I. CHITIN SYNTHETASE AND SENSORY TRANSDUCTION PROCESSES IN *PHYCOMYCES*

II. THE AVOIDANCE RESPONSE, THE HOUSE GROWTH RESPONSE AND THE RHEOTROPIC RESPONSE OF *PHYCOMYCES*

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To my mother Li Ju Chen and

my father Ten Sun Jan

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ABSTRACT

Part I: Chitin Synthetase and the Sensory Transduction Processes in Phycomyces

The response of Phycomyces sporangiophores to various stimuli shows up as changes in the elongation rate of the cell wall, a structure mainly composed of chitin fibrils. The enzyme chitin synthetase was chosen as the subject of this study for the possible role that regulation of its activity might play in the behavioral output.

Properties of the enzyme: A simple assay for chitin synthetase has been developed for the Phycomyces system. Enzyme prepared from Phycomyces was found to catalyze the synthesis of chitin from UDP-N-acetyl-D-glucosamine. The ion requirements, temperature dependence, buffer and pH dependence, and kinetics for this enzyme were investigated. An antibiotic Polyoxin D was found to be a competitive inhibitor of this enzyme.

Cellular localization: To localize the enzyme in the cell: (1) Phycomyces homogenates were fractioned through a series of differential and isopycnic centrifugations. Each fraction was assayed for its specific activities of chitin synthetase and some membrane marker enzymes. The results suggest that chitin synthetase is a plasma membrane bound enzyme. (2) Autoradiography studies of the sporangiophore showed that this enzyme is located mainly in the growing zone of this structure.

Regulations: In vivo, Phycomyces shows a positive growth response to blue light. It is demonstrated that blue light can increase the

chitin synthetase activity *in vitro*. This finding supports the idea that regulation of chitin synthetase activity plays a central role in the responses of *Phycomyces* sensory stimuli. Finally, the possibility of using the chitin synthetase assay as an *in vitro* photoresponse system in the dissection of the sensory transduction processes of *Phycomyces* is discussed.

ABSTRACT

Part II: The Avoidance Response, the House Growth Response, and the Rheotropic Response of Phycomyces

If an object is placed about 1 mm from the growing zone of a Phycomyces sporangiophore growing in air, in about 2 minutes the sporangiophore starts to bend away at a rate of about 2°/minute for as long as half an hour or more. This is called the avoidance response of Phycomyces. The purpose of this study is to find out how a sporangiophore detects a nearby object and avoids it. Electric field, electromagnetic radiation (UV, visible and IR), temperature, humidity and pressure are all excluded as the avoidance eliciting signal. The avoidance response is found to be dependent on the size and the distance of the nearby objects and independent of the compositions and surface properties of the objects.

An air current parallel to the barrier and the sporangiophore can eliminate the avoidance response. In conjunction with this key observation, the Phycomyces responses to wind (rheotropic response and wind growth response) and to enclosure (house growth response) were characterized. The key parameter in all these responses seems to be the air movement in the vicinity of the sporangiophore growing zone. All observations are compatible with the assertion that faster growth is associated with slower wind velocity. To specify in which way air movement can become a signal directly received by the sensor, we propose that the avoidance is mediated by a volatile growth effector emitted somewhere along the sporangiophore and sensed by the growing zone (the chemical self guidance hypothesis). Various possible

alternative forms of the hypothesis were tested experimentally. The only remaining viable one is that the sporangiophore emits volatile growth promoting molecules continuously. The majority of the molecules are readsorbed by the growing zone before they diffuse away, and the barriers modify the distribution of the molecules by altering the ambient wind pattern. Future tests are discussed and a quantitative formulation of the model is presented in Appendices B and C.

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PART I

CHITIN SYNTHETASE AND SENSORY TRANSDUCTION PROCESSES IN *PHYCOMYCES*

Chapter 1

Introduction

INTRODUCTION

(A) *Phycomyces blakesleeanus* as a model system for the study of sensory transduction processes: A brief review.

The sporangiophore (spph) of the fungus *Phycomyces* is a gigantic single cell. It is sensitive to at least 4 distinct stimuli; light, gravity, mechanical stretch, and the closeness of an object. Light is sensed also by the mycelium, it affects the spph initiation (1) and carotenogenesis (2). About 20 years ago, Delbrück and Reichardt (3) started the study of this organism as a model system for sensory transduction processes (S.T.P.). The hope is that the basic mechanisms of S.T.P. may be the same throughout life and some basic facts of general validity might be learned from studying a primitive unicellular organism.

A general review of *Phycomyces* research up until 1969 is provided by Bergman *et al.* (4). Since then progress has been made in several directions. These will be summarized in the following:

(1) Physiology:

Among the *Phycomyces* responses, the light growth response has been by far the best characterized one. Delbrück and Reichardt (3) systematically studied the light growth response. The growth velocity of a symmetrically illuminated spph shows transient changes in response to changes in light intensity. The observation techniques have been greatly refined recently by the development of an automated tracking machine (5, 6) which allows the characterization of the growth response

under a broad range of conditions. The work has been further extended (7) using "white noise" stimuli to obtain a concise mathematical description of the system. These studies have yielded thorough characterizations of the light growth response of wild type and several mutant strains.

Meanwhile, the avoidance response has been investigated by D. Johnson and R. Gamow at the University of Colorado, and by R. J. Cohen, Y. Jan, J. Matricon, and M. Delbrück at the California Institute of Technology. The nature of the signal sensed by the spph is still obscure, but our experiments have considerably narrowed down the possibilities. Details about the study of avoidance response will be described in part II of this thesis.

The combined usage of automatic tracking machine and the white noise techniques optimized the approach of manipulating the environmental parameters and observing the subsequent physiological response.

(2) Genetic dissection of the S.T.P.:

Another approach toward the understanding of S.T.P. is to use genetic techniques. In fact, a major advantage of choosing a microorganism as a model system is that genetic tools might be applicable. The S.T.P. of an organism is to a great extent determined by its genes. Alteration of specific genes can be used as a powerful tool in dissecting the specific steps in the S.T.P. This approach was started by M. Heisenberg in 1966, and later joined by K. Bergman, A. Eslava, E. Cerdá-Olmedo and T. Ootaki. In Phycomyces, mutations can be produced

by using chemical mutagens such as nitrosoguanidine (NTG). Hundreds of behavioral mutants have been isolated so far. Based on physiological data, the mutants were divided into different classes (2). A simple branched pathway model connecting several sensory responses was thereby constructed (Fig. 1). By applying the newly developed heterokaryon-making technique (8), T. Ootaki *et al.* (9) were able to do complementation tests of these *Phycomyces* behavioral mutants. Fifteen photo mutants thus far tested fall nicely into five complementation groups. Two genes (*mad A*, *mad B*) are close to the input end (i.e., photoreceptor end) of the sensory transduction pathway. Two genes (*mad D*, *mad E*) are close to the output end, and one gene (*mad C*) is in between. Although it is entirely possible that more genes are involved in the light response pathway, the picture appears encouraging as it suggests that the total number of genes involved in the *Phycomyces* S.T.P. is small and that an understanding of the S.T.P. at the molecular level is feasible.

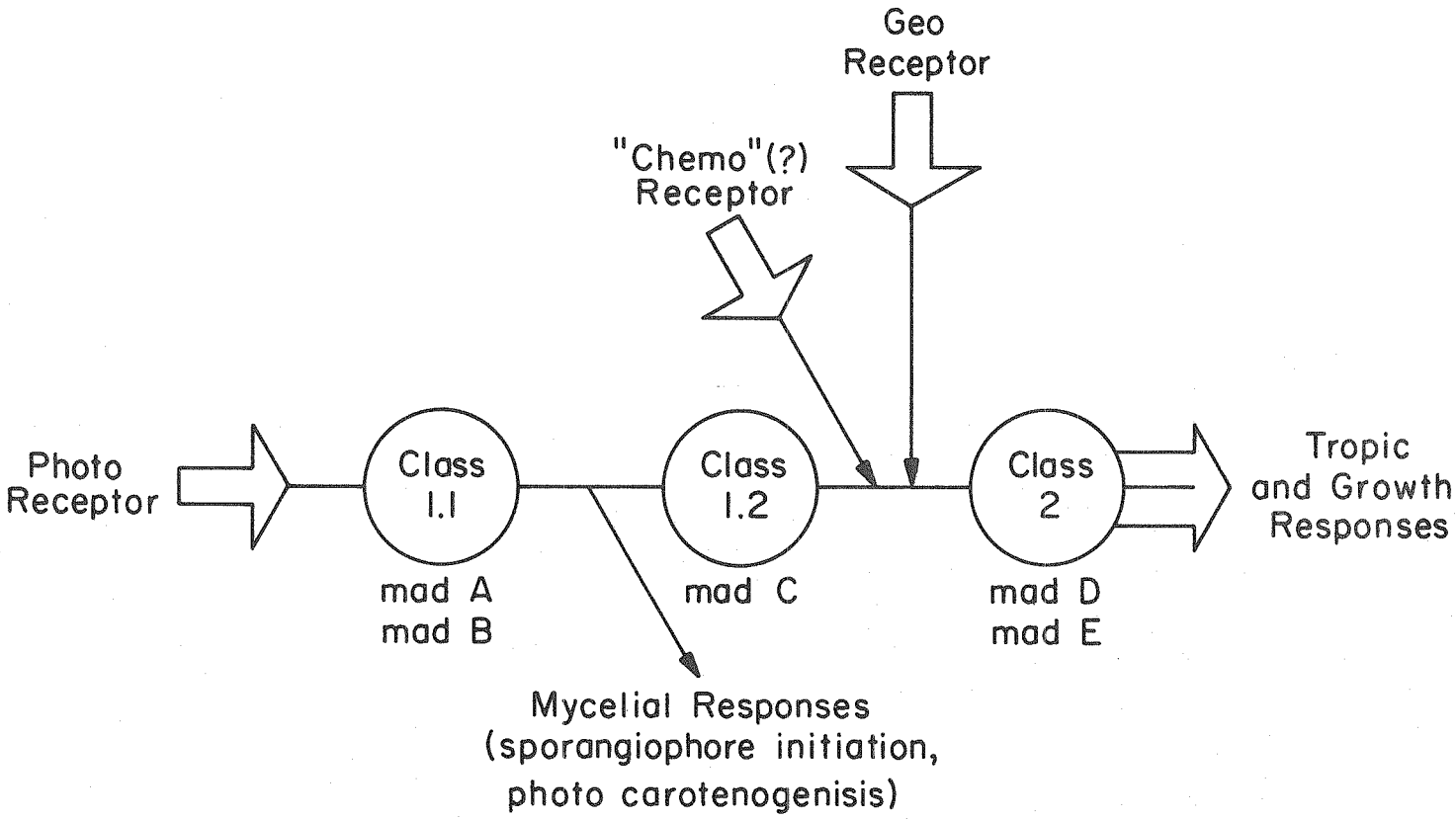
(B) The need of an *in vitro* sensory response system

To understand the sensory transduction processes at the molecular level, it is necessary to identify the products of the genes involved. A general approach to this problem is the "chemical transfer" approach. This approach involves attempts to specifically reverse a mutant phenotype by the addition of one or a series of compounds from wild type. Unfortunately, the spph can take up very few chemicals. For the "chemical transfer" approach to be effective, it becomes necessary to break open the cell. Once the spph is broken,

Fig. 1. A network model for the sensory responses of Phycomyces

The information flow between the three receptors and the three types of responses is represented by arrows. The behavioral mutants are divided into three classes based on the particular site of the network affected [Bergman et al. (2)]. Some of these mutants were tested for genetic complementation [Ootaki et al. (9)]. Five complementation groups were found, defining five genes, designated madA to madE. This grouping of the mutants is consistent with the phenotypical classification scheme of Bergman et al. (2), and is included in the network model.

This figure is adapted from Bergman et al. (2) and Ootaki et al. (9).


Geo
Receptor
"Chemo"(?)
Receptor
Photo
Receptor
Class
1.1
Class
1.2
Class
2
Tropic
and Growth
Responses
mad A
mad B
mad C
mad D
mad E
Mycelial Responses
(sporangiophore initiation,
photo carotenogenisis)

however, we can no longer observe the cell elongation as a manifestation of the sensory responses. Thus, we need an *in vitro* assay of the response to a sensory stimulus as a substitute for the *in vivo* growth response.

(C) Chitin synthetase

The behavioral output, i.e., the variation of the rate of elongation of the spph, implies a variation in the rate of elongation of the cell wall. This latter might be explained by either one or both of the following theories.

1. The variation results from variations of the rate of cell wall synthesis, which could be achieved by regulating enzymes that catalyze the cell wall synthesis or by affecting other cellular constituents that are involved in earlier events of the cell wall synthesis such as the synthesis or transport of substrates of the cell wall synthetases.

2. The variation is due to the change of cell wall extensibility which is mediated by certain enzyme(s) that cleave the chemical bond(s) in the cell wall matrix.

These two viewpoints have been discussed many times. T. Leighton during the 1971 Cold Spring Harbor summer *Phycomyces* workshop suggested that, since chitin was reported to be the major component of *Phycomyces* cell wall (4), chitin synthesis might play an important role in the transduction mechanism for sensory perception (10). Theory 2 has been elaborated by Ortega, Harris and Gamon (11). According to G. Meissner

(personal communication) the spph cell wall is mainly composed of the polyaminosaccharides. Chitin and chitosan (a deacetylated form of chitin) account for about 62 and 13% of the dry weight of the wall, respectively. In addition to chitin and chitosan, the cell wall contains about 3% lipid, 2.5% H_2PO_3, 3% amino acids and 6% of compounds which are extractable with dimethylsulfoxide. Ribose, glucose, fucose, galactose, xylose and mannose account for less than 0.5% of the total cell wall by weight.

No direct experimental support on either of the theories described above has been reported. It has not been shown whether the rate of in vivo chitin synthesis varies during a growth response. Nevertheless, chitin synthetase has been studied with the hope that it can fulfill the need for an in vitro sensory response system according to the following rationale.

1. It seems plausible that the rate of in vivo chitin synthesis varies at least roughly in parallel to a growth response.

2. If chitin synthesis is indeed involved in the growth response, then chitin synthetase, the last enzyme in chitin biosynthesis seems to be the best candidate. Even if the sensory stimuli directly regulate some steps in chitin biosynthesis other than the step catalyzed by chitin synthetase [e.g., transport of the substrate (UDP-GlcNAc) of the chitin synthetase, or chitinase which is an endoenzyme hydrolyzing the chitin chain into shorter chain lengths, thereby creating more reaction sites for chitin synthetase], it is still possible that the effect of regulation can be detected in the apparent chitin synthetase

activity. In Chapter 2, the basic properties and cellular localization of the chitin synthetase of Phycomyces will be described. In Chapter 3, an interesting light effect on chitin synthetase activity will be described.

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Chapter 2

Properties and Cellular Localization of Chitin Synthetase in *Phycomyces blakesleeanus*

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Properties and Cellular Localization of Chitin Synthetase in *Phycomyces blakesleeanus**

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SUMMARY

The response of *Phycomyces* sporangiophores to various stimuli show up as changes in the elongation rate of the cell wall, a structure largely composed of chitin fibrils. The enzyme chitin synthetase was chosen as the subject of this study for the possible role that regulation of its activity might play in the behavioral outputs.

A simple assay for the chitin synthetase has been developed. Membrane preparations from the mycelia of *Phycomyces* were found to catalyze the synthesis of chitin from UDP-*N*-acetyl-D-glucosamine. Both Mg^{2+} and *N*-acetyl-D-glucosamine stimulate the enzyme activity. The pH optimum for the enzyme activity is about 6.5, and the temperature optimum is about 28°. The K_m for UDP-*N*-acetyl-D-glucosamine is 0.6 mM. The antibiotic polyoxin D competitively inhibits the activity at levels which are comparable to those required for the inhibition of mycelial growth. In the presence of polyoxin D (0.1 mM), germinating spores of *Phycomyces* develop into protoplast-like structures. At concentrations up to 0.5 mM, ATP stimulates the enzyme activity and at 2 mM, cyclic 3′:5′-AMP is slightly inhibitory. The enzyme activity was found in the *Phycomyces* mycelia of different ages as well as in the sporangiophores of each of the five developmental stages.

Phycomyces mycelial homogenate was fractionated through a series of differential and isopycnic centrifugations. Each fraction was studied for its (*a*) specific and total chitin synthetase activities; (*b*) morphology under the electron microscope; and (*c*) specific and total activities of the marker enzymes 5′-nucleotidase (plasma membrane), glucose 6-phosphatase (endoplasmic reticulum), succinic-2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium-reductase (mitochondria), catalase (peroxisomes) and acid phosphatase (lysosomes). The fraction which exhibited the highest specific activity contained essentially only membraneous structures. The distribution of the specific chitin synthetase activity coincided with that of 5′-nucleotidase activity and did not coincide with that of any of the other marker enzymes. These results suggest that the chitin synthetase is a plasma membrane-bound enzyme.

Autoradiography studies showed that along the sporangiophore the growing zone (where cell elongation takes place) has higher chitin synthetase activity than the nongrowing zone.

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The sporangiophore of *Phycomyces* has been extensively studied for its behavioral responses (1, 2) to light, to gravity, to mechanical stretches, and to the presence of nearby barriers. In this system we have one distinct behavioral output, namely, the elongation of the sporangiophore. Recently, complementation tests done by T. Ootaki *et al.*[1] show that the class 1-1 and class 1-2 phototropic mutants of *Phycomyces* can be classified into a few complementation groups. This indicates that *Phycomyces* may become the first system in which a pathway between the signal input (light) and the behavioral output (the elongation of sporangiophore) is simple enough to be understood in full detail. The behavioral output, *i.e.* the variation of the rate of elongation of the cell wall of sporangiophore, might be explained by either one or both of the following theories.

1. The variation of the rate of cell wall elongation results from variations of the rate of cell wall synthesis, which could be achieved either by regulating enzymes that catalyze the cell wall synthesis or by affecting other cellular constituents that are involved in earlier events of the cell wall synthesis such as the synthesis or transport of substrates of the cell wall synthetases.

2. The variation of the rate of cell wall elongation is due to the change of cell wall elasticity which is mediated by certain enzyme(s) that cleave the chemical bond(s) in the cell wall matrix.[2]

The major components of the *Phycomyces* cell wall are chitin and chitosan (1). Preliminary observations of the author and M. Forgac that blue light increases the net chitin synthesis *in vitro* suggest that the first theory might be correct. Therefore, chitin synthetase, the last enzyme in the pathway of chitin biosynthesis, was examined because it might play an important role in the output mechanism of the sensory transduction processes in *Phycomyces*. In the present article, the basic properties of chitin synthetase from *Phycomyces* and its localization in the organism are reported.

EXPERIMENTAL PROCEDURES

Materials

UDP-*N*-acetyl-D-1-[^{14}C]glucosamine and Aquasol were purchased from New England Nuclear. Chitinase was purchased

[1] T. Ootaki, personal communication.

[2] J. K. E. Ortega and R. I. Gamow, personal communication.

from Nutritional Biochemical Company. Fine grain autoradiographic stripping plates AR-10 were purchased from Kodak.

Methods

Culture Methods

The wild type *Phycomyces blakesleeanus* strain NRRL 1555(−) was used in all experiments described here.

Liquid Culture—Heat shocked spores (10^4 to 10^5 spores per ml) were inoculated in glucose asparagine plus yeast medium (Medium A) which contains 30 g of glucose, 2 g of L-asparagine·H_2O, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4, 0.25 mg of thiamine-HCl in 1 liter of distilled water. The culture was kept at 21 to 23° with overhead diffuse white light of intensity 10 microwatts per cm^2 and was under constant shaking. The mycelia underwent exponential growth up to about 72 hours after inoculation.

Plate Cultures—Heat shocked spores (10^2 spores per plate) were inoculated into Petri dishes which contained Medium A agar (medium (defined under "Liquid Culture") supplemented with 1% agar). The plates were kept at 21 to 23° with overhead diffuse light of intensity 10 microwatts per cm^2.

Preparation of Chitin Synthetase

Crude Enzyme Preparation—Mycelia harvested from liquid culture were washed thoroughly with cold (0°) homogenization Buffer I (10 mM Hepes,[3] pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 M glycerol). The washed mycelia were then resuspended in about 3 volumes of cold homogenization buffer and then passed through a French press operated at a pressure of 16,000 p.s.i. The procedure of homogenization was repeated. The homogenate was centrifugated at 1,000 × *g* for 3 min. The pellet was washed twice with about 3 volumes of buffer and resuspended in homogenization Buffer I such that the final protein concentration was about 1 to 5 mg per ml. This crude preparation contained mainly cell wall fragments with the plasma membrane still attached.

Plasma Membrane-rich Fraction Preparation—Mycelia harvested from plate culture were homogenized in homogenization Buffer II according to the procedure of Hertel *et al.* (3). The homogenate was fractionated through a series of differential and isopycnic centrifugations (Fig. 1). The microsomal fraction (100,000 × *g*, 90-min pellet) was resuspended in homogenization Buffer II and layered on a discontinuous sucrose gradient formed by layering successively the following sucrose solutions: 11 ml of 50% (w/w), 11 ml of 30% (w/w), and 11 ml of 10% (w/w) sucrose in 1 mM $MgCl_2$. The plasma membrane-rich material collected from the 30% and 50% sucrose interface after the isopycnic centrifugation was pelleted (100,000 × *g*, 90 min) and resuspended in homogenization Buffer I in such a way that the final protein concentration was between 0.5 and 4 mg per ml. It could then be kept at liquid nitrogen temperature for several weeks without losing activity.

Chitin Synthetase Assay

In a standard assay, the reaction mixture contained various amounts of enzyme, 30 mM Hepes, pH 7.2, 20 mM $MgCl_2$, 50 mM *N*-acetylglucosamine, 0.5 mM dithiothreitol, 1 M glycerol, and 0.33 mM UDP-[1-^{14}C]GlcNAc (specific activity, 0.28 mCi per mM) in a total volume of 105 μl. The reaction mixture was incubated at 23 ± 1° for 2 hours with overhead diffuse white light of intensity ~10 microwatts per cm^2. The chitin synthesized was then measured by one of the following two methods.

Method A—The reaction was stopped by adding 1 ml of cold (0°) 5% trichloroacetic acid. One-half hour later, the mixture was filtered through a Millipore filter (pore size 0.22 μm). The filter was then washed thoroughly with 5% trichloroacetic acid and counted in a liquid scintillation counter.

Method B—The reaction was stopped by adding 2 ml of 66% ethanol and the reaction mixture was centrifuged to a pellet (1,000 × *g* for 3 min in the case of assays of crude enzyme and 100,000 × *g* for 90 min in the case of assays of the plasma membrane-rich fractions). The pellet was then washed twice with 2 ml of 66% ethanol and counted. This method is basically the method of Keller and Cabib (22).

Method A and Method B were applied to measure the incorporation of radioactivity in identical reaction mixtures, and the results agreed very well. Method A was a much faster method to use, particularly for the assay of membrane fractions. Therefore, unless otherwise stated, Method A was used in the measurements of chitin synthesis in the experiments described in this article.

[3] The abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; INT, 2-(*p*-indophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium; GlcNAc, *N*-acetyl-D-glucosamine; UDP-GlcNAc, UDP-*N*-acetyl-D-glucosamine.

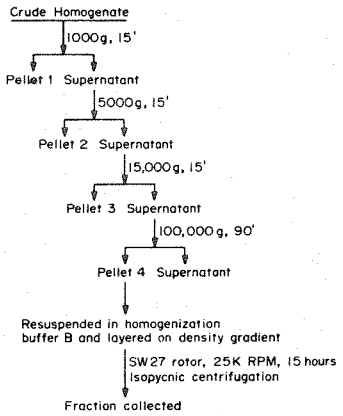


FIG. 1. Cell fractionation procedure.

Purification of Chitinase

Commercial chitinase was purified according to the procedure of Cabib and Bowers (4). The purified chitinase was heated to 70° for 10 min. No chitobiase activity was detectable in this enzyme preparation.

Electron Microscopy

Samples were fixed for 1 hour at room temperature in Karnovsky's fixative (4% formaldehyde, 5% glutaldehyde, 0.008% $CaCl_2$, 0.08 M cacodylate buffer, pH 7.4) overnight at 4°, dehydrated in ethanol (40%, 50%, 60%, 70%, 80%, 90%, 95%, and three times in absolute ethanol), infiltrated in propylene oxide-Epon araldite (50:50) and embedded in Epon-araldite resin (10 parts Epon 812, 10 parts araldite 6005, 24 parts dodecenyl-succinic anhydride and 2% DMP-30) at 60° for 24 hours.

Thin sections were stained with uranium acetate and lead citrate and then examined under a Philips 201 microscope.

Enzyme Assays

The enzymes 5'-nucleotidase, glucose 6-phosphatase and succinic-INT-reductase were assayed according to the procedures of Morré (5). Catalase was assayed with the method of Baudhuin *et al.* (6). Acid phosphatase was assayed according to the procedure of O. A. Bessey *et al.* (7).

Autoradiography

Specimen Preparation—Phycomyces sporangiophores were burst by the method of Oort and Roelofsen (8). The cytoplasm was removed by washing with homogenization Buffer I. The clear cell wall with the plasma membrane still attached was incubated in the standard chitin synthetase reaction mixture for 4 hours. It was then washed extensively in distilled water and mounted on a glass slide.

Autoradiography—The specimen mounted on a glass slide was subjected to autoradiography via the stripping film technique (9). The exposure time was 3 to 10 days. The film was developed and then studied under a phase contrast microscope.

Control experiments were done in exactly the same way except that 0.5 mM polyoxin D was added to the reaction mixture before the chitin synthetase reaction, this control experiment indicated the background contributions to the silver grain distributions.

TABLE I

Requirements for chitin synthetase

The enzyme fraction was prepared as described under "Plasma Membrane-rich Fraction Preparation", except that it was washed twice more with 10 mM Hepes, pH 7.2. The chitin synthetase assay was done under standard conditions except for the variations in the composition of the reaction mixture as indicated. The protein concentration of the enzyme preparation was 1.1 mg per ml.

Reaction mixture	[^{14}C]GlcNAc incorporated/mg protein
	cpm
Complete	84,000
Minus Mg^{2+}	8,000
Minus GlcNAc	21,200
Minus GlcNAc and 1 M glycerol	7,200
Minus Mg^{2+}, GlcNAc and 1 M glycerol	1,000

General Procedures

Protein concentrations were measured by the method of Lowry *et al.* (10). Radioactivity was measured with a Beckman liquid scintillation counter with Aquasol as the scintillation fluid. Column effluents containing free sugars were monitored by the Park-Johnson method (11). Sugars on paper chromatograms were located by the use of aniline diphenylamine spray (Sigma Company).

RESULTS

Properties of Chitin Synthetase

The properties of the chitin synthetase in the crude preparation resembled very much those of the chitin synthetase in the plasma membrane-rich fraction in all aspects that have been checked, *e.g.* the UDP and polyoxin D inhibitions, the effects of ATP and 3′:5′-cAMP on the enzyme, Mg^{2+} and GlcNAc activation, and chitinase digestion of the reaction products. To avoid repetition, only data from experiments using the plasma membrane-rich fractions as the enzyme preparation are presented here.

General Requirements for Enzyme Activity—The requirement for chitin synthetase activity was shown in Table I. Mg^{2+} and GlcNAc greatly stimulate[4] the enzyme activity. Compared to GlcNAc, glycerol is less effective in stimulating the enzyme activity. The enzyme activity increases with the increase of Mg^{2+} concentration in the reaction mixture, reaches maximum when the Mg^{2+} concentration is between 15 mM and 25 mM, and starts to decrease when the Mg^{2+} concentration is above 25 mM. The enzyme activity increases with the increase of GlcNAc concentrations in the reaction mixture and reaches a plateau when the GlcNAc exceeds 0.1 M.

Linearity The amount of chitin synthesized in the reaction mixture increases linearly with time for at least the first 2 hours. It also varies linearly with the concentration of enzyme in the reaction mixture, in the range of 0.5 mg per ml to 5 mg per ml of protein in the enzyme preparation.

Temperature Dependence—The optimal temperature for the enzyme activity is about 28° with half-maximal temperature at about 17° and 42°.

Buffer and pH Optimum—Four buffer systems, namely, Tris-HCl, imidazole-acetate, phosphate, and Hepes were used in the assay of chitin synthetase. The pH optimum is about 6.5 (Fig. 2). Hepes buffer was chosen in the standard assay procedure because it has negligible binding constants for divalent cations and its pH value is less sensitive to temperature changes.

[4] GlcNAc in the chitin synthetase assay mixture is not incorporated into chitin. Therefore, the effect of GlcNAc on chitin synthetase activity is regarded as "stimulatory."

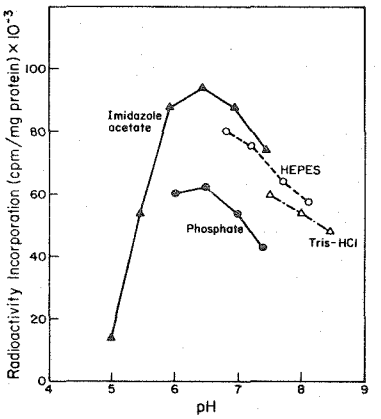


FIG. 2. Dependence of chitin synthetase activity on pH and buffer systems. The enzyme fraction was prepared as described under "Experimental Procedures" except that an aqueous solution containing 1 mM Mg^{2+}, 2 M glycerol, and 1 mM dithiothreitol was used to resuspend the material. The assay was done under standard conditions with the exception of the pH and the buffer used. All buffer solutions were used at a concentration of 50 mM. The protein concentration of the enzyme preparation was 1.1 mg per ml.

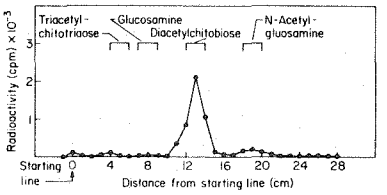


FIG. 3. Paper chromatography of the chitinase digested products of the ethanol insoluble radioactive reaction products. The result presented here is the chromatography by using 1-butanol-pyridine-water (6:4:3) as solvent.

Identification of Reaction Products—Reactions catalyzed by chitin synthetase were carried out in a reaction mixture that contained five times more material than those used in a standard assay procedure. The reaction was stopped by adding 9 ml of 66% ethanol. The reaction product which was insoluble in 66% ethanol was centrifuged at 100,000 × *g* for 90 min. The pellet was then resuspended in 0.3 ml of 0.05 M phosphate buffer, pH 6.3. An aliquot was incubated with 2 volumes of purified chitinase for 4 hours at 37° and then centrifuged (100,000 × *g*, 90 min). No radioactivity was found in the pellet. The supernatant was passed through an AG11A8 (Bio-Rad) column and eluted with water. The fractions containing radioactivity were pooled and concentrated and subjected to descending chromatography on Whatman No. 1 paper with 1-butanol-pyridine-water (6:4:3) or acetic acid-1-propanol-water (1:66:33) as the solvent. In both cases, the radioactive material migrated at the same rate as diacetylchitobiose (Fig. 3). Since the chitinase used digests chitin into diacetylchitobiose, the reaction product was identified as chitin.

Kinetics—The double-reciprocal plot of reaction rate *versus*

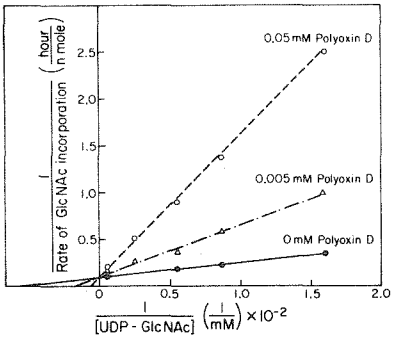


FIG. 4. Polyoxin D inhibition kinetics. The assay was done under standard conditions except for the variation in the concentrations of UDP-GlcNAc and polyoxin D.

substrate concentration is shown in Fig. 4. The apparent K_m for UDP-GlcNAc is 0.6 mM.

Polyoxin D Inhibition—The antibiotic polyoxin D inhibits the chitin synthetase activity in the cell-free system of *Piricularia oryzae* (12) and *Neurospora crassa* (13). The inhibition is competitive with respect to UDP-GlcNAc and specific for the chitin synthetase (13). Studies of the structure (14, 15) of the polyoxins indicate that the mode of action of their inhibition of the biosynthesis of cell wall chitin probably is connected to their structural similarity to UDP-GlcNAc (16). *In vivo*, polyoxin D inhibits the growth and causes swelling of the mycelia of many fungi (13, 17, 18).

In *Phycomyces*, polyoxin D competitively inhibits the chitin synthetase activity (Fig. 4). It also inhibits the mycelial growth of *Phycomyces in vivo* and causes the mycelium to swell. The concentration dependence of the *in vitro* inhibition of chitin synthetase activity is comparable to that of the *in vivo* inhibition of mycelial growth (19).

Effects of Various Chemicals—The inhibition of enzyme activity by polyoxin D is probably due to the structural similarity between the inhibitor and UDP-GlcNAc. Several nucleotide sugars with structures analogous to that of the substrate were also tested for their effects on the enzyme activity. All of UDP-glucose (1 mM), UDP-mannose (1 mM), UDP-xylose (1 mM), UDP-glucuronic acid (1 mM), and UDP-galacturonic acid (1 mM) were found to inhibit about 20% of the enzyme activity. UDP, one of the end products of the chitin synthetase catalyzed reaction, inhibited 75% of the enzyme activity at a concentration of 0.5 mM. ATP at a concentration of 0.25 mM raised the enzyme activity by 33%. However, at concentrations higher than 1 mM, ATP is inhibitory. 3′:5′-Cyclic AMP inhibited 20% of the enzyme activity at a concentration of 2 mM. At concentrations lower than 1 mM, it was ineffective.

Enzyme Activities in Different Developmental Stages of Phycomyces—The life cycle of *Phycomyces* contains distinct developmental stages. Germinated spores develop into mycelia. Two to three days later, sporangiophore growth is initiated. The development of the sporangiophore is conveniently divided into five stages (1). Extensive sensory physiology studies have been done on the Stage IVb sporangiophore (1, 2). Chitin synthetase activity was detected in mycelia growing in exponential phase as well as in sporangiophores in each of the five stages. To assay the enzyme in sporangiophores, the sporangiophores were cut in segments about 1 mm in length, the cytoplasm squeezed out, and the segments washed thoroughly with homogenization Buffer I. The remaining hollow tubes of cell wall with the plasma membrane attached were used as the cellular material in the assay. The properties of chitin synthetase in sporangiophores showed no apparent difference from the chitin synthetase in mycelia in all aspects that have been checked, namely, Mg^{2+} and GlcNAc stimulations, UDP and polyoxin D inhibitions, and effects of ATP and cyclic 3′:5′-AMP on the enzyme activity. Therefore, we assume that the chitin synthetase in sporangiophores and that in mycelia are the same enzyme.

TABLE II

Distribution of specific chitin synthetase activity

The cells were homogenized according to the procedure described under "Crude Enzyme Preparation." The homogenate was then fractionated through differential centrifugation (2,500 × *g*, 10 min; 15,000 × *g*, 30 min, and then 100,000 × *g*, 90 min). Each fraction was assayed for chitin synthetase activity under standard conditions.

Cell fractions	Specific activity[a]
2,500 × *g*, 10-min pellet	0.38
15,000 × *g*, 30-min pellet	0.38
100,000 × *g*, 90-min pellet	0.02
100,000 × *g* supernatant	0.01

[a] The specific activity is expressed as nanomoles of *N*-acetylglucosamine incorporated per min per mg of protein.

Cellular Localization of Chitin Synthetase

Distribution of Chitin Synthetase Activity among Cellular Fractions—The cytoplasm-free cell wall fraction with the plasma membrane still attached which was prepared as described under "Autoradiography" had specific chitin synthetase activity ten times that of the crude cell homogenates.

Mycelia homogenized according to Method A were fractionated through differential centrifugations. Each fraction was assayed for its chitin synthetase activity (Table II). The pellets after the 2,500 × *g* and the 15,000 × *g* centrifugations had high specific activities, while the microsomal and the soluble fractions had low specific activities. The two low speed pellets which contain high specific activities are rich in cell wall fragments to which plasma membrane is still attached.

Homogenization Method B gives a different fractionation of the cellular materials. As the mycelia or Stage I sporangiophore were immersed in homogenization Buffer II for a few minutes, the cytoplasm shrank and the plasma membrane became separate from the cell wall in some regions. This effect was presumably due to osmosis of the cell and did not occur when homogenization Buffer I was used. The distribution of specific chitin synthetase activity among cellular fractions is shown in Table III. The specific activity in the microsomal fraction is much higher than that in the microsomal fraction in Paragraph 2. This increase of the specific activity can be explained by the enrichment of plasma membrane fragments in this microsomal fraction.

Intact mycelia or sporangiophores, when incubated with the reaction mixture for the assay of chitin synthetase, incorporated less than 5% of the radioactivity that would be incorporated into chitin if instead of intact cells equal amount of broken cells were used.

Electron Microscopy—Electron microscopy was used to examine the contents of each cellular fraction listed in Table III with the exception of the soluble fraction. An aliquot from each fraction

TABLE III

Distribution of marker enzyme activities

The cells were fractionated according to the scheme shown in Fig. 1. The experimental details are given under "Plasma Membrane-rich Fraction Preparation" and "Enzyme Assays."

Cell fraction		Protein content mg	Chitin synthetase		5'-nucleotidase (plasma membrane)		Suc-INT-reductase (mitochondria)		Glucose-6-phosphatase (Endoplasmic reticulum)		Catalase (peroxisomes)		Acid phosphatase (lysosomes)	
			% total activity	Specific activity[a]	% total activity	Specific activity	% total activity	Specific activity	% total activity	Specific activity	% total activity	Specific activity	% total activity	Specific activity
1,000 x g 15' pellet		300	51.5	0.38	40.2	2.4	60.5	6.5	18.2	0.9	70	18.8	24.4	0.5
5,000 x g 15' pellet		42	28.8	1.5	43.3	18.5	20	15.3	50.7	15.0	13.8	22.0	44.4	4.9
15,000 x g 15' pellet		11.4	2.5	0.48	1.8	2.7	5.8	16.5	16.9	18.5	5.8	34.0	2.2	1.0
100,000 x g 90' pellet		44	17.2	0.85	14.7	6.0	14.2	10	14.2	3.9	10.4	16.0	29	3.0
--	sucrose gradient 10%	1.2	0.02	0.03	~0	~0	0.45	12.0	2.72	28.0	0.013	0.8	2.5	9.8
--	sucrose gradient 10%/30% interface	5.2	0.96	0.26	0.79	2.7	0.4	3.0	~0	~0	0.1	1.3	4.4	4.0
--	sucrose gradient 30%/50% interface	9	9.9	2.4	12.5	25.0	0.7	2.5	~0	~0	0.1	0.8	3.6	1.6
--	sucrose gradient pellet	3	0.09	0.06	~0	~0	0.44	47.0	7.5	30.8	0.11	2.5	--	--
oo														

[a] Specific activity for chitin synthetase is given as n moles N-acetylglucosamine incorporated per min per mg of protein. Specific activities for the other enzymes listed in Table III are given as n moles substrate degraded per min per mg protein.

[oo] The specific chitin synthetase activity of 100,000 x g 90' supernatant is 0.02 n moles N-acetylglucosamine incorporated per min per mg protein.

was centrifuged at 100,000 × *g* for 90 min. The pellets were then fixed, embedded, thin-sectioned, stained, and examined under an electron microscope as described under "Experimental Procedures" (5). The 30%/50% interface fraction which has the highest specific chitin synthetase activity contains mostly membranous structures and some ribosomes and glycogens. No cell wall fragments, nuclei, or mitochondria were found in this fraction (Fig. 5). The sucrose gradient pellet contained mostly ribosomes, glycogens, and small amount of membrane fragments. The 100,000 × *g* pellet appears in electron micrograph morphology to be somewhere between the above two fractions. The 1,000 × *g*, 5,000 × *g*, and 15,000 × *g* pellets are more heterogeneous in composition as revealed by electron micrographs.

Marker Enzyme Assay—To characterize further the nature of the membrane fragments observed in the electron micrographs, marker enzyme assays were done. The results are presented in Table III. In each assay, for each fraction, three different concentrations of protein in the range of 0.5 mg per ml to 5 mg per ml were used. In all those enzyme assays that were done under the conditions specified under "Materials" and "Methods," the appearance of reaction products or the disappearance of substrates was linearly proportional to the protein concentrations. The specific enzyme activity in any given fraction therefore could be normalized to the protein concentration used in the assay. The following features were observed (Table III).

1. The distribution of chitin synthetase activity correlated well with that of 5′-nucleotidase activity which is widely considered a plasma membrane marker in eukaryotes (20).

2. The distribution of succinic-INT-reductase, catalase, acid phosphatase, glucose 6-phosphatase activities clearly differed from that of chitin synthetase among the cellular fractions.

In summary, the observations concerning the cellular localization of chitin synthetase were:

1. The membrane fragments collected on the sucrose gradient from the 30%/50% interface have high specific chitin synthetase activity. The results of marker enzyme assays suggest that this activity is associated with plasma membrane fragments.

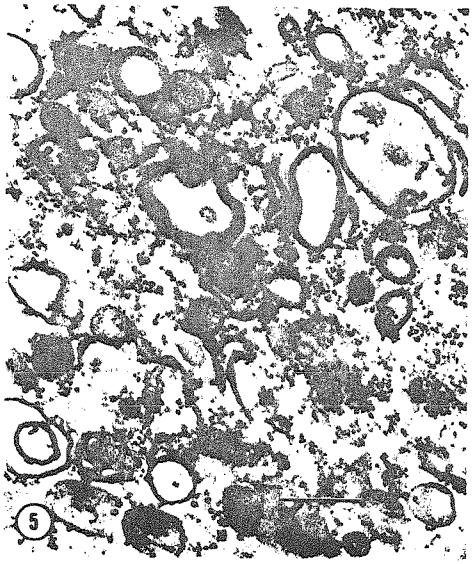
2. The cytoplasm-free cell wall fraction with the plasma membrane attached had both the inside of the plasma membrane and the outside of the cell wall exposed to the radioactivity substrates in the reaction mixture, and the specific activities observed were high. On the other hand, intact cells had only the outside of the cell wall exposed to the reaction mixture, and hardly any chitin was synthesized from extracellular substrates. This suggests that chitin synthetase probably is located in the plasma membrane in such a way that the intracellular UDP-GlcNAc can be used for chitin synthesis.

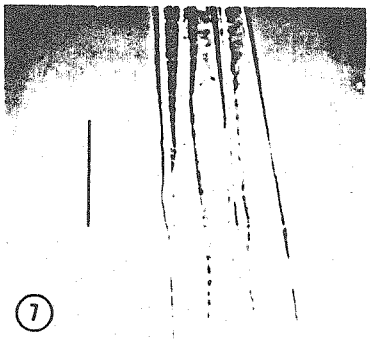
These results suggest that *Phycomyces* plasma membrane is rich in chitin synthetase activity. But the possibility that chitin synthetase activity also exists in other places in the cell such as in the cell wall cannot be excluded.

Autoradiography—Autoradiographic studies were done in pursuit of answers to the following questions of obvious interest. What is the distribution of chitin synthetase activity in the cell? Is the chitin synthetase activity localized only in the growing zone where cell elongation and hence measurable sensory responses occur? The result of autoradiography of Stage IVb sporangiophore is shown in Fig. 6. The columella had very low chitin synthetase activity. The activity is highest in the growing zone. However, there is still noticeable chitin synthetase activity below the growing zone where no cell elongation takes place. Autoradiography of Stage I and Stage II sporangiophores showed the same features, namely, there seems to be higher activity in the region where cell elongation takes place, such as the tip of Stage I sporangiophore and the columella of Stage II sporangiophore, and noticeable activity exists outside the growing zone as well. The polyoxin D control for Stage IVb is shown in Fig. 7. With polyoxin D added in the reaction mixture, very low background level of silver grains was observed. This proves that the distribution of silver grains observed in Fig. 6 indeed represents the distribution of the chitin synthetase activity and not random stickiness of substrates to the cell.

DISCUSSION

The properties of the chitin synthetase from *Phycomyces* resemble those of the chitin synthetase in other fungi (21–24). Mg^{2+} and *N*-acetyl-D-glucosamine stimulate the chitin synthetase activity. UDP inhibits the enzyme activity, and the antibiotic polyoxin D is a potent competitive inhibitor. Polyoxin D should be an ideal chemical for the selection of cell wall synthesis mu-





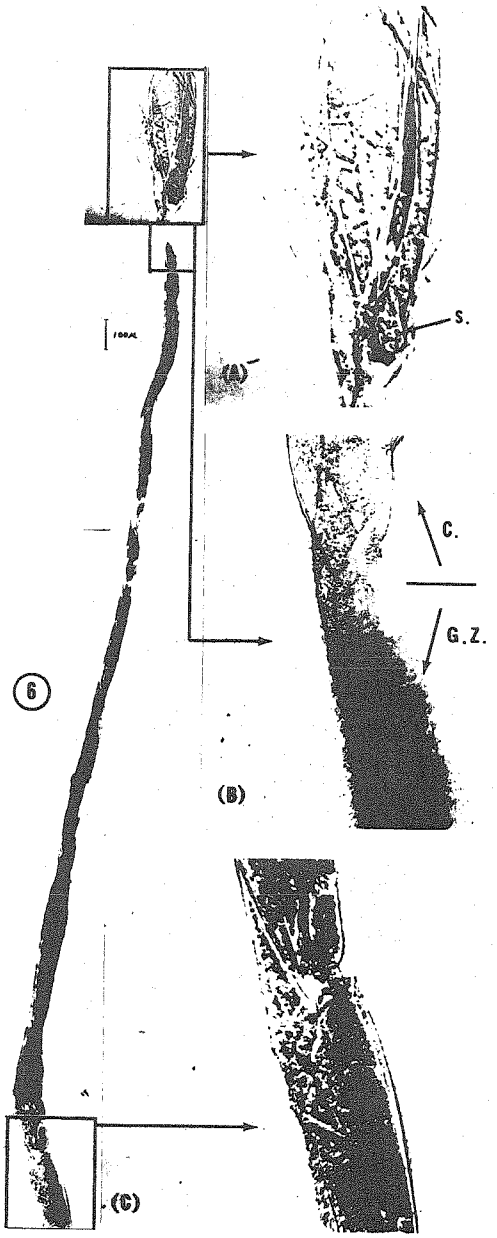


FIG. 5. Electron micrograph of the plasma membrane-rich fraction collected from the 30%/50% interface of the sucrose gradient. This fraction which has the highest specific chitin synthetase activity (see Table III) shows mostly membrane vesicles. Experimental details are given under "Electron Microscopy." The scale line is 0.4 μm. × 39,000.

FIG. 6. Autoradiography of a Stage IVb sporangiophore. Experimental details are given under "Autoradiography." The picture on the *left* shows the autoradiograph of the upper part of a whole Stage IVb sporangiophore. The structure enclosed in the *top box* is the columella. The growing zone extends from about 0.1 mm to almost 3 mm below the columella. Below the growing zone is the nongrowing zone. × 60. *A*, the columella shows very low level of radioactivity incorporation. Some spores (*S.*) still attached to the columella can be seen in the picture. × 160. *B*, the region between columella (*C.*) and growing zone (*G.Z.*) is shown here. The growing zone shows much higher chitin synthetase activity. × 600. *C*, a region of nongrowing zone just below the growing zone. Notice that it has lower but still significant level of chitin synthetase activity. × 210.

FIG. 7. Autoradiograph of the growing zone of a Stage IVb sporangiophore in the polyoxin D-control experiment. Here the autoradiograph was prepared in exactly the same way as the ones shown in Fig. 6 except that 0.5 mM polyoxin D was included in the reaction mixture to inhibit the chitin synthetase activity. The picture shows essentially no silver grains. This experiment confirms that the amount of silver grains shown in Fig. 6 represents the chitin synthetase activity. The scale line is 50 μm. × 600.

tants. It should also be useful in the counter selection procedure designed for selection of certain mutants for which positive selection methods are not available (19).

The *Phycomyces* cell wall outside the growing zone contains many more chitin fibrils than in the growing zone, although there is not much difference in the thickness of the cell wall throughout the sporangiophore (1). The growing zone, which is actively elongating, has high chitin synthetase activity. The nongrowing zone has lower but still significant activity. This suggests that chitin fibrils are continuously synthesized in order to strengthen the cell wall in regions where the elongation had already stopped.

Preliminary experiments by the author and M. Forgac have indicated that high intensity light increases the chitin synthesis in the crude mycelial homogenate. *In vivo*, *Phycomyces* shows a positive growth response to a step up in blue light intensity. The response is normally transient, except in the high intensity range, where the growth velocity increase persists (2). Two identical sets of reaction mixtures were prepared under standard conditions for chitin synthetase assay except for the light intensity. One set was exposed for 2 hours to intense blue light (20 milliwatts per cm^2 at 488 nm) provided by an argon ion laser. Care was taken to control temperature of the samples. The other set was maintained in darkness as a control. Chitin synthesis was about 30% greater in the blue light than in the dark. In similar experiments with the plasma membrane-rich fraction, no such difference in chitin synthesis between intense blue light and darkness was found. Sensitive spectrophotometric measurement did not show any noticeable absorption of light by this fraction in the range 360 nm to 500 nm (the crude mycelial homogenate does adsorb light in this region). Both observations could be explained if this fraction simply did not contain functional photoreceptors. This in turn would suggest that blue light does not affect the chitin synthetase directly. Our current working hypothesis of the molecular mechanism of the *Phycomyces* photoresponse is that after the photons are received by the photoreceptors, some secondary messengers are released. They would presumably be relatively small molecules that could diffuse readily to the site of the output machinery, such as chitin synthetase, and regulate its activity. Further studies of the blue light effect on chitin synthetase activity of photomutants in *Phycomyces* (2) should provide us more insights into the molecular mechanism of the *Phycomyces* photoresponse.

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Chapter 3

Additional data

(A) Light Effects

(1) Introduction:

Chitin synthetase was studied with the hope that it may yield an *in vitro* assay for sensory responses. The immediate question is therefore whether chitin synthetase activity is indeed affected by sensory stimuli. To answer this question, light was chosen as the sensory stimulus because it is easy to quantitate and also because the light growth response is by far the best characterized among all sensory responses. The effect of light on chitin synthetase activity was briefly mentioned in Chapter 2. It will be discussed in more detail in this chapter.

(2) Experimental methods:

(a) Culture methods are the same as described in Chapter 2.

(b) Preparation of chitin synthetase.

(i) Crude mycelial enzyme preparation: Twenty-four-hour-old mycelia were harvested from liquid culture and washed thoroughly with ice cold homogenization buffer I (10 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 1 mM dithiothreitol and 2 M glycerol). The washed mycelia were resuspended in about 2 volumes of homogenization buffer I and then homogenized by using mortar and pestle on ice until most of the cells were broken. This crude homogenate was used for chitin synthetase assay.

(ii) Crude stage 4b spph enzyme preparation. To assay the enzyme in stage 4b spph, the uppermost 1 cm of spph were cut into segments about 1 mm in length. These segments were resuspended in about

3 volumes of homogenization buffer I and used for chitin synthetase assay.

(iii) Plasma membrane-rich fraction preparation: same as described in chapter 2.

(c) Chitin synthetase assay:

To eliminate possible heating of the sample by high intensity light, all assay tubes (0.1 ml) were immersed in a glass beaker with 3 ml water. This arrangement does not alter significantly the optics but provides a large thermal reservoir for the maintenance of constant temperature. The chitin synthetase activities were compared among identically prepared reaction mixtures exposed to various intensities of light. Except for the variation of light intensity, the chitin synthetase assay was done as described in Chapter 2.

(d) Light source:

High intensity light (λ = 488 nm) was provided by a Coherent Radiation model 52G argon-ion laser (Coherent Radiation Lab, Palo Alto, California). The light intensities were measured with a Hewlett-Packard Radiant Flux Meter 8335A with a Model 8334A Radiant Flux Detector Option 013 with T19 Supersil 1 Optical Window (Hewlett-Packard Co., Palo Alto, California).

(B) Results and Discussions

(1) Effects of light on the chitin synthetase activity in crude mycelial preparations.

The result is shown in Table I.

(a) Chitin synthetase activity of wild-type mycelium was 21 ± 5%

greater when illuminated with the 20,000 $\mu W/cm^2$ 488 nm blue light than in the dark. No significant difference was found between assays in room light (10 $\mu W/cm^2$) and those kept in the dark. This result is compatible with the physiological data of stage 4b spph light growth response. A step up in light intensity from darkness to room light intensity causes a transient positive growth response. At the maximum of the response, the growth rate is about 1.6 times that at the steady state. This accelerated growth rate lasts for only about 6 minutes (Foster and Lipson, 1974). Therefore, in a period of two hours with light intensity stepped up from darkness to room light intensity, the total growth of spph is only about 3% more than the total growth of spph kept in the dark. A 3% difference is too small to be detected by our assay. However, a step up in light intensity from darkness to high intensity (20 $\mu W/cm^2$) causes a 30-40% increase in the steady state growth rate. Thus, in a period of two hours, the total growth of a spph in high intensity light is about 30-40% greater than that of a spph kept in the dark. It should be pointed out that the physiological data of the light growth response refer to the spph. No corresponding data about mycelia are available. It is known, however, that the Phycomyces mycelium does respond in other ways to blue light (1). So far, there is no evidence which suggests that the mycelium has a photoreceptor different from that of the spph.

(b) Temperature effect:

The temperature optimum of chitin synthetase activity is about 28°C (Chapter 2). Therefore, it is conceivable that high intensity light

could raise the temperature and thus increase the chitin synthetase activity. To eliminate this possibility, the assay was done in a setup, described in the method section, designed to prevent the sample from heating. Actual measurement indicates a temperature difference less than 0.1°C between samples in the high intensity light and in the dark. However, one can still reason that although the gross temperature of the sample does not rise in the high intensity light, the local temperature near the photoreceptor and/or chitin synthetase could be higher in the high intensity light. This possibility was ruled out by the study of an albino strain C2. C2 has a β-carotene concentration more than one hundred times lower than that of the wild-type. Therefore it absorbs much less light at λ = 488 nm as compared to wild type. If the light effect on chitin synthetase observed was due to local heating, the light effect should be much less in C2 than that in wild type. However, data shown in Table 1 indicate that the light effect on chitin synthetase activity of C2 is the same as that of wild type within statistical error. Thus, it is concluded that the light effect on chitin synthetase activity is not a temperature artifact.

(c) Study of a photomutant:

A direct test for the physiological relevance of the light effect on the chitin synthetase activity is a study of the effect on photomutants. If high intensity light has no effect on the chitin synthetase activity of blind Phycomyces, this would be a good support to the notion that the light effect is closely related to Phycomyces' light responses. Unfortunately, none of the mad mutants available up to date are completely blind. They all give significant positive growth

response to the high intensity light used in this experiment. Nevertheless, one of the mad mutants, C149, was studied. The chitin synthetase activity of C149 mycelium was 16 ± 6% greater in the high intensity light than in the dark. Thus, the light effect on chitin synthetase activity might be less in the case of C149 than in wild type. The difference, however, is too marginal to allow a clear cut conclusion.

(2) Partially purified chitin synthetase preparations:

If the light effect on chitin synthetase described in section (1) is indeed physiologically relevant, then, either the chitin synthetase is directly regulated by light or it requires concerted actions of several molecules, including, e.g., the photoreceptor molecules and molecules which relay the message to the chitin synthetase. The preparation used in the experiments described in section (1) is the crude mycelial homogenate. It probably still contains all the essential molecules in the light growth pathway. Therefore, it is important to examine the light effect on partially purified chitin synthetase preparation to obtain some insight about additional components required for the manifestation of the light effect.

Two preparations were studied.

(a) Dialyzed mycelial homogenate:

The crude mycelial homogenate was dialyzed overnight against an excessive amount of homogenization buffer I at 4°C. The dialyzed material was assayed for chitin synthetase activity in high intensity light and in the dark. No statistically significant light effect was found.

Table I

Light Effect on Crude

Mycelial Chitin Synthetase Activity

Strain/Ratio	$\frac{(S.C.S.A.)^{(1)}_{I=20000\ \mu W/cm^2}}{(S.C.S.A.)_{I=0}}$	$\frac{(S.C.S.A.)_{I=10\ \mu W/cm^2}}{(S.C.S.A.)_{I=0}}$
NRRL 1555(-) (wild type)	1.21 ± 0.05[2] (16)[3]	1.01 ± 0.05 (4)
C2	1.24 ± 0.08 (5)	--
C149	1.16 ± 0.06 (4)	0.99 ± 0.02 (4)

[1] S.C.S.A. = specific chitin synthetase activity

[2] Standard deviation of the mean

[3] Number in parenthesis indicates the number of experiments

(b) Plasma membrane-rich fraction:

The ratio of specific chitin synthetase activity in high intensity light and that in the dark is 0.99 ± 0.05. Again, it is not a statistically significant difference.

These experiments suggest that light does not affect the chitin synthetase directly. The experiment with dialyzed material suggests that dialysis either removes some small molecules which are essential in the manifestation of light effect or inactivates some essential molecules in the pathway.

(3) The crude stage IVb spph chitin synthetase prepared as described in the method section was also tested for light effect. The ratio of specific chitin synthetase activity in high intensity and in the dark was 1.05 ± 0.03, i.e., a light effect of marginal significance. However, the lack of a significant effect can not be taken as a definite indication that light has no effect on the stage IVb spph chitin synthetase activity, because in stage IVb spph, cytoplasm only contributes to a small percentage of the total volume. The rest is the central vacuole which presumably has nothing to do with the light sensory pathway. Thus, unlike the case of mycelial homogenate, in the homogenate of stage IVb spph, the cytoplasm accounts for only small percentage of the total volume. This might account for the lack of a significant light effect in stage IVb spph preparation.

(4) Working hypothesis of the molecular basis of Phycomyces light growth response:

The experimental results so far described lead to the following working hypothesis about the molecular basis of the Phycomyces light growth response:

Upon the absorption of photons by some photoreceptor molecules, certain photochemical events take place which affect the release of some second messenger. These second messengers may be small molecules that diffuse readily to the site of the output machinery including chitin synthetase, and regulate its activity.

A main feature of this hypothesis is the assumption that there exists second messengers which relay the information from input to output. The reason for this assumption is the following:

Phycomyces is a very large cell. After its sensors detect stimuli, how is the message relayed to the site of the output of the S.T.P.? It was shown (unpublished experiments by M. B. Willard and M. Delbrück and others) that there exists a resting potential between the inside and the outside of the spph submerged in water of low salt concentration minus 80-90 mV inside, similar to Neurospora. An attractive speculation is that Phycomyces might make use of this membrane potential to relay the information as in the case of a nerve axon. However, membrane potential changes associated with sensory stimuli have been looked for but have never been observed in Phycomyces. Therefore, the second messenger hypothesis seems to be a plausible alternative.

(B) Effects of Various Chemicals on Chitin Synthetase Activity

The proposed working hypothesis presumes the existence of second messengers which regulate the chitin synthetase activity. Several low molecular weight molecules known as messenger molecules in other organisms were tested for their possible effects on chitin synthetase activity.

(1) Cyclic 3',5' AMP and cyclic 3',5' GMP:

cAMP has been shown to be a key regulatory effector in numerous cellular processes such as the transcription of Lac operon in E. coli (2) and the mediation of hormone action in animals (3). Our working hypothesis, in fact, is formulated in analogy to the two messenger theory of hormone actions (4). More recently, cGMP has been shown to be another important regulatory effector in a number of cellular processes. Goldberg et al. (5) proposed an antagonism between cAMP and cGMP as a general phenomenon.

The effect of cAMP on Phycomyces chitin synthetase is described in Chapter 2. At concentrations lower than 1 mM cAMP is ineffective. At concentrations higher than 1 mM, it is slightly inhibitory (~20% inhibition at 2 mM). This effect is rather small considering the concentration used. The intracellular concentration of cAMP in Phycomyces is about 2 μM (Y. N. Jan and M. Forgac, unpublished data), a thousand-fold less than the concentration required to cause an effect on in vitro chitin synthetase activity. It can be argued that there might be some cAMP-p-diesterase activity in the cellular material which degrades cAMP.

If this is the case, cAMP-p-diesterase inhibitor added together with cAMP should give a more pronounced effect. In some higher organisms theophylline is an inhibitor of cAMP-p-diesterase. Theophylline alone at 2 mM decreases the chitin synthetase activity by about 40%. Together with 2 mM cAMP, they decrease the chitin synthetase activity by about 60%. However, there is no published information concerning Phycomyces' cAMP-p-diesterase and inhibitor. Therefore the significance of the effect of cAMP on Phycomyces chitin synthetase needs further investigation.

Cyclic GMP and dibutyl-cGMP at concentrations ranging from 10^{-10} M to 10^{-4} M have no significant effect on chitin synthetase activity. In most known cases, the intracellular concentration of cGMP is about one-tenth that of cAMP . Therefore we expect the cGMP concentration in Phycomyces to be of the order of 10^{-7} M, which is within the tested range. Thus, cGMP is not likely to be a candidate of the proposed messenger in our scheme.

(2) Acetylcholine and Gaba:

Those neurotransmitters have no significant effect on chitin synthetase activity at concentrations ranging from 10^{-4} to 10^{-6} M.

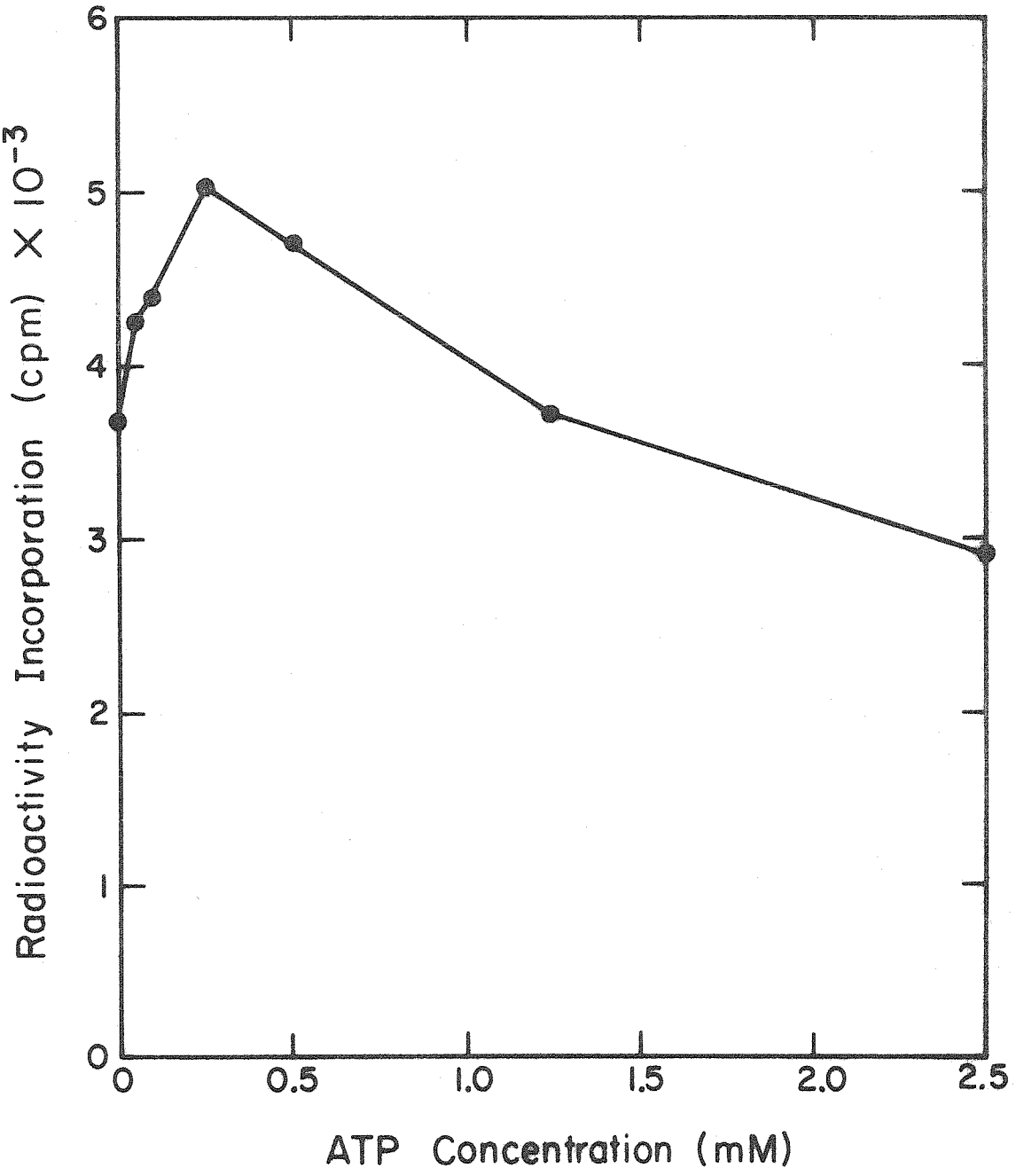
(3) ATP:

Using a crude young mycelial homogenate preparation, T. Leighton (personal communication) discovered that ATP, ADP and AMP at concentrations below 1 mM (but not CTP, GTP, UTP, CDP, GDP, UDP, CMP, GMP, or UMP) stimulate the enzyme. The stimulation is not phosphorylation, or Mg^{++} chelating but appears to be an allosteric effect.

Leighton also found that the chitin synthetase of Class II mad mutants (which are probably output mutants because they are abnormal in all sensory responses) have chitin synthetase with altered heat lability and ATP-stimulation properties. Leighton suggests that ATP might be involved in the regulation of chitin synthetase. We have confirmed the ATP effect on chitin synthetase with the plasma membrane-rich fractions. The results are shown in Fig. 1. ATP is stimulatory at concentrations below 1 mM and inhibitory at concentrations higher than 1.25 mM.

Fig. 2. Dependence of chitin synthetase activity on ATP concentration

The enzyme fraction was prepared as described in Chapter 2. The assay was done under standard conditions except the variation in the ATP concentration. The protein concentration of the enzyme preparation was 1.1 mg per ml.


Radioactivity Incorporation (cpm) X 10^{-3}
0
1
2
3
4
5
6
0
0.5
1.0
1.5
2.0
2.5
ATP Concentration (mM)

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Chapter 4

Discussions

(A) Working Hypothesis of the Molecular Basis of *Phycomyces* Light Growth Response

The experiments discussed in the preceding chapters characterize the properties of the enzyme chitin synthetase in *Phycomyces*. The most interesting finding perhaps is that the enzyme activity can be affected by light *in vitro* and that this effect is not due to heating. Thus, this enzyme may fulfill the need for an *in vitro* response system. If this turns out to be the case, the chitin synthetase assay would be very useful in dissecting the molecular basis of the sensory transduction processes. A list of questions can be formulated to characterize the problem of the molecular basis of S.T.P. more clearly:

(1) What kind of a molecule is the photopigment?

(2) What organelles contain the photopigment?

(3) What chemical reaction happens after a light quantum has been absorbed?

(4) How is the information introduced by this primary photochemical event amplified in a controlled manner?

(5) How does this information get to the output end?

(6) What kind of molecules form the output machinery?

(7) Where is the output machinery localized?

(8) How is the output machinery regulated?

(9) How does adaptation to a wide range of light intensities work?

(1) Input end:

The first three and the last questions deal with the input end of the pathway. Despite considerable efforts, previous attempts to identify and isolate the light receptor have been unsuccessful (1). However, a sensitive new technique for detecting photopigments became available recently. It involves the measurement of light induced absorbance change (LIAC) (2). A LIAC with its action spectrum similar to that for the "blue light response" in other organisms was found in the cells of Dictyostelium discoideum (2) and in the mycelium of Neurospora crassa (3). The difference spectrum of the mycelium of N. crassa before and after the blue light illumination indicates that photoreduction of a b type cytochrome is caused by light (3). In D. discoideum a pigment protein which undergoes a reversible LIAC was purified about 2000-fold from a mitochondrial fraction. Recently, similar LIAC have been found in Phycomyces mycelium (unpublished data of Lipson and Jan in collaboration with Poff and Butler at University of California, San Diego). This technique seems promising for the detection of the Phycomyces photoreceptor pigment(s). Tracing of the LIAC in the fractionated cells might lead to the answer of our second question, i.e., what organelles contain the photopigments. The work of A. Jesaitis (5) provided some insights as to the answer of our second question. It was found that Phycomyces spphs have 15-20% greater sensitivity of light polarized transversely than longitudinally at 456 and 486 nm. By exclusion of other causes this effect has been shown to arise from a linearly dichroic and oriented photopigment.

For the photopigments to exhibit a preferred average orientation, they have to be bound to certain oriented organelles and/or membranous structures. Thus we come to the following tentative answers to the first two questions:

(1) Flavoproteins, possibly coupled to cytochrome, are favored candidates for the photopigments.

(2) The photopigments are probably located either on plasma membrane or in the mitochondria. (In Phycomyces spph the mitochondria are oriented in parallel to the direction of the longitudinal cytoplasmic streaming.) Mitochondria are favored candidates because they were implied to be the locus of the photopigments of D. discoideum.

(2) Intermediate steps:

Nothing is known about the intermediate steps of Phycomyces S.T.P. The following is a speculation based on the very limited information about the input and output ends of the S.T.P. Suppose that chitin synthetase is an important component of the Phycomyces output and is regulated by ATP and/or cAMP, and mitochondria contains the photopigments; then the regulation of ATP synthesis appears to be an important link of the information processing between the input and output. An analogy might be drawn between this speculation and the case in Halobacterium.

The purple membrane of Halobacterium halobium contains only one protein, bacteriorhodopsin, which closely resembles the visual pigments of animals. Cells growing at low oxygen tension start the synthesis of purple membrane. In these purple membrane-containing

bacteria, in the absence of any apparent sources of energy, a proton gradient is generated and maintained across the cell membrane as long as they are exposed to light (6). Oesterhalt and Stoeckenius postulated that the light generated proton gradient arises from a vectorial release and uptake of protons by the bacteriorhodopsin. The gradient may play the central role in the energy coupling process of Halobacteria as suggested in Mitchell's chemiosmotic theory. It has been found (7) that purple membrane patches incorporated into phospholipid vesicles can catalyze light driven proton uptake and ATP synthesis. Thus, this photoreceptor membrane of halobacterium provides an example where reception of light probably induces the establishment of a proton gradient and the synthesis of ATP.

(3) Specific working hypothesis:

Based on the information and speculations so far described, a more specific working hypothesis is now formulated:

After light quanta are absorbed by photoreceptor pigment molecules, assumed to be located in the mitochondria, some chemical reactions take place. As the result of these reactions, second messenger synthesis and/or related pathways are affected. The end products (ATP and/or cAMP) diffuse to the output site (presumably including chitin synthetase) and regulate the output.

(B) Where Do We Go From Here?

This hypothetical scheme of the molecular basis of the light growth response is specific enough for direct experimental tests and thus provides a basis for designing future experiments.

(1) The physiological relevance of light effects on chitin synthetase activity should be established unequivocally:

With the present assay system, the effect of light can be detected only with high intensity light. Thus it presents technical difficulties to determine the action spectrum for the light effect on enzyme activity. Another approach is to look at the effects of light on chitin synthetase activity of blind mutants. Absence of the *in vitro* light effect on chitin synthetase would support the notion that the light effect on the enzyme is relevant to growth response. To counter this possibility of a fortuitous correlation, phenotypical revertants of the blind mutants should be screened for. The revertant should regain the light effect on its chitin synthetase activity.

Unfortunately, among over 100 *mad* mutants surveyed (8, E. Lipson, personal communication), not one meets our requirement of being completely blind at high intensities. This may be due to the fact that all our *mad* mutants are obtained by NTG mutagenesis. The types of mutants expected from NTG treatment are sufficiently leaky to allow photoresponses at high intensities (8). Therefore, unless total blindness is lethal in *Phycomyces*, mutants blind at high intensities might be obtained by other mutagenesis procedures such as the induction of frame shift mutations with an intercalating mutagen like ICR 191.

(2) ATP and cAMP:

The hypothesis postulates that ATP (and/or cAMP) are the second messengers or are involved in the pathway of second messenger synthesis. This should be experimentally testable.

(a) ATP:

The effect of light on the concentration of ATP has been previously investigated. There were large day-to-day variations in the ATP concentrations as measured in small numbers (ten) of sections of stage IVb spph including the growing zone. This finding probably is due to the rapid turnover rate of ATP, estimated as a few seconds. No significant light effect on ATP concentration was observed. These experiments should be repeated with the following modifications:

(i) Larger sample sizes should be used to decrease the variability due to a difference among individual spphs.

(ii) The ATP extraction procedure should be modified, e.g., the cell could be homogenized in cold TCA to prevent variability due to rapid ATP turnover.

(iii) Instead of stage IV spphs, young germlings could be used, because the ratio of growing part to nongrowing part in germlings is much higher.

(b) cAMP:

Preliminary measurements of cAMP, using Gilman's protein kinase binding assay (11) (Jan and Forgac, unpublished data), indicate that light decreases the cAMP concentration in stage IVb spph. The concentration decreases by about 30% within 2 minutes after the onset

of a step up in light intensity from darkness. The concentration returns to the normal dark adapted level within 15 minutes if the light is stepped up to 10 $\mu W/cm^2$. The concentration stays at 30% lower than the dark-adapted level for at least 30 minutes if the light is stepped up to 20000 $\mu W/cm^2$. The time course of this light effect is analogous to the light growth responses of Phycomyces, i.e., a transient response to a step up of intensity to 10 $\mu W/cm^2$ and a steady state response to a step up of intensity to 20000 $\mu W/cm^2$.

Similar findings of reduced cAMP concentration by light was also found in the photoreceptors of the frog (12).

(3) Chemical transfer experiments:

If the physiological relevance of light effects on chitin synthetase activity could be established, the chemical transfer experiment described below would be of great interest:

(a) Identification of second messenger molecules:

Of two identical cultures of Phycomyces cells, one is kept dark-adapted while the other is exposed to light. After the light/dark treatment, each culture is quickly homogenized in cold TCA to denature all the proteins. After TCA is removed (e.g., by ether extraction), this pair of homogenates can be tested on a partially purified chitin synthetase preparation to see whether there is a differential effect on the enzyme activity. If a differential effect is detected, this light/dark pair of homogenates can be fractionated by various biochemical techniques to pin down the chemical responsible for the difference. These chemicals would be candidates for second messengers.

(b) In vitro complementation tests for light growth response mutants:

Once the appropriate photomutants are available, various combinations of cellular components from wild type and blind mutants can be investigated for the light effect on chitin synthetase activity. This would provide a means to identify the chemical lesions of the photomutants, thereby serving as a method of dissecting the light growth response pathway.

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Part II

The Avoidance Response, the House Growth Response, and the Rheotropic Response of *Phycomyces*

1. Introduction

If an object is placed about one mm from the growing zone of a sporangiophore growing in air, in about 2 minutes the sporangiophore starts to bend away at about 2°/min for as long as half an hour or more. At no time during this response is any contact made with the object. This behavior has been called the avoidance response.

The avoidance response was first described by Elfving (1) in 1881 and extensively studied by him and by Steyer, Errera, Jost, Slotte and others (reviewed by Elfving (2)). It was rediscovered by Shropshire in 1962 (3) and studied during summer workshops at Cold Spring Harbor, 1965-1968. Through these studies, many facts about this response have been established. These were described in the 1969 Phycomyces review (3), and at that time summarized as follows:

"So far, only negative evidence is available as to the source of information for the sporangiophore. The following facts appear to be definite
(i) If the sporangiophore is placed between two closely opposed barriers or inside a tube with internal diameter of a few millimeters it shows a transient positive growth response.
(ii) The avoidance response occurs in complete darkness.
(iii) It occurs at 100% humidity.
(iv) Neither the material nor the color of the barrier have a strong influence on the response: glass, wood, plastic, black tape, or a crystal, transparent for infrared radiation of a black body at room temperature, are equally effective.
(v) The solid barrier can be replaced by a glass rod (diameter, 150 μm), by a horizontal human hair (diameter, 75 μm) or by a horizontal silk thread (diameter, 15 μm). In the experiments with horizontal cylindrical objects, the latency is independent of the diameter of the object, but the thinner the object the closer it has to be placed and the more localized is the response. Heating a horizontal copper wire anywhere between 0.1°C and several °C does not modify the effect."

Based on these observations, visible light or ultraviolet radiation could be excluded as the stimulus for the avoidance response. Beyond this, however, nothing conclusive could be said about the mechanism underlying the avoidance response. For example, although qualitatively the sporangiophore avoided barriers of various colors or materials placed at the same distance from the sporangiophore, it was not shown in those experiments whether these barriers are quantitatively equally effective in causing the response. Therefore, these data leave open the question whether infrared radiation or electrostatic forces, for instance, might play an important role.

The above mentioned experiments, although they failed to prove how the sporangiophore senses the barrier, nevertheless suggested the following hypothesis:

A volatile growth effector is emitted by the organism. The barrier causes a concentration gradient across the sporangiophore and therefore the differential growth rate. Bilateral barriers result in symmetric changes in concentrations, and hence cause a transient growth response.

This hypothesis will be referred to as the chemical self-guidance hypothesis (CSGH). In recent years the CSGH has dominated the research on the avoidance response and most of the efforts have been centered on testing and modifying this idea.

Since 1968, the study of the avoidance response was continued independently by D. L. Johnson and R. I. Gamow at the University of Colorado, and by R. Cohen, J. Matrican, M. Delbrück, and the author

at the California Institute of Technology. None of the Caltech results have been published. We will quote freely from this material, where appropriate.

Johnson and Gamow (4), in an important study of the avoidance response, published in 1971, concluded that the response is independent of the orientation in relation to gravity, does not occur in still air, and that its manifestation requires both the movement of air and a barrier. They proposed that a growth stimulating gas, probably water vapor, is emitted from the sporangiophore and that the movement of air and the barrier combine to cause a defined asymmetry of the gas which in turn induces an asymmetry in growth. These authors were the first to recognize the importance of random winds, which we confirm. We disagree with them on the proposal that water vapor is the gas in question and show that exceedingly small air movement in combination with a barrier are sufficient to induce an avoidance response. The following is an account of our efforts in attempting to answer the basic question: "How does the sporangiophore sense a nearby object?"

2. General Methods

Unless otherwise stated, the following is the standard method used in all experiments.

(I) Culture conditions

Sporangiophores of wild type Phycomyces strain NRRL 1555(-) were grown in shell vials (12 mm diameter x 35 mm high) containing 4% potato dextrose agar (Difco) and 5 µg/ml thiamine (Sigma Co.). To produce healthy sporangiophores, an average of 5 heat shocked spores were inoculated into each vial. The vials were incubated enclosed in glass jars at 22 ± 1°C with overhead diffuse white light of intensity about 10 $\mu W/cm^2$ until the first crop of sporangiophores appeared. The vials were then removed from the jars and incubated in a light box at 22 ± 1°C with overhead illumination (a few $\mu W/cm^2$). The box was humidified to 60-80%. The sporangiophores were plucked daily so that a fresh crop was ready the next day. Usually only the second, third and fourth crops of 2-3 cm long stage 4b sporangiophores were used for the experiments.

(II) Physiological experiments

At least 30 minutes before each experiment, a vial containing a 2-3 cm long vertical (straight) sporangiophore was selected and transferred to the experimental setup. Thirty minutes usually is long enough for the sporangiophore to adapt to the new environment and to reach a steady state of growth.

For growth measurement, the position of the top of the sporangium was measured to ± 2 μm using a measuring microscope fitted with a Filar micrometer (Gaertner Science Corp., Chicago, Illinois). The angular deviation of the sporangiophore from the vertical was measured using a goniometer accurate to ± .5°. A Sony Videorecorder model PV 120 U modified for time lapse (1:60) recording proved convenient for experiments where only the bending of the sporangiophores is of interest. All the experiments were carried out between 21° and 25°C either under overhead diffuse white light of intensity about 10 $\mu W/cm^2$ or in the darkroom illuminated with physiologically inactive red light.

(III) Apparatus for the avoidance response

The design is shown in Fig. 1. The basic idea of the design is to allow fine movement of the sporangiophore and the barrier while keeping the chamber air tight.

The sporangiophore stands in the middle of this air tight chamber made of transparent lucite. The vertical position of the sporangiophore can be adjusted by a micrometer screw A. The horizontal positions of two parallel barrier mountings are independently controlled by micrometer screws B and C. Barriers made of different materials are attached to the barrier mountings. Unless otherwise stated, barriers are 2.2 cm x 2.2 cm lucite or cover glass. The standard size of the chamber is 6.2 cm x 6.2 cm x 6.2 cm. Chambers of similar design but different size have also been used.

(IV) Apparatus for wind experiments

Unless otherwise stated, pumped room air (diaphragm pump) was used as air current source. Tygon tubings and glass tubings were used to conduct the air stream. The air flow rates were measured by calibrated flowmeters (Matheson #R615B and R615A). Observation wind tunnels were made of glass constructed so as to insure laminar flow of the air stream (Fig. 2).

For some of the experiments the humidity was regulated by directing the air stream through various salt solutions (e.g., saturated NaCl solution provides air with relative humidity $\sim$ 65%, and distilled water provides air with relative humidity close to 100%). These procedures are detailed in the text and in the figure captions.

(V) Measurement of air movements

Velocities between a few mm/sec and 150 cm/sec can be estimated by timing the motion of cigarette smoke. For velocities lower than a few mm/sec, the following method is used: Cigarette smoke is introduced into the apparatus in which the velocity of the air movements is to be determined. The velocities of smoke particles are determined by measuring the time for the particles to cross a transverse HeNe laser beam about 1 mm in diameter. The laser (Spectra physics, operated at physiologically inactive wavelength 632.8 nm) has a low output power (0.5 mW) such as not to heat up the apparatus to induce convection, but is bright enough to allow the smoke particles to be seen. The velocity of such a particle falling freely under gravity is insignificant. This velocity was determined by timing the particle

movement near a horizontal barrier within a house. In this geometry the vertical component of air convection near the surface of a horizontal barrier is negligible. Therefore the vertical component of the movement of the particle provides a measure of its free fall velocity. The velocity was found to be at most a few μm/sec.

Fig. 1. The standard apparatus for avoidance response experiments

The apparatus is made of lucite. Legend: B, bearing; BA, barrier; B.M, barrier mounting; M, micrometer; S, sporangiophore.

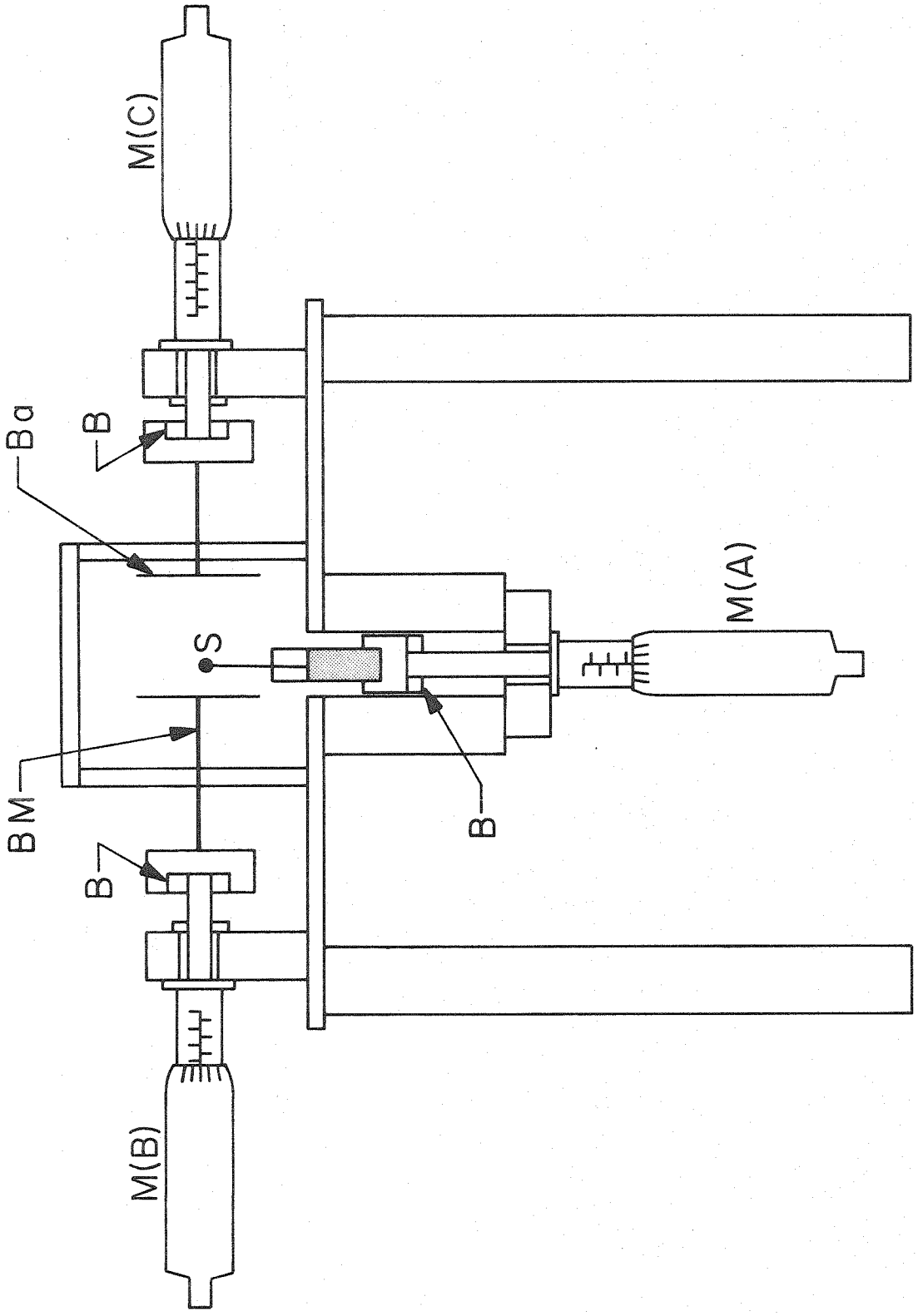
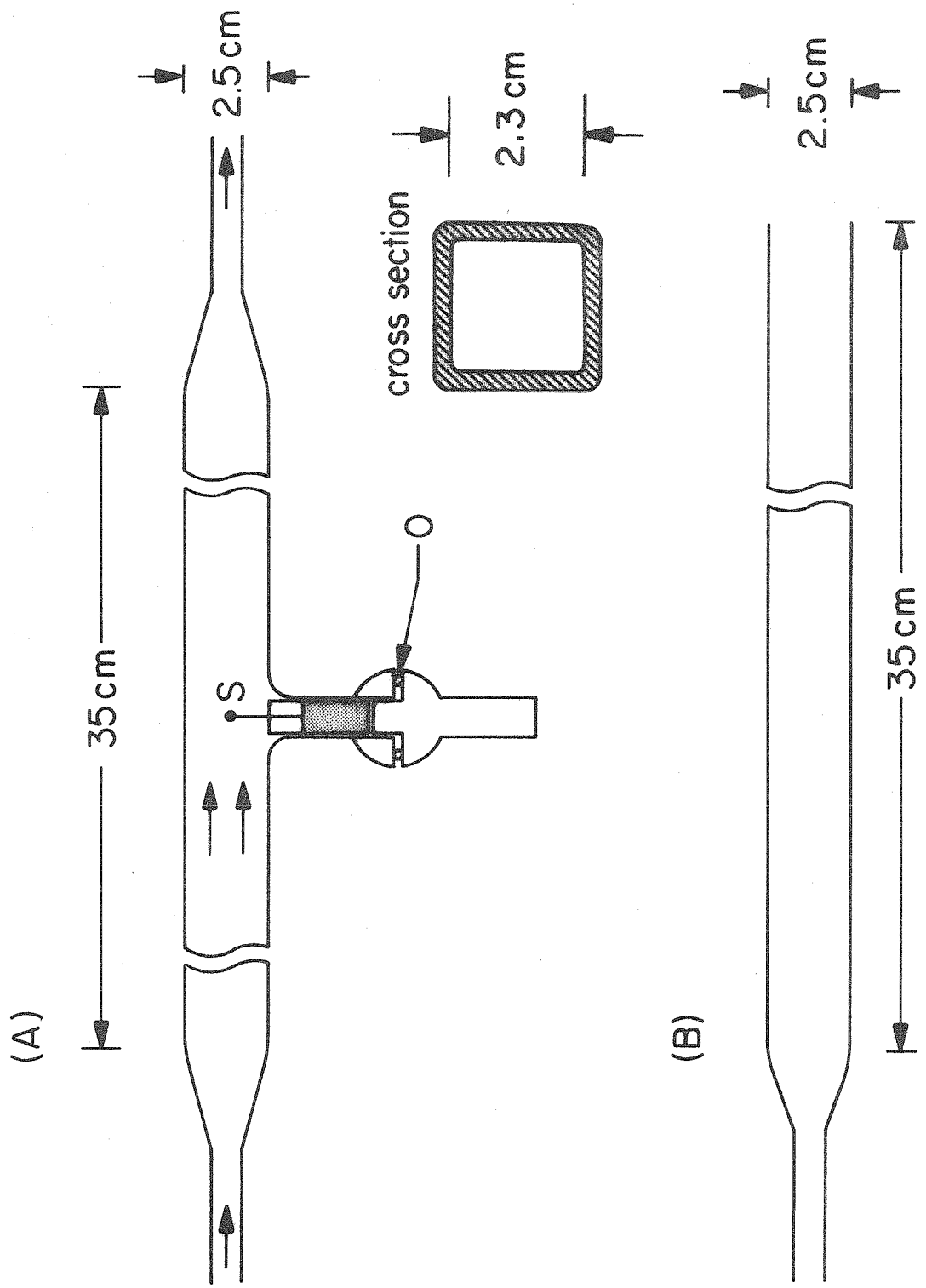

M(C)
Ba
B
M(A)
S
BM
B
B
M(B)

Fig. 2. Wind tunnels

The wind tunnels are made of glass. Legend: O, o-ring; S, sporangiophore.


(A)
35cm
2.5cm
S
O
cross section
2.3cm
(B)
2.5cm
35cm

3. Experiments, Results and Interpretations

I. Outlines of the experiments and interpretations

The present working hypothesis for avoidance is less explicit than the Chemical Self-Guidance Hypothesis. It consists of three postulates: (1) The sporangiophore possesses a very sensitive mechanism for detecting air movements; (2) Slower air movement, in any direction, around the growing zone promotes a positive growth response; (3) The effect of barriers is to slow down air movements. Thus a single barrier will produce a gradient of the air movements in the vicinity of the sporangiophores growing zone causing a sporangiophore to bend away from the barrier. Evidence will be presented to support these postulates. A brief abstract is first given to provide an overall view of the strategy.

The original intent of our study was to understand the avoidance response, but it soon became evident that air movements play an important role. Therefore, the air current (wind) effects had to be characterized first. It was found (Section IIA) that the sporangiophore grows into the wind. This effect will be referred to as rheotropism. The bending rate decreases monotonically with decreasing wind velocity and becomes insignificant for wind velocity below 1 cm/sec. This observed effect is compatible with our hypothesis, since a faster lateral wind velocity causes a greater difference of the gradients of the tangential velocity of air movement between the leeward and the windward side of a sporangiophore, and hence a faster

bending rate. Another wind effect is the wind growth response (Section IIB). The sporangiophore shows a transient negative growth response to a step up in wind velocity both for longitudinal and lateral winds. This effect is quite prominent at lateral wind velocities of 3-10 cm/sec, where the tropic response is barely noticeable. This finding is also compatible with our hypothesis, since the step-up in wind velocity is large compared to random air motions due to imperfections in thermostasis.

The strongest support of the notion that wind plays the crucial role in the avoidance response is the observation that an air current (15-30 cm/sec) parallel to the barrier and the sporangiophore can eliminate the avoidance response (Section III). The interpretation of this experiment is that the superimposed wind is much faster than random winds in the vicinity of the sporangiophore and the barrier. Therefore the difference in air movements around the sporangiophore caused by the barrier becomes a much smaller fraction of the absolute magnitude of the air movements, and is insufficient to cause an avoidance response.

Since the air movements in the laboratory vary randomly from time to time and can introduce uncontrolled rheotropic responses, our quantitative studies of the avoidance response were almost exclusively done in closed chambers (Section IV). The dependence of the avoidance response on the distance (Section IVC) as well as the size (Section IVD) of the barrier was established. Qualitatively, the dependence is correlated with the degree of asymmetry of air movements around the sporangiophore caused by the barrier.

Various substances including activated charcoal were tested for their effectiveness in causing the avoidance response. They all act alike (Section IVB). This suggests that the barriers act purely by their aerodynamic effects, not by adsorbing or reflecting a gas. The possibility of a barrier adsorbing or reflecting radiation, or inducing electrostatic effects can also be ruled out.

A house placed over a sporangiophore causes a transient positive growth response. This effect was discovered by R. J. Cohen during his study of the avoidance response and will be referred to as the house growth response. The house growth response is believed to be due to the same cause as the avoidance response. The house probably acts by quieting the random wind in the laboratory. The house effect occurs not only when the sporangiophore is originally placed in open air, but also when the sporangiophore is already enclosed in a bigger completely closed house (the house-within-a-house effect). In the light of the hypothesis, this effect can also be explained if an adaptation to various wind velocity levels, similar to light-level adaptation, is postulated. The quietness inside a house decreases with the size of the house. Therefore, the second house further quiets the wind and hence causes a growth response.

To make the model concrete, we must specify in which way air movements can become a signal directly received by the sensor. Speculations have been centered around the idea that the avoidance response is mediated by a volatile growth effector emitted somewhere along the sporangiophore and sensed by the growing zone (i.e., the chemical

self guidance hypotheses). Since the response is remarkably independent of the composition of the barriers, all the barriers must act alike on the effector. There are three alternative ways the barrier might affect the distribution of the gas. The first two alternatives assume that all barriers adsorb the gas X to the same extent, or that they all reflect the gas X in a similar fashion. The alternative of adsorption versus reflection is coupled to an alternative concerning the effect of the gas X on growth. If barriers adsorb the gas X, the gas X must inhibit growth, since growth speeds up on the side proximal to the barrier. Conversely, if barriers reflect the gas X, the gas must stimulate growth. Both the promotor-reflection and the inhibitor-adsorption models have been disproved by experimental tests. In fact attempts to establish the existence of an effector have invariably yielded negative results. The only remaining viable alternative of the chemical self guidance hypothesis is that the barriers modify the distribution of the gas X near the spph by altering the ambient random wind. The majority of the effector molecules must be readsorbed by the spph growing zone before they diffuse away. This model can reasonably account for all of our findings. The detailed formulation and proposed experimental tests are given in Section VIB.

Finally, specific tests ruling out possible roles in the avoidance response played by humidity, electrostatic effects, pressure and temperature will be described in Section VII.

II. Wind effects

A. Rheotropic response

Figure 3 shows the experimental setup. A sporangiophore was put in the wind tunnel A. The air current can be controlled by a pump and a valve. This figure also shows a typical tropic response of the sporangiophore to air current (velocity = 12 cm/sec). About 2 minutes after the air current is turned on, the sporangiophore starts to bend into the air current at a rate of 1°/min. The bending lasts for 30 minutes or more.

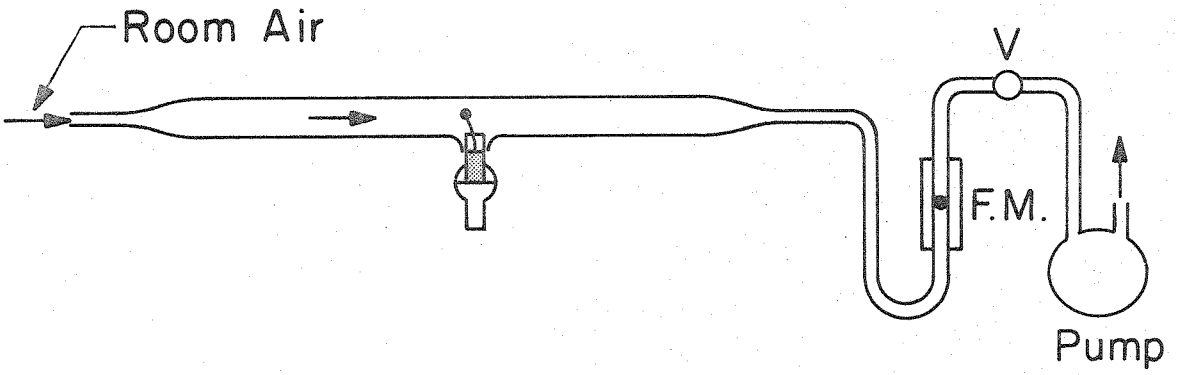
The bending rate as a function of air current velocity (or Reynolds number Re) is shown in Fig. 4. The bending rate increases linearly with increasing air current velocity up to Re $\simeq$ 0.6, then much more slowly. The tropic response virtually disappears at velocities $\leq$ 1 cm/sec (Re = 0.07).

To interpret the rheotropic response, we begin with general remarks on the aerodynamic situations involved. Reynold's number, $Re = ud/\nu$ (u = velocity of air stream, d = diameter of the object, ν = kinetic viscosity), may be conceived as a measure of the ratio of inertial flow to viscous flow. The attention will be focused on the sporangiophore growing zone which can be considered as a long cylinder with diameter of 0.01 cm. The kinematic viscosity of air at 20°C is 0.15 cm^2/sec. The Reynold's numbers for the air stream velocities used range from 0.07 to 10 and are shown in Fig. 4. Very large Reynold's numbers (> 100) imply turbulence; between 5 and 100 there is a wake. Below 5 there is laminar flow (5, 6). For the

sporangiophore growing zone, at velocities of the order of 1 cm/sec, there will be no turbulence and the flow will be essentially laminar with a very slight asymmetry between the leeward and the windward sides. Therefore, the asymmetry between the leeward and windward sides will be very minute. According to our hypothesis, there should be very little bending. The wake develops as the velocity increases. At the highest velocity (150 cm/sec) tested in these experiments, there should be a significant wake but still no turbulence on the leeward side. Therefore, the asymmetry of tangential velocity gradient between the two sides of a sporangiophore should increase with increasing velocity. Our hypothesis predicts that the bending rate should increase accordingly. That prediction agrees with the data shown in Fig. 4. In Fig. 4 there appears to be a break at Re $\sim$ 0.6 on the curve of bending rate versus Reynolds number. The significance of this break is not clear since the wind pattern does not change appreciably until Re $\sim$ 5 is reached and a wake begins to appear.

Fig. 3. Rheotropic response experiment

The experimental setup is shown on the top of the figure. The wind tunnel shown in Fig. 2a is used here. Room air is sucked through the wind tunnel by the pump. The air speed is controlled by the valve. On the bottom, data from a typical experiment are plotted. The air speed used in this experiment was 12 cm/sec. Legend: F.M, flowmeter; V, valve.


Room Air
V
F.M.
Pump

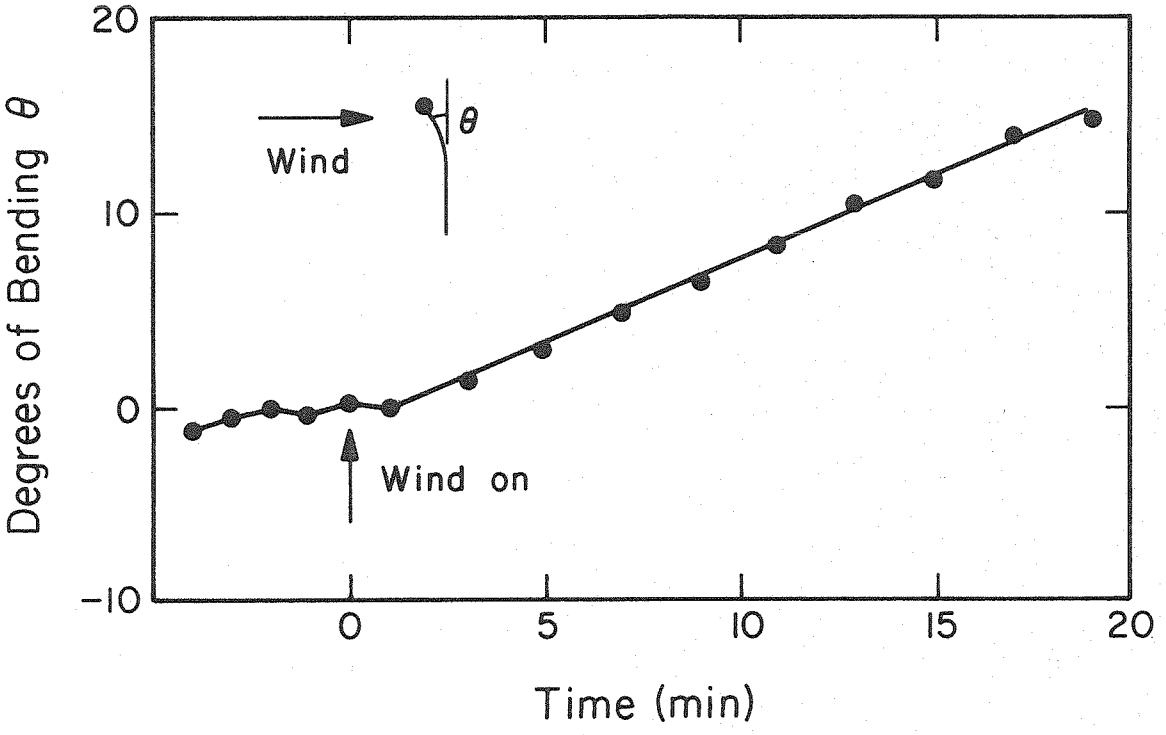
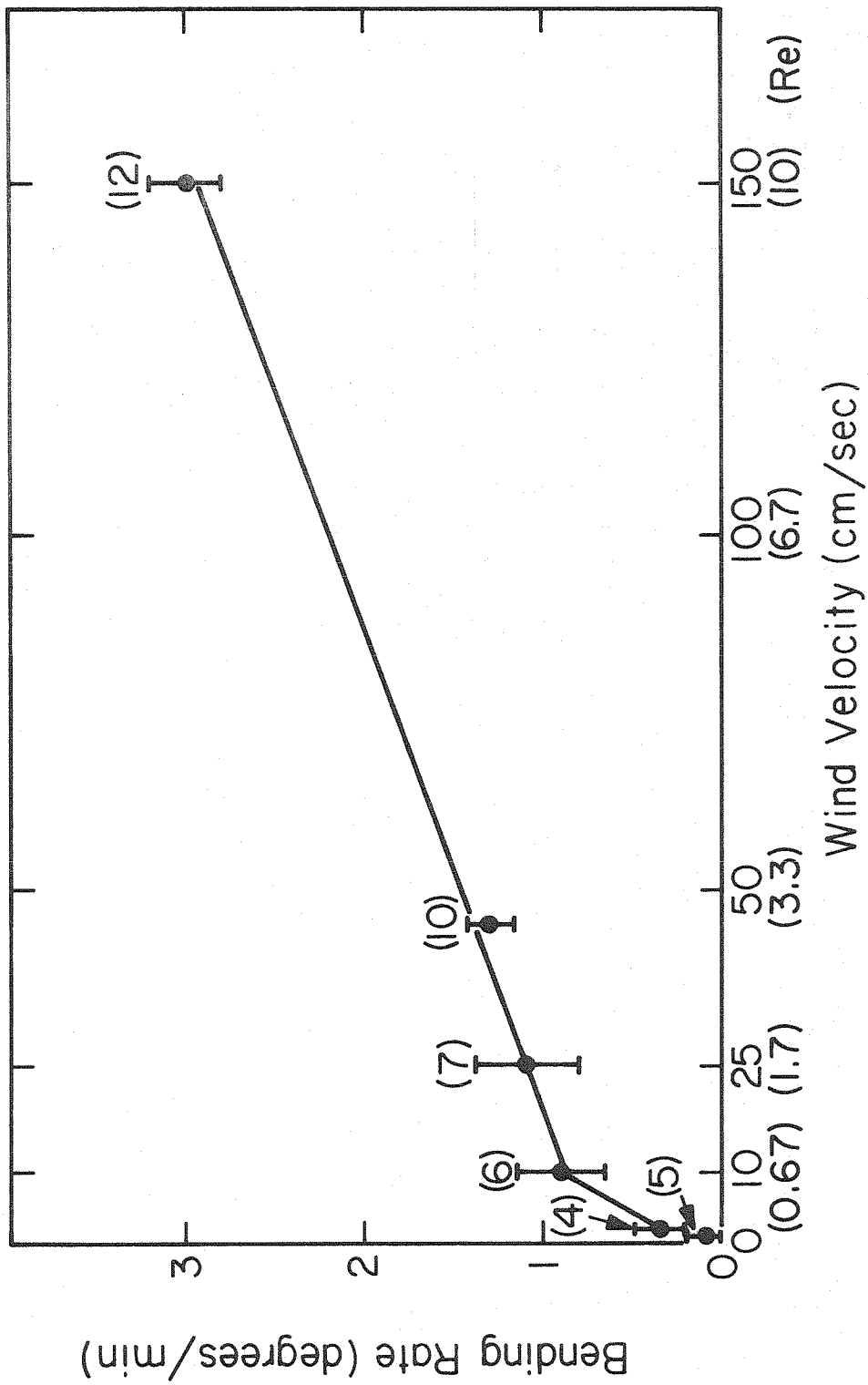

20
10
0
-10
0
5
10
15
20
Degrees of Bending θ
Time (min)
Wind
θ
Wind on

Fig. 4. The dependence of bending rate on lateral wind velocity

The experiments are done by using the setup shown in Fig. 3. The rate of sporangiophore bending into the wind increases with increasing lateral wind velocity. The number in the parenthesis above each data point indicates the number of sporangiophores used. Error bar indicates standard deviation of the mean. The number in the parenthesis below each wind velocity along the abscissa is the corresponding Reynold's number (Re).


Bending Rate (degrees/min)
0
1
2
3
(4)
(5)
(6)
(7)
(10)
(12)
0
10 (0.67)
25 (1.7)
50 (3.3)
100 (6.7)
150 (10) (Re)
Wind Velocity (cm/sec)

B. The wind growth response

One of the two major assumptions in our working hypothesis is that air movements near the sensor promote a negative growth response. The main evidence to support this assumption is the finding that deliberately applied air currents of low speed cause a transient negative growth response of the sporangiophore.

In the experiments, laminar room air currents (10-15 cm/sec, generated by a pump) pass the sporangiophore either transversely, or longitudinally from above, or longitudinally from below. In each case there is a transient negative growth response after the air current is turned on and a transient positive growth response after the air current is turned off. Figure 5 shows a typical growth response to a transverse air current. The setup is the same as that shown in Fig. 3. It should be pointed out that in the case of the lateral wind the sporangiophore simultaneously shows a tropic response. We turned the wind on for only 7 minutes in each cycle. Therefore, the total bending is only 5-6° which can cause only negligible effects on the measurement of the growth rate in the vertical direction. The sporangiophore tends to straighten itself during the period when the wind is off.

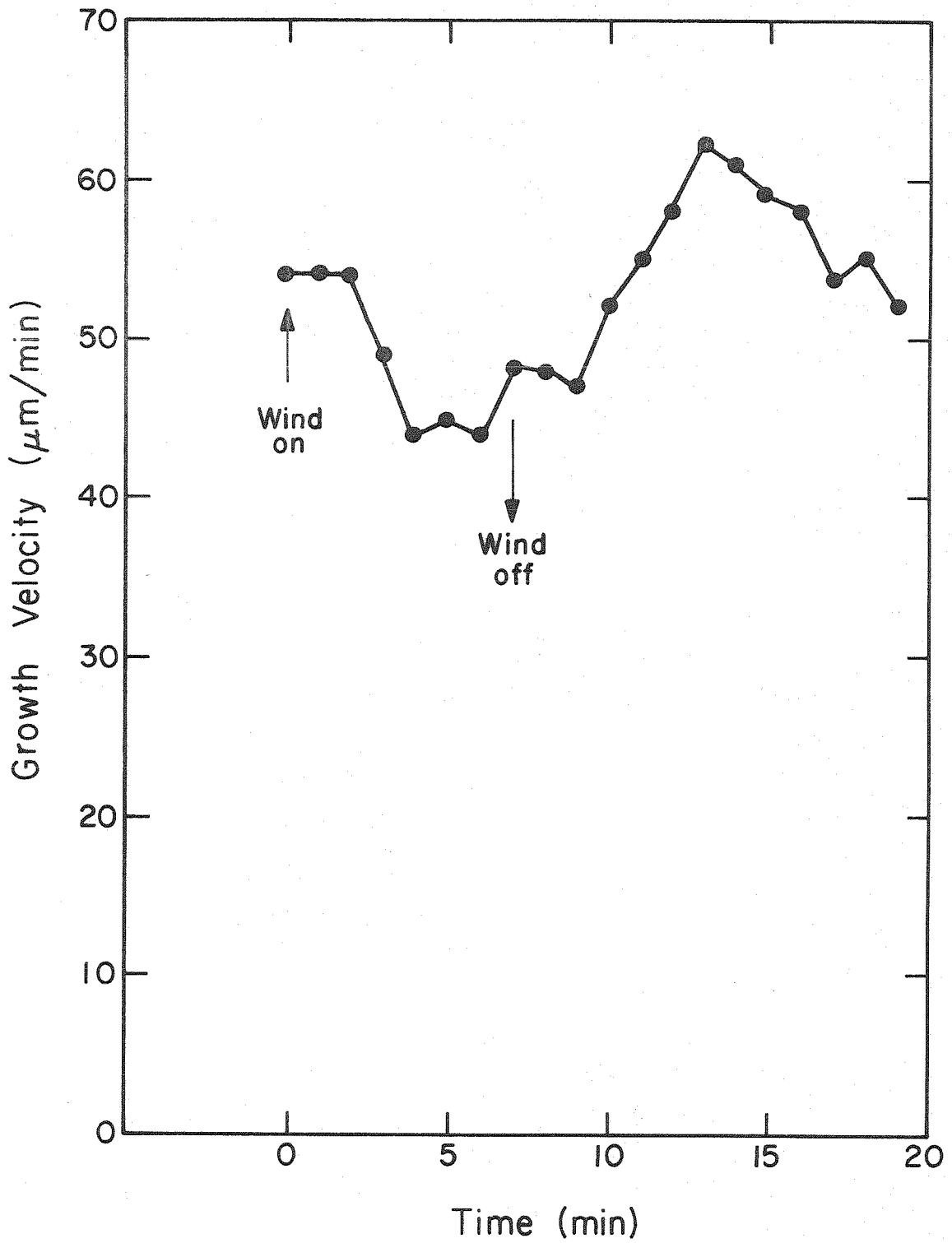
The dependence of the wind growth response on the speed of the transverse wind was studied. For a speed as low as 3 cm/sec, there is still a clear-cut wind growth response comparable to the one shown in Fig. 5. The effect diminishes when we decrease the wind speed down to 0.3 cm/sec, which is comparable to the residual random wind velocity inside the wind tunnel. The conclusion drawn from this

experiment is that a step up of wind velocity from a few mm/sec to a few cm/sec is sufficient to elicit a significant negative growth response.

A note of caution should be given here. The nature of the air current makes a tremendous difference. If we use compressed air from the tap, the wind effect is either opposite in sign or absent. This is probably due to the fact that air from the compressed air tap differs from the room air to which the sporangiophore is adapted. The possible differences may include temperature, humidity and especially chemical composition. R. J. Cohen (personal communication) has tested a great number of volatile substances and has found that very many produce positive or negative growth responses, some at very low concentrations of the agent.

Fig. 5. Growth response to a lateral wind

The experimental setup is shown in Fig. 3. The lateral air current (3 cm/sec) was turned on and off at the times indicated. This was done periodically, 7 min on, 12 min off for a total of 5 periods (95 minutes). The average is plotted.


70
60
50
40
30
20
10
0
Growth Velocity (μm/min)
Wind on
Wind off
0
5
10
15
20
Time (min)

C. Are wind effects actually responses to stretch?

The stretch responses

(i) The response to an asymmetric stretch was discovered by Dennison (7) and has been described by him as follows:

"When a lateral force (above a certain threshold) is applied to the sporangium, either by the direct action of a calibrated glass filament or by the indirect action of the centrifuge, an immediate passive bend is formed in the growing zone. Within 2 min of the formation of the passive bend, a rapid tropic reaction begins. It consists of rapid bending about 5°/min which continues for about 5 min. The direction of this tropic response is always opposite to the stimulating passive bend." (3)

The exact threshold of the stimulus strength for this response has not been determined. However, the threshold can be estimated: Moving a sporangiophore to the horizontal position does not cause a stretch response. In this situation, the lateral force on the sporangium due to gravity is calculated to be 0.063 dyne ($F = mg = [980 \times (4/3\pi R^3) \times \rho]$, where $R = 0.025$ cm, the radius of the sporangium, and $\rho = 1$ g/cc, density of the sporangium). A centrifugal force of 3 g applied laterally to the sporangium is enough to elicit a strong stretch response (7). Therefore, the threshold for the lateral stretch response is estimated to be between 0.063 and 0.19 dyne.

(ii) The response to a symmetric stretch

When the mechanical stimulus is applied symmetrically along the cell axis of the sporangiophore by hanging a weight from an inverted specimen, a growth response is elicited. Stretching the cell causes a transient negative growth response, and relaxing the stress causes a transient positive growth response (8). The threshold

of the stimulus strength for the stretch response is estimated to be 0.5 dyne (8).

Are wind responses actually responses to stretch?

Wind effects might be thought to be mediated indirectly, through the drag forces exerted by the wind. This conjecture can be excluded because

(i) The drag forces of the longitudinal wind on the sporangiophore are calculated in Appendix A. The calculation shows that the wind velocities employed in the wind growth response experiments cause drag forces far below the threshold of the stretch responses. Even at 100 cm/sec the drag force of longitudinal wind is more than a factor 10 below threshold (0.5 dyne).

(ii) The drag forces of the lateral wind on the sporangiophore are also calculated in Appendix A. The calculation shows that for a lateral wind velocity as high as 100 cm/sec the drag force is still below the estimated threshold of the lateral stretch response.

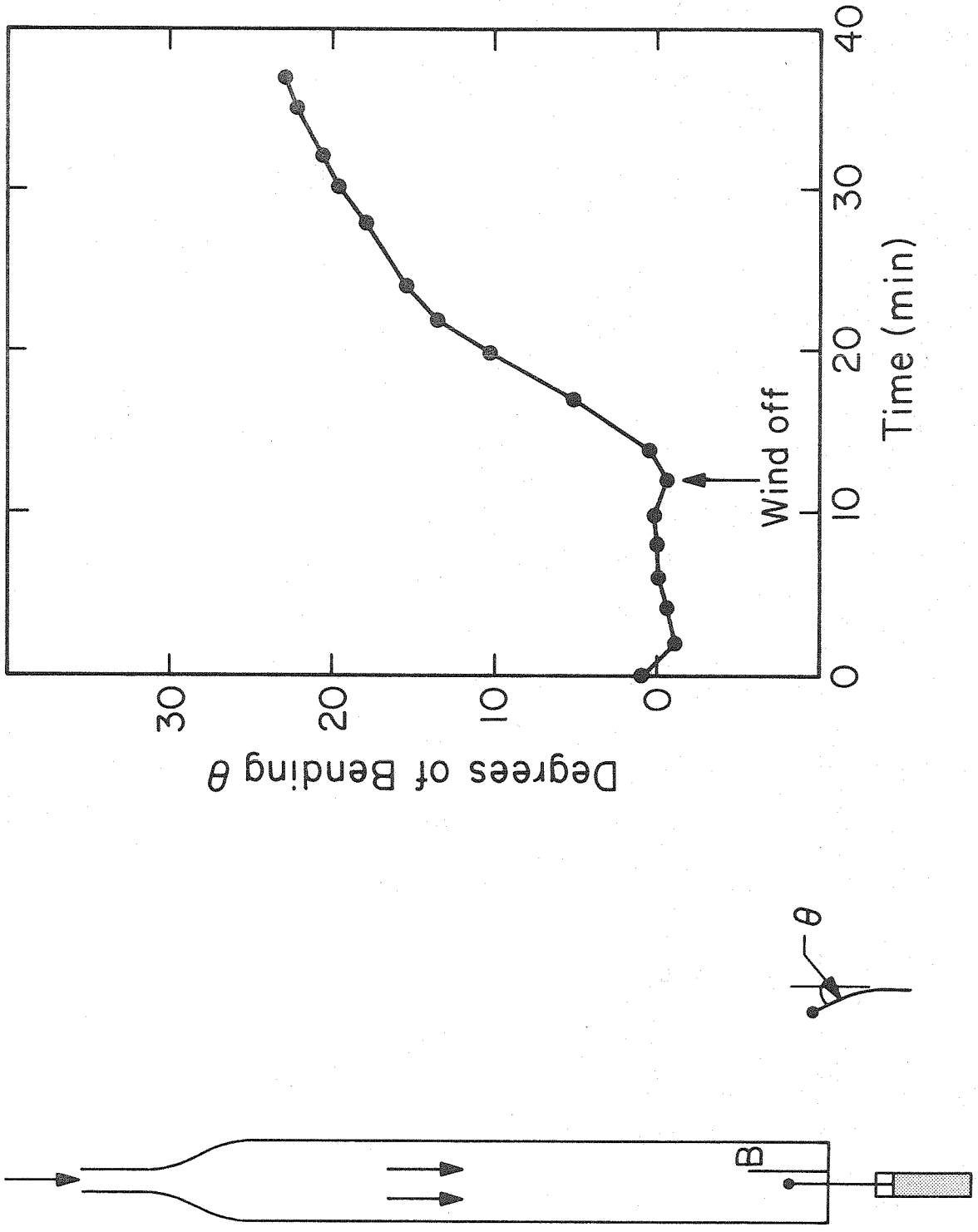
(iii) Suppose the wind effects were actually responses to stretch caused by wind. Then longitudinal wind upward and downward should cause opposite growth responses, since they stretch and relax the sporangiophore respectively. However, wind causes negative growth responses regardless of the direction of the wind.

III. Elimination of the avoidance response by an air stream parallel to the barrier and the spph

In the last section, we have shown experiments demonstrating the wind effects on the growth of the sporangiophore. The important question is whether these wind effects and the avoidance response actually have the same cause. If these two effects have the same cause, we would expect that air currents can interfere with the avoidance response. The experiment to test this possibility is shown in Fig. 6. From the open end of a vertical wind tunnel B a laminar downward (or upward) air current of a speed of 15-30 cm/sec passes the sporangiophore. The sporangiophore is adapted to the air current for at least 30 minutes. At t = 0, a glass barrier (2.2 cm x 2.2 cm) is moved to 1.2 mm away from the sporangiophore. If there is no air current parallel to the barrier, the sporangiophore shows a normal avoidance response. With the air current parallel to the barrier, the sporangiophore does not avoid the barrier as long as the air current persists. When the air current is turned off, the sporangiophore starts to avoid with a latency of 2-3 minutes. This experiment clearly demonstrates that air currents can interfere with the avoidance response.

Fig. 6. Elimination of the avoidance response by an air stream parallel to the barrier and the sporangiophore

The experimental procedure is described in the text. Tap air blown downward and room air sucked upward gave similar results.


Degrees of Bending θ
0
10
20
30
Wind off
Time (min)
0
10
20
30
40
θ
B

IV. Characterization of the avoidance response

A. Avoidance response in a closed chamber

We begin our consideration with some general remarks about the aerodynamic situation involved.

The speed of random air movements in a normal laboratory is in the range of 10-100 cm/sec. We know from the study of the wind effects that air movements of this magnitude can introduce uncontrolled growth as well as rheotropic responses. The velocity of the air movement within a house is a function of time as well as space. After a house is closed, within a minute, the air movements inside the house quiet down, and a slow quasi-steady state convection is established. The air movement pattern varies slowly with a time constant of a few minutes, presumably due to slight temperature differences between different wall areas.* Although the direction of the air velocity near a sporangiophore may vary, the magnitudes are more or less constant for a given house. Thus, the "quietness" for each house can be characterized by its "characteristic air speed"

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* The sporangiophore metabolizes at a high rate and transpires water into the air. As a consequence its temperature might be different from that of the environment and a microcirculation in the vicinity of the growing zone might thereby be generated. However, this has been shown not to be the case. A self-generated microcirculation has never been observed by direct measurements. Also, both calculations and direct measurements indicate that the possible temperature differences generated by a sporangiophore are less than 0.3°C, and calculation shows that such small temperature differences can only give microcirculation with velocities less than 0.08 mm/sec.

defined as the average speed of air movements near the sporangiophore inside that house. This "characteristic air speed" (v) decreases with decreasing house sizes. Inside the standard avoidance apparatus (6.2 cm x 6.2 cm x 6.2 cm), the characteristic air speed is 1-3 mm/sec.

Figure 7 shows a typical avoidance response of the sporangiophore to a barrier in the standard avoidance apparatus. The two barriers are initially both far away (> 15 mm) from the sporangiophore. At t = 0, one barrier is moved to a distance of 1.2 mm from the sporangiophore. After a latency of about 2 minutes, the sporangiophore starts to bend away for as long as 20 minutes or more.

The maximal bending rate occurs between the 4th minutes and about the 14th minutes. The bending rate is quite constant during this period. Thereafter, the bending rate starts to decrease, presumably because the growth zone gets farther from the barrier due to bending. The response can be kept up indefinitely in a tropostat where the distance between the growth zone and the barrier is kept constant. We can thus define a "maximal bending rate:"

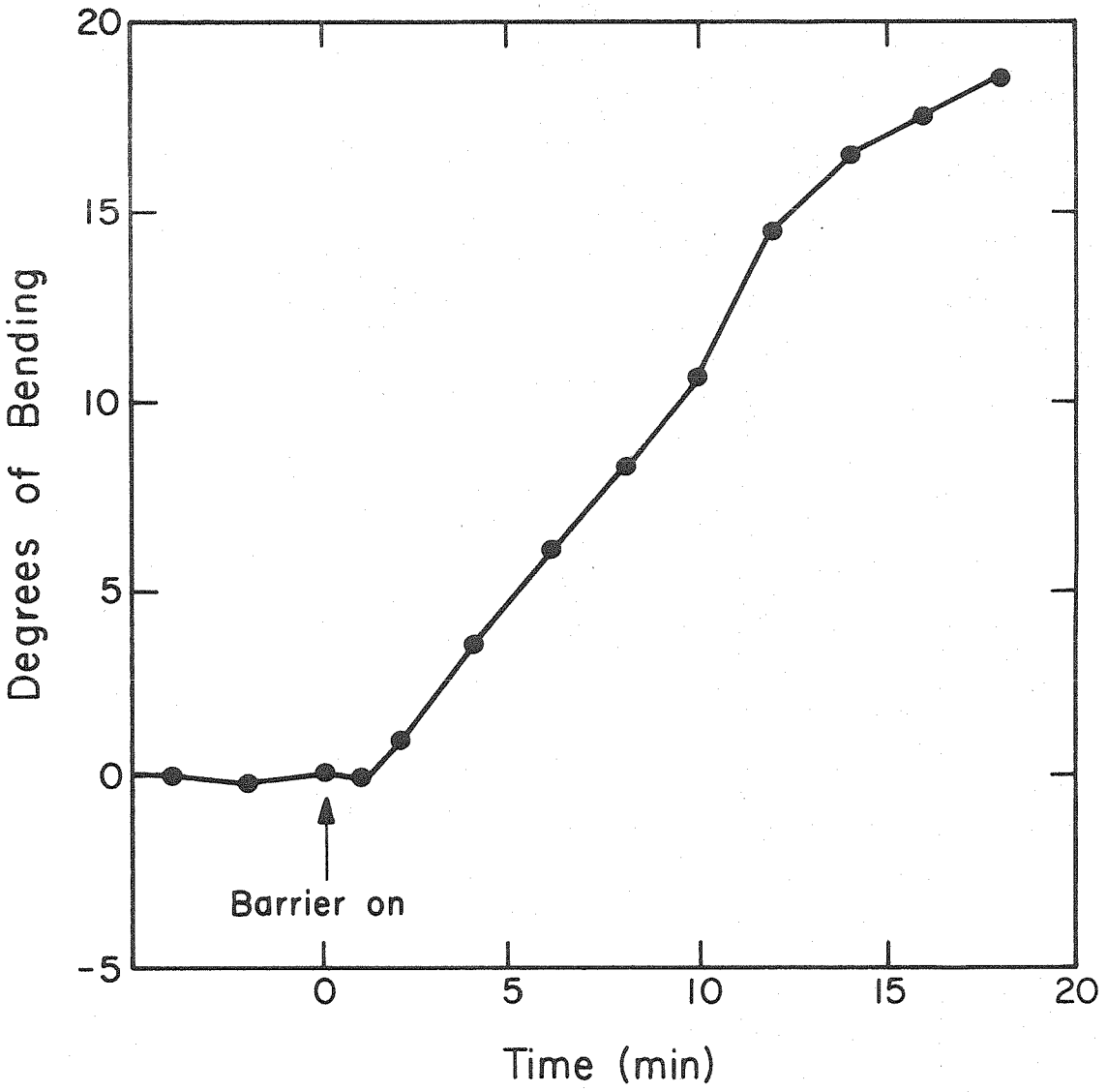
$$\left(\frac{d\theta}{dt}\right)_{max} = \frac{\theta(t=14) - \theta(t=4)}{10} \quad \frac{\text{degrees}}{\text{min}}$$

to characterize any particular avoidance response. In the case shown in Fig. 7,

$$\left(\frac{d\theta}{dt}\right)_{max} = 1.3°/\text{min}$$

Fig. 7. Avoidance response in a closed chamber

Inside the air tight standard avoidance apparatus, a glass barrier (2.2 cm x 2.2 cm) was moved to 1.2 mm from the sporangiophore at the time indicated by the arrow. The sporangiophore bent away from the barrier after a latency of 1-2 min.


20
15
10
5
0
-5
Degrees of Bending
Barrier on
0
5
10
15
20
Time (min)

B. Independence of barrier material

Type I experiments: Unilateral stimuli -- The experiments were done in a sealed house under standard conditions, except for the material of the barrier. In the case of liquid barriers, the entire apparatus was tilted 90°, a beaker was filled with the specified liquid and the liquid surface served as the barrier. The barrier was then raised to the vicinity of the sporangiophore which now assumed a horizontal position for the avoidance response measurements.

Two points should be made here. First, the sporangiophore also has negative geotropic response, i.e., it will bend away from the direction of gravity. Since the latency of geotropism is longer than 30 minutes, the avoidance response will be over before geotropism gets started. Secondly, the avoidance response is independent of the orientation of the sporangiophore and the barrier with respect to gravity, i.e., the avoidance response is the same whether the sporangiophore and the barrier are both vertically or both horizontally oriented (Johnson and Gamow (4), and confirmed by us). The barriers tried were all similarly effective in causing an avoidance response, i.e., in the sealed house, any barrier (diameter > 10 mm) placed 1 mm away from the sporangiophore causes the sporangiophore to bend away from the barrier at a rate 1 to 3°/min for about 20 minutes and with a latency of 2 to 4 minutes.

The different materials used include a) solids: glass, quartz, plastic, teflon, wood, black tape, aluminum, brass, magnet, a crystal

transparent for infrared radiation of a black body at room temperature, activated charcoal, $CaCl_2$ and KOH pellets, and b) liquids: water, concentrated sulfuric acid, paraffin oil and FC 43 (a fluoro carbon oil, perfluorotributylamine). Collectively they cover extreme ranges of the following parameters:

(a) Visible light (radiation) absorption properties

(b) Ultraviolet radiation absorption properties

(c) Infrared radiation absorption properties

(d) Dielectric properties

(e) Magnetic properties

(f) Surface adsorption properties (activated charcoal, $CaCl_2$ and KOH pellets, and teflon)

(g) Affinity to water (hydrophobic versus hydrophilic)

Type 1 experiments tell us that the barriers of different material all cause avoidance responses in a similar fashion. To test with a higher degree of confidence whether barriers made of different materials are really the same as far as avoidance is concerned, Type II experiments were done.

Type II experiments: Bilateral stimuli -- Taking dielectric properties as an example, let us consider two equal-sized plate barriers, one made of plastic with dielectric constant $\varepsilon \sim 3$ and one made of aluminum with $\varepsilon \sim \infty$, mounted onto the two barrier mountings. At the start of the experiments, they are brought in parallel to an equal distance ($\sim$ 1.5 mm) from the sporangiophore from opposite directions. The sporangiophore's angle of deviation from the vertical orientation

is measured. In this setting, the double barriers are dielectrically highly asymmetric. If the avoidance response depends on the dielectric constants of the barriers, we would expect the sporangiophore to bend consistently away from one of the two barriers. No such tropic response is found. Thus we conclude that the dielectric properties of the barriers are not important parameters in the avoidance response. Similar experiments involving pairings of glass versus activated charcoal and ordinary glass versus infrared absorbing glass were done to test whether the sporangiophore can distinguish between barriers of different gas adsorption properties or different infrared absorbing properties. In each case, the sporangiophore does not show preference in bending. Thus our conclusion is that for a great variety of barrier materials the avoidance response elicited is identical. This strongly suggests that the avoidance response is independent of the electric, magnetic, gas adsorbing, electromagnetic radiation absorbing (including ultraviolet, visible, and infrared light) properties of the barrier. This result is the main reason which leads to the idea that the barriers act purely by their aerodynamic effects.

C. Distance dependence of avoidance response to large barriers

The $(\frac{d\theta}{dt})_{max}$ as function of the barrier distance is shown in Fig. 8. The experiments were done under standard conditions, varying the distance (d) between the sporangiophore and the barrier. The $(\frac{d\theta}{dt})_{max}$ increases as the distance between the barrier and the sporangiophore decreases to an extrapolated maximum of 1.7°/min at zero distance. It drops to zero at a distance of about 12 mm, decreasing more or less linearly with distance.

Latency: As the distance between the barrier and the sporangiophore increases, the latency for the onset of the average response also increases. The latency is 2 ± 1 min for d = 1.2 mm and 3 ± 1 min for d = 3.5 mm. This result is similar to the finding of Foster and Lipson (9) that the latency of the Phycomyces light growth response increases with decreasing stimulus intensity, and is indeed similar to almost any response in any organism that has a latency.

Dependence on size of the house: Johnson and Gamow (4) reported that the avoidance response does not occur in still air. In their experiments, a sealed (2.5 cm x 2.5 cm x 7.5 cm) glass house was used to minimize air currents. They report that in this house there is only very weak (if any) avoidance response, at least one order of magnitude weaker than that found in the presence of normal laboratory air movements. Contrary to their report, we find very significant avoidance responses in sealed houses. Inside a sealed (6.2 cm x 6.2 cm x 6.2 cm) house, the $(\frac{d\theta}{dt})_{max}$ of a sporangiophore avoiding a (2.2 cm x 2.2 cm) glass barrier at a distance of 1.2 mm is at least half of that

of the corresponding avoidance response found in open air. The magnitude of $(\frac{d\theta}{dt})_{max}$ is not very sensitive to the size of the house. It may decrease slightly with decreasing size of the house. For example, the $(\frac{d\theta}{dt})_{max}$ of a sporangiophore 1.2 mm away from a 2.2 cm x 2.2 cm barrier inside a (2.5 cm x 2.5 cm x 2.5 cm) house is 1.0° ± 0.25° (3 experiments) as compared to 1.25° ± 0.2° inside a (6.2 cm x 6.2 cm x 6.2 cm) house (6 experiments).

Interpretation of the distance dependence of $(\frac{d\theta}{dt})_{max}$: According to our hypothesis, the key factor should be the air movements in the vicinity of the sporangiophore. These movements were studied carefully by the procedure described in section 2V. The air movement around the sporangiophore was found to vary with the distance (d) between the sporangiophore and the barrier.

d = 1 mm: The air movements around the sporangiophore in one particular experiment is shown in Fig. 9A, as an example. There is a "quietness" (vertical wind velocity) gradient extending from the barrier outward with the air velocity on the surface of the barrier being zero. The region between the sporangiophore and the barrier is rather quiet (velocity < 100 μm/sec). The air speed increases as the distance from the barrier increases. Thus, the effects of a barrier are twofold: (1) It quiets down the nearby air movements. (2) It causes a velocity gradient and hence an asymmetry of air movements on the proximal and the distal sides of the sporangiophore.

$d \geq 5$ mm: The asymmetry of the air movements near the two sides of the sporangiophore decreases as the distance between the sporangiophore and the barrier increases. At d = 5 mm, the air speed at a position 1 mm to the proximal side of the sporangiophore is only slightly less than that at 1 mm to the distal side of the sporangiophore (1 mm/sec versus 1.5 mm/sec). At d = 10 mm, no noticeable difference is found. At this distance also the avoidance response disappears. Thus the distance dependence of the asymmetry of air movements between the two sides of the sporangiophore correlates well with that of the bending rates.

Fig. 8. Dependence of the avoidance response on the distance of a large barrier

The experiments were done under standard conditions, varying the distance between the sporangiophore and the barrier. The $(\frac{d\theta}{dt})_{max}$ decreases more or less linearly with the distance. The number in the parenthesis above each data point indicates the number of sporangiophores used. Error bar indicates standard deviation of the mean.

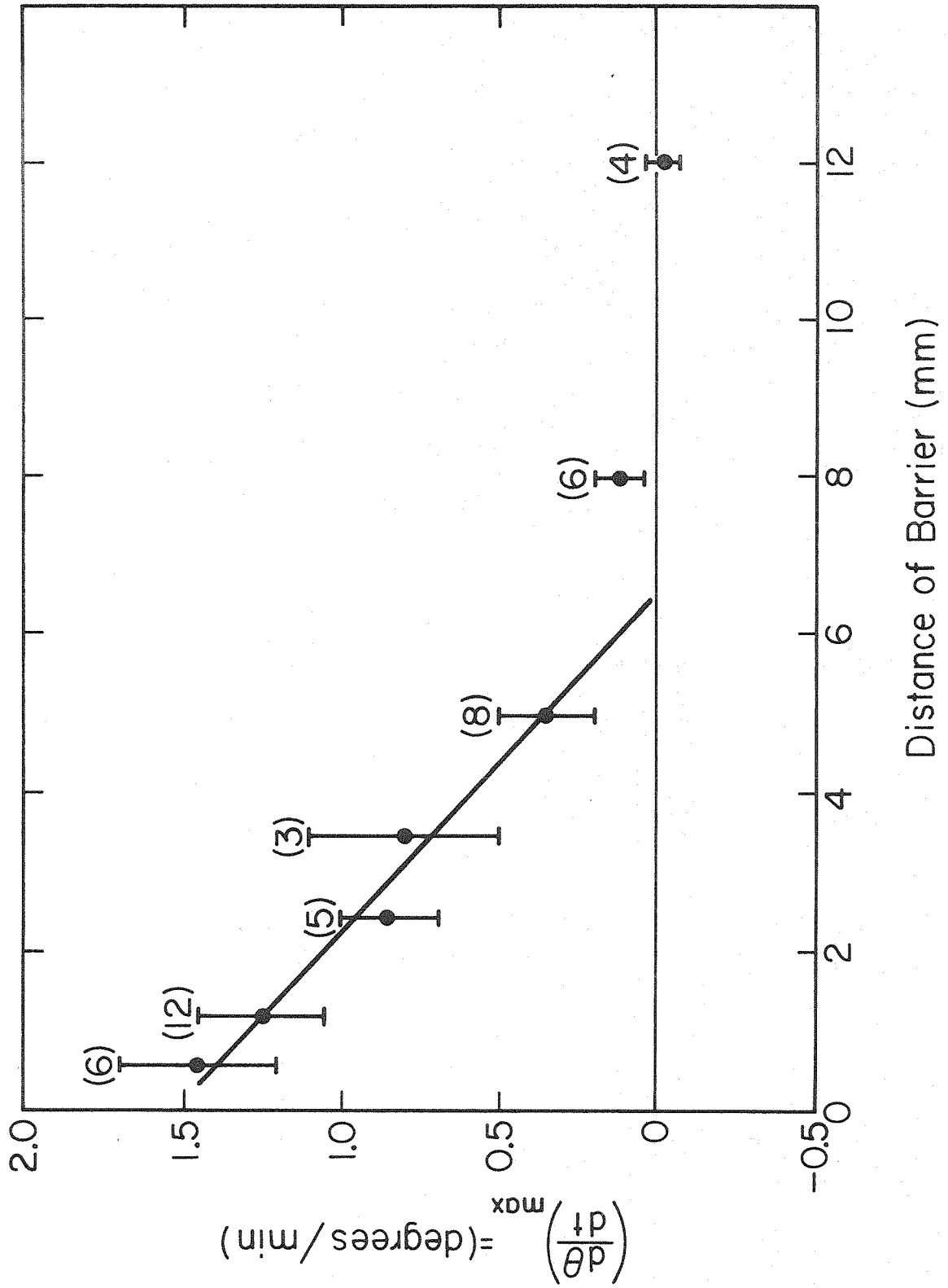
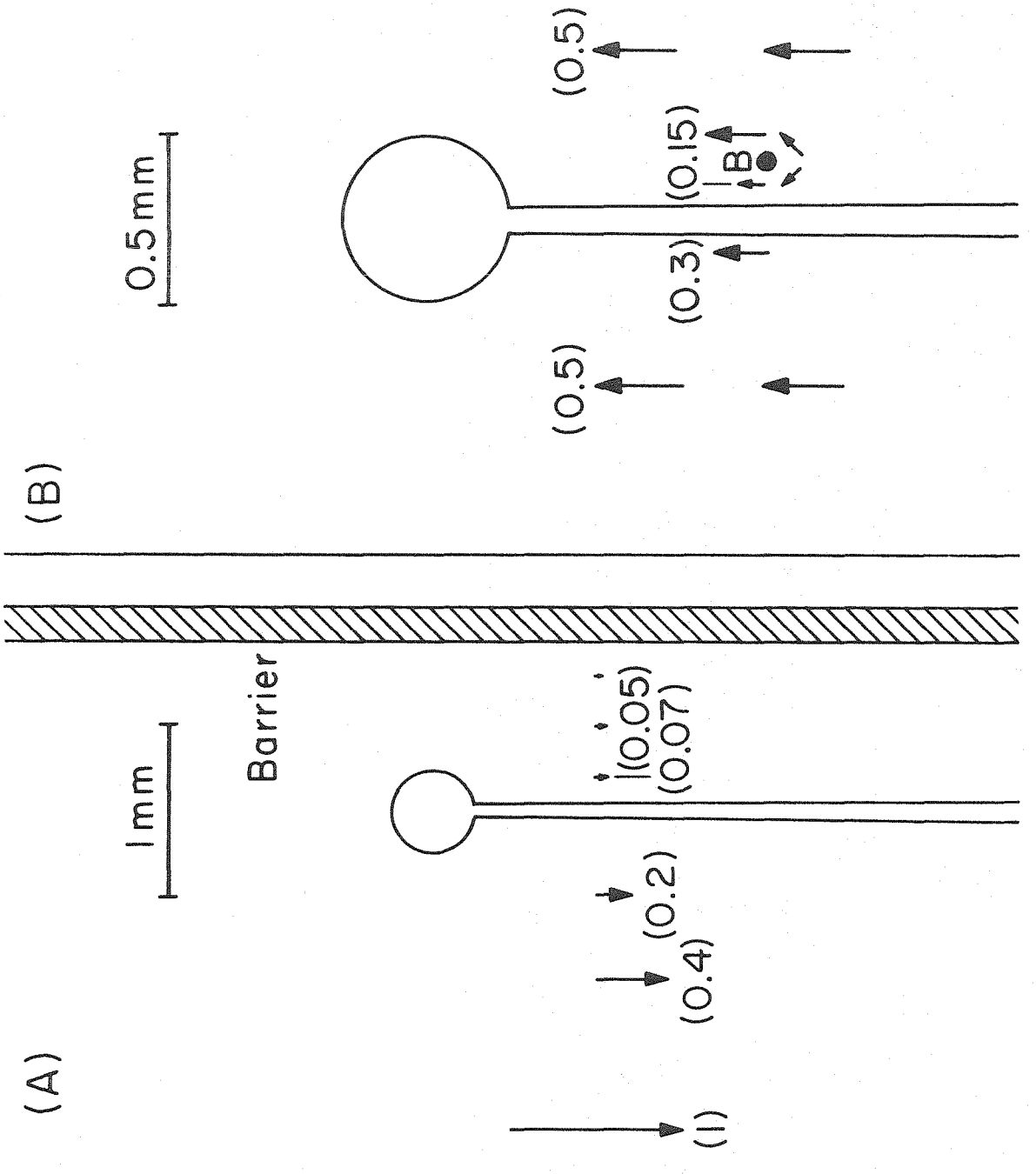

2.0
1.5
1.0
0.5
0
−0.5
0
2
4
6
8
10
12
(6)
(12)
(5)
(3)
(8)
(6)
(4)
$\left(\frac{d\theta}{dt}\right)_{max}$ =(degrees/min)
Distance of Barrier (mm)

Fig. 9. The effect of barriers on the air movements near the sporangiophore growing zone

The arrows indicate the velocity of the air movement at the points in space, including direction. The number in parentheses indicates the magnitude of the velocity in mm/sec. (A) illustrates the effect of a large barrier 1 mm away and (B) illustrates the effect of a thin wire (diameter 50 μm) 0.1 mm away from the sporangiophore. In both cases, the barriers quiet the air movement in the region between the sporangiophore and the barrier.


(A)
(1)
(0.4)
(0.2)
(0.05)
(0.07)
1mm
Barrier
(B)
(0.5)
(0.15)
B
(0.3)
(0.5)
0.5mm

D. Distance dependence of avoidance response to thin barriers

Experimental setup: The experiments were done in a standard avoidance apparatus with the barrier mountings replaced by a thin wire (tungsten or Nylon, 50 μm diameter and about 4 cm long). The wire is placed horizontally so that it is perpendicular to the sporangiophore as well as to the focal plane of the measuring microscope.

The location of the sensor: The thin wire has to be adjacent to the middle of the growing zone (∿ 1 mm below the sporangium) to elicit a significant avoidance response. Thus, the location of the sensor for the stimulus is established. As in the case of light, the sensor is at the same location as the site of the response, i.e., in the growing zone.

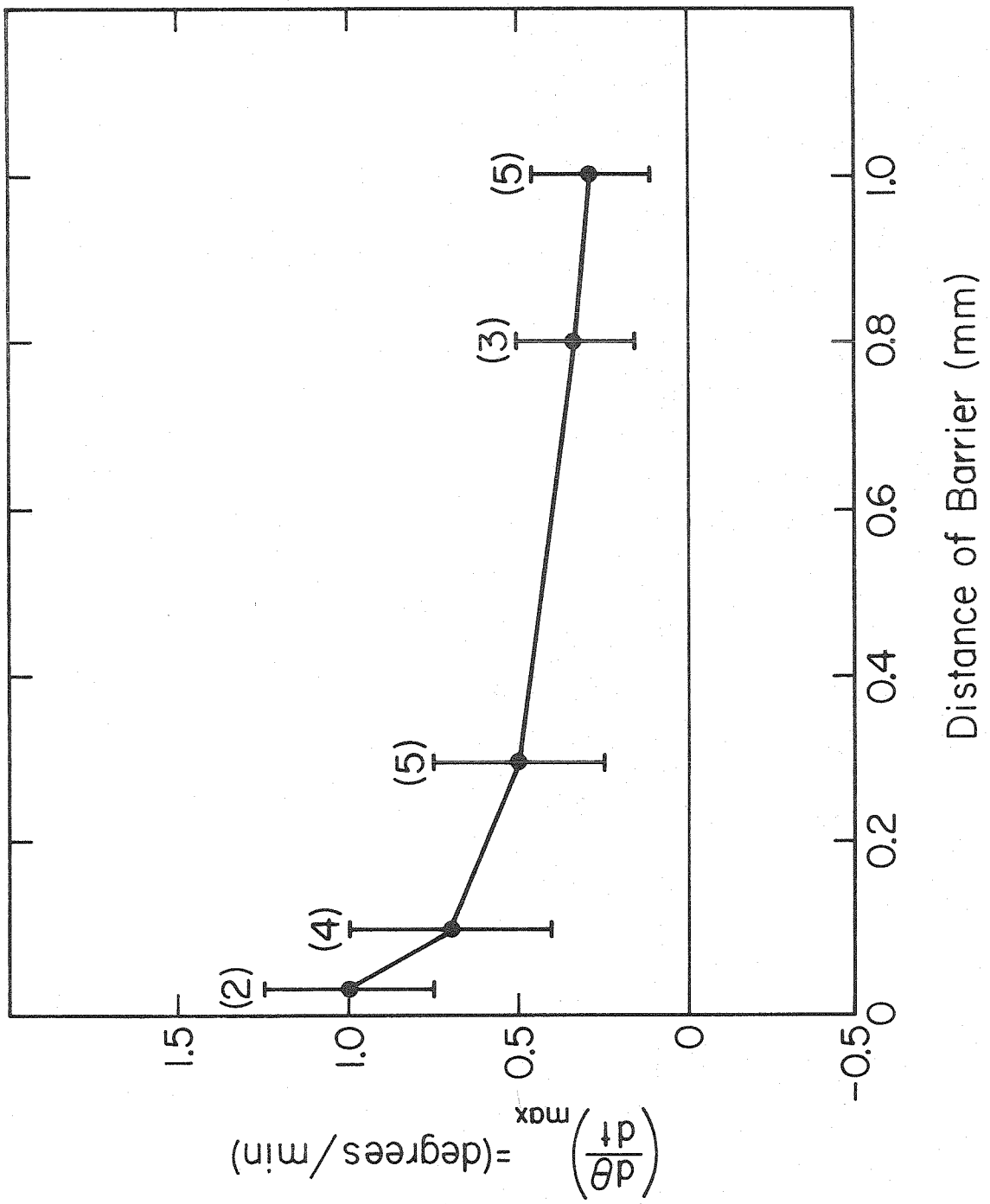
At the beginning of the experiment, the barrier is moved to approximately 0.7 mm below the sporangium so that the barrier stays for a few minutes adjacent to the middle of the growing zone while the sporangiophore grows at a rate of about 0.05 mm/min. The result is shown in Fig. 10. The bending rate decreases monotonically with increasing barrier distance. The sporangiophore can sense the presence of a thin wire as far as 1 mm away. To explain this remarkably weak distance dependence, the effect of the thin wire on the air movement near the sporangiophore was examined.

A representative pattern of the air movement around the sporangiophore and a thin wire (diameter 50 μm) horizontally opposing the middle of the growing zone is shown in Fig. 9B. The distance between the wire and the sporangiophore is 0.1 mm. The thin wire causes measurably asymmetry on the air movements. The air speed in

regions between the wire and the sporangiophore is lower than that on the distal side. This asymmetry might explain the bending caused by the thin wire. The Reynold's number is very small (Re $\sim$ 0.001) for the flow rate occurring inside these houses. Simple aerodynamic considerations indicate that the flow will be seriously disturbed up to distances comparable to the dimension of the object δ. At greater distances (d) the velocity disturbance falls off as δ/d. The fact that the velocity disturbances only fall off inversely with distance (instead of with the square of the distance or even higher powers) probably explains the amazingly weak distance dependence of the avoidance response to a horizontal wire.

Fig. 10. Dependence of the avoidance response on the distance of a thin barrier

The experimental procedure is described in the text. The number in the parentheses above each data point indicates the number of sporangiophores used. Error bar indicates standard deviation of the mean.


(2)
(4)
(5)
(3)
(5)
1.5
1.0
0.5
0
−0.5
0
0.2
0.4
0.6
0.8
1.0
Distance of Barrier (mm)
$\left(\frac{d\theta}{dt}\right)_{max}$ =(degrees/min)

V. House growth response

A. House effect

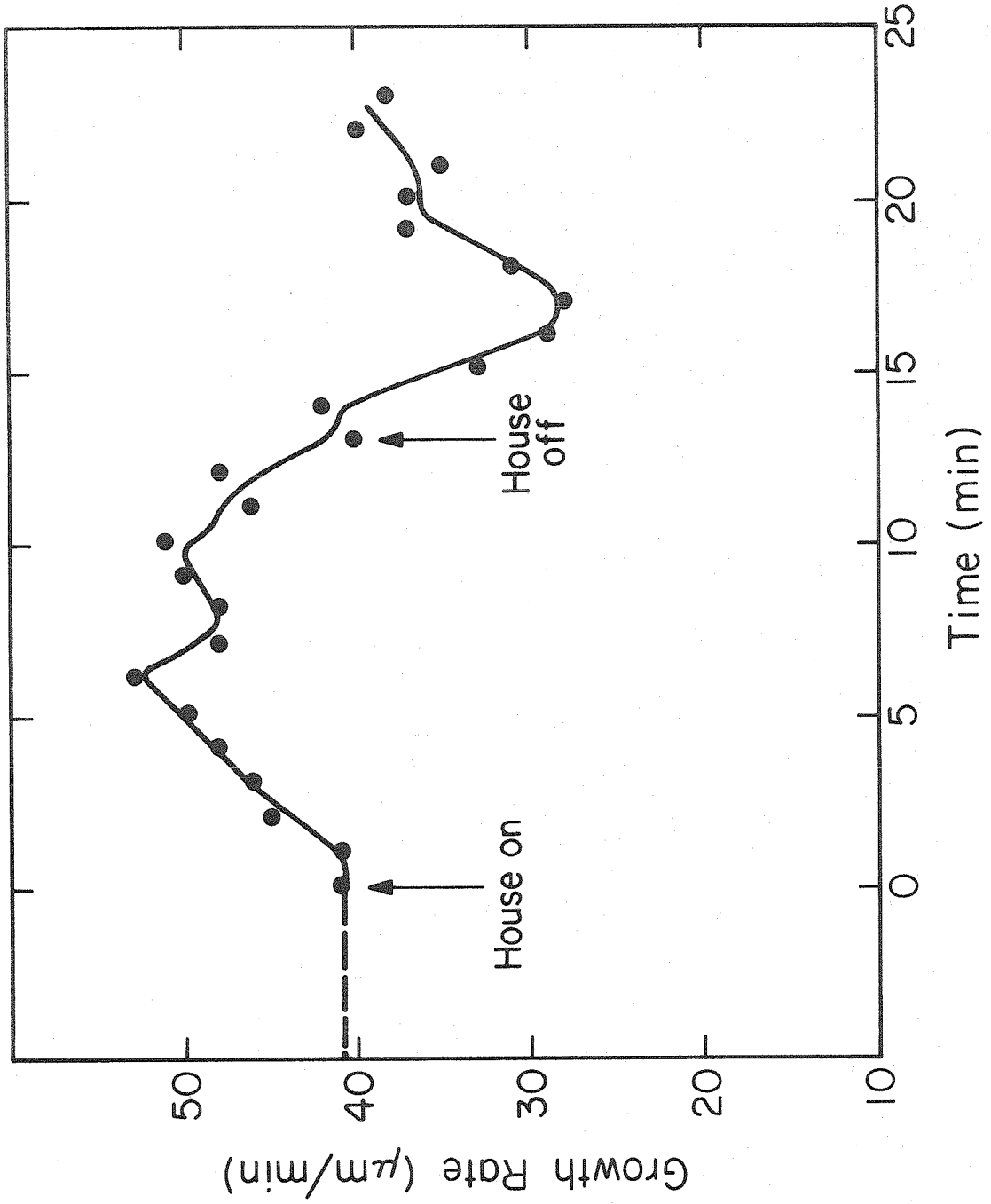
The house effect, discovered by R. J. Cohen, is shown in Fig. 11. In this experiment, a (5 cm x 5 cm x 7.5 cm) plastic house placed over a single sporangiophore previously standing in open air, causes a transient positive growth response with a latency of about 2 minutes, and a negative one upon removal of the house. This effect does not seem to be very sensitive to the size of the house. We placed over a sporangiophore in open air plastic or glass houses of various sizes (2.5 cm x 2.5 cm x 2.5 cm, 2.5 cm x 2.5 cm x 7.5 cm, 5 cm x 5 cm x 7.5 cm, 7.5 cm x 7.5 cm x 7.5 cm, 10 cm x 10 cm x 10 cm). In each case, there was a quantitatively similar positive growth response when the house was put on and a negative one when it was removed. The characteristic air speeds (defined in Section IV) are 2-5 mm/sec, 1-2 mm/sec, 0.5-1 mm/sec, 0.5 mm/sec and 0.02-0.1 mm/sec for houses of size 10 cm x 10 cm x 10 cm, 7.5 cm x 7.5 cm x 7.5 cm, 5 cm x 5 cm x 7.5 cm, 2.5 cm x 2.5 cm x 7.5 cm and 2.5 cm x 2.5 cm x 2.5 cm respectively. From these values, we concluded that (1) there is a trend of decreasing air movements (increasing quietness) with decreasing house sizes, and (2) in no case is the air completely quiet. The slowest air movement observed was about 20 μm/sec inside a 2.5 cm x 2.5 cm x 2.5 cm house.

The house effect can be explained by the quieting down of random wind due to enclosure. The average air speeds drop from faster than 10 cm/sec in the open laboratory air down to less than 1 cm/sec

in the large house (10 cm x 10 cm x 10 cm) and to less than 1 mm/sec in the small house (2.5 cm x 2.5 cm x 2.5 cm). Houses of different sizes cause similar house effects. This might be explained by assuming that a tenfold increase in quietness saturates the response, which agrees with the wind growth response data.

Fig. 11. House growth response

The house (5 cm x 5 cm x 7.5 cm) was lowered or raised over the sporangiophore at the times indicated by the arrows. This was done periodically, 13 min on and 10 min off for a total of 3 periods (69 minutes). The average is plotted.


Growth Rate (μm/min)
50
40
30
20
10
House on
House off
0
5
10
15
20
25
Time (min)

B. House-within-a-house effect (J. Matricon)

The house effect occurs not only in open air, but also when the sporangiophore is already inside a bigger completely closed house. The setup is shown in Fig. 12. The inner house could be slipped over the sporangiophore from the outside through a hole. The sporangiophore gives a growth response to this inner house in a manner similar to the house effect in open air. Originally we interpreted the house effects by assuming that the house acts by confining a diffusing growth effector. However, that interpretation was inconsistent with many other experiments to be discussed in detail in Section VI. Our present interpretation of this experiment is the following:

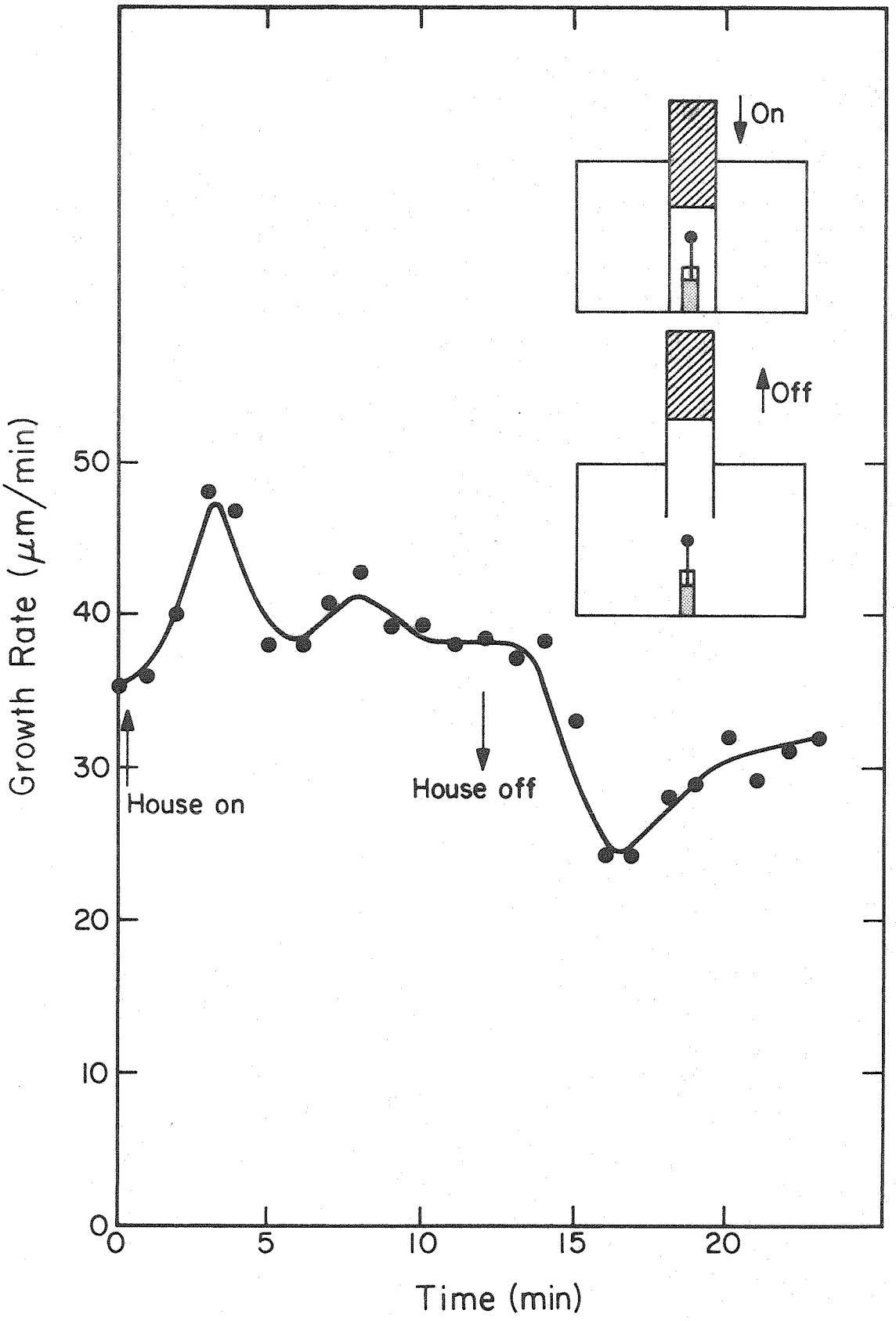
With the inner house lifted, the characteristic air speed in the large outer house is about 4-6 mm/sec. With the inner house lowered the characteristic air speed inside this double house is about 0.5-0.8 mm/sec. The outer house alone reduces the air speed near the sporangiophore from faster than 10 cm/sec to 4-6 mm/sec and causes a positive growth response. After the sporangiophore has adapted to the new level of quietness, the lowering of the inner house further reduces the air speed by a factor of about 8 and hence causes a second positive growth response to this inner house.

Adaptation: The house effect is a transient one, similar to the growth response to a step up in light intensity. Therefore the sporangiophore must adapt to the level of quietness, and this adaptation must occur as in the case of light, with a time constant of minutes, not seconds, since it takes summation of lateral wind for a minute

to produce a tropic response. The avoidance response to a barrier is a sustained response until the specimen has grown away from the barrier, or indefinitely if the experiment is carried out in a tropostat, where the barrier during the response is kept close to and parallel to the growing zone at all times. To explain this sustained response we are faced with the same alternative as in the case of the responses to light: averaging of adaptation around the circumference; or strictly local adaptation combined with the effects of spiral growth. The mad mutants of class 2 show similarly perturbed avoidance and phototropic responses, especially those that show a very prolonged light growth response and a correlated very weak phototropic response.

Fig. 12. House-within-a-house growth response (J. Matricon)

The inner house (2.5 cm x 2.5 cm x 9 cm) could be slipped over the sporangiophore from the outside through a hole on the roof of the outer house (15 cm x 15 cm x 12.5 cm) 0.5 mm wider than the inner house. Both houses are made of lucite. The characteristic air speed in the outer house is 4-6 mm/sec. With the inner house lowered the air speed inside this double house becomes 0.5-0.8 mm/sec.


On
Off
Growth Rate (μm/min)
50
40
30
20
10
0
0
5
10
15
20
Time (min)
House on
House off

VI. The chemical self-guidance hypothesis and specific tests of various forms of it

The experiments described so far seem to agree with our working hypothesis stated at the beginning of Section I. Air movement probably plays the crucial role in the wind effects, avoidance response and the house growth response. To make this hypothesis more concrete, however, we must specify the signal actually received by the sensor in the avoidance response.

In this section we will examine the chemical self-guidance hypothesis, i.e., the hypothesis which assumes that the sporangiophore emits a volatile growth effector (gas X), which mediates the avoidance response. In the framework of this hypothesis, for the barrier to cause bending of the sporangiophore, it must somehow alter the distribution of gas X around the sporangiophore. There are two alternatives. The barrier can alter the gas X distribution either (a) by limiting diffusion or (B) by altering convection near the sporangiophore.

A. The barrier modifying diffusion of the effector

We will examine first the alternative that the barrier acts by limiting the diffusion of gas X. From experiments shown in Section IV (b) we know that the avoidance response is independent of the composition of the barrier. Therefore we must assume either that all of these barriers adsorb the gas X (effector) similarly or that they all reflect it similarly. The alternative, adsorption versus reflection, is coupled to an alternative concerning the effect of the gas X on the growth of the sporangiophore. If barriers adsorb the gas X,

the gas must inhibit growth since growth speeds up on the side proximal to the barrier. Conversely, if barriers reflect the gas, the gas must stimulate growth. We have therefore two alternative models, i.e.,

(α) The growth promotor-reflection model

(β) The growth inhibitor-adsorption model

(α) Disproof of the growth promotor-reflection model

Before discussing experimental tests of this model, the mode of effector emission should be considered.

The mode of emission and the background concentration problem

A sporangiophore in a closed house (6.2 cm x 6.2 cm x 6.2 cm) gives identical avoidance response to a barrier regardless of whether the barrier is introduced to the vicinity of the sporangiophore immediately after the sporangiophore is put into the house, or at some time (up to many hours) after the sporangiophore is put into the house. A normal avoidance response even occurs when the test sporangiophore and fifty additional sporangiophores have been in the closed house for several hours before the barrier is moved up to the specimen. This experiment shows that the sporangiophore would have to emit gas X continuously. However, it also indicates that the simple concept of continuous emission of a persistent gas cannot be correct. If it were true, the background concentration of gas X would keep building up. A steady state background concentration can only occur if the gas disappears, either because it decays or because the barrier and the walls adsorb it.

Since for the moment we assume that all barriers reflect the gas X, we must also assume that the gas X decays into something which no longer promotes sporangiophore growth. A lower limit for the half life time of X can be estimated from the distance dependence of the avoidance response as presented in Section IV(C). The data showed that a barrier at a distance of 5 mm or more can cause an avoidance response. This implies that the gas X should live long enough for a significant portion of the gas to diffuse to the barrier and back, i.e., a distance of 1 cm or longer. This diffusion process takes $\tau = x^2/2D$ seconds (x = distance, D = diffusion coefficient of gas X). Put in x = 1 cm and D = 0.15 cm^2/sec, we obtain τ = 3 sec. Thus, we estimate that the half life of gas X should be at least a few seconds.

Experimental tests disproving the promotor-reflection model

(1) Avoidance response to "adsorptive" barriers

According to the model under discussion, a barrier alters the effector concentration by reflecting the effector. A barrier made of strongly adsorptive materials should be less effective in causing avoidance or might even cause negative avoidance. However, strongly absorbing materials such as activated charcoal barrier are as effective as any other barriers (e.g., plastic or glass barriers) in causing an avoidance response (Section IV B).

(2) Avoidance response to thin barriers

The experiments using thin wires as barriers (Section IVD) speak strongly against the promotor-reflection model. A thin wire (diameter $\sim$ 50 μm) causes detectable avoidance responses at a distance of 1000 μm. In this situation a thin wire which reflects would

cause an extremely small perturbation in the distribution of the effector gas at the barrier itself, and a fortiori near the growing zone.

(3) The failure of detecting the effector by bioassay

A direct test of the promotor-reflection model is to demonstrate the existence of the effector. Experiments designed to show the existence of an effector, however, have invariably yielded negative results.

Experiment 1: The experimental setup is shown in Fig. 13. The room air stream (or compressed tank air) flows down on the tested sporangiophore at a constant speed of 15 cm/sec. Changes of the composition of the air stream were made by simultaneously switching two 3-way valves, S_1 and S_2. Switching takes less than 0.5 sec. Pathway 2 consists of a glass chamber containing about 1000 stage 4b sporangiophores. According to the promotor reflection model, the air stream passing through pathway 2 should carry the growth promoting effector. Pathway 1 does not contain any sporangiophores other than the tested sporangiophore. The model predicts a positive growth response when the air stream is switched to pathway 2 and vice versa. The data (Fig. 13) show no growth response upon switching. In this experiment, a wide range of concentrations of gas X were tested by varying the number of stage 4b sporangiophores and the air stream speed. In each case, no growth response was found upon switching. Therefore this negative result cannot be explained by saying that the concentration

of gas X carried by the air stream is not right in causing a growth response.

Experiment 2 (R. J. Cohen): The setup is the same (Fig. 12) as the "house-within-a-house" experiment described in Section V except that between the inner house and the outer house there were a few thousand sporangiophores uniformly spaced such that a few dozen sporangiophores were within 2.5 cm of the tested sporangiophore. According to the promotor-reflection theory, the effector concentration outside the inner houses should be higher than inside. Since many sporangiophores were within 2.5 cm of the tested sporangiophore, there should be an increase in the effector concentration upon lifting the inner house even if the effector decays with a half life of about 1 sec. Therefore, there should be a reversed "house-within-a-house" effect, i.e., there should be a positive growth response instead of a negative one when the inner house is lifted. Experimental results showed a normal "house-within-a-house" effect.

(β) The growth inhibitor-adsorption models

The experiments described above speak strongly against the promotor-reflection model, but they are compatible with the other alternative, i.e., the growth inhibitor-adsorption model. However, the inhibitor-adsorption model can also be rejected because

(1) It can not explain the wind growth response.

The air current will sweep away the emitted gas and therefore decrease the gas concentration near the growing zone. According to the growth inhibitor-adsorption model, the effector inhibits growth.

Therefore the air current should cause positive growth responses. This contradicts the experimental results (Section II B).

(2) Two sporangiophores repel each other.

Two sporangiophores were tested for their effects upon each other in a situation of accurate parallel alignment (Fig. 14). The midplane between the sporangiophore is a plane of symmetry and should act like a virtual reflecting plane irrespective of the distribution of sources and sinks on the sporangiophore. Therefore, if all real barriers including sporangiophores adsorb, this virtual barrier should reflect. In this experiment, we should see a reversal of the avoidance response, causing the sporangiophores to move toward each other.* The experiment showed clear mutual avoidance of the aligned sporangiophores, thus disproving the adsorption-inhibition model.

We conclude that the barriers act neither by adsorption nor by reflection of a gas X.

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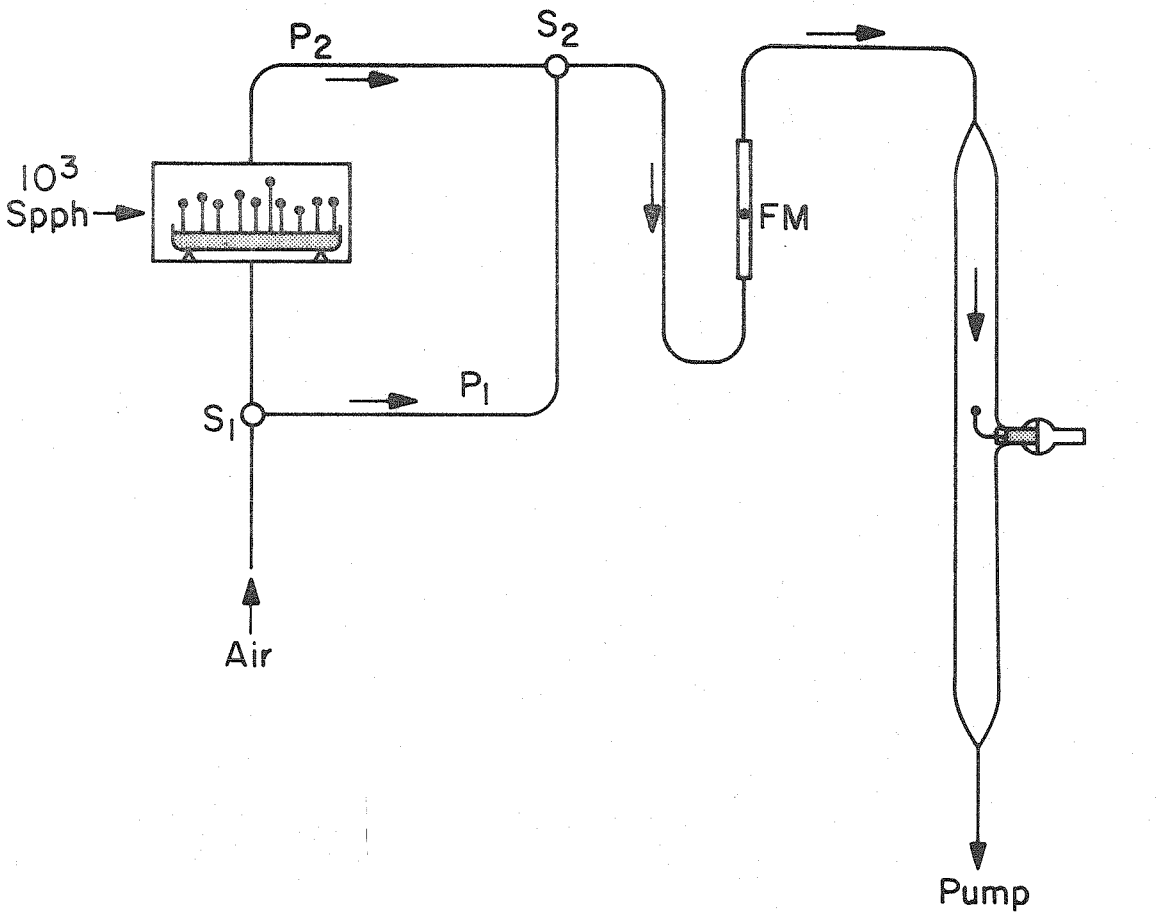
*Two sporangiophores repel each other regardless of their sex.

Fig. 13. Attempted bioassay of growth effector emitted by many sporangiophores

The experimental setup is shown on the top of the figure. Pathway 2 (P_2) includes a glass chamber containing about 1000 stage IV sporangiophores. The air current produced by suction was switched between the two pathways at times indicated. This was done periodically, 10 min P_1, 10 min P_2, for a total of 5 periods (100 minutes). The average is plotted. No growth response upon switching was observed. Legend: P_1, pathway 1; P_2, pathway 2; S_1, switch 1; S_2, switch 2; F.M., flowmeter.


P2
S2
10^3
Spph
FM
P1
S1
Air
Pump

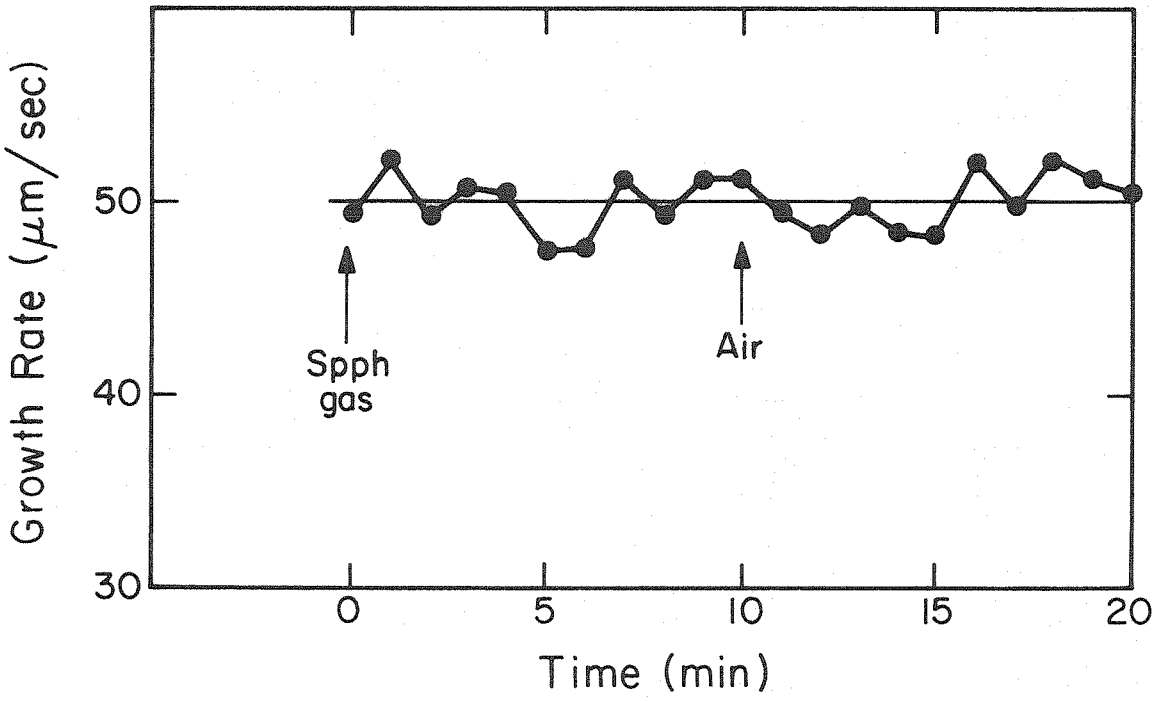
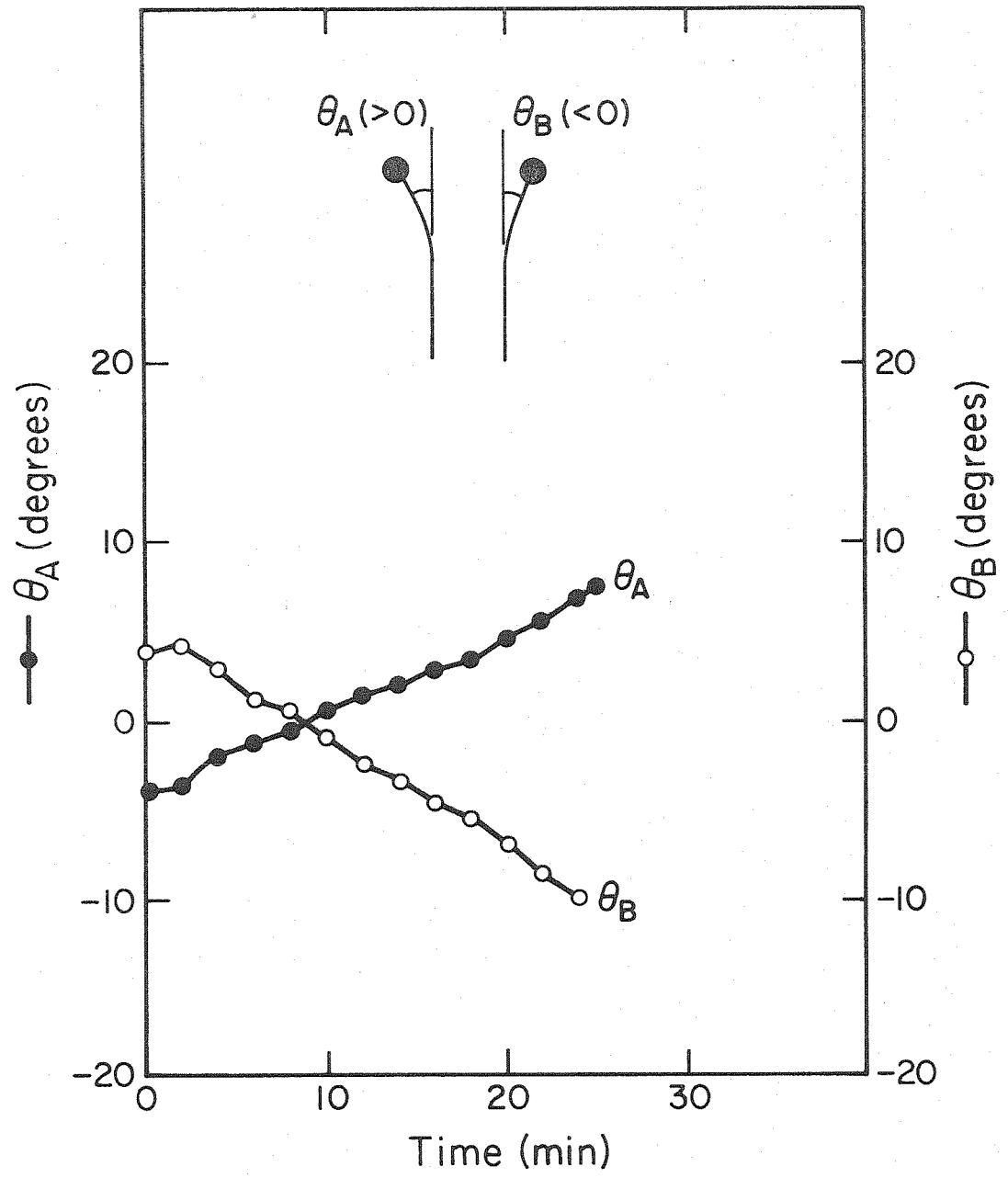

Growth Rate (μm/sec)
50
40
30
Spph
gas
Air
0
5
10
15
20
Time (min)

Fig. 14. Two sporangiophores repel each other

At t = 0, two sporangiophores growing in different vials inside the standard avoidance set-up were brought to a distance of 0.6 mm from each other, and were accurately aligned so that they were parallel to each other. The sporangiophores bent away from each other.


θA (>0)
θB (<0)
θA (degrees)
θB (degrees)
θA
θB
20
10
0
-10
-20
0
10
20
30
Time (min)

B. The barriers as aerodynamic obstacles

From the preceding discussion, we conclude that in causing the avoidance response, the barriers do not act by modifying diffusion. An alternative and perhaps the only remaining viable form of the CSGH is to postulate that barriers affect the distribution of the effector by altering the ambient wind pattern near the sporangiophore.

The localization of the emitter

To explore this hypothesis, we start with an argument which permits us to specify the localization of the emitter. We know from the experiments described in Section IV that the sporangiophore can not distinguish an activated charcoal barrier from a glass barrier at a distance of 0.5 mm from the sensor, suggesting that the majority of the effectors do not reach the barrier. If the emitter is 0.5 mm or farther away from the sensor, the emitted molecules must diffuse at least 0.5 mm to reach the sensor. A significant portion of the effector molecules will then reach the barrier and hence the difference in the adsorptivity of barriers ought to matter in causing the avoidance response. This contradicts our experimental observation. Therefore, the distance between the emitter and the sensor must be much less than 0.5 mm. Thus, we are led to the conjecture that the emitter is closely interspersed with the sensor, and that the growing zone readsorbs the emitted effectors at such a rate that most of the effectors fail to escape from the vicinity of the growing zone.

The readsorption model

Based on the above considerations, we formulate the readsorption model with the following assumptions:

(1) The sporangiophore emits a volatile growth promoting substance X at a constant rate. The emitter of X is interspersed with the sensor in the growing zone. The emission points themselves might be the sensing points, sensing the net emission from these points.

(2) The growing zone readsorbs the emitted effectors at such a rate that most of the effectors fail to escape from the vicinity of the growing zone.

(3) The local growth rate of the sporangiophore decreases when the local adsorption rate of X at the surface of the growing zone diminishes.

The major difference between the present model and the previously disproven promotor-reflection model (Section VI A (α)) lies in the assumption (2) of the present model, which implies that the barrier can not act by modifying diffusion.

The present model can be treated mathematically. The concentration distribution of effector can be determined by solving a diffusion-convection equation with an appropriate boundary condition. A boundary condition at the surface of the growing zone is formulated according to the assumptions of the model (Appendix B):

$$A - kC(a,\theta) = -D\frac{\delta C(r,\theta)}{\delta r}\Big|_{r = a}$$

Here, cylindrical coordinates are used. $C(r,\theta)$ is the concentration distribution of X; A is the emission rate of X per unit surface area;

k is the adsorption rate constant; and D is the diffusion constant of X in air. $kC(a,\theta)$ is the rate of adsorption of effector per unit area at the surface of the growing zone. This term indicates the strength of the growth promoting stimulus.

The diffusion-convection equation for slow lateral winds ($Re < 1$) is solved using the Oseen approximation (Appendix C). The concentration of effector turns out to be higher on the leeward side, predicting the sporangiophore bending into the wind. Lateral winds and hence random winds also decrease the average concentration of the effector near the growing zone. Thus the quieting of winds will result in an increase of concentration of the gas X near the sensor, and an increase in the growth rate. This model also reasonably accounts for responses to thin wire barriers, which may be expected to produce a quieting of winds, strictly locally.

Quantitatively, these effects depend critically on the value of the adsorption coefficient k, in relation to the sporangiophore radius a, and the diffusion coefficient of gas X in air, D. The critical parameter is the dimensionless number $\mu = ak/D$, which is proportional to the readsorption rate. It compares a, the radius of the sporangiophore, to D/k, a length which measures the mean distance to which effectors diffuse before they are readsorbed (Appendix B). The physical meaning is the following:

Consider the fraction $P\mu$ (b) of molecules whose greatest excursion from the source before readsorption is less than b. In the vicinity of the emitting surface $P\mu$ (b) has the form

$$P\mu(b) = \frac{1}{1 + 1/\mu \log(b/a)}$$

If $\mu << 1$, $P\mu(b) \sim 1$, most molecules can reach distance b. If $\mu >> 1$, $P\mu(b) << 1$, most molecules will be readsorbed before they can reach b. We require that the majority of the molecules do not reach a nearby barrier, substituting the numbers into this form we obtain the explicit requirement that $\mu \geq 5$ (Appendix B).

On the other hand the asymmetry of the quantity of effector molecules readsorbed by the leeward and the windward side of the sporangiophore is approximately $\frac{ua}{2D} \times \frac{1}{1 + \mu} = \frac{Pe}{2} \frac{1}{1 + \mu}$ (Appendix C); i.e., for small Peclet number Pe, the asymmetry increases with air speed and decreases with the adsorption rate constant. Thus for $\mu>>1$, the wind can cause very little asymmetry. For lateral wind velocity of 3 cm/sec and $\mu = 5$, the asymmetry is about 3 parts in a hundred. Since the sporangiophore exhibits rheotropic response to 1.5 cm/sec lateral wind, according to our calculations, the sporangiophore should be able to detect an asymmetry of one or two parts in a hundred between the leeward and the windward sides. Thus, for $\mu \sim 5$ we obtain both a reasonable efficiency of readsorption to account for the activated charcoal experiments and a reasonable asymmetry in the presence of lateral wind.

The constant k (= $\mu D/a$) has the dimensions of velocity and is related to α, the probability that a molecule is adsorbed at any one encounter with the surface by the relation $k = \alpha D/L$, where L is the mean free path between collisions with air molecules. Thus, for $\mu = 5$, we have $\alpha = 10^{-3}$ (where $L = 10^{-6}$ and $a = 5 \times 10^{-3}$ cm). In other words, our model requires the probability that a molecule be

adsorbed at any one encounter with the growing zone to be of the order of 10^{-3}.

We postulated that the local growth rate of the spph is regulated by the local adsorption rate of the effector (assumption 3). But we did not specify how the spph detects the adsorption rate. One alternative is to assume that the spph has some receptor organelle on the surface of the growing zone which enables it to detect the effector concentration. Another alternative which seems more economical from the standpoint of Phycomyces is the assumption that the emitter and the detector are the same structure, in other words, that the spph senses the net emission of effector. An increase in adsorption rate decreases the emission and hence causes higher growth rate. According to this alternative, any material which can be adsorbed to the emitter has the potential of decreasing the emission rate of the effector and causing a growth response. This might explain R. J. Cohen's finding that many volatile substances can cause growth responses of spph. This also cautions that a substance which can cause a growth response is not necessarily the growth effector emitted by the spph.

Future experimental tests of the readsorption model

For a critical examination of the model, the experiments proposed in this section should be performed. Some of these experiments have already been tried. More data are needed to check the predictions of this model.

(i) In this model, the barriers act as aerodynamic barriers. Therefore, the model predicts that in an absolutely quiet house the avoidance response should vanish. The slowest random air movement we have been able to achieve so far is 0.02-0.1 mm/sec, inside a small house (2.5 cm x 2.5 cm x 2.5 cm). This speed probably is still significant. We might be able to lower the air speed further by using better designed houses. Two important parameters in the designing of the houses are the temperature stability and the size of the house.

(ii) Mutant selection: We do not have an efficient way for screening avoidance mutants. However, an efficient method for screening mutants with abnormal rheotropic responses (non-rheotropic and super rheotropic) has been developed recently. A mutant which exhibits non-rheotropic behavior can belong to one of the three classes:

(A) "Stiff" mutants which have defects in the output machinery and thus exhibit abnormal behavior in all the tropic responses.

(B) Slow growers which will give slow and very weak tropic responses.

(C) Specific rheotropic mutants which are abnormal only in the rheotropic responses but not in phototropism.

After screening about 30,000 nitrosoguanidine mutagenized colonies, 8 non-rheotropic mutants were obtained. Six belong to class A and two belong to class B. No class C mutant has been found yet. The mutation rate for non-rheotropism appears to be low compared to that for obtaining stiff mutants in nitrosoguanidine mutagenesis. Heavier dosage of nitrosoguanidine and/or mutagenesis by other mutagens such as frame shift mutagens should be tried.

Analysis of specific rheotropic mutants should provide us insights into the nature of the avoidance response. For example, our hypothesis assumes that most of the effectors are readsorbed before they can escape the sporangiophore growing zone. This might explain our failure to detect effector by bioassays using wild-type Phycomyces sporangiophores. Among the rheotropic mutants, however, we might expect some mutants which overproduce the effector, or mutants which have defects in the readsorption mechanism. Enough effector molecules might be collected from these mutants to induce a growth response in the wild-type sporangiophore in the bioassay experiments.

In summary, we have excluded the chemical self guidance hypotheses postulating the barrier as a diffusion modifier. The alternative form of the chemical self guidance hypothesis postulating the barrier as an aerodynamic obstruction can reasonably account for the experimental results. Possible further experimental tests have been discussed.

VII. Specific tests ruling out various alternative hypotheses

A. Electrostatic effects

(a) Electrostatic bending

It is a common observation that sporangiophores are sensitive electrometers since any charged body (a rubbed plastic, for instance) causes strong attraction by virtue of induced charges. The notion therefore should be considered that the sporangiophore always carries small charges, or net polarization of charges, that the sporangiophore may induce countercharges or polarization in the barrier and that the mechanical force so generated causes subliminal bending. This notion was tested by examining controlled electrostatic deflections of sporangiophores situated between vertical condenser plates (separated by 0.4 cm and charged up to ± 400 V (Fig. 15). Maximal electric field causes visible passive bending (about 2°), but no significant tropic stretch responses to this passive bending. In contrast, the avoidance response evoked by either one of the same condenser plates (uncharged), placed close to the sporangiophore, occurs without visible preliminary bending. This experiment eliminates electrostatically induced bending as the cause of the avoidance response.

(b) Electrostatic detection

It is known that some electric fish can detect weak electric fields with their extremely sensitive electroreceptors. It has been suggested (10, 11) that fish with weak electric organs set up electric field in the water and detect any distortion of the field caused by the presence of objects with different conductivity. It is conceivable

that Phycomyces might use electrostatic effects as the cue for the avoidance response. There exists a resting potential between the inside and the outside of a sporangiophore submerged in water of low salt concentration, minus 80-90 mV inside, and similar ones have been found in Neurospora (unpublished experiments by M. B. Willard and M. Delbrück). This membrane potential will normally be cylindrically symmetrical around the cylinder axis. The symmetry might be destroyed by any dielectric brought into the vicinity of the sporangiophore, and these dielectrics could thus affect membrane processes controlled by membrane potentials, and could thereby cause an asymmetry in growth. This hypothesis is ruled out on the basis of three experiments:

(1) The experiments illustrated in Fig. 15, i.e., the sporangiophore does not respond to a constant electric field (up to 1000 V/cm).

(2) Type 1 experiments described in Section VI: Semiquantitative comparison of the avoidance of a mineral oil ($\varepsilon \sim 2$) meniscus with that of a water ($\varepsilon \sim 80$) meniscus, both used as horizontal barriers, showed no detectable difference.

(3) Type 2 experiments described in Section VI: The sporangiophore does not show tropism when sandwiched between dielectrically highly dissimilar barriers (plastic with $\varepsilon \sim 3$ vs. aluminum with $\varepsilon \sim \infty$). A similar but more critical experiment is described in the following:

Two parallel horizontal cover glasses (0.1 mm thick, $\varepsilon \sim 5$) 3 mm or 1.5 mm apart gave no tropic response for a horizontal sporangiophore placed symmetrically between them (Fig. 16). A brass plate ($\varepsilon = \infty$) 1 mm thick was then laid on top of the upper cover glass, making the double barriers dielectrically highly asymmetric. Unlike the

type 2 experiments, in this case the surface properties of the two barriers facing the sporangiophore are the same. Therefore, the only difference of the two barriers is the dielectric constant. Still no tropic response was seen.

Fig. 15. The sporangiophore in a constant transverse electric field

The experimental setup is shown on the left hand side of the figure. The sporangiophore is situated at the center between two brass plates inside a closed house. A voltage source is connected to the two brass plates and a constant electric field thereby applied to the sporangiophore transversely. The data are shown on the right hand side of the figure. The lower curve gives the voltage difference between the brass plate on the right and the brass plate on the left. The upper curve shows the angular deviation of the sporangiophore from the vertical direction. The angular deviation is defined as positive when the sporangiophore bends toward left. These data indicate that the sporangiophore does not show any appreciable tropic response to a constant electric field. Legend: V, voltage source; S, switch; O, o-ring.

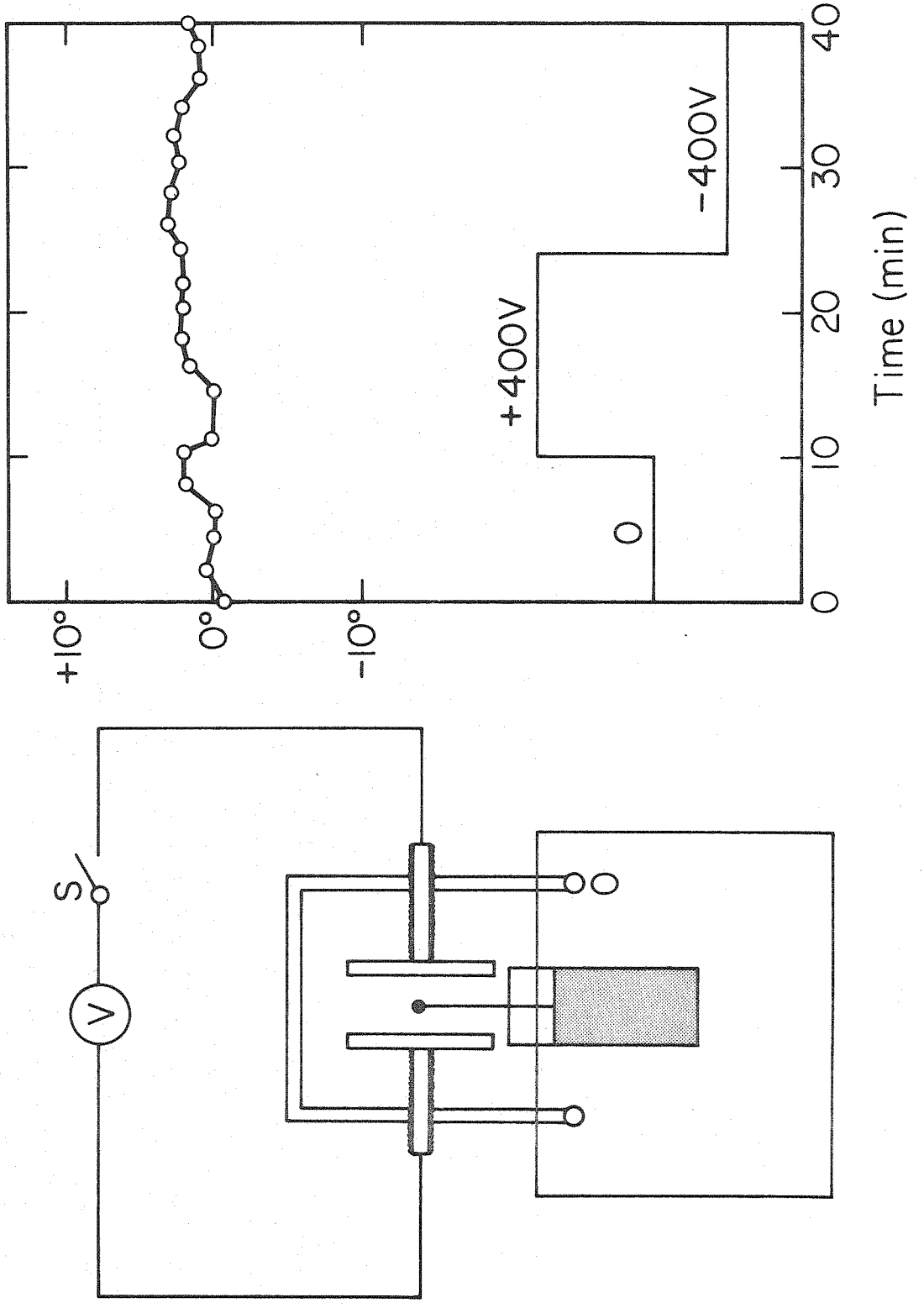
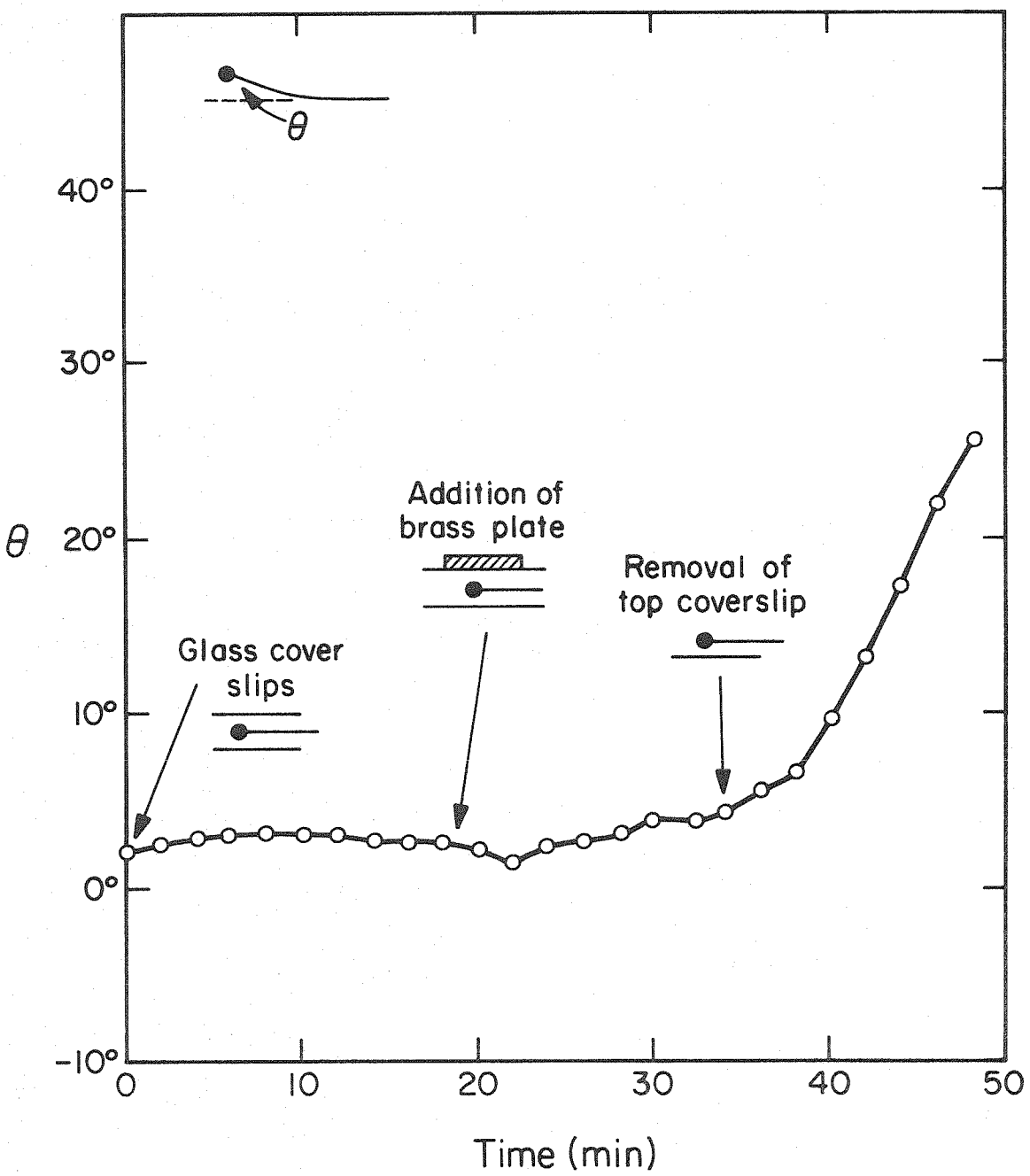

+10°
0°
-10°
0
+400V
-400V
0
10
20
30
40
Time (min)
S
V

Fig. 16. The sporangiophore in a dielectrically highly asymmetric environment

The procedure is described in the text. The sporangiophore does not exhibit a tropic response when placed between two barriers of drastically different dielectric properties. θ indicates the angular deviation from the horizontal direction, with bending upward being defined as a positive deviation.


θ
40°
30°
20°
10°
0°
-10°
θ
0
10
20
30
40
50
Time (min)
Addition of brass plate
Removal of top coverslip
Glass cover slips

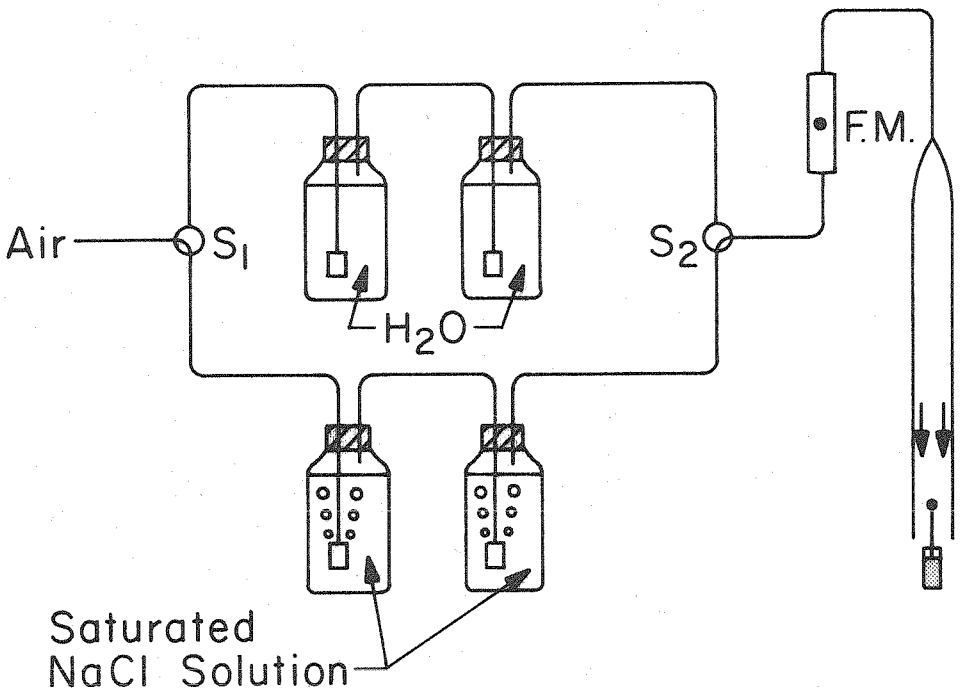
B. Humidity effect

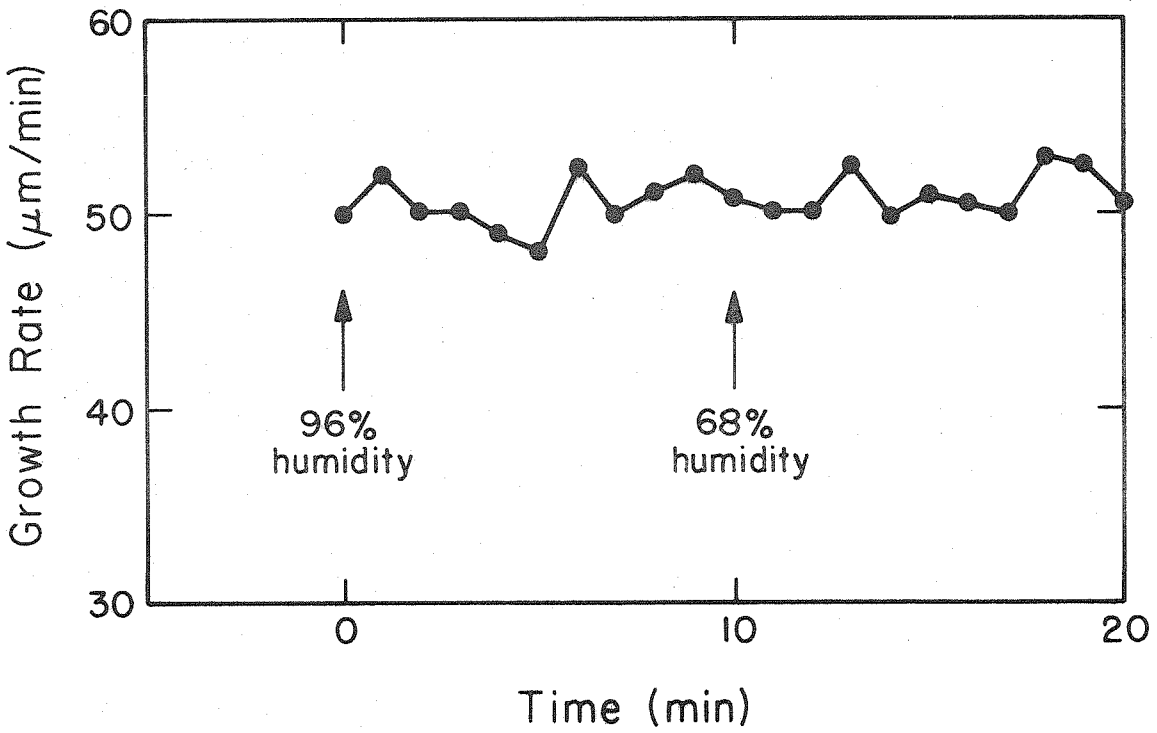
Johnson and Gamow (4) proposed that water vapor is the cause of the avoidance response. We do not believe so. In experiments involving vertical downward air current of 3 cm/sec, if the humidity of the air current is alternated between 68% and 96% in 20 minute cycles, no measurable growth responses to the change in humidity were observed. Another experiment which can not be explained easily by the water vapor hypothesis is that the avoidance response in the presence of 100% humidity is qualitatively indistinguishable from that in the presence of low humidity (30-50%).

One of the arguments of Johnson and Gamow in favor of water vapor as the cause of the avoidance response is based on a series of experiments by Thimann and Gruen (12) showing a negative tropic response of the sporangiophore to the local application of a small drop of distilled water to the surface of the sporangiophore. The time course and the magnitude of the response are similar to those of the avoidance response. We have found, however, that this effect is not specific to water. The same effect occurs in response to covering one side of the growing zone with vacuum grease or protein solutions. It would appear that these effects represent extreme cases of avoiding a barrier. For instance, we may be dealing with local increase in the concentration of gas X, due to blocking its escape via evaporation.

Fig. 17. Humidity experiments

The experimental setup is shown in the upper part of the figure. Uniform air current alternating from pathways containing either water or saturated NaCl solutions, with relative humidity 96% and 68% respectively, was blown on the sporangiophore. The data plotted in the lower part of the figure are average values of 5 periods. No growth response upon switching to different relative humidity was detected. Legend: S, switch; F.M., flowmeter.


Air
S1
H2O
S2
F.M.
Saturated
NaCl Solution

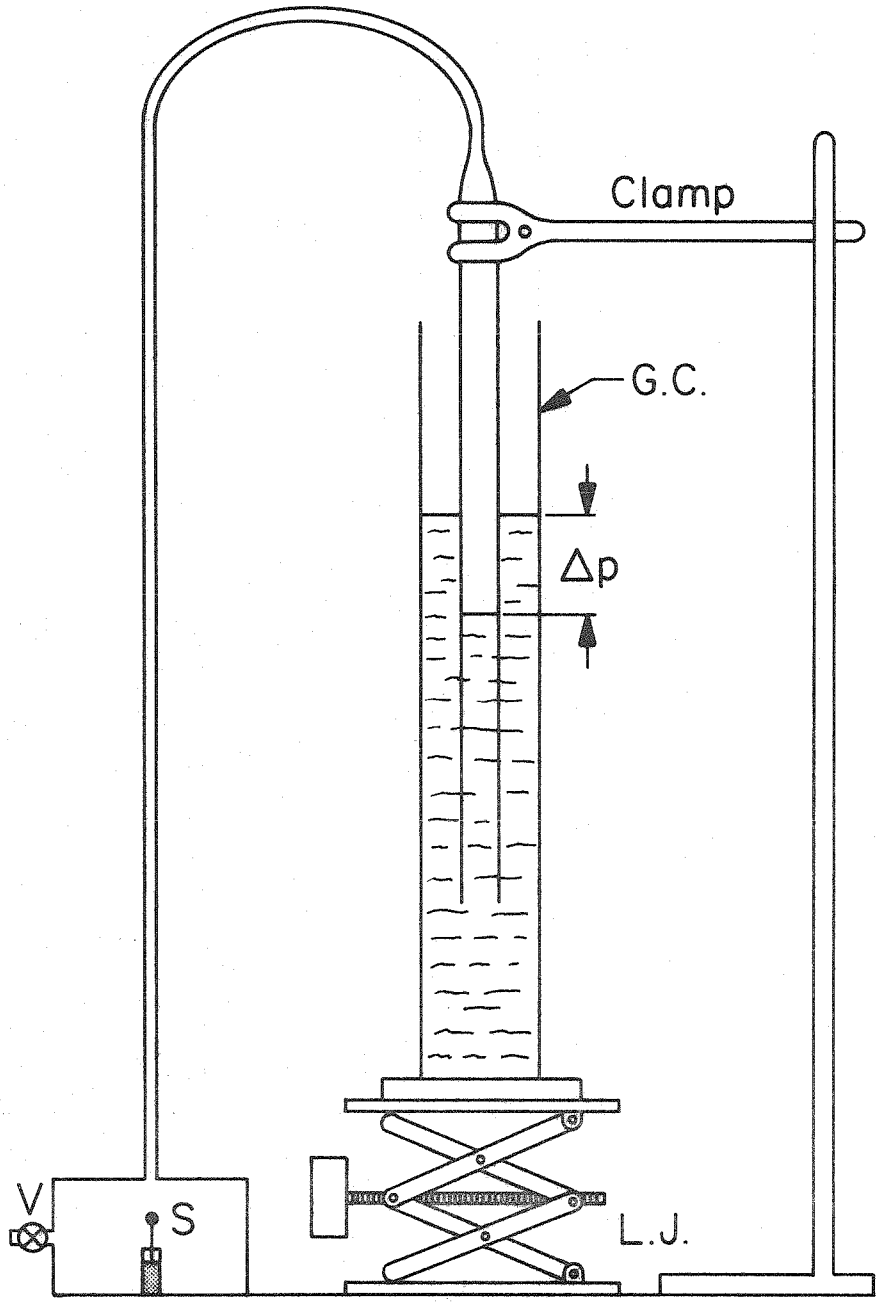

Growth Rate (μm/min)
60
50
40
30
96%
humidity
68%
humidity
0
10
20
Time (min)

C. Pressure effect

Air currents are driven by pressure gradients. For a sporangiophore in an air current, there must be a small pressure difference between the leeward and the windward side. Therefore an alternative explanation of the rheotropic response is that the sporangiophore can detect pressure difference across itself and give a tropic response to the pressure difference. This possibility was tested in three experiments involving pressure steps between different values of pressure (1 atm + 25 cm water vs. 1 atm, 1 atm + 5 cm water vs 1 atm - 5 cm water, and 1 atm + 0.5 cm water vs. 1 atm - 0.5 cm water respectively). No measurable growth responses were detected. The experimental setup for alternating the pressure is shown in Fig. 18. The pressure change is accomplished by adjusting the vertical position of the water-containing graduate cylinder. The pressure changes cover the range of pressure differences we might encounter in the wind experiments.

Fig. 18. Apparatus for pressure experiments

The sporangiophore is situated in an airtight glass chamber. The pressure in the chamber is controlled by adjusting the vertical position of the water containing graduate cylinder. Legend: G.C., graduate cylinder; S, sporangiophore; V, valve; L.J., lab jack; Δp, pressure difference between the inside of the chamber and the atmosphere.


Clamp
G.C.
Δp
V
S
L.J.

D. Temperature effect

Another hypothetical scheme by which the sporangiophore could detect the nearby air movements is that the sporangiophore detects a minute temperature change caused by the air movement. It is known that the sporangiophore transpires at a high rate. Thus, higher wind velocity near the sporangiophore might increase transpiration and thereby lower the temperature of the sporangiophore slightly, and hence decrease the growth rate. Therefore the sporangiophore might detect nearby air movements by detecting temperature changes provided it has a very sensitive temperature detection mechanism. This explanation is unlikely since humidity changes give no growth responses. It is contradicted also by the following direct test: A sporangiophore situated vertically at the center between two large reservoirs, kept at 27°C and at 19°C respectively and located 1 cm apart, does not bend toward either reservoir.

4. Discussion

The experiments discussed in the preceding sections establish many facts about the wind effects, the house growth response and the avoidance response of Phycomyces sporangiophores. We find that:

(1) The sporangiophore shows a rheotropic response (into the wind) to lateral wind of velocity $\geq$ 1 cm/sec.

(2) A step up in wind velocity (wind of any direction) causes a negative growth response of the sporangiophore.

(3) Fast wind (15-30 cm/sec) parallel to the barrier and the sporangiophore eliminates the avoidance response.

(4) The avoidance response occurs in a closed chamber.

(5) The avoidance response is independent of the dielectric, magnetic, gas-adsorbing, electromagnetic radiation-absorbing (ultraviolet, visible, and infrared) properties of the barrier.

(6) The sensor of the avoidance response is situated near the middle of the sporangiophore growing zone.

(7) The magnitude of the avoidance response (defined as the maximal bending rate) decreases approximately linearly with increasing distance of the barrier.

(8) Enclosure of the sporangiophore causes a positive growth response (house growth response).

(9) If the avoidance response is mediated by a volatile substance, the barriers are not reached by diffusion away from the immediate vicinity of the sporangiophore growing zone.

(10) The electrostatic field, humidity, pressure, mechanical stretch, and temperature have been excluded as possible signals which mediate the avoidance response.

By measuring the air movements in these experiments, we found that the air movement seems to be the key parameter involved in all three effects: the wind effect, the house growth response, and the avoidance response. All our experiments appear to be consistent with the working hypothesis stated at the beginning of Section I. However, the physical nature of the signal has not been identified yet. The hypothesis which appears most plausible to us is the postulate that the avoidance response, wind effects and the house growth response are all mediated by a volatile growth effector emitted and detected in the growing zone of the sporangiophore (CSGH).

We have examined several simple forms of CSGH and found that they are incompatible with experimental results, notably the invariable failure in our attempts to detect this hypothetical gas. The present model postulates that the sporangiophore emits and readsorbs a volatile growth promoting effector and the barrier affects the effector distribution by acting as an aerodynamic obstacle. This model can reasonably account for the avoidance response, the wind effects and the house growth response. It also explains the apparent dilemma that we failed to detect the hypothetical gas.

The avoidance response certainly has a great survival value for Phycomyces. In nature, the Phycomyces mycelium is likely to be found in some dark, damp cracks. For its spores to be dispersed, the

sporangiophore has to find its way out. If there is light, the sporangiophore can use light as a cue. However, if it is in darkness, the avoidance mechanism can provide a way for the sporangiophore to get out.

In nature the avoidance response is not unique for Phycomyces. It has been observed also in cellular slime molds (13). The behavior of the fruiting bodies of slime molds is amazingly similar to that of the sporangiophore of Phycomyces. They repel each other, avoid barriers as well as thin rods, and bend into wind. The avoidance in slime molds is also independent of the material of the barrier except for activated charcoal, to which the fruiting bodies bend toward instead of away from it. This finding has been interpreted as strong evidence supporting the hypothesis that the avoidance response is mediated by a gas. However, it is claimed that the culminating fruiting bodies are extremely sensitive to temperature difference. The bending toward charcoal could be interpreted as orientation toward warmer body, unless a control using as the barrier a black object other than charcoal is done. The bioassays attempted by Bonner and Dodd were similar to the ones we tried (Section VI), and were equally unsuccessful. So were the attempts to identify the hypothetical gas.

Another similar case is the "group" effect and rheotropic responses of developing Fucus eggs (14, 15). The cell polarity of the developing eggs of the brown alga Fucus furcatus was found to be determined rheotropically in sea water. At pH 6.5, the cells tend to form their rhizoidal pole downstream. Qualitatively, the downstream

response concurs with the positive group effect, i.e., the phenomenon that nearby zygotes tend to initiate their rhizoids toward each other. The effect is pH dependent. These phenomena led to the hypothesis that they are mediated by a growth stimulating substance emitted by the egg. However, when the authors tried to analyze their results quantitatively, the simple-minded picture broke down. They ended with a very complicated model involving two hypothetical effectors, one small and one large (molecular weight greater than 10^7!), without any direct evidence for the existence of such molecules.

The general picture emerging from the studies of the organisms which grow away (or toward) each other (or another object) and also show rheotropic responses is that the phenomenon seems to be mediated by a diffusible substance. Further analysis then reveals many difficulties which cannot be explained by a simple picture. Among the difficulties, the most notable one seems to be the invariable failure to detect the hypothetical diffusible substance. These failures probably can not be all attributed to improper experimentaion. Instead, they tend to suggest to us that these organisms employ some subtle and elusive mechanism to detect a nearby subject. Although the model we proposed can reasonably account for the experimental results, more critical tests such as the ones proposed in Section VI are needed to tell whether our model provides the answer.

Appendix A

The drag force on the sporangiophore

Since forces greater than 0.5 dyne are known to cause a stretch response (8), it is important to calculate the drag force of the wind on the sporangiophore to see whether the rheotropic response and the wind growth response might be attributable to the mechanical stress. The relevant part of a sporangiophore is a sphere (sporangium, 0.05 cm in diameter) and a cylinder (upper half of growing zone, 0.01 cm in diameter, $\simeq$ 0.1 cm in length).

In the case of longitudinal wind, the drag force on the sporangiophore is simply the drag force on the sphere (sporangium). In the case of lateral wind, the drag force is the sum of the drag force on the sphere (sporangium) and that on the cylinder (growing zone).

The drag force D can be expressed as

$$D = C_D(\rho v^2/2)A$$

where C_D: drag coefficient $C_D(Re)$, which decreases with the Reynold's number Re up to $Re = 10^3$.*

v: velocity of the wind

ρ: density of the air, 1.2×10^{-3} g/c.c.

A: projected area of the object normal to the wind direction

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*C_D values as function of Re are taken from Handbook of Engineering Mechanics (1962), (ed. W. Flügge) pp. 25-81, McGraw-Hill, New York.

Velocity (cm/sec)	Drag Force on the Sphere (dyne) (for longitudinal and lateral wind)	Drag Force on the Cylinder (dyne) (for lateral wind)	Total Drag Force for Lateral Wind (dyne)
1	0.00014	0.00007	0.00014
10	0.0017	0.00017	0.002
100	0.036	0.008	0.044

These numbers are to be compared with the estimated thresholds for the forces eliciting stretch responses:

longitudinal pull: 0.5 dyne lateral pull: 0.1 dyne

Around the thresholds of the wind responses ($\sim$ 1 cm/sec) the drag forces are about 1000 times smaller than those required to produce stretch responses.

Appendix B

The Re-adsorption Model

An emission-adsorption problem: How strong must re-adsorption be so that adsorption on a barrier nearby becomes negligible?

An adsorbing barrier at a distance small compared to the length of the growing zone (3 mm) catches those effector molecules which reach it. It thereby diminishes the concentration of the effectors near the surface of the sporangiophore. We require this effect to be small since the adsorbing quality of the barrier seems to be irrelevant. What constraint does this requirement impose upon the strength of readsorption? To obtain an answer to this question we will approximate the situation by considering a concentric cylindrical barrier. We will solve the diffusion equation between two concentric infinite cylinders, with the inner cylinder the sporangiophore and the outer cylinder the barrier. The diffusion equation is

$$\frac{d^2}{dr^2} C(r) + \frac{1}{r} \frac{dC(r)}{dr} = 0 \qquad (1)$$

where C(r) is the concentration distribution of effector.

We consider an inner cylinder (radius a) which emits A molecules/cm^2/sec into the space r>a. The molecules are adsorbed on this same surface at a rate kC(a), where k is the adsorption rate constant. The net emission is therefore A-kC(a). In a steady state in still air this net emission must be carried away at a rate $-D\frac{\delta C}{\delta r}\Big|_{r=a}$, where D is the diffusion constant of the effector X in air.

Our boundary condition at the surface of the inner cylinder is therefore

$$A - kC(a) = -D\frac{\delta C}{\delta r}\Big|_{r=a} \qquad (2)$$

If the emitting surface is surrounded by a totally adsorbing surface which has the shape of a concentric cylinder with radius $b > a$, at that surface we have

$$C(b) = 0 \qquad (3)$$

The general solution of equation (1) with (3) satisfied is

$$C_{a,b}(r) = B \log \left(\frac{b}{r}\right)$$

The solution of (1) which satisfies (2) and (3) is

$$C(r) = (A/k) \frac{\log (b/r)}{\log (b/a) + 1/\mu} \qquad (4)$$

where $\mu = ak/D$. μ is a numerical constant which is proportional to the readsorption rate. It compares a, the radius of the sporangiophore, to D/k, a length which measures the mean distance to which effector molecules differ before they are readsorbed.

From (4) we find that the concentration of the effector at the surface of the sporangiophore (r=a) is

$$C(a) = (A/k) \frac{1}{1 + 1/\mu \log (b/a)} \qquad (5)$$

In the absence of a barrier ($b = \infty$) this concentration is A/k. The presence of a cylindrical barrier at r=b reduces the concentration to the fraction

$$P_{\mu}(b) = \frac{1}{1 + 1/\mu \log (b/a)}$$

This function measures the fraction of molecules not adsorbed by a barrier at $\underline{b}$, and thereby the fraction of molecules whose greatest excursion from the source before readsorption is less than $\underline{b}$. This function is plotted in Fig. B for various values of μ.

The curves show clearly the effect which the numerical value of μ has on confining the effector molecules to the neighborhood of the source. For instance, if we require that less than 10% of the molecules reach a barrier at $\underline{b} = 5\underline{a}$ we must have $\mu \geq 5$. They also show that for $\mu = 5$ readsorption is weak enough to allow 50% of the effector molecules to escape to a distance greater than $0.2\underline{a}$, thereby giving random air currents to modify the distribution of effector molecules around the sporangiophore. This aspect is analyzed in Appendix C.

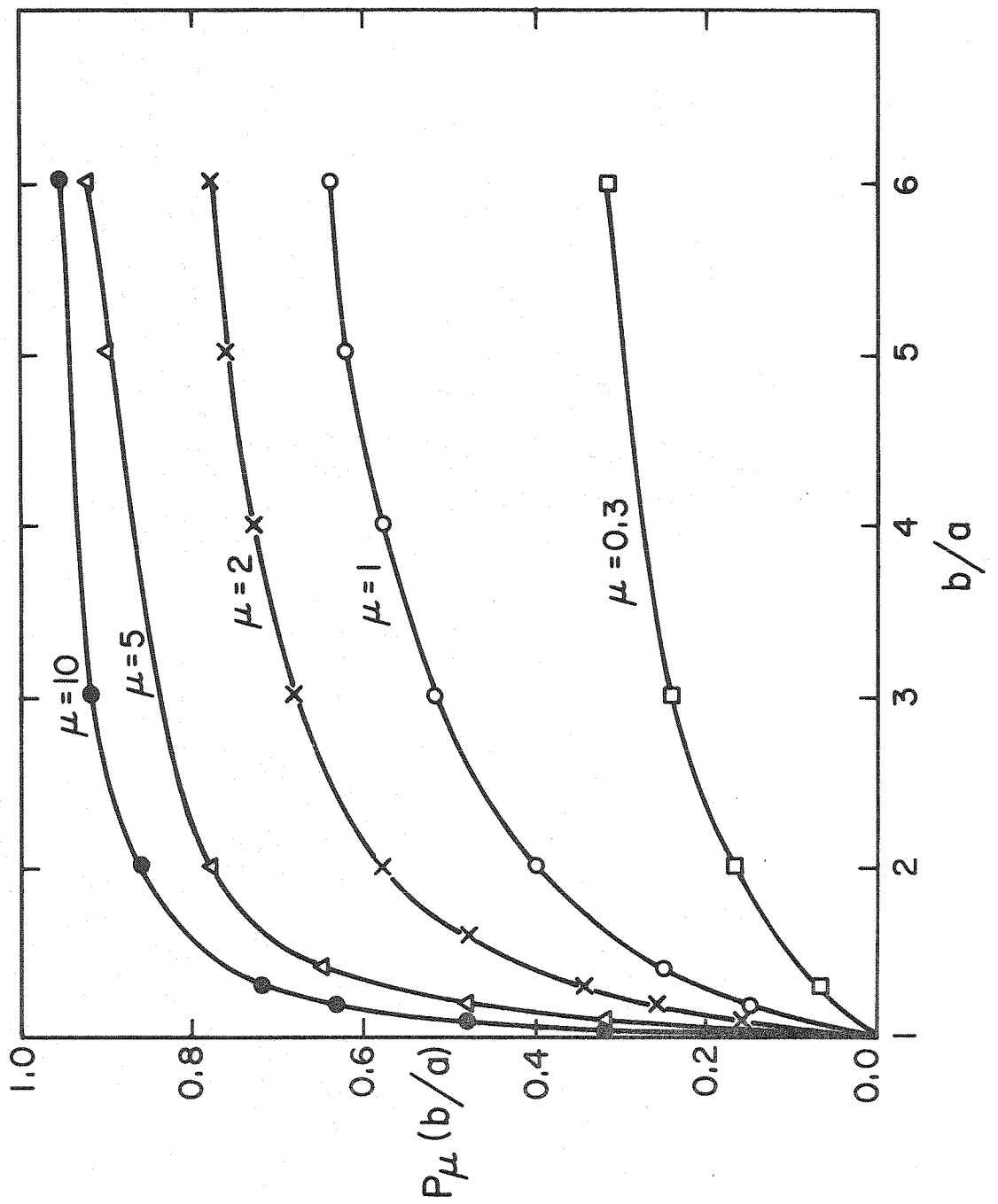
Fig. B. The distribution of molecules around the cylindrical emitter in the readsorption model. The function plotted is

$$P_{\mu}(b/a) = \frac{1}{1 + 1/\mu \log(b/a)}$$

where a = radius of the cylindrical emitter adsorber.

P(b/a) = cumulative distribution of maximal distances from the axis less than b. Note that in the cylindrical case all molecules will be readsorbed in the absence of a barrier.

$\mu = ak/D$ = a numerical constant which measures the mean excursion of the molecules from the axis, D/k, in relation to the radius of the source, a.


1.0
0.8
0.6
0.4
0.2
0.0
Pμ (b/a)
1
2
3
4
5
6
b/a
μ=10
μ=5
μ=2
μ=1
μ=0.3

Appendix C

The Effects of Lateral Wind in the Readsorption Model

We wish to calculate the effect of a lateral wind on the distribution of effector molecules around the emitting and adsorbing cylinder, and thereby on the readsorption of the effector. We solve the diffusion-convection equation in the Oseen approximation, using the appropriate boundary conditions. The solution will be obtained as a converging series of Bessel functions. The first term yields the change in concentration of effector averaged over the circumference of the cylinder. The second term yields the asymmetry of the distribution of leeward versus windward.

The constants a, A, k, D have been defined in Appendix B.

The Oseen approximation (6) of the diffusion-convection equation ignores wind velocity components in the y direction and assumes:

u = nominal airstream velocity

(1) $$u\delta c/\delta x = \left(\frac{\delta^2 c}{\delta x^2} + \frac{\delta^2 c}{\delta y^2}\right) D$$

Our Boundary conditions are (as explained in Appendix B):

(2) $$-D\,\frac{\delta c}{\delta r} = A - kc, \text{ on } r = a$$

$$c \to o \text{ as } r \to \infty$$

(3) Introduce $\lambda = u/2D$, a reciprocal length

(4) The Peclet number is $Pe = \frac{ua}{D} = 2\lambda a$; it will be assumed

that:

$$Pe << 1$$

(5) Introduce $c = e^{\lambda x}\phi$. Equation (1) becomes the Bessel equation:

$$(6) \qquad \frac{\delta^2\phi}{\delta x^2} + \frac{\delta^2\phi}{\delta y^2} - \lambda^2\Phi = 0$$

The general solution of (6), satisfying (2), is:

$$(7) \quad \phi = \beta_o \, Ko(\lambda r) + \beta_1 \cos\theta \, K_1(\lambda r) + \beta_2 \cos 2\theta \, K_2(\lambda r)\ldots$$

For $\lambda a << 1$ we will have $\beta_o \, Ko(\lambda a) >> \beta_1 \, K_1(\lambda a) >> \beta_2 \, K_2(\lambda a)\ldots$

K_o, K_1, K_2 ... are modified Bessel functions of the second kind.

At the boundary $r = a$ we have ($\lambda a << 1$)

$K_o = \log(2/a\lambda) - \gamma$; $K_1 = 1/\lambda a$ γ = Euler's constant = 0.577

$K_o' = -1/\lambda a$; $K_1' = -1/\lambda^2 a^2$

The boundary condition (2) in terms of ϕ and in polar coordinates becomes:

[since $\frac{\delta c}{\delta r} = e^{\lambda x}(\frac{\delta\theta}{\delta r} + \lambda\phi \cos\theta)$]

$$(2') \quad -D\left(\frac{\delta\phi}{\delta r} + \lambda\phi\cos\theta\right) = A\, e^{-\lambda a\cos\theta} - k\phi$$

Substituting (7) into (2') we obtain (2'')

$$-D\,[\beta_o \lambda K_o' + \beta_1 \lambda K_1' \cos\theta + \lambda \beta_o K_o \cos\theta]$$
$$= A\,[1 - \lambda a\cos\theta] - \beta_o k K_o - \beta_1 k K_1 \cos\theta$$

To determine β_o and β_1 we separately equate to zero the terms without and with cos θ

Without cos θ:

$$-D\beta_o \lambda K_o' = A - \beta_o k K_o$$

$$(8) \qquad \beta_o = \frac{A}{k K_o - D\lambda K_o'} = A \frac{a}{D} \frac{1}{\mu K_o - a\lambda K_o'}$$

With the help of β_o we can determine how the average adsorption of X (≡ stimulation) depends on the wind velocity. The adsorption will be

$$(9) \qquad S \equiv kc = \frac{A k K_o}{k K_o - D\lambda K_o'}$$

$$= A \frac{1}{1 - \frac{D\lambda}{k} \frac{K_o'}{K_o}}$$

$$= A \frac{1}{1 + \frac{D}{ka} \frac{1}{K_o}}$$

$$= A \frac{1}{1 + \frac{1}{\mu K_o}}$$

For $\mu \ll 1$ (weak adsorption) S will be a fraction of A and will logarithmically decrease with increasing wind velocity.

For $\mu \gg 1$ (strong adsorption) S will be nearly equal to A and will depend very little on wind.

We turn next to terms with cos θ in (2")

$$-D\lambda K_1'\beta_1 - D\lambda\beta_o K_o = -\lambda aA - \beta_1 kK_1$$

or $$\beta_1(kK_1 - D\lambda K_1') = -\lambda aA + D\lambda\beta_o K_o$$

$$\beta_1 = \frac{-\lambda aA + D\lambda\beta_o K_o}{kK_1 - D\lambda K_1'} = \frac{\frac{-\lambda a^2}{D} A + a\lambda\beta_o K_o}{\mu K_1 - a\lambda K_1'} \tag{10}$$

$$\frac{\beta_1}{\beta_o} = \frac{a\lambda K_o - \lambda a(\mu K_o - a\lambda K_o')}{\mu K_1 - a\lambda K_1'} \tag{11}$$

$$= a^2\lambda^2 \frac{K_o(1-\mu) - 1}{1 + \mu}$$

We wish to calculate the dissymmetry of the adsorption, leeward/windward. These adsorptions will be proportional to the concentrations $c(a,\theta = 0)$ and $c(a,\theta = \pi)$. Thus we need to calculate the ratio:

$$R = \frac{c(\theta=0)}{c(\theta=\pi)} = \frac{e^{\lambda a}(\beta_o K_o + \beta_1 K_1)}{e^{-\lambda a}(\beta_o K_o - \beta_1 K_1)}$$

$$= e^{2\lambda a} \frac{1 + \frac{\beta_1}{\beta_o}\frac{K_1}{K_o}}{1 - \frac{\beta_1}{\beta_o}\frac{K_1}{K_o}} \tag{12}$$

From (11) we have

$$\varepsilon = \frac{\beta_1}{\beta_o}\frac{K_1}{K_o} = a^2\lambda^2 \frac{K_o(1-\mu) - 1}{(1+\mu)\lambda a \cdot K_o} = a\lambda \frac{1 - \mu - 1/K_o}{1+\mu}$$

$|\varepsilon|$ is certainly $<< 1$. It is negative or positive depending on whether $\mu << 1$ or $\mu >> 1$.

Therefore we obtain

$$R = (1+2\lambda a)(1+2\varepsilon) = 1 + 2(\lambda a + \varepsilon)$$

$$= 1 + 2\lambda a \left(1 + \frac{1 - \mu - 1/K_o}{1+\mu}\right)$$

$$= 1 + 2\lambda a \frac{2 - 1/K_o}{1+\mu}$$

This quantity is > 1 (more on leeward side)

if $K_o > 1/2$

or $\log 2/a\lambda - 0.577 > 1/2$

or $\log 4/Pe > 1.077$

or $Pe < 1.36$, i.e., in all our cases

The dissymmetry R-1

(1) is approximately proportional with wind speed

(2) is appreciable only if μ is not too large compared to unity, i.e., when readsorption is not too strong. On the other hand, if $\mu << 1$ little of A is readsorbed and most of the emitted effector would be wasted and could be affected by barriers. From Appendix B, we obtained that $\mu \geq 5$ since μ cannot be too large compared to unity. Therefore we concluded that $\mu \sim 5$. For $\mu \sim 5$

we obtain both a reasonable efficiency of readsorption and a reasonable dissymmetry in the presence of lateral wind.

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