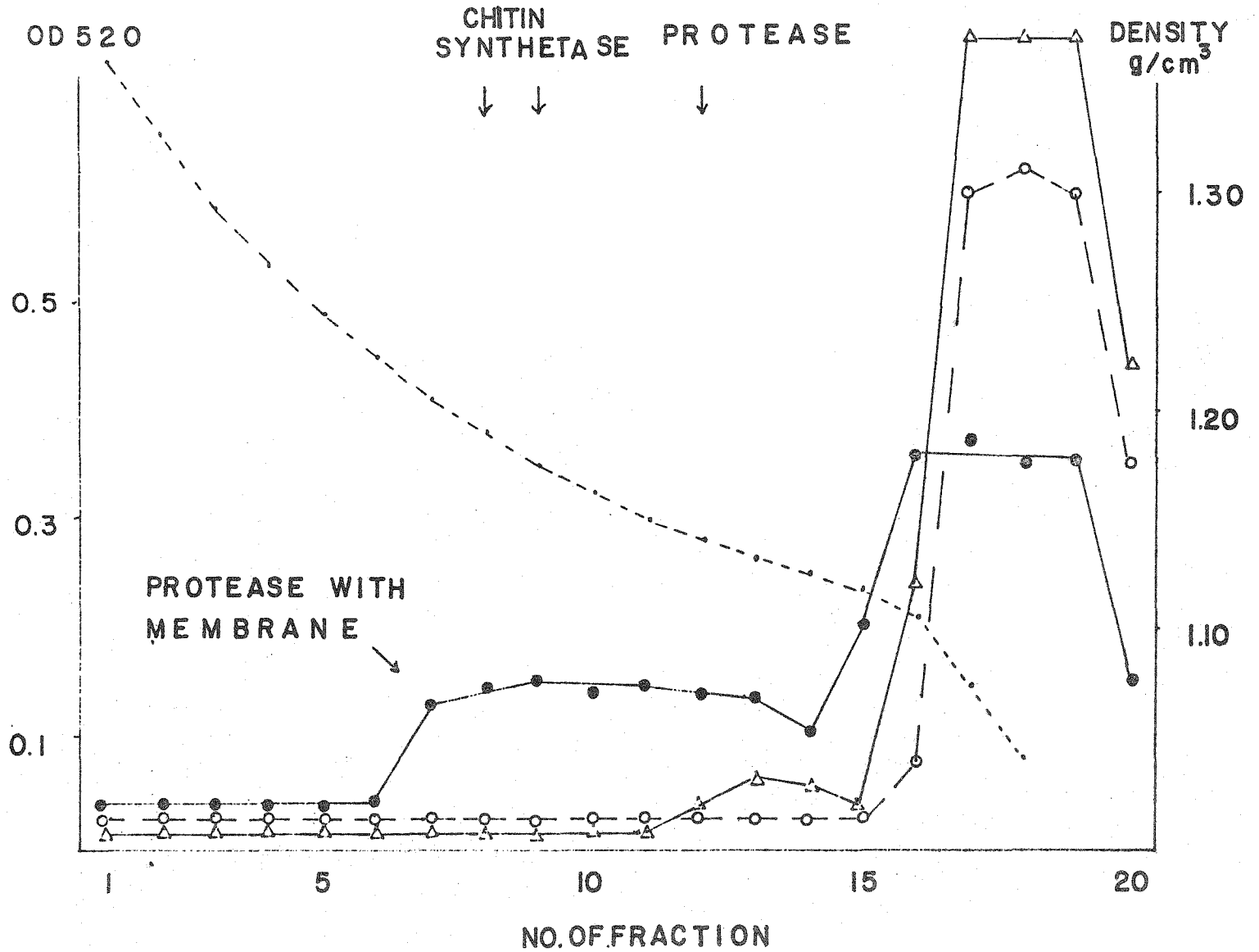
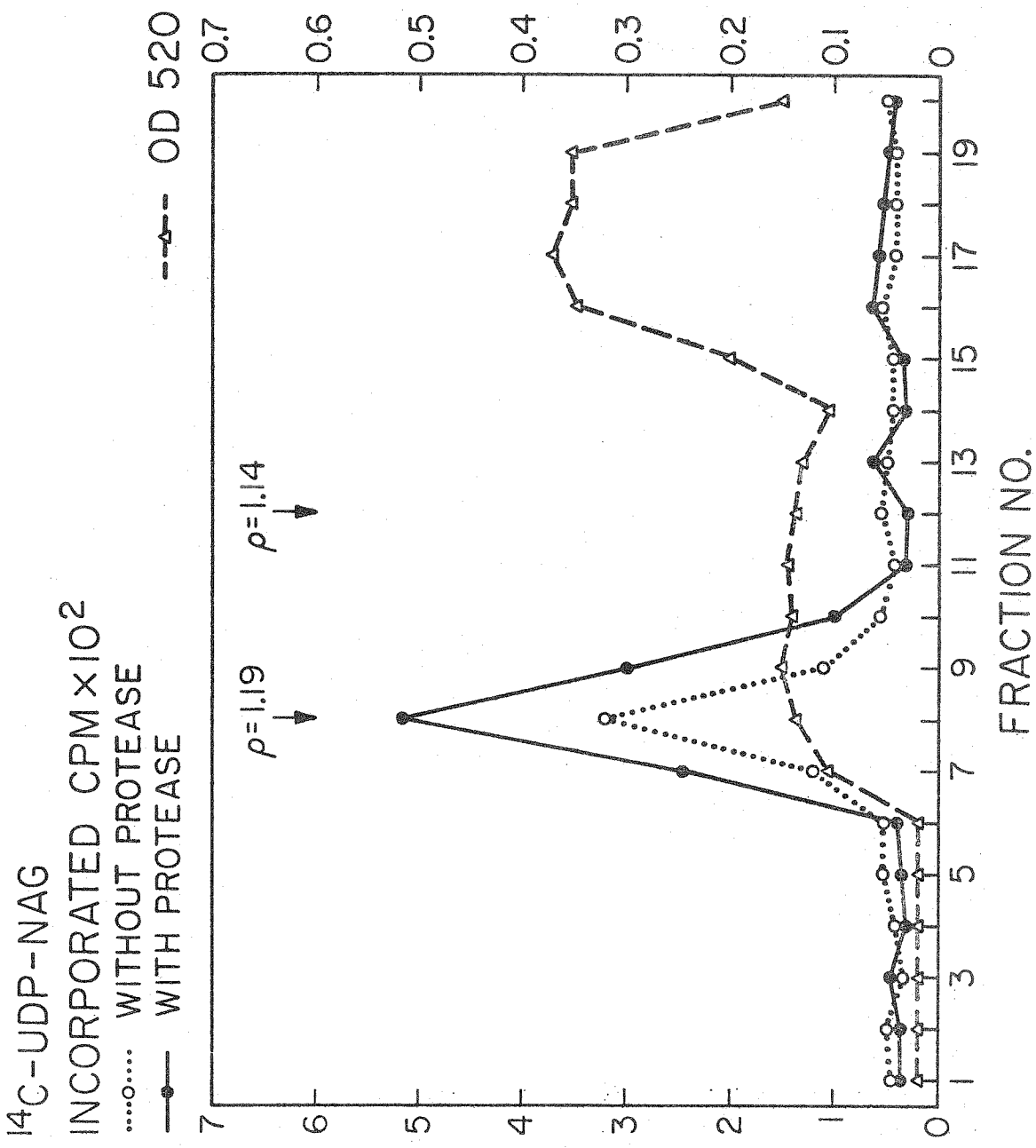

OD 520
CHITIN SYNTHETASE
PROTEASE
DENSITY g/cm3
0.5
0.3
0.1
1.30
1.20
1.10
PROTEASE WITH MEMBRANE
1
5
10
15
20
NO. OF FRACTION

Figure D 10: Increase in chitin synthetase activity after adding a serine protease (B-DI) to a crude plasma membrane fraction and running it on a 10 - 58 % metrizamide gradient. The proteolytic activity of the fractions is plotted as --▲-- ; the same fractions give higher activities of chitin synthetase (—●—) than the ones from a gradient run without protease (···o···).


^{14}C-UDP-NAG
INCORPORATED CPM×10^2
..... WITHOUT PROTEASE
—●— WITH PROTEASE
--△-- OD 520
ρ=1.19
ρ=1.14
FRACTION NO.
0
1
2
3
4
5
6
7
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1
3
5
7
9
11
13
15
17
19

2.4) Solubilization Attempts

The main difficulty in the chitin synthetase activation experiments probably resides in the fact that we have not separated the non-active form of the enzyme from the active one.

Various attempts have been made to produce a zymogen fraction (i.e. a chitin synthetase sample that shows activity only after proteolytic treatment). The membranous fractions were exposed to various chaotropic reagents (sodium perchlorate,sodiumthiocyanate, potassium bromide) and/or several detergents (Brij 36, Nonidet P-40, Tween 80) without being able to achieve the desired separation.

We must conclude that if there is a chitin synthetase zymogen it is attached to the membrane in the same way as the active enzyme.

Chapter E

Discussion

DISCUSSION

1) A Short Review of Cell Wall Synthesis

The presence of chitin in sporangiophores of Phycomyces was established in 1936 by Van Iterson et al. (7o), chitosan was detected in 1954 by Kreger (71), both in the mycelium and in the spph. The most recent analysis is by Meissner and is reported on page 4.

Bartnicki-Garcia noted in 1968 (72) that the presence of chitin and chitosan in the cell wall is unique for the zygomycetes.

How are the various components organized in a cell wall? Fungal walls consist of microfibrils embedded in an amorphous matrix; fungi are not different from plants in this respect. Chitin fibrils are 15 - 20 nm in diameter, and in spphs the wall itself is 600 nm thick, i.e. about 100 times thicker than the plasmalemma (2). A detailed review about the multilaminate structure is given by Arnson (73).

No model has been developed for fungal cell walls, but it might be helpful to consider the following suggestion about the molecular organisation of a plant cell wall by Albersheim (74) :

The main component is cellulose; forty β-1,4-glucan chains are hydrogen bonded to form cellulose fibers;

they are linked together by three other polysaccharides in the following way: many xyloglucan molecules adhere to the surface of the fibers, each xyloglucan is bound to a single arabinogalactan chain, which in turn binds to rhamnogalacturonan in such a way that each cellulose fiber is connected to several rhamnogalacturonan chains. In this model extension of the cell wall ("growth") might be regulated by enzymes acting on bonds between the crosslinking polysaccharides. A comprehensive analysis of cell wall synthesis thus has to deal with at least the following questions:

a) How and where are the polysaccharide chains initiated and elongated?

b) How and where occurs the fabrication of fibrils?

c) How are new fibrils incorporated into the cell wall?

d) How are the fibrils interconnected?

It is safe to say that the first question can be considered closer to an answer than the other ones.

With this model and a multilaminate structure in mind we can discuss various ways to influence cell wall extension ("growth").

A cell wall softening enzyme (possibly a protein disulfide reductase (72)) might either cut connections

between fibers (and the turgor pressure then might push a synthesizing machinery into this hole) or a chitinolytic enzyme might cleave fibrils thereby producing more reaction sites for chitin synthetase.

Another way is to supply more substrate for this enzyme and to assume it finds a way to incorporate the new material. A third possibility to regulate cell wall synthesis consists in activating the chitin synthetase, e.g. by limited proteolysis.

These views have been considered in Phycomyces with respect to the primary target of the light information channel: chitin synthetase or a chitinolytic enzyme? A soluble chitinase is present in Phycomyces as described by Cohen (75).

To approach this question Ortega et al. (76) have investigated the mechanical properties of spphs. They detected increased extensibility after a light stimulus. It is not possible though, to deduce the primary effect of light from this result, for the softening of the wall matrix might be the step that follows the increased pro - duction of chitin fibrils, to assist their incorporation.

For the extension growth of pea seedlings Murray et al. (77) present evidence for a role of glucosidases

causing wall plasticization; but the authors point out that these enzymes could exert an indirect control and their reactions may only be concomitants of growth.

The dimorphism exhibited by various fungi (yeast-like or mycelial growth) seems to be connected to the activity of a cell wall loosening enzyme, as discussed by Gander (78). The occurence of an active protein disulfide reductase (first described by Nickerson (85)) in the yeast form, and its low activity in the mycelial form, is consistent with the conclusion that this enzyme is necessary to provide sufficient plasticity for bud formation.

One characteristic of many filamenteous fungi is the presence of chitin in the cell wall; another is the apical extension of the hyphal wall. This apical incorporation of UPD-NAG can be switched to an overall incorporation (wall thickening and no extension) in Aspergillus nidulans by addition of cycloheximide (79); this finding can be explained by assuming that chitin synthetase is dispersed throughout the cell and is activated under normal circumstances only at the apex. Cycloheximide has no influence on the total synthesis of the wall polysaccharides. The overall activation seems to be connected with the availability of a cytoplasmic transport system. If no

cycloheximide treatment was applied an assembly of vesicles was observed near the hyphal tip (80).

These remarks highlight the many unsolved questions in cell wall synthesis. Various attempts have been made to establish modes of regulation. The possible role of proteolytic enzymes is investigated in this thesis.

Other approaches have concentrated on cyclic nucleotides and adenosine phosphates. A role of cAMP in plants is suggested by the finding that theophylline, a common inhibitor of phosphodiesterases in cell homogenates, is known to stimulate plant growth (81). In Phycomyces Jan (6) and Cohen (90) showed that there is a variation of the level of cAMP after a light signal and the time course of this variation is similar though slightly faster than the light growth response.

The role of ATP deserves more attention at the moment; there is evidence that in Candida albicans the control over the synthesis of chitin (and mannan) is partly provided through controlling the activity of phosphofructokinase by adenosine phosphates (82). Low activity of this enzyme results in more available substrate for chitin synthetase; this in turn leads to apical extension of the mycelium. The requirement for NADPH for cell division is reported to be higher than for mycelial growth. It is

shown that dimorphism and cell wall composition are related: In C. albicans the mycelial wall contains 3 times as much chitin as the yeast-phase wall.

Thus chitin synthesis may be positively regulated by controlling phosphofructokinase while increased NADPH generation allows cell division to occur.

Other interesting observations on the control of cell wall synthesis are reported from the water mold Blastocladiella emersonii (83). UDP-NAG is shown to inhibit the first pathway-specific enzyme of hexosamine biosynthesis.However, during germination of the zoospore this end-product inhibition is overcome: activation of chitin synthetase increasesutilization of UDP-NAG and the flux of metabolites in the pathway to UDP-NAG and a chitin wall is quickly constructed.

The activation of the chitin synthetase itself seems to depend on the fusion of vesicles with the plasma membrane as reported by Soll et al. (84); this observation can be interpreted as favoring Cabib's view (8), that activation of the chitin synthetase bound to the plasma membrane requires fusion of protease carrying vesicles with the plasmalemma.

However, the vesicles may only contain presynthesized chitin fibrils which get incorporated after fusion

with the cytomembrane. In order to distinguish between these possibilities a closer cooperation between biochemistry and electron microscopy is necessary. One has to know the activities of the vesicles that can be seen.

This discussion has led us back to the idea that limited proteolysis may be part of the cell wall synthesis regulating machinery. It should be noted, though, that in the case of yeast and Blastocladiella we are considering regulations related to particular stages of the life cycle of the organisms, and not, as in Phycomyces, related to rapid responses to external stimuli.

2) Serine Proteases and Inhibitors

In recent years the investigation of the participation of proteases in biological control processes in microorganisms has been initiated in several laboratories. Previously a great deal of knowledge had been accumulated for mammalian cells, showing that limited proteolysis is a general mechanism in many important and fast acting physiological functions. Examples are the enzyme cascade of blood coagulation, complement activation, and fibrinolysis (20).

The proteases involved can be characterized as serine proteases and acid proteases (both endopeptidases), and exopeptidases. Their activation usually means the conversion of a zymogen to an active enzyme by the splitting of a single peptide bond.

The presence of the corresponding types of proteases in microorganisms has been investigated during the last ten years and proteases similar to the various forms mentioned have been detected.

A generalization that arises from the literature is that in microorganisms like Neurospora crassa (27-29, 36) and many species of yeast (47-50) the inactive precursor of a protease is a protease- inhibitor complex rather than a zymogen. This finding initiated a series of

inhibitor purifications in various laboratories.

Fig. E1 illustrates the two possibilities. The route to the active enzyme via an enzyme-inhibitor complex can also be taken through inhibitor digestion. This would be "reversible" if the inhibitor is present in excess.

Figure E1: Control of enzyme activity by zymogen activation (irreversible) and/or by dissociation of an enzyme-inhibitor complex (reversible).

So far very little has been achieved in assigning proteases definite roles in physiological processes. The investigations by Cabib about the regulation of chitin synthetase in yeast were the first case in which limited proteolysis could be shown to initiate physiological events.

Our investigations on Phycomyces generally agree with the results from other microorganisms. Some new features have to be pointed out.

Three serine proteases are detected, inhibited with comparable high association constants by the same inhibitor protein. These proteases have specificities different from trypsin and chymotrypsin. The high molecular weight serine protease of Phycomyces exhibits elastase type specificity.

The inhibitor of these proteolytic enzymes is present in excess. It forms a 1:1 complex with each of the three proteins. Dissociation of the complex has been achieved only under denaturing conditions.

No inhibitor protein was detected for the A type protease; the enzyme itself is comparable to the acid proteases studied by Subramanian et al.(62) with respect to MW and pH optimum. Protease A activity might be controlled by another mechanism (e.g. by a local change in the pH), but it should be pointed out that three inhibitor proteins for A type proteases have been described in yeast (38,49).

No protease with type C (carboxypeptidase) activity was found, using various assays described in the literature. Wolf et al.(69) isolated a mutant in yeast which has no C type carboxypeptidase but is indistinguishable from WT otherwise. It can be concluded that

whatever physiological role there is for protease C this function can be covered by other enzymes of corresponding or overlapping specificity.

Aminopeptidases were discovered in N. crassa by two types of assays by Siepen et al.(36); no activity of that kind could be detected in Phycomyces.

3) Chitin Synthetase in Phycomyces

The serine proteases affect chitin synthetase activity, but since no purified synthetase is available we have no way to tell whether this enzyme itself is the substrate. Monitoring the effect of proteases on chitin synthetase preparations by comparing samples taken after various times of incubation by their SDS gel electrophoresis patterns shows visible changes in at least 15 bands.

We must point out another difficulty in relating our biochemical results to the known physiological ones: the biochemical studies reported here were undertaken with chitin synthetase from young mycelia, before these have formed spphs, rather than with material from sporangiophores. We chose to work with germlings because these have a 10-fold higher specific chitin synthetase activity. The physiological growth rate responses occur only in the spph.

Though one could assume a persisting mode of regulation for chitin synthetase it is also possible that a special machinery is constructed that enables the physiological responses once the cell enters the spph stages. The fact that mycelia do not exhibit (and do not need) a light growth response would argue in favor of this idea.

One way to continue the experiments would thus be to apply the proteases to chitin synthetase preparations from spphs.

Our data indicate that for the mycelial enzyme the situation in Phycomyces turns out to be more complicated than the one in yeast, though even in this organism there is no unambiguous situation (see below). That the regulatory processes are different (at least in details) must be expected since the enzymes are used differently by the corresponding cells.

The yeast enzyme is most of the time inactive and turned on at a certain point in the cell cycle. It synthesizes chitin on the side of the plasma membrane on which it finds the substrate (the cytoplasmic side) and does not reach through the membrane; this aspect was confirmed by Duran et al.(25).

These authors show that chitin synthetase zymogen is attached to the yeast plasma membrane and it is found to be accessible only from the inside of the cell. The association of chitin synthetase zymogen with the plasmalemma is the basis for the explanation of localized activation of the enzyme and initiation of septum formation. However, a low-density particulate fraction containing chitin synthetase was reproducibly found to account for

20% of the activity. This activity might be linked to particles of lower density (compared to the plasma membrane) or consists of precursor structures, which will end up in the plasmalemma in due time. The solution of this problem must await further characterization of the low density fraction.

The Phycomyces chitin synthetase must at all times participate in the cell wall production. The zymogen, therefore, if involved in the physiological responses, would have to be available at any time. The chitin is synthesized on the external side of the membrane and if the transport of substrate through the membrane is part of the chitin synthetase function the enzyme has to span the plasma membrane.

The results indicate that chitin synthetase is subject to activation by limited proteolysis; but a detailed description would require a better understanding of the interaction of subcellular fractions. In vivo a protease released from its compartment will probably immediately be trapped by an inhibitor; it can only act by either staying in its vesicle which in turn fuses with another one or by being transported into the target compartment.

4) A Hypothesis for the Regulation of Chitin Synthetase.

The evidence we have collected refers to *in vitro* reactions with soluble proteins and the steps illustrated in Fig. E2 are known to occur under these circumstances. We might postulate that vesicles carrying the corresponding proteins coalesce in a way that would allow the cascade to proceed. This chain involves the three different ways of enzyme activation we discussed earlier. It extends the original hypothesis of Cabib (8) and was first suggested by Holzer (23).

The cascade leaves open how the vesicles might be directed to the corresponding sites and how they might be induced to fuse . It is known, however, that a cell is equipped with mechanisms for moving around numerous organelles. The model explains acceleration of chitin synthetase, it does not indicate how the process would be slowed down. Excess protease could cause destruction but it is difficult to see how such a procedure could be accurately controlled *in vivo*.

The scheme does not include any inhibitors of the chitin synthetase itself,i.e. inhibitors that do not influence proteolytic activities. Such a protein has been described by Lopez-Romero *et al*.(65) for the yeast form of *M. rouxii*.Preliminary tests did not detect inhibition of

that kind in Phycomyces.

It is very likely that more components than the ones included in the schematic description are involved in the actual regulatory processes, because the construction of chitin fibrils itself must be a complicated mechanism, let alone the correct assembly of these fibrils into the wall.

Some progress has been made by Ruiz-Herrera et al.(54,55). They show that by incubating chitin synthetase granules with substrate (UDP-NAG) and activator (NAG), a network of chitin microfibrils is produced as observed in the electron microscope. Microfibrils with a granule at one end were a common appearance.The granules are roughly ellipsoidal in shape and measured 350-1000 Å in diameter; individual microfibrils were measured to be about 150 Å wide and up to 2 μm long.

The authors interpret their findings as experimental support to the "end-synthesis theory of microfibril elaboration via a terminal enzyme granule" (54). This suggestion was originally advocated by Preston (89) for the formation of cellulose microfibrils in plant cell walls and is now extended to chitin fibrils of fungi.

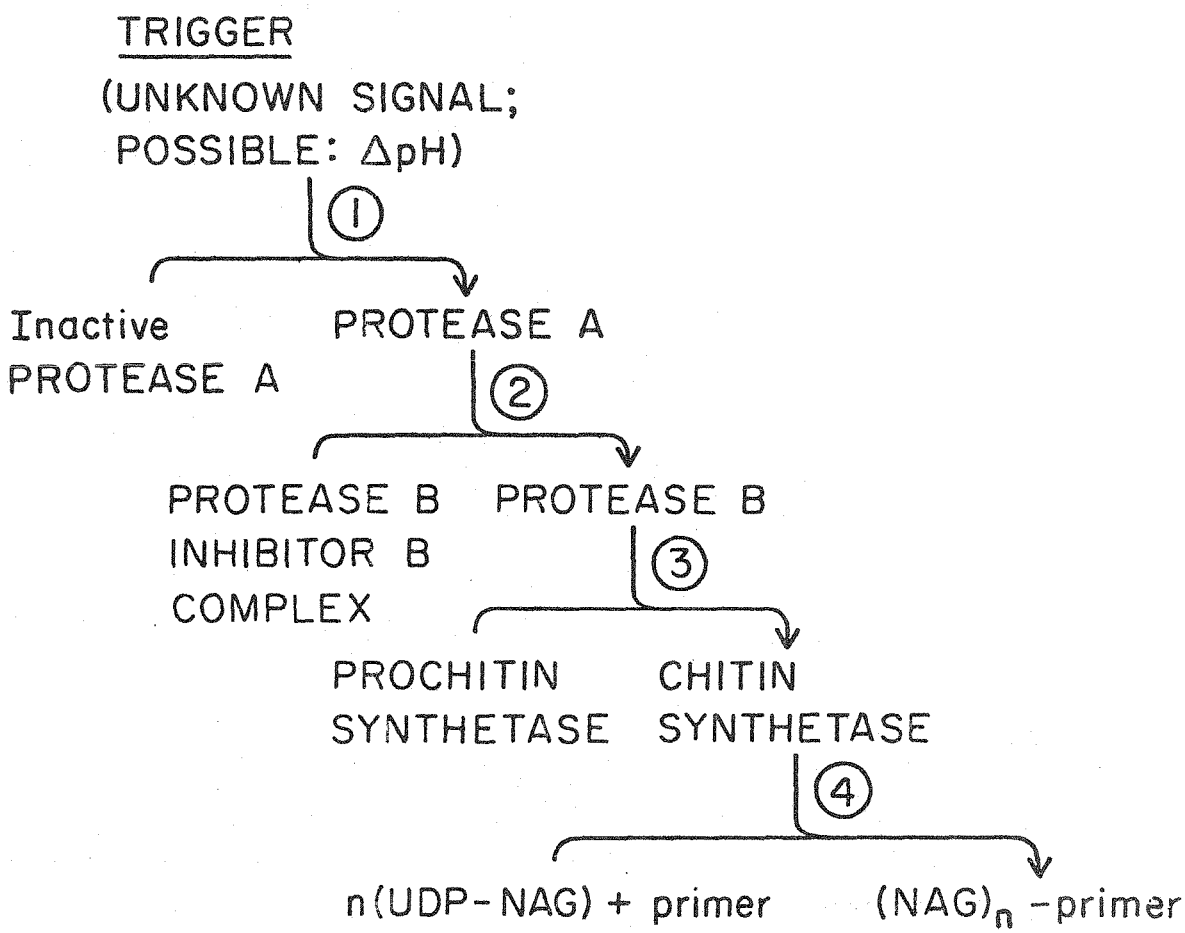
Figure E2: Hypothetical cascade for the activation of chitin synthetase in microorganisms. The shown reactions are known to occur *in vitro*.

1)Activation of protease A

2)Protease A destroys inhibitor B

3)Protease B converts chitin synthetase zymogen into the active enzyme

4)Chitin is synthesized


TRIGGER
(UNKNOWN SIGNAL;
POSSIBLE: ΔpH)
①
Inactive
PROTEASE A
PROTEASE A
②
PROTEASE B
INHIBITOR B
COMPLEX
PROTEASE B
③
PROCHITIN
SYNTHETASE
CHITIN
SYNTHETASE
④
n(UDP-NAG) + primer
(NAG)n -primer

5) Directions for Future Work

The study of serine proteases with respect to their substrate specificities and their relation to the homologous set of mammalian serine proteases provides interesting problems for future research, especially since identification of some physiological roles begins to emerge. Serine proteases inactivate tryptophan synthetase in yeast (39), are involved in the activation of nucleases in Neurospora (30), and, possibly, of chitin synthetase in fungi.

How do serine and acid proteases interact? To answer this question it would be helpful if the proteases could be isolated in larger quantities, for instance by affinity chromatography. One might be able to weaken the protease-inhibitor complex by chemical modification of the inhibitor just enough to be able to dissociate the complex with mild treatment. Another way to construct a weak complex might be to use, say, yeast proteases with Phycomyces inhibitor. Experiments in this direction will be done in the immediate future in collaboration with H. Holzer and his group in Freiburg, West-Germany.

The conditions under which vesicles either release their enzymatic contents or coalesce with other vesicles has to be investigated. Preliminary studies are necessary to develop procedures to isolate intact various vesicular

fractions.

The question whether inhibitory proteins control directly the activity of chitin synthetase has to be pursued further.

The interaction of the proteases has to be investigated: under which conditions do they destroy each other's inhibitor? What is the pH dependence of inhibitor hydrolysis? Proteases A and B are both in the same subcellular fraction. Are the two enzymes in the same vesicles or in separate ones? If they are in the same compartment, how are they connected? Are there other proteases (e.g. aminopeptidases) detectable in the cell that could influence the regulating serine proteases?

The solution of these problems will contribute to the understanding of protease action as essential elements in complex biological phenomena.

Appendix

Cytochrome c in *Phycomyces*

CYTOCHROME C IN *PHYCOMYCES*

1) Introduction

> "To oxidize food molecules all organisms from yeast to man require a variant of cytochrome c. Differences in this protein from species to species provide a 1.2-billion-year record of molecular evolution."

This quotation from Dickerson (34) outlines the importance of the studies of cytochrome c. This "ancient protein" (34) is located in the mitochondria of all aerobic cells; it accepts electrons from cytochrome b and transfers them to the cytochrome oxidase. During this process the iron in the molecule shifts between the ferrous and ferric states.

Cytochrome c is an ideal molecule for comparative studies. It is widely distributed in nature and readily extractable from biological materials. It has a convenient total length and is amenable to sequencing by standard methods. Its rate of mutation is optimal for gross phylogenetic studies of large taxa.

The 3D structure of this protein is perfectly conserved in all eukaryotes. In man and in *Neurospora* it looks essentially alike though the proteins differ in 44 out of 104 (man) or 107 (*N. crassa*) positions. The primary structure (the amino acid sequence) is the information left by the evolutionary record.

The procedures for isolating cytochrome c have been worked out for many classes of organisms. By determining the amino acid sequence of a representative of a large group it is possible to understand its relation to others.

This approach was used by Nolan and Margoliash (86) to argue in favour of the distinctness of fungi from the animal and plant kingdom. (Fungi are nutritionally distinct. Plants are characterized by photosynthesis, animals by ingestive and fungi by absorptive nutrition (87)). The comparative studies of cytochrome c concluded that these proteins are homologous, and that they arise from homologous gene loci (86,88). The available sequences then suggest the fungi form a phylogenetic line distinct from the animal and plant kingdom. As an example wheat was found to be more closely related to man than to fungi from the point of view of its cytochrome c (86).

Phycomyces belongs to a well defined taxonomic group of fungi. The fungi of this group reproduce asexually by nonmotile sporangiospores, and sexually by forming a zygospore through the fusion of gametangia. The name zygomycetes is derived from this morphological characteristic.

As was pointed out in Chapter E the cell wall of zygomycetes is mainly composed of chitin and chitosan.

This is another indication for the unity of this class. Unfortunately the relation to other groups of fungi is still a mystery (87), and no conclusions about their origin can be presented on the basis of morphological data.

Comparative studies on cytochrome c might change this situation. Until now sixty different complete sequences have been determined from 67 species of eukaryotes (88). Among those are six fungi: Saccharomyces oviformis, Debaromyces, Candida krusei (three species), Neurospora crassa (these four belong to the ascomycetes), Humicola lanuginosa (a deuteromycete), and Ustilago (a basidomycete). It should be noted that baker's yeast is the only known species that has polymorphs or isozymes of cytochrome c (64,88).

Though none of the listed fungi belongs to the zygomycetes, we may get a hint as to the distance of Phycomyces from the represented classes by comparing the amino acid sequences. This is done by constructing the amino acid difference matrix according to Dickerson and Timkovich (88), where each entry gives the number of amino acids which differ between two species.

This can be done after the sequence has been established. So far only the amino acid composition of cytochrome c of Phycomyces has been determined by us along with the N-terminal amino acid (alanine). We report on this in

the following paragraph.

Special acknowledgements are necessary in this case. Nearly all the preparative work was done by Kathleen Kong, a summer student. She never gave up hope that all the yellow stuff in the test tubes would eventually yield a red protein.

Special thanks are due to Bruce Black, Vince Farnsworth, and Jeff Hubert in Dr. Lee Hood's laboratory . They helped with machines, chemicals, and good advice.

2) Purification of Cytochrome c

The cells were grown and harvested as described in chapter C. The cytochrome was extracted following essentially the procedure outlined by Scott and Mitchell (33).

The harvested cells were frozen in liquid N_2 and ground in a Waring blender repeatedly with liquid N_2 being poured over the powder; 2ml of .05 M Tris-HCl (pH 8.6) was added per gram tissue. The pH was changed to 10.5 with concentrated ammonia and the sample stirred in ice for 15 min. The pH was adjusted back to 8 with glacial acetic acid. The material was centrifuged at 2000 x g x 1o min. and the pellet washed with the Tris-HCl buffer. Both supernatants were combined and centrifuged at 20.000 x g x 30 min. This supernatant was dialyzed overnight against 100 volumes of 5 mM ammonium phosphate, pH 7, containing 0.1 mM $K_3Fe(CN)_6$. The dialysate was passed through an SP-Sephadex column equilibrated with 50 mM ammonium phosphate, pH 7. A red band forms at the top of the column. The cytochrome c was eluted with a linear salt gradient (0-0.5 M NaCl). All buffer solutions contained o.1 mM $K_3Fe(CN)_6$.

The cytochrome c was monitored by measuring OD 410. Dialysis and ion exchange chromatography were re-

peated several times. The material was lyophilized and filtered on G 50 Sephadex equilibrated with 200 mM ammonium phosphate. The cytochrome c peak was lyophilized and found to be homogeneous with respect to molecular weight (SDS gel electrophoresis). A typical result gave 1 mg of cytochrome c per 150 g (wet weight) of original cell material. A determination of the overall purification factor is hampered by the fact that the material (before column chromatography) is of brown-yellow color.

A 25-fold purification was achieved from the first to the last column step.

3) Amino Acid Analysis

The amino acid analysis was performed with the Durrum D-500 automatic amino acid analyzer by the methods described by Spackman et al.(64) after hydrolysis with 6 N HCl at 110°C; samples were taken at 24,48,72 hours.

The results are listed in Table API, which also gives the amino acid compositions for three other species of fungi (taken from (63)).

There is one trimethyllysine residue. This fact was determined by including mono-,di-, and trimethyllysine in the usual standards run in the amino acid analyzer and by adding 10 nmoles of trimethyllysine (MW 781.9) to the sample of Phycomyces cytochrome c, where it coeluted with the previously unidentified peak under investigation.

Cysteine was determined after perchloric acid oxidation; tryptophan has not been determined until now. Since the other fungal cytochromes have one tryptophan residue we assume tentatively the same for Phycomyces.

A prerequisite of sequencing is to check whether the N-terminal end is blocked. A Dansyl-Edman degradation was performed according to Gray (35). No blocking could be detected.In the process the N-terminal amino acid was identified as alanine, an ambivalent amino acid. Phycomyces joins the other fungi very well: Saccharomyces

oviformis starts with threonine, Candida krusei with proline. The N-terminal amino acid of Neurospora crassa is glycine.

Table AP1 : Amino acid composition of cytochrome c of *Phycomyces* in comparison with *S. oviformis*, *N. crassa*, and *C. krusei*.

	Phycomyces	C.krusei	S.oviformis	N.crassa
Asp	12	8	11	13
Thr	8	7	8	9
Ser	8	6	4	3
Glx	9	10	9	8
Pro	4	7	4	3
Gly	12	12	12	15
Ala	12	12	7	9
Val	6	3	3	1
Met	1	3	2	2
Ile	3	3	4	5
Leu	6	6	8	7
Tyr	3	5	5	4
Phe	5	4	4	6
His	2	4	4	2
Me_3lys	1	1	1	1
Lys	11	11	15	13
Arg	4	4	3	3
Cys	2	2	3	2
Trp	1 (?)	1	1	1
Total	110	1o9	108	107

	# of hydrophobic amino acids		
	With aromatic rings	nonaromatic rings	total
Phycomyces	9	16	25
S.oviformis	10	17	27
N.crassa	9	15	24
C.krusei	10	15	25

Table AP2: Comparison of the number of hydrophobic amino acids in the cytochrome c of four fungal species.

4) Discussion

The number of 110 amino acids in the cytochrome c of Phycomyces is in good agreement with known totals. The fungi are the only group whose number of amino acid residues varies from species to species (between 107 and 111). Higher plants all have 112 residues, insects all have 108, mammals all have 104 residues.

In detail the composition varies strongly between the fungi, but if one counts the number of hydrophobic amino acids with aromatic or nonaromatic rings they turn out to be rather similar. This is demonstrated in Table AP2. More detailed evaluation of differences has to await the full sequence.

With respect to the methylated lysine residue Phycomyces joins other fungi, they have ε- N - di- or tri-methyllysines at positions 72 or 86, or at both positions (91). Higher plants have modified residues at both positions.

It is known that this methylation of lysine occurs posttranslationally. The reason for this modification is probably to protect the positive charge at the corresponding positions even at high pH. Lysine residues are clustered in two positively charged regions which are essential for the mechanism of action of cytochrome c (34). Methylation assures the presence of these charges.

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