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Phycomyces

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INTRODUCTION

This monographic review on a fungus is not addressed to mycologists. None of the authors has been trained or has otherwise acquired a general proficiency in mycology. They are motivated by a common interest in the performances of signal handling exhibited by the sense organs of all organisms and by the desire to attack these as yet totally obscure aspects of molecular biology by the study of a microorganism with certain desirable properties.

The sporangiophore of the fungus Phycomyces is a gigantic, single-celled, erect, cylindrical, aerial hypha. It is sensitive to at least four distinct stimuli: light, gravity, stretch, and some unknown stimulus by which it avoids solid objects. These stimuli control a common output, the growth rate, producing either temporal changes in growth rate or tropic responses.

We are interested in the output because it gives us information about the reception of the various signals. In the absence of external stimuli, the growth rate is controlled by internal signals keeping the network of biochemical processes in balance. The external stimuli interact with the internal signals. We wish to inquire into the early steps of this interaction. For light, for instance, the cell must have a receptor pigment as the first mediator. What kind of a molecule is this pigment? Which organelle contains it? What chemical reaction happens after a light quantum has been absorbed? And how is the information in-

troduced by this primary photochemical event amplified in a controlled manner and processed in the next step? How do a few quanta or a few molecules trigger macroscopic responses? Will we find ourselves confronted with devices wholly distinct from anything now known in biology?

For light the dynamic range of Phycomyces, covering nine powers of ten in intensity, is similar to the range handled by the vertebrate eye. At the lower end of this range, the sensitivity is probably as great as the quantum noise limit permits, though not "one quantum per cell" as Wassink and Bouman (213) at one time conjectured.

For the other stimulus qualities, the sensitivities are inadequately analyzed. For gravity it is a matter of some importance that here is a system operating without obvious statoliths. A detailed analysis has become feasible since a mutant has been found that responds much more strongly than does the wild type. As a stretch sensor (a sensitivity first encountered by Dennison in connection with geotropic studies), Phycomyces is unique among the mechanosensitive ones with respect to the geometry and accessibility of the transducer. The avoidance response, a gross effect, has been noted intermittently since the 1880's. It is unique in the elusiveness of the physical stimulus involved: temperature? humidity? some other gas? a combination of several of these? The efforts in recent years of various persons (Shropshire, Kenehan, Heisenberg, Matricon, all unpublished) to narrow the possibilities have brought to light a number of new and surprising aspects of this response, but have not nailed down the physical stimulus.

This review, then, is addressed to those who aim to push sensory physiology to the limits of molecular biology. We believe that what can be learned from Phycomyces is relevant to this next phase of our quest for a mechanistic understanding of life. It is true that Phycomyces does not admit the use of the method which sensory physiologists have come to consider the *sine qua non* of their trade: the study of electrical signals. In spite of some scattered efforts, no such signals have been detected in Phycomyces.

There is, however, much room for similarities in earlier stages of the transducer chain, before the growth responses of Phycomyces and the receptor potentials of animal sensory cells, and it is to these as yet obscure stages that we think Phycomyces work can make a contribution of general relevance. It is here that membranes, microtubules, and other "solid state" structures may form a common ground in the amplifying and regulating mechanisms of signal handling.

Quite aside from its spectacular responses to various stimuli, the sporangiophore of Phycomyces recommends itself as an interesting object for other aspects of cell biology. Some of these aspects relate to an astounding property, discovered gradually over a long period, but fully elaborated only by Gruen (99). When severed from its parental mycelium at an early stage and supplied with nothing more than water and oxygen, this cell can run through its entire life cycle, increase its volume manyfold, and differentiate a sporangium, a columella, and spores. Here is a system that cries out for studies on the level of molecular genetics. One would like to analyze the contribution of nuclei and mitochondria to the control of metabolism and development of the whole sporangiophore, and to the life history of these organelles themselves. A great deal of basic genetic, biochemical, and ultrastructural work with Phycomyces has to be done to exploit these possibilities.

Phycomyces has been of interest to biologists for more than a century in various connections. First and persistently because of its giant sporangiophore and its striking phototropism; since 1904, because Blakeslee (16) discovered sexual self-incompatibility in some of the "Mucorineae" (including Phycomyces) and in this connection coined the terms "homothallism" and "heterothallism"; later, because Burgeff (37) and Schopfer (181) discovered the requirement of Phycomyces for vitamin B_1 (thiamine, aneurin). Schopfer developed this discovery into a quantitative B_1 assay procedure, widely used for a decade.

In this review we have attempted to be comprehensive as to subject matter and selective as to quality by bringing together and sifting work on all aspects of this organism, published over more than a century, often in journals not commonly found in libraries. A complete bibliography of Phycomyces literature has been compiled by Shropshire and Dennison.

Some previously unpublished data have been incorporated into this review. Some of these data will be presented more fully in papers which are in press or in preparation and are referred to as such. Others are not likely to appear soon in the context of other papers. These are mostly data acquired during the summer workshops at the Laboratory of Quantitative Biology in Cold Spring Harbor in 1965–68. We have included only data that we think are unambiguous and technically reliable and have given sufficient details to permit their reproduction by others. These data are referred to by the reference number 100 and the name of the observer (or his initials if he is one of the authors of this review).

Each section of this review was drafted by an author or authors whose names are given in the table of contents. General editing and coordina-

tion was done by M. Delbrück, but each author saw and corrected a semifinal version of the whole review and the final version of the sections credited to him.

We are very much indebted to E. S. Castle, A. Frey-Wyssling, H. Gruen, S. K. Malhotra, J. Matricon, M. Plempel, and R. M. Thornton for critical comments on various sections of this review, and to Patricia Reau for harmonizing substance and form.

1. Systematic position of Phycomyces, natural history

We will begin with a brief orientation regarding the position of Phycomyces within the general scheme of life. As a true fungus it has well-defined nuclei and mitochondria, is dependent for energy on preformed organic nutrients, and has two life cycles: a sexual one, involving highly resistant spores, designed to resist unfavorable seasons, and a vegetative one, involving spores designed for efficient dispersal in large numbers (Fig. 1–1). Also, in common with all other fungi, except for the class of Oomycetes, it has a cell wall whose principal structural element is a system of fibrils composed of chitin (poly-*N*-acetylglucosamine) rather than cellulose (poly-glucose; Section 36).

Phycomyces belongs to the lower fungi, the Phycomycetes. This group, which used to have the status of a "class," is now recognized as a taxonomic artifact. It lumps together fungi whose common feature as opposed to the classes of higher fungi (Ascomycetes and Basidiomycetes) is a negative one: the *absence* in the sexual life cycle of a distinct *dikaryotic* phase. Whereas in the Asco- and Basidiomycetes the *fusion of cells* of opposite mating type (plasmogamy) is followed by a period in which the nuclei of the two types divide in synchrony before finally *nuclear fusion* occurs (karyogamy), no such separation of the two steps occurs in the lower fungi.

This negative characteristic, however, is not a good reason for lumping the lower fungi together. These fungi have accordingly been reassigned by taxonomists to a group of classes, characterized by the type of flagellation of the vegetative spores.

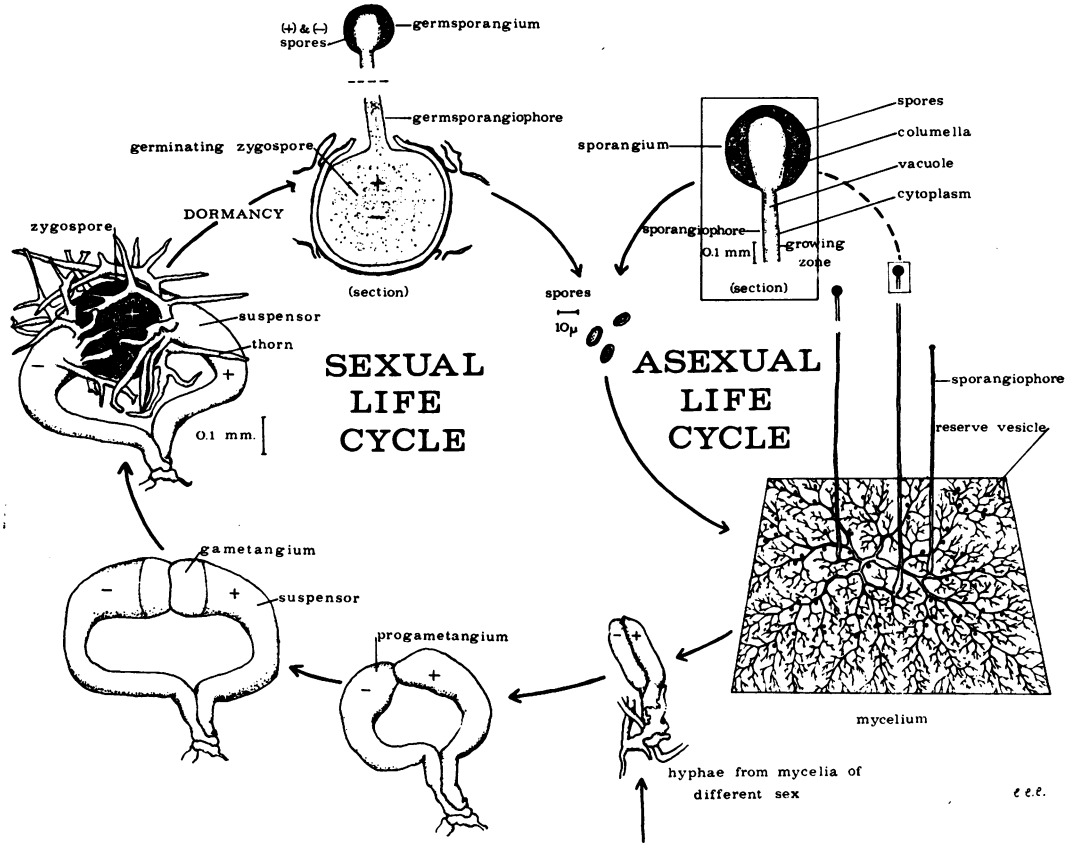


FIG. 1–1. *Asexual and sexual life cycles and the principal structures of Phycomyces.*

This flagellation is highly characteristic as to number, mode of insertion, and type (whiplash or tinsel) and serves to create classes with taxonomic validity. The relation of these classes to each other or to corresponding ones of algae or protozoa is dubious.

In this scheme, then, Phycomyces belongs to the class Zygomycetes, characterized by (i) vegetative spores *without* flagella and nonmotile, thus adapted to a terrestrial saprophytic life [because of the nonmotility, this class is also referred to as Aplanatae (3)], and (ii) a mycelium that is almost completely nonseptate (coenocytic).

Phycomyces belongs to the order Mucorales, characterized by sporangia, i.e., by the development of spores endogenously, as opposed to spores which are formed terminally from hyphae, thus exogenously produced: conidia.

Phycomyces belongs to the Mucoraceae, the largest and probably most primitive of the families of the Mucorales. Here the formation of the sporangium involves the development of a *columella*, as follows: as the sporangiophore bulges out at the tip to form a sporangium, the nuclei and most of the other cytoplasmc materials migrate to the periphery. Inside this mass, a new cell wall is formed which fuses with the cell wall of the sporangiophore at the base of the sporangium. This newly formed pear-shaped cell wall is the columella.

Phycomyces belongs to a subfamily whose multinucleate vegetative spores are formed from sets of pre-existent nuclei and do not develop from mononucleate protospores. Hence, the spores can be heterokaryotic if the mycelium is heterokaryotic. Detailed tests have shown that the spores are in fact as heterokaryotic as might be expected if the nuclei were randomly mixed at the time of spore formation. The mode of formation of spores in the *germ sporangium* is not clear (Sections 29, 30).

The genus Phycomyces was named and created by Kunze (126). It is unique with respect to the enormous size and strength of the sporangiophore, the stalk that carries the sporangium upward. This coenocytic structure is the principal object of interest to us.

Currently there are three species recognized in this genus, only two of which are available, viz., *P. blakesleeanus* and *P. nitens* (8, 35). Practically all recent work has been done with strains of *P. blakesleeanus* and most of the data reported in this review refer to it. The principal difference between the two species is the spore size, those of *P. nitens* being larger. The two species interact sexually, and this interaction leads to the formation of zygospores which germinate and form germ sporangia with spores. The spores, however, are sterile (35).

Previous to 1925, *P. blakesleeanus* and *P. nitens* had not been distinguished, and both were referred to as *P. nitens* (Kunze). Fortunately, this confusion is immaterial since the physiology of the two species seems to be very similar. In this review we will omit the specific designation and refer to *Phycomyces blakesleeanus* (Burgeff) simply as Phycomyces. Little is known about the natural history of Phycomyces. During the last 150 years, it has been found growing on the walls and timbers of an oil mill in Finland, in oil mills in Saxony, on litter under beech trees in England, on refuse on a warehouse floor in Hull, England, on a decaying wasp nest in Ireland, on dead wood in Urbana, Ill., on human feces in a cave near Rome, Italy, and on peaches in a refrigerator in Cold Spring Harbor, N.Y.; it has been isolated numerous times from mouse, rabbit, and horse dung.

The enormous size of the sporangiophores (compared to that of related genera) has given rise to speculations that may be epitomized by a quotation from a lengthy paper by the Abbé J. B. Carnoy (44), who conjectured that the reason for the great length of the sporangiophore is "de produire, dans les Mucorinées, cette variété que Dieu s'est plu à répandre sur ses oeuvres les plus infimes comme sur les créatures les plus élevées."

LIFE CYCLE

2. Spore germination

The vegetative spores of Phycomyces are single, ellipsoid, nonmotile cells, which contain several nuclei and are surrounded by a thick wall. Their size is one of the principal morphological differences between the species; those of *P. blakesleeanus* are 8 to 13 by 5 to 7.5 μm; those of *P. nitens* are considerably larger (8).

When placed in an environment unfit for growth, the spores remain dormant. Their low metabolic activity permits survival for long periods. Air-dried spores can be kept viable in loosely stoppered tubes in the refrigerator for at least 4 years (100 W.S.); water-suspended spores can likewise be kept for over a year. The spores can be lyophilized, and in this way they have remained viable for at least 23 years (84, 155). Well-sporulated agar cultures may be conserved for several years refrigerated (108), frozen (42, 43), or covered with mineral oil (100 W.S.).

Under suitable circumstances the spore germinates. After 1 hr (at 20 C) its microscopic appearance begins to change. The spore swells, its volume doubles every 2.5 hr, and its shape tends to globose. Numerous minute vacuoles appear, coalesce, and about 5 hr later form a single large

vacuole. Very soon thereafter, one or two, rarely three, germ tubes emerge from the spore and constitute the first hyphae (88). The nuclei, clearly distinguishable in the spore, start dividing before the formation of the germ tubes, are distributed over the growing cell, and change their aspect to become less conspicuous in the hyphae (5, 119).

Very few dormant spores germinate immediately when provided with the nutrients required for growth (salts, carbon source, nitrogen source, thiamine). In minimal medium sterilized by filtration, only 3.6 or 1.5% of the spores germinate (176, 194), but many different treatments are known to greatly increase this fraction. The transition from a dormant to a germinating spore requires an activation. The activated spore is defined by its capacity to germinate immediately when provided with the required nutrients.

The method most commonly used to activate spores is heat shock. Temperatures of 48 to 53 C for 3 min result in the germination of about 95% of the spores subsequently plated out (174). The temperature of 48 C appears to be optimal, producing 95% germinability for treatments of up to 2 hr. The optimal conditions for heat-shocking are near the survival limit of the spores, and the use of slightly higher temperatures or longer exposures may reduce drastically the germinating capacity.

Heat-activated spores not provided with suitable conditions revert to the dormant state. A minimal growth medium is not needed for germination; a glucose and phosphate solution serves nearly as well, and a glucose solution alone permits 18% of the spores to germinate. The rate of deactivation depends on the environment, being rather fast in water-suspended spores at room temperature but much slower in the refrigerator or in dried spores. Deactivated spores can be reactivated by a new heat shock, but this process cannot be repeated indefinitely, as exemplified by the following experiment: if heat shocks (3 min, 50 C) and deactivations (10 hr, 25 C, initially decreasing germination to about 20%) are applied alternately to a water suspension of spores, the deactivation caused by each cold period diminishes progressively until a state of permanent activation is reached; however, if the process is continued thereafter, the spores die (103).

Activation may also be obtained with many chemicals, like acetic acid, propionic acid, pyridine (163), acetaldehyde (177), glyoxylic, pyruvic, or butyric acid (194), hypoxanthine (162), and different natural extracts (160, 163, 171). For example, activation of at least 90% of the spores is achieved by treatment with 0.1 M ammonium acetate at 30 C for 15 min or by using the same compound at a concentration of 0.001 M in the medium. Acetate activation is irreversible: the spores can be kept in water for more than 1 week without appreciable loss of germinability (22). Acetate is considered responsible for the activation observed when plating the spores in autoclaved media, adding certain autoclaved chemicals or even by using autoclaved cotton plugs for the tubes (23, 164, 194). This effect can be quite striking; 90% of the spores germinate in a glucose-phosphate medium that has been autoclaved for 1 hr.

The process of activation of Phycomyces spores is imperfectly understood. The situation has some similarity to the activation of the ascospores of Neurospora (200). Oxygen is not required during the activation, but it is essential for vacuolization and formation of the germ tube (103, 218). The respiration of the resting spores in water is very low (0.19 μliter of oxygen per mg of tissue per hr). In nonautoclaved minimal medium, it increases considerably (1.9 μliters/mg); in minimal medium after heat-shocking, it is still higher (11.4 μliters/mg). Heat-shocking itself rapidly increases respiration, but, if nutrients are lacking, this increase is transient (173).

Germinating spores release pyruvic acid, ethyl alcohol, and acetaldehyde into the medium (174, 176). A homogenate of spores, if heat-shocked, still produces pyruvic acid (178). On the basis of these observations and studies with different metabolic inhibitors, Rudolph proposed that heat-shocking stimulates primarily an intense glycolysis. Pyruvic acid and its immediate transformation products are released to the medium because they are accumulated to such an extent that even the increased respiration cannot metabolize them (175, 178). These compounds are themselves activators of germination, but the reactions that they trigger are unknown. Acetate activation is accompanied by increased permeability of the cell wall evidenced by sensitivity to heavy-metal poisoning, and by numerous structural changes, particularly the swelling of the epispore, or middle layer of the wall, and marked alterations of the mitochondria (22).

Most authors consider that spores are germinated when they are clearly vacuolized after 18 hr of growth (103), and viable counts of spore suspensions may be obtained in this manner if the proportion of viable spores is not too small. Reliable viable counts have been obtained from the proportion of tubes supporting growth when a set of them is plated with a sample containing on the average 0.2 to 2 viable spores and assuming a Poisson distribution (107), but this method is cumbersome because it involves the use of at least 500 tubes for a good determination. Plate-counting is inaccurate owing to the rapid spread of the

mycelium; the colonial mutants selected to bypass this inconvenience add peculiarities and complications of their own. The observation that low *p*H induces colonial growth in all strains (100 S. H. Goodgal) can be used for counting. Adequate determinations are obtained when the spores are placed in a top layer of glucose-asparagine-yeast agar (107) on a plate of the same agar adjusted to *p*H 3.2 with HCl (100 E.C.O.).

3. Mycelial growth

The mycelium of Phycomyces (Fig. 1–1) may be grown in aerated, liquid media or on solid substrates. To obtain on agar large mycelia capable of supporting the development of strong sporangiophores, two to five spores/cm^2 should be plated. In liquid culture, good germination is found at concentrations up to 10^6 spores/ml.

Following germination (*see* Section 2) the hyphae grow and branch rapidly. Growth occurs only at the hyphal tips. The rate and direction of growth and branching of the hyphae and hence the rate of mycelial spread varies with the strain and with the culture conditions. The spread of the mycelium is severely retarded in many mutant ("colonial") strains. Similar colonial growth is produced with acid media (100 S. H. Goodgal). Media and conditions may be compared by the use of racing tubes. At 20 C, on potato-dextrose-agar medium, the mycelial front advances at a constant rate of about 2 cm/day.

The mycelium spreads out radially and symmetrically from the original spore site. No cross walls separate various parts of the mycelium. Rare exceptions to this rule occur on the periphery, probably in response to damage. The hyphae never anastamose (88). The importance of these two observations is discussed in Section 29.

The effects of *local* conditions on the growth of a hypha have been incompletely analyzed. One would like to know the role of the availability of nutrients and oxygen, of the *p*H, of metabolic products of neighboring hyphae, etc.; further, whether the effect is tropic (causing a change in direction of growth), trophic (causing a change in growth rate), or morphic (causing modification of the branching pattern).

The latest detailed study of these questions is that of Schmidt (180), undertaken before the advent of chemically defined media. Schmidt's study established the occurrence of positive aerotropism and of chemotropism of uncertain sign. Clearly, one is dealing with questions of chemoreception, quite similar to those studied so successfully in bacteria with the aid of specific mutations (2).

After approximately 24 hr of growth, "reserve vesicles" are formed (34, 88, 97). These are large round evaginations of the hyphae and contain cytoplasm with many oil droplets. Since they are drained of material and shrink in size during sporangiophore formation, it is assumed that they contain material held in reserve for the sporangiophores. Another organ, the "rhizoid," has been reported but its function is unknown (97).

Growth measurements of liquid cultures of Phycomyces in chemically defined media were important for the bioassay of thiamine. A systematic study of the optimal growth conditions is is that of Burkholder and McVeigh (38). They found (i) a broad optimum *p*H centered at 4.0 (no growth with the *p*H adjusted to 2.7 or 7.3), and (ii) a broad optimum temperature of 15 to 25 C in high thiamine (10^{-6} M). In low thiamine (10^{-7} M and lower), there is a distinct maximum at 15 C. Thus, the breakdown of thiamine may influence the amount of growth found at high temperatures. Growth is still appreciable at 3 C.

No effect of light on mycelial growth has been detected. Light does, however, quantitatively affect the level of carotenoid synthesis (63) and sporangiophore initiation (Section 23g).

4. Sporangiophores

After mycelium has grown on an agar surface for 2 to 3 days, sporangiophore growth is initiated. This development can be prevented by growing mycelium in liquid culture with agitation. Regarding the effect of light on sporangiophore initiation, *see* Section 23g. Regarding the effect of gamones in suppressing sporangiophore initiation, *see* Section 30a.

The development of a sporangiophore (Fig. 4-1) is conveniently divided into five stages, described by Castle (54) as modified from Errera (85).

(a) **Stages of development and dimensions.** *Stage I.* The sporangiophore grows upward from the mycelium as a simple pointed tube. It grows at the tip at 1 to 2 mm/hr. The tip of the cell does not rotate, in contrast to later stages (55).

Stage II. The tip of the sporangiophore swells and the bright yellow sporangium is formed. During this stage the stalk does not lengthen or twist. The time of transition from stage I to stage II varies greatly with growth conditions (Section 6).

Stage III. A period of several hours during which there occurs neither stretching of the sporangiophore nor enlargement of the sporangium.

Stage IVa. The elongation of the sporangiophore is resumed and increases slowly. The sporangium quickly becomes dark brown or black. Twist begins in the counterclockwise direction as seen from above.

Stage IVb. Approximately 90 min after the

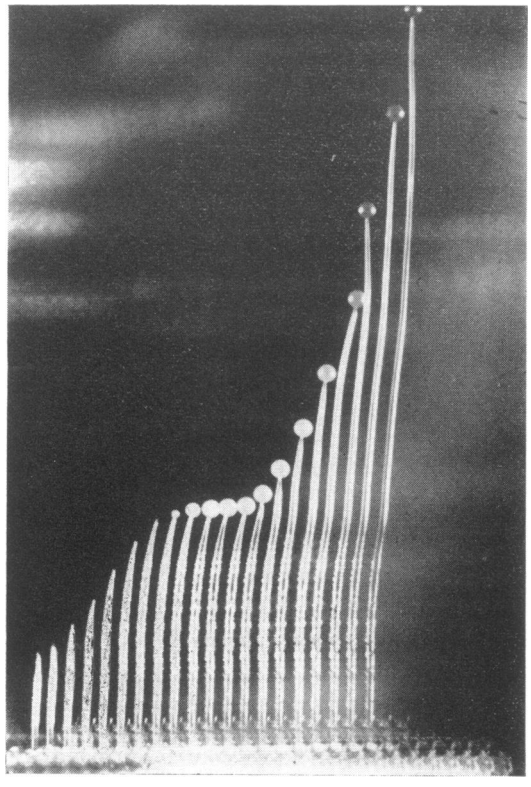


FIG. 4–1. *Stages of development of sporangiophores. Twenty photographs of the same sporangiophore, taken at 1-hr intervals. The sporangium is about 0.5 mm in diameter. During the last hour the sporangiophore elongated about 3 mm. It will continue to elongate in this stage (IVb) at an approximately constant rate until it has reached a height of about 10 cm. (Photo Lois Edgar.)*

onset of growth, the twist reverses to clockwise and reaches a steady rate of about one revolution of the sporangium in 30 min. Stage IVb is generally used in studies of the sporangiophore's sensory apparatus. In this stage the growth rate becomes relatively constant once it has reached a rate of 3 mm/hr (Fig. 4–2).

The stage IV sporangiophore can reach 15 to 20 cm but its useful life is usually shorter owing to limitations of mechanical stability. The sporangiophores are typically about 100 μm in diameter. There is a very gradual taper from about 170 μm near the base to about 100 μm a few millimeters from the sporangium and a steeper one to 70 μm at the sporangium.

A central vacuole is present along the entire length of the sporangiophore. In the growing zone, typical values are: radius of vacuole, 20 μm; thickness of protoplasm, 30 μm; thickness of cell wall, 0.6 μm. Below the growing zone, the protoplasm is thinner, about 5 to 10 μm in sporangiophores 4 cm long and 1 to 2 μm in sporangiophores 8 cm long.

If the stage IV sporangiophores are picked at night, a new crop of stage IVb is available in the morning. A culture may be used in this way for as long as 1 week.

(b) **Effects of temperature and humidity.** The growth rate of the sporangiophore increases exponentially with temperature from 7 C or lower up to about 25 C (45). Above 27 C, the growth rate drops abruptly. In the exponential range, the growth rate doubles for a ΔT of 8 C, on the average, but in individual cases ΔT may vary between 4.5 and 11 C.

The growth rate also depends on the humidity (212); it increases with increasing humidity. This dependence has not been worked out quantitatively. Corresponding to this dependence, a positive transient growth response to a step-up in humidity and a "hydro-tropic" response to a humidity gradient has been reported by Walter. These experiments were, however, technically crude and gave very erratic results. They deserve repetition and elaboration, especially in connection with studies of the "avoidance response" (Section 28). Humidity has a strong effect on the *rate* of several of the tropic responses. These rates become very low in low humidity and are reasonably constant above about 60% relative humidity, but again no quantitative study exists.

In the work just described it has been overlooked that the temperature of the sporangiophore may be different from that of the surrounding air. The temperature difference is principally determined by three factors, which can be estimated:

(i) Heat production Q^M through metabolism.

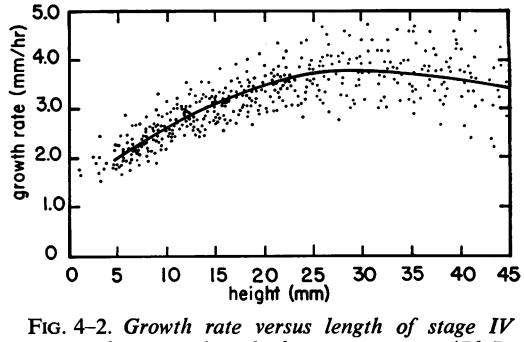


FIG. 4–2. *Growth rate versus length of stage IV sporangiophores. Adapted from Dennison (Ph.D. Thesis). The points represent measurements on sporangiophores grown at various intensities, from complete darkness to* $\log_2 I = -3.8$. *No dependence of growth rate on intensity was found.* $T = 20 \pm 2$ C. *Abscissa: height above stage II length.*

With an O_2 consumption of 10^{-10} moles/min cm sporangiophore (Section 10) and a heat release of 5×10^4 cal/mole of O_2, one obtains $Q^M = 5 \times 10^{-6}$ cal/min cm sporangiophore.

(ii) Heat loss Q^T due to transpiration. With a transpiration rate at 75% relative humidity of 1 nliter/min cm sporangiophore (Section 9) and a heat loss of 540 cal/ml of water, one obtains $Q^T = 540 \times 10^{-6}$ cal/min cm sporangiophore. Q^T is about 100 times larger than Q^M, so that the latter is negligible.

(iii) Heat gain (or loss) Q^c due to convection. Q^c per minute is (183).

$$Q^c = 6 \times 10^{-3}(\Delta t/2r)^{1/4} A \Delta t$$

where Δt is the temperature difference between the sporangiophore and the bulk air, r = radius, A = surface of sporangiophore. Putting $Q^T = Q^c$ leads to a $\Delta t \cong -1$ C.

It would be of considerable interest to make actual measurements of Δt, by radiometric microscopy. This would permit one to effectively monitor short-term transpiration rate changes during growth responses.

The fact that the growing zone wall twists quite fast during growth was discovered astonishingly late (136). Despite much study and speculation there is no quantitative explanation for spiral growth.

Twist is present throughout most of the growing zone, but some subtle differences in the distribution of growth and twist have been observed (64). Twist is thus not merely a passive product of growth.

Using an "iron lung," Roelofsen (167) was able to study the elastic properties of the growing zone by varying its internal pressure. The sporangiophore elongated and twisted with increasing pressure. Since the twist per millimeter of elongation was much lower in this passive situation than during growth, twist is in part due to an active process, presumably intussusception of cell wall units. The nature of these "units" is unknown.

(c) **Growing zone.** The growth of the sporangiophore is limited to a region that in stage IVb extends for 2 to 3 mm below the sporangium. Starch markers placed in this region move upward with a velocity which decreases with their distance from the sporangium. The lower end of the growing zone is taken as the point at which starch markers cease to move upward. Since this cessation of growth happens very gradually, the "length" of the growing zone is poorly defined.

In stage I sporangiophores the growing zone extends only 1 to 2 mm down from the tip (55).

(d) **Incorporation of matter from mycelium.** The incorporation of matter from the mycelium into the sporangiophore in the presence of ample nutrients extends through the entire duration of stage I and ends during the time of sporangium formation. This statement is based on:

(i) *Measurements of dry weight.* Gruen (99) found about 45 μg/cm for stage I, and about 120 μg for stages II, III, and IV, irrespective of length.

(ii) *Measurements of ^{32}P uptake.* David (100) (Fig. 4–3) finds about 0.25 μg of P/cm for stage I, and about 0.5 μg of P for stages II, III, and IV, irrespective of length. It is likely that the bulk of the ^{32}P is in polyphosphates rather than in nucleic acids (Section 12).

With respect to phosphate uptake, a more far-reaching statement can be made: nearly all of the phosphate in the sporangium is taken up from the medium by the mycelium before initiation of the sporangiophore (Table 4–1). This suggests that the ^{32}P is incorporated into polyphosphate in mycelium and that this subcellular particulate is subsequently transferred as such into sporangiophores and sporangia. Nuclei may also be transferred as subcellular particulates into sporangiophores because: (i) no dividing nuclei have been seen in stage I sporangiophores (100 Ute Wagenmann); and (ii) the sporangium contains progressively fewer viable spores the earlier during stage I the sporangiophore is plucked, as shown in Fig. 4–4 (100 C.N.D. and M.H.). The generality of the phenomenon by which sporangiophore sub-

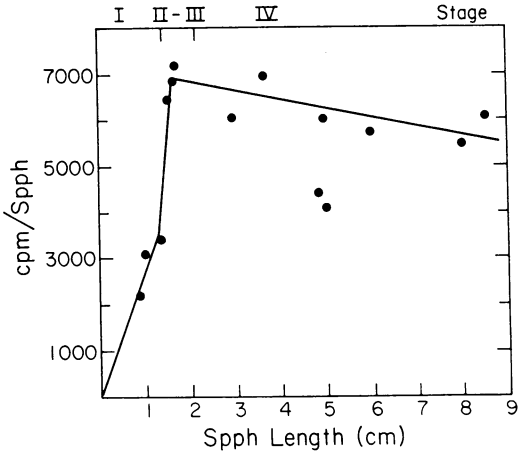


FIG. 4–3. *Total phosphorus content of sporangiophores at various stages (100 C.N.D.). Growth medium: glucose-asparagine, 6 μg of P per ml. ^{32}P as phosphate was added to give specific activity of 0.006 μc/μg. Sporangiophores of various lengths and in various stages were plucked and radioactivity counted. About one-half of the phosphorus is taken up during stage I, the other half during sporangium formation; there is no net increase during stage IV. The final P content of a sporangium is about 0.25 μg = 0.4% of dry weight.*

cellular particulates appear to be synthesized in the mycelium needs further investigation. The ability to label single sporangiophores and to separate subcellular particulates in single sporangiophores (Section 12) makes such investigations possible.

(e) **Growth in water** (100 M.H.). When submerged in water, sporangiophores grow (196) and exhibit negative phototropism. The growth rate in pure water is poorly reproducible; often the sporangiophores die after 20 min.

Reproducible growth can be obtained by buffering at *p*H 5 to 7 with 0.005 M buffer (phthalate at *p*H 5, phosphate at *p*H 6 and 7) and adding 0.2 M NaCl. Under these conditions the growth rate is 1.5 to 2.0 mm/hr and can not be stepped up by improved oxygenation.

(f) **Spore liberation** (113). In Phycomyces the spores are liberated from the sporangium by dehiscence, i.e. by a rupture of the outer wall of the sporangium at its line of contact with the columella (Fig. 1–1). This rupture does not take place spontaneously, but only upon contact of the sporangium with a solid surface or with a drop of water. The ruptured sporangium is a sticky droplet. Sporangia sticking to dust can be carried away by wind or insects; those sticking to grass, by browsing animals. The sporangium wall does not liquefy in Phycomyces, in contrast to other Mucoraceae, but the spores of a sporangium may get dispersed by rain water after dehiscence.

TABLE 4–1. *^{32}P found in sporangium when label is added to mycelium either before sporangiophore initiation or some time during stage I*[a]

Sporangiophore no.	Approx length of sporangiophore when label was added	Time of sporangium formation after ^{32}P addition	Counts/min in sporangium
	mm	*hr*	
10	0[b]	40	4,150
14	0	44	2,950
11	0	44	1,460
12	0	44	2,145
13	0	52	1,560
6[c]	10–15	5	190
7[c]	10–15	5	260
5[c]	5	8	560
9[c]	3	9	880

[a] ^{32}P as phosphate (0.1 ml, 2.5×10^5 counts/min) was added at 0 hr on top of the mycelium in a vial containing 3 ml of low-phosphate (6 μg/ml) glucose-asparagine medium. At the time of ^{32}P addition, there were several stage I sporangiophores of various lengths; other sporangiophores came up after ^{32}P addition. All sporangiophores were allowed to mature and the time of sporangium formation noted (in hours after ^{32}P addition). In stage IV the sporangiophores were plucked, sectioned. and counted. The sporangium was counted separately from the top section of the sporangiophore. The data show that the bulk of the ^{32}P in a sporangium is taken up by the mycelium befoer sporangiophore initiation.

[b] Sporangiophore not yet initiated at time of ^{32}P addition.

[c] Stage I sporangiophore at time of ^{32}P addition.

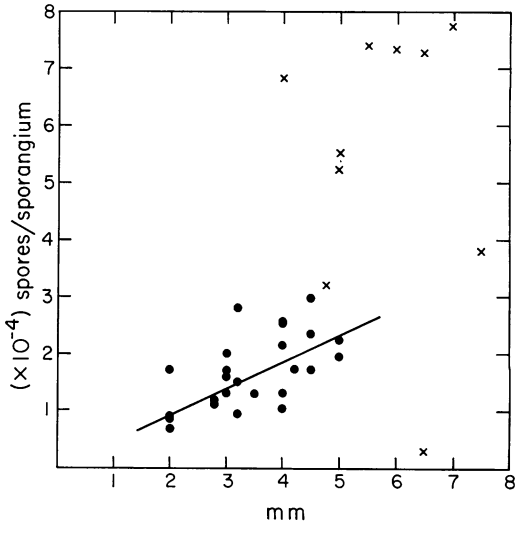


FIG. 4–4. *Viable spores in mature sporangia obtained from sporangiophores plucked in stage I or II, and kept with base in water until maturation (100 C.N.D. and M.H.). Abscissa: length of sporangiophore at time of plucking. Ordinate: number of viable spores at time of maturation (black sporangium). The lengths at maturation ranged between 10 and 32 mm. Symbols: ●, sporangiophores in stage I at time of plucking; ×, sporangiophores in stage II or III at time of plucking.*

5. The sexual cycle

The strains of Phycomyces can be classified as either (+) or (−) by the definition that two strains have a different sign if they can interact to form special structures called zygospores. These groups correspond to sexes and the zygospores are products of sexual fusion, as first recognized by Blakeslee (16), who called this situation heterothallism.

Phycomyces is isogamic, that is, both sexes contribute symmetrically to the sexual process (195). The attribution of the signs (+) and (−) to the two groups of strains is arbitrary and does not imply any relation to the concepts of male and female. Strains of opposite sex are indistinguishable in practice except by their sexual reaction. Many reports of presumed secondary sexual characters have been refuted as environmental or racial differences unrelated to sex. It is

possible, however, that some differences exist, as, for example, in the distribution of nuclei per spore (120).

When vegetative hyphae of different sex grow near one another, a number of chemical and morphological changes occur in them. The hyphal tips swell, carotene production increases, more oxygen is consumed, and the transformed hyphae, or zygophores, of each sex grow towards the other and enter into contact (34, 39, 149).

Burgeff (34) showed that these processes occur if the two sexes are separated by a celloidin membrane and concluded that diffusible substances are produced by each sex and detected by the other. Plempel has carried out extensive investigations of these substances in Phycomyces and other Mucorales. Each sex continually produces a specific diffusible substance, called progamone, which, upon detection by the other sex, induces in it an increase in metabolic activity, and the production of another specific substance, the gamone, but does not cause morphological changes (144, 149). The progamones are substances of high molecular weight, possibly proteins, and they may produce their effects through the derepression of certain genes of the recipient (100 M. Plempel).

The gamones are also water-diffusible substances and cause the hyphal tips of opposite sex to transform into zygophores. The gamones of Phycomyces and several other Mucorales have been isolated, are active at concentrations of 10^{-7} M, and are very similar in different species and sexes (146, 148, 150). It has been shown by Van den Ende (209) and Reschke (Tetrahedron Letters, *in press*) that in *Blakeslea trispora* the (+)-gamone activity is associated with each of a group of closely related C_{18} compounds, the trisporic acids. The structure of these compounds had been determined by Caglioti et al. (40) and by Cainelli et al. (41). The trisporic acids are structurally related to the carotenes. There exist conflicting claims whether the excess production of carotenes observed during mating is induced by the progamones (149) or by the gamones (204, 209). The structure of the (−)-gamone has not yet been established but it appears to be similar to that of the (+)-gamone (Reschke, *in press*). Phycomyces mutants blocked in their carotene synthesis exhibit incomplete sexual reactions (107) and produce little or no gamone (100 M. Plempel).

The zygophores induced by the action of the gamone produce a new pair of hormones, the zygotropic factors. In *Mucor mucedo* the zygotropic factors diffuse through the air and cause zygophores of opposite sex to accelerate and direct their growth towards higher concentrations of the hormone. The concentration gradient is made steeper by the short life of these factors in the presence of oxygen (7, 145, 147, 151). The zygophores of Phycomyces, however, grow submerged in the substratum (16) and it has been suggested that its zygotropic factors diffuse in water instead of air (151). The complex sequence of interactions of these three pairs of hormones is illustrated in Fig. 5–1.

The zygophores of opposite sex, after making contact, develop coralloid swellings and grow into the air, thickening considerably and finally forming a ring, each half of it composed by the distal region of one zygophore, the progametangium (Fig. 1–1). A transverse wall inside each progametangium separates a cell, the gametangium, from the rest of the mycelium; the wall between the two gametangia disappears and a single cell is formed, the zygote, whose abundant cytoplasm and thousands of nuclei are derived from the two mycelia. The zygote matures into a zygospore, swelling to a spherical shape and forming a thick, black outer wall. The adjacent portions of the zygophores on both sides of the zygospore, the suspensors, produce fine, dichotomic black spines or thorns disposed all around the zygospore (16, 121, 129, 138).

The zygospore remains dormant for at least 3 months and frequently longer. Efforts to shorten this period have failed (17, 182), leading to the suspicion of an internal clock, relatively independent of external events. Contaminations by asexual spores or regeneration of mycelium left around

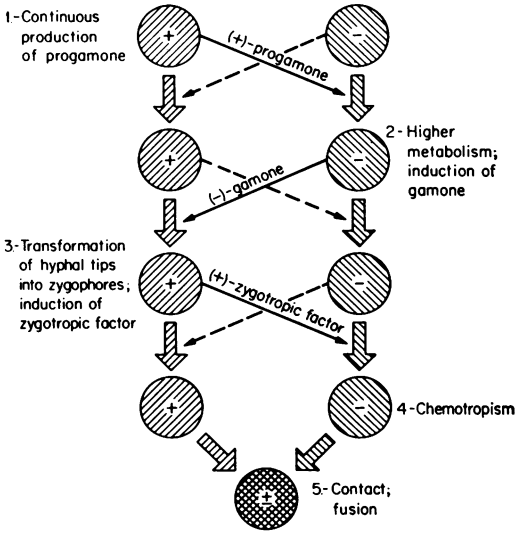


FIG. 5–1. *Sequence of interactions involved in the sexual reactions of Phycomyces. Symmetrical effects are produced by the hormones represented by broken lines.*

the zygote sometimes have been mistaken for early sexual germinations.

The germination of the zygospore is not influenced by nutrients and is usually carried out on simple water-agar. A sporangiophore, quite similar to the normal sporangiophores of the asexual cycle, emerges from the zygospore without producing a mycelium. The prefix germ- is used to designate the products of zygospore germination. The germsporangium carries spores, similar to those of the asexual cycle, and genetic recombination can be shown to have occurred (Section 30).

Phycomyces blakesleeanus does not hybridize successfully with members of other species; its distinction from *P. nitens* was based mainly on the existence of a sterility barrier (35). It exhibits strong sexual reactions with *Mucor mucedo* and, to a lesser extent, with *Absidia glauca*, but these reactions do not lead to the formation of zygospores (16, 18, 34).

6. Experimental studies of development and regeneration

The normal development of Phycomyces has been described in Sections 2 to 5. At certain moments in its life, a new pattern of events is brought about by the interplay of internal and external circumstances. We will consider here the introduction of developmental changes by environmental alterations or mechanical manipulations.

Germination of the spores is triggered by a combination of internal conditions and external physical and chemical agents. The spore usually develops a mycelium. A rare exception is caused by the addition of neutral red to the medium, producing swelling of the spore and the appearance of a single unbranched hypha that corresponds to a very small sporangiophore and carries a small sporangium with spores (88). This case resembles the normal germination of the zygospore.

Mycelial growth can be maintained for a long time in aerated liquid media or by transplantation of the mycelium to a new plate. Little differentiation takes place during continuous mycelial growth, but, in response to appropriate stimuli, mycelia can undergo extensive differentiation, forming either sporangiophores or sexual structures.

The onset of sporangiophore formation, representing a complete change in the morphology and economy of the fungus, is influenced by the chemical composition of the medium and other environmental factors. It is accelerated by starvation, by illumination (32; Section 23g), and by high density of spore seeding; it is retarded by high initial *p*H of the medium (172). The sexual reaction inhibits the formation of sporangiophores, and the growth of sex heterokaryons is accompanied by a reduced and delayed sporangiophore yield. Usually in the Mucorales the growth of the sporangiophores requires a humid atmosphere; in Phycomyces, however, they elongate normally in dry air, if the substrate is kept moist (114).

Phycomyces can develop a large number of dwarf sporangiophores, generally less than 1 mm long (38, 70, 138). The production of normal or dwarf types is decided mainly by the density of spores seeded into the medium. The availability of more than 2 μliters of minimal medium per germinating mycelium leads to normal growth, and less than 0.1 μliter to dwarf growth. Mixed populations may be obtained at intermediate densities, low oxygen concentration, or poor nitrogen source. A poor nitrogen source in a cold environment (13 C), or darkness alone, gives rise to dwarfs even at low densities of spores (172, 205).

Temperature-sensitive mutants able to form sporangiophores at 16 C but not at 26 C have been isolated (100 E.C.-O.). Exposure of these mutants to 16 C for at least 5 hr after mycelial growth at 26 C for 3 days is sufficient to permit sporangiophore formation. The cold period is not effective if it coincides with inhibition of protein synthesis by cycloheximide. Thus, the synthesis of temperature-sensitive proteins is necessary to remove the developmental block. The required age of 3 days may represent a metabolic clock and the mutations in question may determine a genetic system for sporangiophore initiation.

The transition between the different stages in the growth of the sporangiophore depends on a complex regulating mechanism, as reflected by the "trapped air effect" (172). If several culture vials are placed in a closed chamber with a volume of about 200 ml, the sporangiophores initiated on the second day do not form sporangia but remain in stage I and continue to elongate at a normal rate for many hours. This effect is *not* due to lack of oxygen (as shown by filling the chamber with pure oxygen) and *not* due to the production of CO_2 (as shown by absorbing CO_2 with KOH). It appears to be due to a gas that can be adsorbed by charcoal (100 D.S.D.).

The zygophores, modified hyphae which carry out the sexual fusion, bear some resemblance to sporangiophores. They may in fact be converted to sporangiophores, even after contact with the other sex has been made, if the sexual reaction is not completed (34, 138).

Small and infertile aerial hyphae, known as pseudophores, are found in sex heterokaryons, and related forms appear in abortive sexual reac-

tions occurring under conditions of high osmotic pressure. Many of them are transformed spontaneously into dwarf sporangiophores (17, 34, 138).

Many altered conjugation forms occur with variable frequency. Among the rarest are the "azygospores," or zygospore-like structures formed by a single gametangium, and the zygospores produced by a single sex-heterokaryotic mycelium. Both forms are sterile, but they are reminiscent of fertile zygospores produced by the homothallic species of Mucorales (17, 34, 129, 138).

Excessive dryness and heat are lethal to dormant zygospores. Some injuries and treatments of the zygospore may lead to formation of a mycelium instead of a sporangiophore (33, 110, 182).

Phycomyces has a surprising capacity for regeneration. Its chance of survival is considerable even if the cell is cut open and the cytoplasm squeezed out.

Burgeff (32) inserted half a sporangiophore inside another of a different genetic constitution, mixed their cytoplasms, incubated in a humid, dark place, and obtained regeneration in the form of sporangiophores carrying viable and frequently heterokaryotic spores.

Weide (214) modified this type of manipulation by squeezing the cytoplasm of young sporangiophores onto a glass slide. Cytoplasmic drops from different sporangiophores can be fused to form a large mass, several millimeters in diameter. Often, particularly if the mass touches a piece of cell wall of one of the sporangiophores, a surrounding wall is produced that leaves out only a small layer of dead cytoplasm. Such masses frequently regenerate small sporangiophores carrying viable spores, or, if submerged in nutrient solution, they regenerate a mycelium. Heterokaryons are produced mixing cytoplasm of sporangiophores of diverse genetic type. Foreign substances can be added to the regenerating material, and in this way nuclei of one strain have been added to the cell contents of another, producing functioning heterokaryons (Section 12).

Differentiated parts of the thallus have a remarkable plasticity. A sporangiophore regenerates a mycelium if it is submerged in a nutrient solution. A young sporangium develops a mycelium under the same conditions. An older sporangium develops only spores even when it is broken up and its contents are poured into another cell or medium. The irreversible differentiation takes place in the late stage II sporangium before its parceling into spores and formation of the columella (93).

Many different kinds of damage, such as scratching, cutting, corroding with chemicals, and pressing with a string, result in regeneration of the sporangiophore. Healing consists in the coagulation of the damaged cytoplasm, the movement of a ring of living cytoplasm towards the scar, and the formation of a new wall between the coagulate and the ring of cytoplasm. Normal growth may be resumed in the new wall or in its neighborhood (93, 122). The large measure of local autonomy along the sporangiophore, noted by Gamow and Goodell (88*a*) facilitates the regeneration of isolated segments of the sporangiophore.

The normal sporangiophore of Phycomyces is unbranched and carries a single sporangium. Branched sporangiophores carrying two or more sporangia are rarely found in culture, but some chemicals induce them, for example, naphthalene (214). Certain morphological mutants branch frequently, and their sporangiophores resemble those of *Mucor spinosus*. Grehn (96) showed that slight damage to the apical part of plucked sporangiophores results in many types of regeneration and branching, often of great regularity.

7. Plucked sporangiophores

Sporangiophores are not separated from the mycelium by a cell wall (99). Nevertheless, they can be severed from the mycelium without losing turgor because of the high viscosity of the cytoplasmic "plug" which fills the basal end. The plucking operation is most consistently successful in stage I, progressively less so in later stages, in proportion to the development of the vacuole.

Gruen (99) found that a sporangiophore plucked at an early stage not only retains its turgor, but even grows, develops, and reacts normally for many hours when supplied with just water at its basal end. The potential value of this discovery for studying growth, differentiation, photoresponses, and metabolic mechanisms of sporangiophores isolated from the mycelium has hardly been exploited.

The technique of using isolated or plucked sporangiophores has been useful for measurements of osmotic quantities and "iron lung" cell wall studies (167), streaming and metabolism (Sections 7 and 10), water uptake (Section 9), and staining of organelles (Section 11).

(a) **Techniques.** Sporangiophores should be plucked carefully from the mycelium. One method is to use pointed tweezers to pick up the sporangiophore near its base a few millimeters up from the points of the tweezers. With experience, nearly 100% of the plucked sporangiophores will grow.

Sporangiophores grow well with their bases in distilled water, or in lightly buffered media at any *p*H between 3 and 7.6. In contrast to mycelia, they do not acidify the medium during

growth (102). They can be propped up against a glass slide over a plate of water with or without Vaseline. If Vaseline is used to stick the sporangiophore on the glass slide, it should be kept away from the growing zone. They will also grow submerged in water, floating on the surface of water, or inside water-filled capillaries.

If sections of the sporangiophore are to be analyzed chemically, as for adenosine triphosphate (ATP; 188), or for the distribution of radioactivity (Section 9), one wants to cut at precise points along the sporangiophore. One easy way is to freeze the sporangiophores on dry ice covered with aluminum foil and to section with a razor. A drop of water applied to the cut sections freezes, making them easy to pick up.

(b) **Growth of whole sporangiophores** (99). Plucking modifies long-term growth and the time scale of maturation. Sporangium formation occurs more synchronously (99). The sporangiophore's stimulus-response systems are unaffected (100 E.W.G.).

Normal growth rates of sporangiophores are about 40 to 50 μm/min during stage IV. Sporangiophores plucked during stage I grow about 40 μm/min in stage IV; if plucked during early stage IV they grow at rates of 25 to 40 μm/min. Plucked sporangiophores are thinner than attached ones. The earlier they are plucked, the thinner they are and the fewer viable spores they contain in stage IV (Section 4d).

Sporangiophores plucked in stage IV grow only an additional 20 hr or so. Plucked in stage I they grow for about 60 to 80 hr, and attain a length of 8 to 9 cm. During this period their dry weight decreases from 80 to 70 μg. In contrast, attached sporangiophores grow for 130 hr or more, and attain a length of 14 to 15 cm. During stage I their dry weight increases proportionally with length.

Differences between the growth of attached and plucked sporangiophores express themselves only in experiments lasting many hours. The mycelium must supply something to the attached sporangiophores that is responsible for these long-term differences. Gruen (102) found it *not* to be a constituent of synthetic or potato-dextrose media, and suggested that it is an as yet unidentified factor synthesized in the mycelium. Supplementation with a complete nutrient medium leads to regeneration of mycelium at the base of the sporangiophore, a feature conspicuously absent during growth in water.

(c) **Growth of isolated top sections.** When oxidative phosphorylation and glycolysis in plucked sporangiophores are inhibited in all of the stalk except the top 3.5 mm, the growth is not inhibited for 3 or 4 hr. Within this period the sporangiophores give normal bending and growth responses to light. If the metabolism in more of the stalk is left uninhibited, the sporangiophores grow normally for a longer time and have a greater net growth. The same pattern of growth rates and net growth is obtained by pinching the stalk shut in various places. Thus, the growing zone is functionally autonomous, but if it is isolated from the rest of the stalk it appears to eventually deplete something normally supplied from below (88*a*).

8. Protoplasmic streaming

Protoplasmic streaming occurs both in the hyphae and in the sporangiophores of Phycomyces. Streaming has been divided into two types. (i) Contractile-hydraulic streaming appears to result from a hydraulic pressure system. Organisms with such systems (such as the amoebae) are composed of ectoplasm which is gel-like and which is believed to contract, driving the sol-like endoplasm forward. (ii) Active shearing-type streaming is involved when movement in both directions takes place within or along long thin cytoplasmic strands. There are often a great many such strands per cell. This type of streaming is seen in higher plants, radiolaria, and foraminifera.

Phycomyces sporangiophores display the latter type of streaming. In the lower portions of thin or old sporangiophores, the flow of particles in both directions along seemingly single strands of protoplasm can be readily observed by phase-contrast or interference microscopy. In most of the sporangiophores, the axes of these strands are parallel to each other and are generally parallel to the axis of the sporangiophore, but they can deviate by as much as 15° from the axis and this angle may vary along the sporangiophore (137). It is not clear whether the particles move along the strands of protoplasm or whether the strands move and carry the particles, like conveyor belts.

In foraminifera (Allogromia) the strands in question are the filopodia, protoplasmic threads extending out through the cell's exoskeleton into the aqueous environment (4). Extraneous particles added to the water ride along the strands when they become attached to them. Each strand must contain at least two substrands moving or causing motions in opposite directions, since one never observes strands on which particles move in one direction only. Electron microscope pictures show that most filopodia do in fact contain several substragds, each enclosed by a cytoplasmic membrane (217). Moving pictures of the streaming in the filopods of Allogromia (118) show striking similarities to the

streaming in Phycomyces, suggesting that in Phycomyces, too, the particles are passively carried on the surface or inside of strands.

The rate of streaming in Phycomyces is about 2.5 μm per sec upward and about 3.0 μm per sec downward. The rates of streaming are usually higher in the lower parts of sporangiophores (averaging 2.0 μm/sec near the top and 3.3 μm/sec 1 or 2 cm below; 152). Except at the ends of the sporangiophore, subcellular particles seldom permanently change direction. Even though one particle may appear to bump into another going in the opposite direction, forcing it to change direction, such changes of direction are in most but not all cases temporary.

The main function of streaming appears to be the transport of subcellular particles (such as mitochondria, lipid droplets, glycogen). It does not move bulk water up the sporangiophore because the rate of water transport is too great to be carried upstream by the cytoplasm (Section 9).

9. Water uptake, water transport, and transpiration of sporangiophores

The average water loss through attached sporangiophores growing on an agar plate was first measured by Grehn (97). Grehn limited the evaporation from the agar by covering it with paraffin oil and measured the water loss by repeated weighings. The value obtained per sporangiophore was 0.29 nliter/min at 14 C and at an unspecified relative humidity.

Using individually plucked sporangiophores with their bases sealed into a small manometer system, uptake values were found from 1.2 nliters/min a few hours after the sporangiophore reached stage IVb to 0.1 nliter/min 10 to 12 hr later (100 K.W.F.). In these experiments the relative humidity was held constant at 75%.The measured uptake rates ranged from seven times the volume growth rate at the maximum growth rate to a little more than one time the volume growth rate for old sporangiophores. Thus, for healthy, growing sporangiophores the water uptake is fast relative to the volume increase. When a sporangiophore grows more slowly than normal, the absolute rate of water uptake is less, but not to the same degree as if the uptake were proportional to growth rate.

David and Foster (100) grew plucked stage IV sporangiophores with their bases immersed in ^3H_2O for periods of 4.5, 6, and 16.7 hr and measured the uptake of ^3H_2O into the sporangiophore. They obtained average values of 0.62 to 0.28 nliter/min (per sporangiophore). The same decrease in uptake rate with age of the sporangiophore and the same order of magnitude of uptake as in the manometer measurements were observed. Possible diffusion of unlabeled water out of the immersed base of the sporangiophore was not measured.

Most of the transpiration is expected to be through the growing zone, because a waxy cuticle (53) is present everywhere on the cell wall except in the growing zone. However, Gamow and Goodell (88*a*; Section 7) found with pinched-off sporangiophores that water can be taken up through any point of the cell wall. Thus, a portion of the water is probably transpired along the nongrowing part of the sporangiophore. The distribution of transpiration along the sporangiophore has not been experimentally determined.

Because of the taper of sporangiophores mentioned in Section 4, from 170 μm near the base to 70 μm near the sporangium, an uptake rate of 1 nliter of water per min corresponds to a much larger upstream rate near the growing zone (4 μm/sec) than near the base (0.6 μm/sec). In this calculation we have neglected possible transpiration along the sporangiophore and have included both vacuole and protoplasm in the cross-section through which the upstream occurs.

In experiments in which the uptake of tritiated water was measured, the movement of the ^3H_2O front was in agreement with these rates. However, diffusion is important so that in 16.7 hr, although the main front was at 2 cm (average uptake 0.3 nliter/min), the entire length of a 4.5-cm sporangiophore was partially labeled. The observed distribution of ^3H_2O appears to be adequately explained by diffusion, bulk transport, and the geometry of the sporangiophore.

These measurements indicate that it takes a number of hours before material is brought by bulk transport and diffusion from the base to the growing zone.

10. Oxygen consumption

(a) **Rate of oxygen consumption and ATP level in sporangiophores.** Plucked stage I and IV sporangiophores have a steady-state level of oxygen consumption of 2.5 and 5 $\times 10^{-10}$ moles of O_2 min^{-1}, respectively (100 E.W.G.). The oxygen consumption of the stalk of 2 to 3 cm long stage IV sporangiophores is about 10^{-10} moles of O_2 cm^{-1} min^{-1} and does not vary significantly along the stalk. The sporangium consumes about 2 $\times 10^{-10}$ moles of O_2 min^{-1} (100 E.W.G.).

No increases in oxygen consumption associated with growth responses, including those induced by "sunrise" periods (Section 14) have been found (21, 70, 88*a*). It might be thought that such increases are limited to the growing zone and that the background level of oxygen

consumption by the whole sporangiophore is so high as to blanket increased consumptions of the growing zone. However, corresponding experiments involving tests of O_2 consumption of upper portions only (Section 7c) gave equally negative results (100 E.W.G.). We infer that the extra consumption associated with growth responses is relatively small.

The concentration of extractable ATP in sporangiophores has been determined by Shropshire and Gettens (189) and by Shropshire and Bergman (188). The average amount of ATP is about 2×10^{-11} moles per cm length of sporangiophore, corresponding to a concentration of 10^{-3} M in the cytoplasm. The findings reported in the earlier paper of a transient increase in this level after a light stimulus could not be substantiated in later more extensive and more precise measurements reported in the second paper. Comparing the steady-state level with the oxygen consumption rate given above and assuming that 6 ATP are produced per O_2 consumed, one arrives at a turnover time for ATP of about 2 sec. In view of the shortness of this time, the determination of extractable ATP depends critically on the extraction procedure, especially on the speed and effectiveness with which the reactions producing and consuming ATP are arrested. It is therefore not surprising that individual determinations show rather large fluctuations of the ATP level.

(b) **Rate of oxygen consumption in mycelium.** Mycelium has an average rate of oxygen consumption per mg (wet weight) of about 7×10^{-10} moles per min, similar to that of an equal weight of sporangiophores. The rate varies with the stage of development of the mycelium grown on solid medium (70). The total rate of consumption rapidly rises to a maximum during the development of the first large crop of sporangiophores (3.5 days after inoculation) and then slowly declines. The sporangiophores themselves contribute only about 15% to the total consumption.

(c) **Respiratory quotient.** de Boer (70) also studied the respiratory quotient (CO_2 given off/O_2 consumed) of the developing mycelium and sporangiophores on different media. On carbohydrate-rich media, the respiratory quotient was higher than 1 (up to 1.25). The theoretical respiratory quotient for the complete oxidation of carbohydrates is unity, for lipid less than unity. The experimentally determined value greater than unity calls for a special explanation. It could be caused by an excess liberation of CO_2 or by a process which liberates oxygen (thus counteracting the net oxygen consumption rate). Excess liberation of CO_2 could be caused by glycolysis if the production of lactic acid is followed by decarboxylation. de Boer, however, did not find CO_2 liberation under anaerobic conditions. He therefore postulated that the mycelium is converting carbohydrates into lipids, a process that also releases CO_2. In keeping with this interpretation, de Boer found for mycelium grown on a lipid-rich medium the expected value 0.7 to 0.8.

(d) **Dependence of sporangiophores upon oxygen.** Many fungi can grow anaerobically for long periods of time, but Phycomyces sporangiophores do not have this capacity. When oxygen is removed from sporangiophores by placing them in nitrogen, streaming and growth rapidly stop. Growth of sporangiophores stops within 10 min if the growing zone is placed in nitrogen and slows down to about 60% of normal at 50% of normal oxygen concentration (100 C. R. Hamilton). Under nitrogen, streaming in the upper part of the sporangiophore stops in 1 or 2 min. Streaming in the lower part of the sporangiophore may continue for 30 or 40 min on the energy supplied by glycolysis (88*a*).

de Boer (70) has shown that Phycomyces does not give off CO_2 anaerobically. The respiratory quotient does not change when the oxygen concentration is reduced to 10% of normal. The end products of glycolysis appear to be unmetabolizable and to build up in the cytoplasm under anaerobic conditions.

CYTOLOGY

11. Kinds of particulates and their distribution

Ultrastructural studies of sporangiophores suffer from two difficulties: a thick cell wall, which impedes the rapid infusion of fixatives, and a large vacuole, which is inherently unfixable. A variety of methods have been tried by a number of investigators and the best results have generally been obtained with glutaraldehyde fixation as the first step. The pictures show the usual, and some unusual, organelles well enough and give a general impression of the disposition of these organelles. However, they also clearly show that a considerable amount of disorganization and dislocation has taken place during fixation. An improved method of fixation therefore remains an important desideratum.

In this section we summarize the findings published by Peat and Banbury (140), Thornton (206, 207), and unpublished work from this laboratory (100 S. K. Malhotra; 100 P.V.B., M.Z.).

Between the central vacuole and the cell wall is a cytoplasmic layer 30 μm thick in the growing

zone and thinner lower down. The cytoplasm contains numerous nuclei, mitochondria, lipid droplets, glycogen granules, and other particles, all of them presumably carried along by protoplasmic strands (Section 8).

In stage I the growing tip is devoid of nuclei, and many nuclei destined for spore formation are found 20 to 25 μm below the apex (Fig. 11-1). Below these nuclei is a region of granular cytoplasm containing many globules and "dense bodies" but few mitochondria or nuclei. Below this region there are many vesicles which finally merge with the vacuole.

These vesicles, which often contain regular cytoplasmic constituents in various stages of disintegration, have been interpreted by Thornton (206) as "autophagic vesicles," i.e., as vesicles which engulf cytoplasmic materials destined for destruction and disgorgement into the vacuole. These vesicles start out as small cisternae which coalesce around a portion of cytoplasm to be disposed of, forming a double membrane which is later reduced to a single membrane. These vesicles are not found in the mycelium but begin to form in the earliest stages of sporangiophore development.

The vacuole contains proteinaceous crystals and cellular debris. In stage IV sporangiophores, there is found around the vacuole near the columella a residue of the granular cytoplasm of stage I which contains no nuclei or mitochondria. Nuclei are also excluded from the periphery of the growing zone (100 S. K. Malhotra).

Properties of the major cytoplasmic constituents, irrespective of stage of the sporangiophore, are as follows:

1. The nuclei contain five or six chromocenters and are about 2 μm in diameter. No dividing nuclei have been seen in any stage of development of the sporangiophore (201, 100 Ute Wagenmann).

2. Mitochondria occur throughout the sporangiophore. In the growing zone they have well-developed cristae. Near the growing tip of stage I sporangiophores, the cristae are extremely long and appear to separate the mitochondrion into several compartments. Mitochondria in the base may have another form.

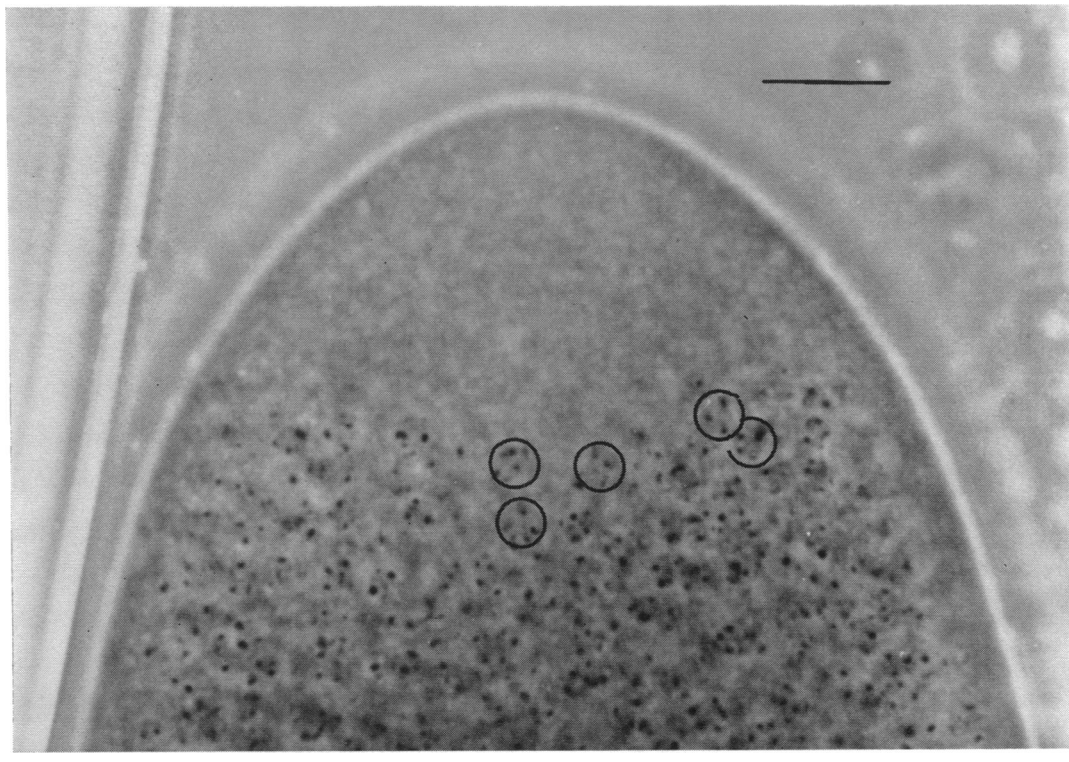


FIG. 11–1. *Tip of stage I sporangiophore (100 Ute Wagenmann). Fixed and stained with aceto-orcein and aceto-carmine. Scale, 10 μm. The picture shows a terminal zone free of nuclei; lower down a zone with many nuclei, each with five to six chromocenters. Some of these nuclei have been outlined by circles.*

3. Lipid globules are ubiquitous. Large globules accumulate in stage I tips and are incorporated into the sporangium. In stage IV, small ones are found in the growing zone and progressively larger ones lower down. Many of these globules carry a crystalline monolayer array of ferritin molecules (Section 33), possibly embedded in a membrane matrix on a portion of their surfaces (Section 12). In wild type the globules contain β-carotene and the larger ones are conspicuously yellow. In electron micrographs they exhibit varying degrees of density.

4. Glycogen is ubiquitous, except in hyphal tips and stage I tips (207). The glycogen particles are about 80 nm in diameter.

5. Bundles of endoplasmic reticulum are found in the cytoplasm, especially in the growing zone. These are frequently associated with nuclei and seem to be a proliferation of the nuclear envelope. Near the tip of stage I sporangiophores there are some fine tubules. Ribosomes, both free and attached, are found associated with the endoplasmic reticulum.

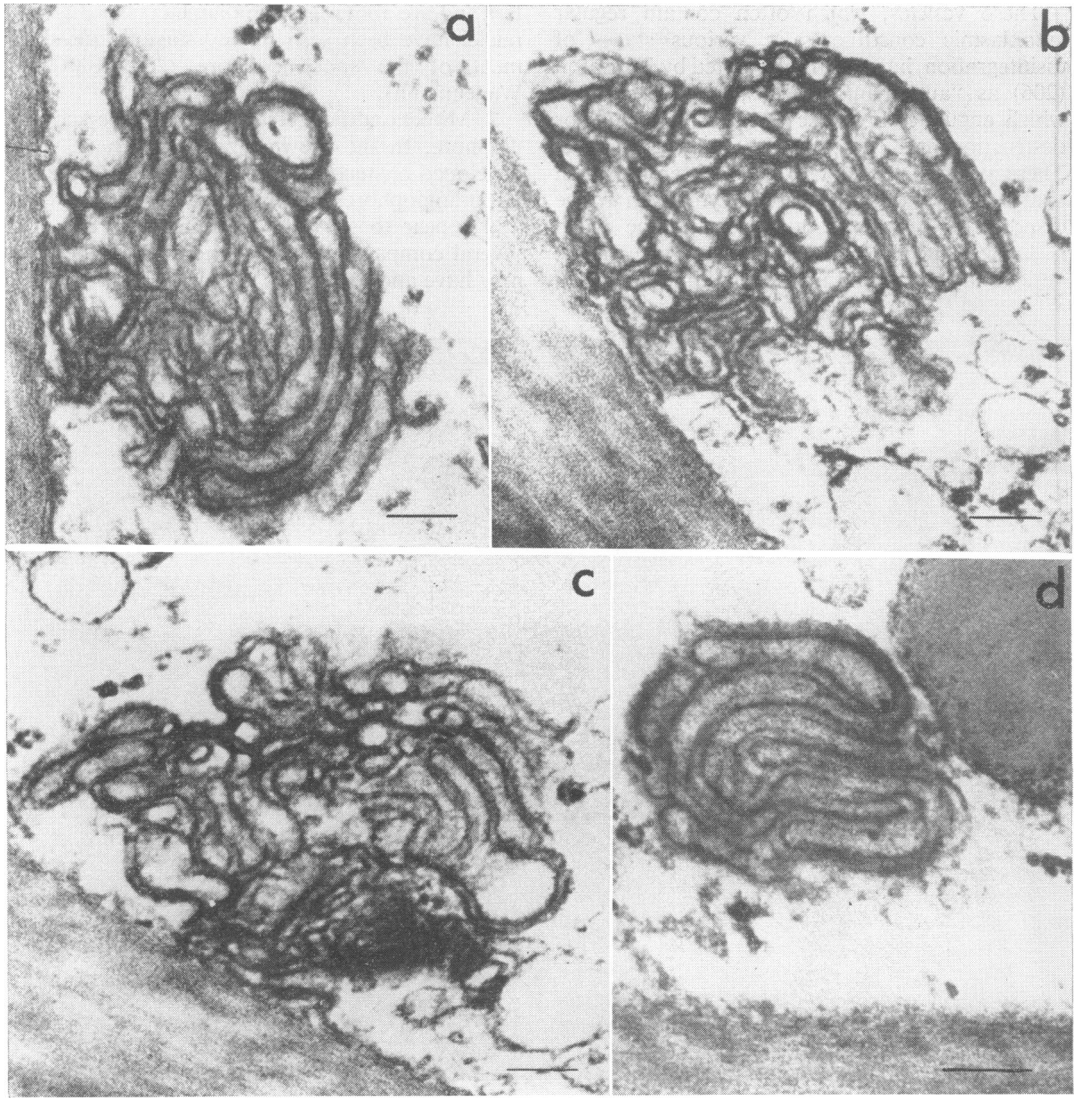


FIG. 11–2. *Malhotra particles (100 S. K. Malhotra). Multiply folded double membrane structures, inside cytoplasm, attached or close to plasmalemma. (a, b, c) Sections through the same structure; (d) through a different structure. Stage IV sporangiophores, growing zone. Glutaraldehyde fixation, postfixed with OsO_4, scale 0.1 μm.*

6. Multivesicular bodies containing many small vesicles and "myelin figures" are found near the plasmalemma. A multifolded membranous structure (Fig. 11-2), resembling retinal rods, has been seen close to the plasmalemma in thin sections made by S. K. Malhotra (100).

7. Frequently, small vesicles resembling lomasomes (131*a*) are seen between the plasmalemma and the cell wall.

12. Intracellular centrifugal separation of organelles in stage I sporangiophores

In this section we summarize data obtained by a new technique. These data will be published in a forthcoming paper by Zalokar (J. Cell Biol., *in press*).

(a) **Technique.** Cell organelles of Phycomyces can be separated by centrifugation of living sporangiophores. Separation succeeds best in stage I, which is rich in cytoplasm and has a relatively small vacuole. One can also centrifuge stage IV sporangiophores, but because of their length part of the cytoplasm is injured and coagulates into shapeless plugs when it is moved centrifugally along the vacuole. The wall of the sporangiophore serves as a centrifuge tube, but, since it is not very rigid, it has to be supported by a buoyant medium. The sporangiophore is plucked by its base and placed, with the tip down, into a capillary tube containing 40% Ficoll (Sigma) at the bottom and filled with 10% Ficoll. The tube is then placed into an adapter for a Spinco centrifuge tube. Fluorochemical KF3 supports the capillary by flotation and prevents breakage under high centrifugal forces. Centrifugation is done in a swinging-bucket Spinco centrifuge (SW39 rotor) at a maximum speed of 35,000 rev/min.

At low centrifugal forces, 100 × *g*, only part of the cytoplasm flows to the bottom, leaving behind the peripheral layer attached to the wall. At higher forces, ~2,000 × *g*, all the cytoplasm sediments and is separated from vacuolar fluid and from lipid droplets, which collect at the centripetal end. Even prolonged centrifugation at this speed does not separate cell organelles. The separation of organelles succeeds best if the sporangiophore is centrifuged for 10 min at 50,000 × *g* and then for 20 min at 100,000 × *g*. Longer centrifugation times make no significant improvement; lower forces do not suffice to stratify cell organelles, which probably fail to be broken from cytoplasmic connections.

When centrifuged sporangiophores are mounted horizontally on a microscope slide, the layers remain separate long enough for microscopic observation, although some mixing does occur. The layers cannot be fixed with cytological fixatives without distortion. Fixatives penetrate into the sporangiophore mainly through its tip and when they diffuse further down into the sporangiophore, they displace its contents. For the same reason, it is difficult to perform cytochemical reactions. For electron microscopy, the best fixation was obtained with glutaraldehyde (5% in 0.1 M phosphate buffer, *p*H 6.8), post-fixed with OsO_4, or with a mixture of glutaraldehyde and acrolein in cacodylate buffer (0.1 M, *p*H 7.0; F. A. Burr, *personal communication*). Thin sections were made from material embedded in Vestopal (Jäger Co., Geneva) and then stained with uranyl acetate and lead citrate.

(b) **Description of layers.** In the sporangiophore of stage I, the following layers could be observed, starting from the centrifugal end (Fig. 12-1):

1. Polyphosphates: this layer consists of droplets, confluent into one big mass at the tip. Polyphosphates give a metachromatic reaction with toluidine blue and methylene blue (purplish color as opposed to bluish coloration of other cytoplasmic structures). With lead acetate, followed by H_2S, they turn black, due to lead sulfide

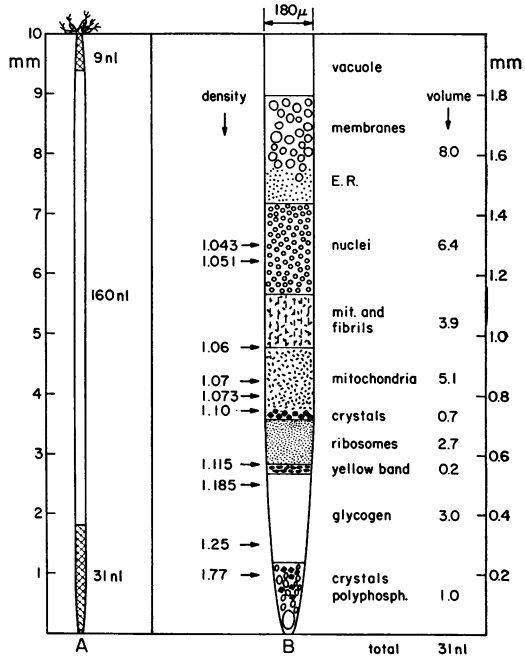


FIG. 12-1. *Intracellular centrifugal separation of organelles in stage I sporangiophores. (A) Whole sporangiophore, about 1 cm long, with organelles heavier than water packed in tip section, lipids in base section. (B) Tip section, showing layers of various organelles, with estimated packed volume of each. Arrows at the left indicate positions that oil drops of known density (indicated by numbers) take up during centrifugation.*

precipitation where lead phosphate had been formed. Sodium molybdate reaction, specific for inorganic phosphates, gives faint bluish color. It appears that the layer contains other substances besides polyphosphates. In electron micrographs, at least two types of droplets were observed. It was not possible to determine which corresponded to polyphosphates.

2. Polyphosphates and protein crystals: the upper part of the polyphosphate layer appears granular due to many protein crystals.

3. Glycogen: this layer appears optically empty and, by electron microscopic observation, consists of tightly packed glycogen granules 80 nm in diameter. They give a reddish-brown color with iodine and positive periodic acid-Schiff (PAS) reaction.

4. Intensely yellow granular layer: this layer is usually a narrow band (disc), consisting of granules of various sizes. Electron micrographs revealed that it is rich in ferritin, arranged in the typical two-dimensional pattern (Section 33) folded in various ways. It appears as if a membrane, covered with ferritin molecules, had been stripped from the surface of the lipid droplets and centrifuged down. However, no membrane could be seen in the electron micrographs.

5. Ribosomes: these appear brownish-yellow in transmitted light under the microscope. They give typical staining with toluidine blue, acridine orange, and other basic stains, indicating a high concentration of nucleic acids. Electron micrographs showed that the ribosomes are closely packed and devoid of cytomembranes.

6. Crystals: the major part of the proteinaceous crystals sediments in a layer between the ribosomes and the mitochondria. The crystals are of various sizes and are probably rhombohedral. They give positive reactions for proteins, negative reactions for carbohydrates and lipids. In the normal cell, most of the crystals are in the vacuole near the tonoplast. In electron micrographs, at high magnification, they display a very regular crystal lattice pattern.

7. Mitochondria: this layer gives positive reactions for succinic dehydrogenase with tetrazolium salts and for cytochrome oxidase by Nadi reagent. In electron micrographs, one can see typical mitochondria, measuring 0.5 to 2 μm, loosely packed, with ribosomes filling the interstices.

8. Ill-defined material comprising the upper half of the mitochondrial layer: besides mitochondria, this layer contains irregular granules, often carrying some carotenoid pigments. Sometimes one can detect a fibrillar structure, with fibrils parallel to the longitudinal axis, largely obscured by granulation. At 50,000 $\times$ g, some components of this layer sediment above the nuclei and move down only at 100,000 $\times$ g. This layer was not studied closely. This layer and layer 10 may contain the autophagic vesicles of Thornton (206).

9. Nuclei: these can be recognized easily by their morphology and positive Feulgen reaction. No other layer gives a positive Feulgen reaction; the nuclear layer is negative for succinic dehydrogenase, indicating complete separation of nuclei and mitochondria. In electron micrographs the spaces between nuclei are filled with small vesicles, apparently part of the endoplasmic reticulum.

10. Irregular particles above nuclei: these particles could not be resolved properly under the light microscope, but they appear to be composite cytoplasmic aggregates, containing vesicles, granules, and some carotenoids. A clear layer sometimes separates above the nuclei, composed of vesicles of endoplasmic reticulum. The coarse granular zone shows, under electron microscopic observation, many profiles of unit membranes.

11. Vacuole: appears optically empty, but at high magnification one can observe in it very small granules in Brownian motion. After fixation, a substantial precipitate forms, indicating that the vacuolar sap contains many solutes.

12. Lipoprotein: the layer contains irregular light-scattering vesicles and oil globules colored with carotenoids. Electron microscopy shows that the main component of this layer is cytomembranes. Ferritin, which covers the lipid droplets in undisturbed cells, can also be found in this layer, probably still associated with lipids.

(c) **Buoyant density of layers.** Oil droplets of known specific gravity introduced into the cell before centrifugation indicate the density of various layers. The oil must be sufficiently inert not to damage the cytoplasm. Ethyl and butyl phthalate are not toxic and can be mixed to give densities between 1.043 (butyl phthalate) and 1.129 (ethyl phthalate). Oil droplets were introduced into sporangiophores which had been centrifuged at 50,000 $\times$ g for 10 min and cut open through the vacuolar layer without loss of cytoplasm. After injection through the open end, the opening was sealed with molten bee's wax; the sporangiophore was supported in a small capillary containing 20% Ficoll and centrifuged at 50,000 $\times$ g for 20 min. The oil droplets settle in areas of equivalent density, indicated in Fig. 12-1.

Since the oil droplets displace rather large volumes, they indicate the *average* density of the layer, i.e., cell organelles plus interstitial suspen-

sion medium, and not the density of the particles in that layer. A closer approximation to the cell organelle density is obtained by injecting fine particles of approximately the same size as the organelles. Bacteria and phage were used as such density indicators. These can be injected into the cell and centrifuged without apparent interaction with the cytoplasm. *Escherichia coli* (density 1.07) settled between the ribosomes and the mitochondria. Bacteriophage (density 1.475) settled below the ribosomes and above the glycogen. After Feulgen staining the injected organisms can be recognized easily.

(d) **Microsurgery.** Centrifugal separation of cell organelles makes it possible to assay their enzymatic and other properties in living cells, as compared with particles isolated from broken-cell preparations. There is no direct proof that the particles remain undamaged by the centrifugation, since centrifuged sporangiophores do not continue to grow and do not regenerate. It is known, however, from other experiments that cells can survive centrifugal forces of several hundred thousand times gravity.

Cytochemical tests show that mitochondria can still perform their respiratory functions and it should be possible to detect activities of other enzymes. Since it is difficult to obtain penetration of reagents through the cell wall, methods are needed to study enzymatic reactions of particles isolated from centrifuged cells. Several methods of isolation are possible. (i) The tip of the sporangiophore is cut and the contents ooze out in order of sedimentation (analogous to piercing a centrifuge tube at the bottom). The material can be collected under mineral oil and separated with microneedles. All layers behave as viscous liquids and do not pack into solid sediments. (ii) The sporangiophore is cut at appropriate levels (analogous to sectioning of a chromatographic column). By this method, one particular layer is isolated, but the others are disturbed by cutting. (iii) A cut is made through the vacuolar layer and layers are sucked out with a micropipette (analogous to pipetting from the top of a centrifuge tube).

With this last method the nuclear layer was aspirated and transplanted into a normal sporangiophore. When the donor nuclei were from the red *carR21* strain, which produces lycopene, and the host was the albino *car-5*, the presence of functional nuclei was indicated by pigment production. In many cases, heterokaryons were obtained, producing yellow sporangiophores (Section 29b). After plating their spores, albinos, reds, and yellow heterokaryons were found. This experiment proves that the nuclei survived centrifugation and that the trait concerned is controlled by a nuclear gene. It opens ways of testing genetic and other functions of organelles by injecting them into appropriate hosts.

(e) **Search for photoreceptor.** If the photoreceptor is located on a special cell organelle, it should sediment in a particular layer. It should be recognizable by its color and characteristic spectrum. We scanned absorption spectra of different centrifuged layers with a Cary microscope-spectrophotometer. We could not find any region giving a spectrum corresponding to the action spectrum of the photoresponse. The only distinct absorption peaks were due to carotenoids, cytochrome, and a slight indication of flavine. To avoid absorption by carotenoids, the mutant *car-10* was used primarily. In most layers, light-scattering by small particles was intense. The polyphosphate layer showed an absorption near 420 nm, due to cytochrome or related compounds. Glycogen had rather low scattering, but showed no peak. The yellow layer gave end absorption in the blue, undoubtedly due to ferritin, without discernible peaks, definitely not matching the action spectrum. Ribosomes showed intense light-scattering, appearing brownish under the microscope. The mitochondria had a very strong absorption peak near 420 nm, due to cytochrome. The position of the peak varied according to the oxidation state of the cytochrome, between 418 and 422 nm. Reduced cytochrome showed also a low peak at 525 nm. Besides cytochrome, a low shoulder was observed around 445 nm, probably due to flavines. Nuclei have a distinct cytochrome peak at 422 nm and a slight indication of flavine at 445 nm. No absorption bands were observed in the vacuole. The lipid layer gave a typical spectrum of carotenoids, predominantly β-carotene in wild type and lycopene in mutant *carR21*. Crystals, which have been suggested by Thimann's (202) group to be the photoreceptor, scattered light strongly, but showed no absorption peak in the visible region.

This centrifugation technique, combined with microspectrometry, is useful in the study of pigments which are present in sufficient quantities and sediment with a particular cell fraction. The receptor pigment escaped detection, either because its absorption is so low that it can not be measured above background or because it remains attached to the cell wall, or because it did not sediment in a distinct layer and remained scattered through many layers.

SENSORY PHYSIOLOGY (LIGHT)

13. Light growth response

Of the responses to light of Phycomyces, the growth response is simplest to analyze though

possibly physiologically irrelevant. This response was discovered and extensively studied by Blaauw (12–15). He recognized that the control of the growth rate by light is much more simply expressed by the growth response than by phototropism. In his enthusiasm he declared (15) that the connection between the tropic and the growth response had now become an empty problem: "Das Problem an sich des Phototropismus ist leer geworden. Weitere theoretische Betrachtungen über dieses Problem würden uns nur noch länger von der Forschung der wirklichen und dadurch inhaltsreichen Erscheinungen des Wachstums fernhalten. Im Phototropismus selbst doch liegt kein Problem; denn der Phototropismus ist eine reine Wachstumsercheinung. Aber das Wachstum ist als Lebenserscheinung ein Problem voller Tiefe." Sections 15–17 will be concerned with the problem of phototropism.

To demonstrate the growth response, the specimen is irradiated symmetrically around the vertical axis and this symmetry is maintained at all times. The stimulus program involves only temporal variation of the light intensity. The output is a temporal variation of the growth rate.

Two practical arrangements for achieving symmetry of irradiation are: (i) unilateral irradiation of a rotating specimen (at least 2 rev/min), or (ii) bilateral irradiation.

With constant symmetric irradiation, the specimen on the average grows parallel to the axis of symmetry. With bilateral irradiation at right angles to this axis, all directions are in labile equilibrium (71); i.e., light does not exert an orienting effect. For this reason it is preferable to have the light come in at an angle less than 90° to the axis of symmetry. If the angle is too small, the specimen may show high amplitude hunting around the direction of equilibrium (75). In practice, an angle of 60° from the axis of symmetry gives reasonably steady growth (for some mutants, like *car-10*, this question needs reinvestigation).

The growth response has been studied almost exclusively in stage IVb, but one report (130) shows a growth response for stage I.

In stage IV the growth rate at constant intensity of irradiation settles down to a constant value. This value is independent of the intensity, from complete darkness to bright sunlight, and amounts to about 50 μm/min. Growth responses can be elicited by single pulses, by step-ups in intensity, by periodic light programs, and by exponential increases in intensity (sunrise, Section 14).

A single pulse of light produces a transient response in the growth rate beginning after about 3 min, a positive phase lasting from the 3rd to the 7th min with a maximum at 5 min, followed by a shallower, more extended negative phase, presumably compensating quantitatively for the positive phase. Thus, there is no net gain in growth, only a redistribution in time. This statement is difficult to prove rigorously for individual pulse stimuli, but it has been checked quite carefully for periodic pulse or step-up step-down programs. These do not change the average growth rate (100 M.D.).

A step-up in intensity is followed by a growth response similar to that evoked by a pulse but lacking in the negative phase.

The responses are graded. The response rises from its minimum measurable value to a saturation value when the pulse size is increased by a factor of about 30. The response is a function of the pulse size, S, the product of *intensity* and *duration*. This product rule is valid up to times of about 1 min, and has been tested down to 1 sec (71).

A step-down in intensity produces a negative growth response which is shallower than the saturated positive growth response and has a latent period which is longer by at least 1 min than that of the positive growth response (49).

14. Light and dark adaptation, sunrise experiments

The growth response depends strikingly on the intensity to which the specimen had been adapted before the stimulus. We define the level of adaptation, A, either as the actual intensity to which the specimen had been adapted or as the virtual intensity to which it would have to be adapted to give a corresponding response to a standard stimulus. Thus, our definition of the level of adaptation has the dimension of intensity and we can express adaptation by saying that the responses are a function of S/A. This ratio has the dimension of time. It tells how many minutes worth of adapting light flux have to be compressed into a short stimulus to give a response of a particular magnitude (71). $S/A = 2$ min gives a barely measurable response, $S/A = 60$ min a saturating response. Measurements of the kinetics by which the level of adaptation changes have shown that this level follows approximately the equation

$$dA/dt = (I - A)/b \qquad (14\text{–}1)$$

where b is the time constant of adaptation; i.e., if a specimen is brought to a certain level, A, and the adapting light is then turned off, the level of

adaptation will drop by a factor e during this time. Time constant b equals about 5 min at 20°. None of the mutants so far differ appreciably with respect to b.

Equation 14-1 implies that during a pulse stimulus, i.e., a stimulus whose duration is short compared to b, the increase in the level of adaptation is equal to $It/b = S/b$. The increase is thus a function of the product of intensity times time, with a proportionality constant involving the same time constant as the one which characterizes dark adaptation.

It is useful to visualize the implications of equation 14-1 for two frequently used periodic stimulus programs.

During a periodic stimulus program consisting of *short* stimuli of size S, given at intervals of time t, the level of adaptation, after a few cycles, settles down to a periodic variation in which it jumps during each stimulus from the prestimulus level:

$$A_- = (S/b)/(e^{t/b} - 1) \qquad (14\text{–}2)$$

to the poststimulus level

$$A_+ = (S/b)/(1 - e^{-t/b}) \qquad (14\text{–}3)$$

and then returns to A_- during the interval t (Fig. 14–1A).

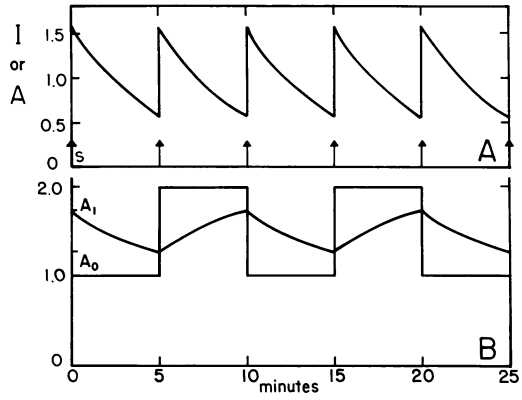


FIG. 14–1. *Theoretical level of adaptation as a function of time during two commonly used periodic stimulation programs (equation 14-1). The diagrams show the periodic variations in adaptation which are established after the programs have been running for several cycles. In A), the program consists of a pulse every 5 min (equation 14-2, 3). The ordinate represents intensity or adaptation units. The pulse has the dimension intensity × time and was chosen equal to five intensity units × minutes. In B), the intensity switches between two levels, I_0 and I_1 every 5 minutes (equation 14-4, 5, 6). I_1/I_0 was chosen equal to 2. In both cases, the time constant b is assumed to be 5 min.*

During a different periodic program, the incident intensity alternates between the intensities I_0 and I_1, staying at each intensity for a time t. The level of adaptation again settles down after a few cycles to a periodic variation between the levels A_0 and A_1. At the end of the I_0 period, it reaches (Fig. 14–1B)

$$A_0 = (I_0 + I_1 e^{-t/b})/(1 + e^{-t/b}) \qquad (14\text{–}4)$$

and at the end of the I_1 period it reaches

$$A_1 = (I_1 + I_0 e^{-t/b})/(1 + e^{-t/b}) \qquad (14\text{–}5)$$

The fluctuations ΔA and ΔI are related by

$$\Delta A = \Delta I\,(1 - e^{-t/b})/(1 + e^{-t/b}) \qquad (14\text{–}6)$$

Thus, ΔA is always smaller than ΔI, and for t = 5 min this ratio is a little less than one-half.

During a step-up of intensity, the level of adaptation approaches the new level with the time constant b. The growth response to a step-up is transient because of this adaptation phenomenon and not because of any lack of substrate needed to support a sustained higher growth rate. A sustained higher growth rate can in fact be elicited if the step-up is repeated every 1 or 2 min. This simulated exponential increase in intensity is similar to though somewhat faster than the actual intensity changes at sunrise. A sustained increase in growth rate can be maintained for as long as the exponential intensity increase is continued.

Figure 14–2 shows examples of such sunrise experiments (100 W.S.). In experiments a and b, the intensity was raised from $\log_2 I = -8$ to $+9$, in seventeen steps; in c, from $\log_2 I = -3$ to $+9$ in twelve steps, each step doubling the intensity (see Section 38 for intensity scale). In a, the doubling time was 1 min; in b, 2 min; in c, 4 min. In a and b, the growth rate begins to rise 4 min after the beginning of the sunrise, reaches a maximum of nearly double V_0, then settles down to 1.6 V_0, and stops there until a few minutes after the end of the rise, even though the intensity remains high. If the doubling time is lengthened to 4 min (c), no growth response is observed. If the rise to this very high intensity is made much faster, a new phenomenon is observed (Section 21).

What has been said about the growth response and the adaptation kinetics is valid over an intermediate range, the "normal range," from about $\log_2 I = -8$ to $+1$. In the low range, below $\log_2 I = -8$, the time constants both of the growth response and of adaptation become greater (100 M.D. and Lois Edgar). For the high range, *see* Section 21.

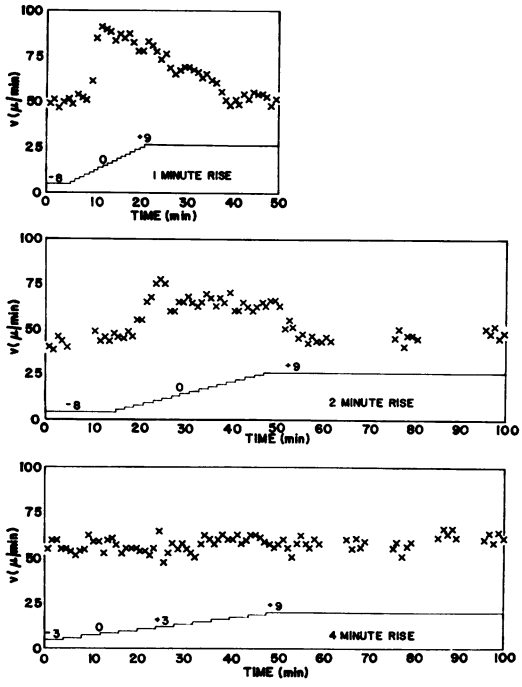


FIG. 14–2. *Sunrise experiments. Symmetric illumination. Intensity doubled every 1 min (top), 2 min (middle), 4 min (bottom). Note prolonged supranormal growth rates in top and middle during "sunrise," and return to normal growth rate a few minutes after reaching and maintaining the highest intensity.*

15. Phototropic response

In Phycomyces, only the sporangiophores are phototropic. In some fungi, especially parasitic ones, the hyphal tips are negatively phototropic (89). In contrast to Fucus eggs (116), the direction in which the germ tube emerges from the vegetative spores in Phycomyces is not influenced by the direction of light or its polarization. Nevertheless, there is reason to suspect that a receptor pigment is present throughout the mycelium, and that light can exert a controlling function there, although not on growth (Section 23g).

The tropic response of the sporangiophore is normally positive towards the light source. If the tropic response is elicited without changing the total flux, e.g., by stopping rotation (*see* Fig. 15–3), its latency is about 6 min, considerably longer than that for the growth response (58). If it is elicited in conjunction with a moderate growth response, its latency is close to that of the growth response, about 3 min (46). In contrast to the growth response, the tropic response is *steady*. As long as the geometrical relation between the growing zone and the light source is kept constant, the bending rate also stays constant after a short latency and initial transient phase (Fig. 15–1). This constancy can be demonstrated crudely by putting the sporangiophore on a turntable and rotating it at about 3°/min (Fig. 15–2), or, for more sophisticated measurements, by using the tropostat (79).

In the normal range ($\log_2 I = -8$ to $+1$), the steady bending rate is independent of the intensity. Above $\log_2 I = +1$, the bending rate drops off rapidly, reaching 0 at about $\log_2 I = +6$; below $\log_2 I = -8$, it drops off slowly, reaching 0 at about $\log_2 I = -25$ (157; Fig. 21–2). Thus, phototropism occurs over a total intensity range of about 30 intensity units, or a factor of 10^9.

Although the steady bending rate is independent of the light intensity in the normal range, dark and light adaptation are involved. In fact, the tropic response can serve in a simple way to measure the kinetics of dark adaptation. If a specimen is first adapted to a symmetrical high intensity and then tested for tropic response at low intensity, there is a delay during which the general level of adaptation drops from the high to the low level. The measurement of this delay time is the simplest procedure for analyzing dark adaptation kinetics (Fig. 15–3).

Once the steady state of the tropic response is reached, however, we *cannot* interpret it by saying that the specimen over its whole cross-section is adapted to the locally prevailing light intensity. If it were, the growth rate would be uniform all around the specimen and no differential growth could be maintained. To avoid this difficulty, Castle (60) developed a limited theory which implies that light adaptation is *uniform over the cross-section* even though the light distribution is not uniform. The level of adaptation seems to correspond to some average of the intensity prevailing in any cross-section. This postulate accounts for a variety of situations and is therefore useful for tying these observations together.

To arrive at this theory we begin with the observation (57) that the tropic response can be produced under conditions in which the net growth response, averaged across the entire cell, is zero. A light program is used in which the net flux of light is kept constant, i.e., by switching at time zero from a symmetrical bilateral irradiation to a unilateral irradiation with doubled intensity. Under these circumstances the average growth rate does not change at all. It becomes azimuthally asymmetric:

$$v(\Psi) = \bar{v}\,(1 + \epsilon \cos \Psi)$$

where Ψ is the azimuth angle. In a typical case $\epsilon = 0.05$; i.e., the growth rate on the proximal

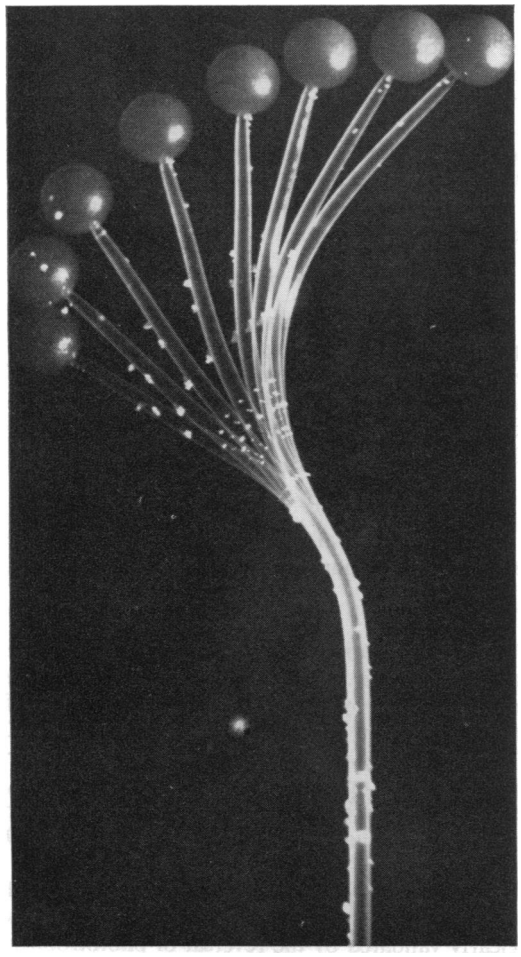


FIG. 15–1. *Steady-state phototropic response in air. Pictures taken at 5-min intervals. Growth rate, 60 μm/min. Tropic rate, 3°/min. Starch grains attached as markers on the sporangiophore and on the sporangium demonstrate the extent of the growing zone (about 2 mm) and spiral growth (about 60°/5 min at sporangium, progressively slower lower down). Scale: sporangium diameter, 500 μm. (Photo Lois Edgar.)*

side is 5% below average and on the distal side 5% above average.

The bending rate, $d\alpha/dt$ is related to ϵ:

$$d\alpha/dt = \epsilon\bar{v}/r \qquad (15\text{–}1)$$

where r is the radius of the sporangiophore. For a typical case we may have $\bar{v} = 50$ μm/min and $r = 50$ μm. With $\epsilon = 0.05$ this leads to a bending rate of 0.05 radians/min = 2.9°/min.

The aim of our theory (a somewhat modified and extended version of Castle's approach) is to establish an expression for the *growth* dissymmetry as a function of the dissymmetry of the *irradiation*. It states that the growth speed dissymmetry of a specimen, exposed to several light sources of unequal intensity disposed in one plane normal to the sporangiophore, is given by the equation:

$$\vec{\epsilon} = \frac{K \sum_i \beta_i I_i p_i (f_i + a_i)}{\sum_i \beta_i I_i p_i} \vec{e}_i \qquad (15\text{-}2)$$

where K = proportionality constant; $\vec{e}_i$ = unit vector in direction of light from source number i; I_i = intensity of beam i; p_i = peripheral screening attenuation factor; β_i = relative efficiency of the receptor pigment for light of quality i; f_i = a parameter expressing the manner in which the sporangiophores evaluate the dissymmetry of light due to focusing, i.e., the focusing advantage of beam i ($f_i > 0$ for sporangiophores in air); and a_i = a parameter expressing the manner in which sporangiophores evaluate the dissymmetry of light due to attenuation by scattering and by absorption, i.e., the attenuation disadvantage of beam i ($a_i < 0$).

Equation 15–2 summarizes a number of aspects of the tropic response and may point the way to a more refined analysis of the chain of transduction from signal to response.

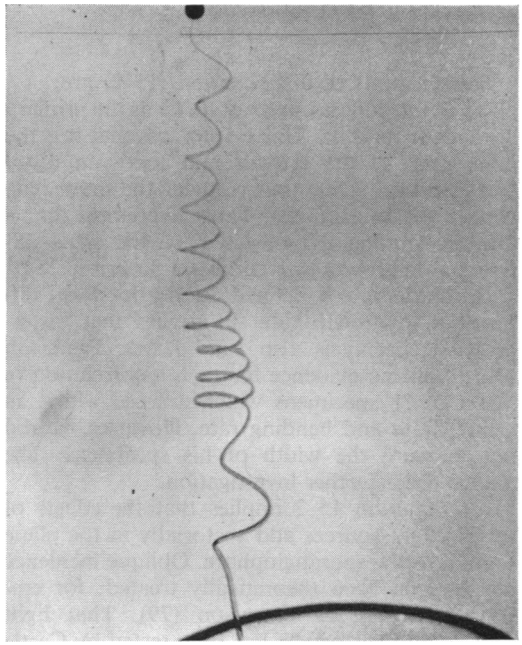


FIG. 15–2. *Phototropic helix. Culture vial mounted on turntable rotating at 3°/min while illuminated from front. Duration of experiment 22 hr. Growth rate, 3 mm/hr. (Photo D. S. Dennison.)*

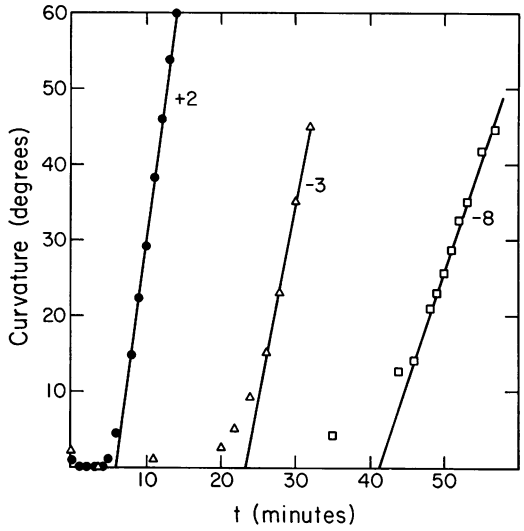


FIG. 15–3. *Dark adaptation measured with phototropic response. The sporangiophore was rotated while illuminating with $\log_2 I = +2$. At $t = 0$, rotation was stopped and the intensity dropped by 5 or 10 $\log_2$ units. Angle of bend plotted versus time. For the control, the intensity remained at $\log_2 I = +2$. The latency (extrapolated back from the steady bending state) without dark adaptation is 6 min; with down adaptation of 5 $\log_2$ units, it is 23 min; with 10 units, 41 min. This gives a time constant of 3.5 min/$\log_2$ unit, or 5 min per factor e. T = 20.6 ± 0.1 C; relative humidity, 64%.*

Some aspects of this equation (15–2) are:

(a) It introduces ϵ and not $d\alpha/dt$ as the primary dependent variable. This emphasizes that it is the differential in the growth rate (between distal and proximal sides) that is under the immediate control of the stimulus. Thus, it predicts that a thinner sporangiophore will have the same differential in growth rate and bend faster.

(b) Variable ϵ is defined as the *fractional* differential in growth rate. It implies that faster-growing specimens also bend faster. Dennison (79) found no evidence for such a correlation in a test of 38 specimens which differed widely in growth rate and bending rate. However, he did not measure the width of his specimens. The matter needs further investigation.

(c) Equation 15–2 implies that the effects of several light sources add vectorially in the plane normal to the sporangiophore. Oblique incidence has not yet been theoretically treated; for empirical results, *see* Dennison (79). That light sources add vectorially has been tested by Castle (60), using sources of equal spectral quality. Equation 15–2 simplifies in this case to $\epsilon = K(I_1 - I_2)(I_1 + I_2)$. This agrees with his experiment.

(d) For a single monochromatic source, β and pI drop out of equation 15–2. *Since the wavelength does not influence focusing appreciably, it should affect the steady tropic response only through its effect on the attenuation.* Thus, measurements of ϵ as a function of λ on single specimens should lead to the most direct determination of a.

(e) For opposed monochromatic sources, the equilibrium condition $\epsilon = 0$ leads to the relation

$$\beta_1/\beta_2 = p_2I_2(f_2 + a_2)/p_1I_1(f_1 + a_1) \quad (15\text{–}3)$$

The focusing advantage f is practically independent of the wavelength, while the attenuation a is strongly dependent on it. This effect of attenuation can be neglected only if a is small compared to f. Under these conditions equation 15–3 simplifies to

$$\beta_1/\beta_2 = p_2I_2/p_1I_1 \quad (15\text{–}4)$$

If the peripheral screening is small or is the same for both monochromatic sources, then

$$\beta_1/\beta_2 = I_2/I_1 \quad (15\text{–}5)$$

This is the relation used to determine the tropic action spectrum. Such an action spectrum can be contaminated by internal screening pigments if the attenuation a is not small compared to f. The screening pigment could be the receptor pigment itself or other pigments. In Phycomyces, self-screening is negligible, and screening by other pigments is slight for wavelengths above 300 nm (72).

(f) Equation 15–2 states that focusing and attenuation act antagonistically. This idea is clearly validated by the reversal of phototropism below 300 nm, and, more subtly, by the comparison of the behavior of various pigment mutants and of wild type (Section 17). The assumption that f and a are additive quantities is not justified in extreme cases such as stimulation with λ = 280 nm. Here attenuation is complete, i.e., *no* light reaches the distal side. This attenuation is due to gallic acid (3,4,5-trihydrobenzoic acid). Dennison (76) discovered that sporangiophores contain about 5 to 10 mg of gallic acid per ml, probably in the vacuole and in the autophagic vesicles (Section 11), and that this substance causes the rapid rise in optical density below 300 nm (Sections 24 and 35).

Empirically, we find at 280 nm in the steady state (tropostat): $d\alpha/dt$ = 17°/min = 0.3 radians/min. With respect to equation 15–1, this means that stimulation of one side only gives a growth rate dissymmetry $\epsilon = 0.3$. Equation 15–2 is clearly not applicable in this case. When there is no light to be focused, it is meaningless to speak of focusing advantage.

16. Dioptric effect

The early workers on phototropism, in the days before the basic principles of photochemistry had been worked out, thought that the plant could directly sense the direction of light. A great step forward was taken by Blaauw (12), who conjectured that the lens properties of the cylindrical sporangiophore were related to sensing the direction of light, since the transparent sporangiophores do produce a rather sharp focusing line. Buder (28, 29) tested this notion by immersing sporangiophores in mineral oil with a refractive index (1.45) considerably higher than that of the cell, thus converting a converging lens into a diverging one. He found that the specimens now bent away from the light, a finding supporting this conjecture. Still, doubt could be raised since the mineral oil changes the accessibility of oxygen, elimination of water, and possibly other parameters as well as the dioptric effect.

Shropshire (187) attacked the problem more directly. He placed a cylindrical glass lens (diameter, 0.16 mm) parallel to the sporangiophore (diameter, 0.12 mm) at a distance of 0.14 mm. Parallel light was focused by the glass lens so that the light path within the sporangiophore became divergent (Fig. 16–1). This caused negative phototropism. Shropshire's results seemed to put Blaauw's conjecture beyond dispute. Ironically, however, Shropshire rediscovered in the course of these experiments a strange and long forgotten phenomenon, the "avoidance response" (Section 28). Similar negative bending away from the glass lens was found even *without* light! Though this bending is quantitatively always less than the observed negative phototropism, it complicates the interpretation since a doubt remains about the interaction of the two responses. Thus, none of the proofs that the light distribution in the cell is critical for phototropism is definitive by itself. The strongest evidence is implicit in the analysis presented in the next section.

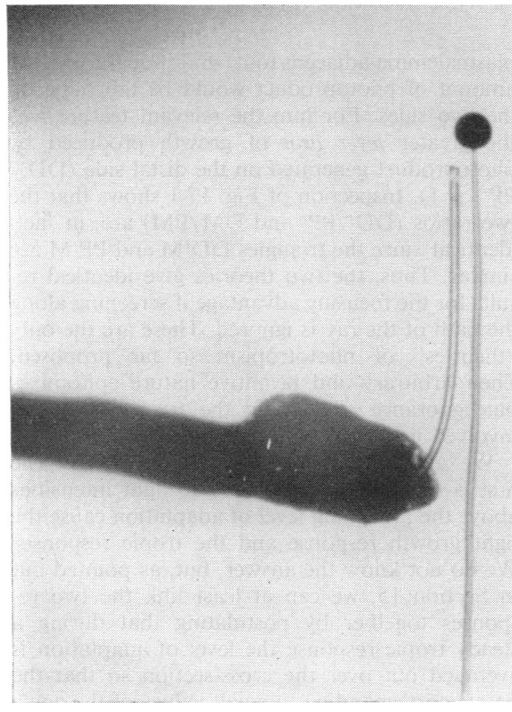


FIG. 16–1. *Shropshire's set-up for "proof of dioptric effects" and rediscovery of avoidance response. The sporangiophore shows a negative phototropic response if illuminated through cylindrical glass rod. It also shows a negative tropic response (avoidance) to the glass rod in complete darkness.*

17. Sporangiophores immersed in fluids of refractive index n(neutral) (n_n)

Since the direction of the phototropic response can be reversed by immersing the specimen in a medium of high refractive index, it might be expected that specimens immersed in a medium of some intermediate index would be phototropically neutral. Shropshire (187) verified this prediction, using mixtures of fluorocarbon oils covering a range $1.28 < n < 1.41$. Shropshire found, using light of 440 nm, a sharply defined neutrality point. Its value was $n_n = 1.295$, appreciably below the refractive index of either cytoplasm (1.36) or vacuole (1.34). Consistent is the earlier finding of Stifler (196) that sporangiophores immersed in water ($n = 1.333$) exhibit negative phototropism. The difference between n_n and the refractive index of the cell is in the expected direction because a residual focusing advantage compensates for the attenuation of light on its path between proximal and distal receptors. Zankel et al. (220) tested this idea further by determining n_n at two wavelengths chosen to give a large difference in attenuation. They chose wavelengths of 495 nm, where the β-carotene in the cell has an absorption maximum (λ_{max}), and 510 nm, where this absorption has dropped by a factor two and the receptor pigment is still active. A shift of n_n in the expected direction was found ($n_n^{495} = 1.306$; $n_n^{510} = 1.312$), but it was smaller than expected. Further information was obtained with the advent of the pigment mutants (Section 29). Especially useful are the albino mutants *car-5* and *car-10*, which contain very little β-carotene. These two mutants differ in the amounts of attenuation due to scattering; *car-5* shows the same scattering as wild type, whereas *car-10* has less, about 60%.

Another mutant which is useful in this context is *carR21*, whose scattering is above that of wild type but whose absorption is due to lycopene instead of β-carotene, with λ_{max} shifted from 495 to 515 nm. Table 17-1 lists n_n as determined for each of these strains at appropriate wavelengths (100 M.H. and K.W.F.).

To analyze these results, recall equation 15-2 which for phototropic neutrality implies $\epsilon = 0$ and therefore equality of focusing advantage and attenuation disadvantage:

$$f = -a \qquad (17\text{-}1)$$

To test the validity of this equation (and thereby one aspect of equation 15-2), we need independent estimates of f and a. The phototropically neutral refractive index n_n can serve to partially determine f, and spectrophotometry can serve to partially determine a. Both quantities can be estimated except for a proportionality constant, a different one for f and a. This procedure suffices to verify equation 17-1.

We must first decide what the measured values of n_n tell us about the residual focusing advantage at tropic equilibrium. Focusing does not create a dissymmetry in the *total* flux of light passing through the proximal and distal halves of the sporangiophores; it merely concentrates the light in the distal half of the sporangiophore near $\beta = 0$ (Fig. 17-1A). Which aspect of this concentration of the light causes a dissymmetry of the growth rate? Castle (46) conjectured that the relevant aspect is the difference in the average *path length* that the light traverses on the distal relative to the proximal side (DM/PM $\geqslant$ 1). Castle assumed that the cell was filled with protoplasm and homogeneously distributed receptor pigment. A greater path length would therefore imply more photoproduct. Buder (30) proposed a different idea. He conjectured that the receptor pigment is confined to the cell wall (or the plasmalemma adjacent to it) and thought that the amount of photoproduct would be the same on the two sides. For him the relevant feature was the greater *lever arm* of growth produced by photoproduct generated on the distal side (DD'/PP' $\geqslant$ 1). Inspection of Fig. 17-1 shows that the two ratios (DD'/PP' and DM/PM) are, in fact, identical since the triangles DD'M and PP'M are similar. Thus, the two theories give identical results for the focusing advantage if screening along the path of the ray is ignored. These are the only "theories" of phototropism so far proposed. Their arbitrary and primitive nature epitomizes our ignorance concerning the true relationship involved in this transduction process.

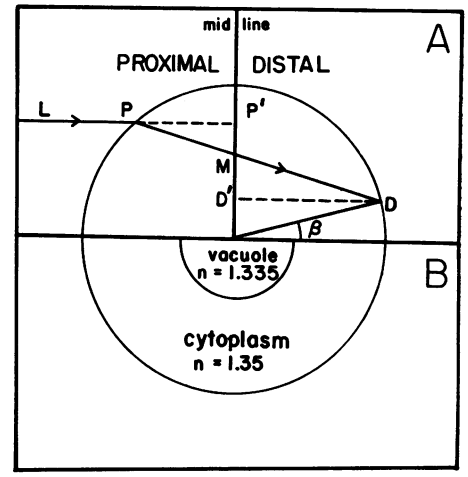


FIG. 17-1. *Sporangiophore as a cylindrical lens. (A) Ray L impinges from the left and is refracted at the first surface. The "path length" theory considers the ratio DM/PM as a measure of the dissymetry; the "lever arm" theory uses instead the ratio DD'/PP'. The maximum value of β is about 20°. (B) The model used for computing focusing advantages as a function of n_1, the refractive index of the immersion fluid.*

TABLE 17-1. *Comparative n_n values for wild-type and mutant strains*

Strain	Wavelength	n_n	Δn^a	$a^{opt b}$	$a^{opt}/\Delta n$
	nm				
carR21	515	1.274	0.086	0.324	3.8
Wild type	495	1.306	0.054	0.230	4.3
Wild type	510	1.312	0.048	0.187	3.9
car-5	495	1.314	0.046	0.149	3.2
car-10	495	1.334	0.026	0.090	3.5

[a] $\Delta n = 1.36 - n_n$.

[b] Values for a^{opt} taken from Fig. 24-1, where it is expressed in OD units and converted to "fraction lost by absorption and scattering."

We are confronted with two problems. The first is the general one: How do light intensities above the prevailing level of adaptation cause the light growth response and the tropic response? We do not know the answer, but, as pointed out in Section 15, we can at least link the two responses together by postulating that during a steady tropic response the level of adaptation is averaged out over the cross-section so that the points corresponding to small values of the angle β (Fig. 17-1A) are constantly exposed to the condition $I/A > 1$. This approach establishes a qualitative relationship between the two responses. The second problem is to establish a quantitative relationship. Not even the sign of the tropic response can be deduced from this connec-

tion with the growth response. It is true that within the irradiated sector on the distal side $I/A > 1$ but this sector is narrow (in air about 40°), while the remaining 140° of the distal side are not irradiated except for scattering. No *a priori* argument predicts for the distal side a net advantage from focusing. The conjectures of Castle and Buder are merely devices to evaluate the dissymmetry of the light in some plausible way so as to give a number of the right sign. It is unlikely that the way the sporangiophore evaluates the dissymmetry is closely related to either of these devices. A plot of the measured values of n_n verus a^{opt} extrapolates to an ideal value of $n_n = 1.36$ for $a^{opt} = 0$. Therefore, we can take this refractive index as the one which the sporangiophore would evaluate as $f = 0$. This is indeed a plausible value for the following reason. Calculations have been made (100 K.W.F.) for a model sporangiophore with a radius of 50 μm, vacuole radius 20 μm, cytoplasm thickness 30 μm (Fig. 17-1B), refractive index of vacuole 1.335 and of cytoplasm 1.350.[5] For this model sporangiophore, the focusing advantage was evaluated by using a modified path length hypothesis: the receptor pigment was assumed to be distributed uniformly throughout the cytoplasm and absent from the vacuole. Reflections at the first surface were taken into account. The ratio of the total absorption by receptor pigment in the distal half to that in the proximal half, minus one, was taken as the computed dissymmetry f(comp). These calculations gave the following results:

f(comp) = 0 for $n_1 = n$(cytoplasm)
·(n_1 = refractive index of immersion fluid)

(The presence of the vacuole with a refractive index slightly lower than that of the cytoplasm makes no difference in this case because the dissymmetry produced by that fraction of the rays which passes through the vacuole is insensitive to the convergence of the rays incident on the vacuole, and it is very small. The more marginal rays, which pass through cytoplasm only, contribute the major portion to the dissymmetry.)

For small deviations of n_1 from 1.35 the values of f(comp) varied *linearly* with this deviation

$$f(\text{comp}) = 1.27\Delta n$$

Calculations based on the lever arm hypothesis also gave a linear dependence of f(comp) on Δn, though with a slightly larger proportionality constant. Therefore, we accept Δn as a quantity proportional to f.

We now turn to a closer consideration of the attenuation part of equation 17-1. Spectrophotometry permits us to measure the sum of the attenuation due to scattering and that due to true absorption.

Table 17-1 lists the attenuations as taken directly from Fig. 24-1. These values of a^{opt} are nearly proportional to the values of Δn. The $a^{opt}/\Delta n$ ratios are given to show this proportionality.

This is as far as our analysis can go at the present time. We have no absolute values of either the focusing advantage (as explained above) or of the attenuation (as measured by the sporangiophore). For the attenuation this is true because we do not know the relative disposition of the receptor pigment and the absorbing and scattering structures. What portion of these structures lies between proximal and distal receptor pigment (where it is relevant) and what portion lies peripheral (where it is irrelevant)? Further, the optical measurement of scattering depends on the numerical aperture of the detector used. Only radiation which is scattered outside this aperture is counted as scattered.

Thus, Δn is at best proportional to the true value of f, and a^{opt} at best proportional to the true value of a. That they are approximately proportional to each other is a partial vindication of equation 17-1.

It should be noted that scattering is equally or more important than true absorption, as shown by the comparison of *car-5* and *car-10*. Although neither albino mutant absorbs light measurably, *car-10* scatters light about 60% as much as *car-5* and its Δn differs proportionally.

Similarly, comparing wild type at 495 and 510 nm, the small difference in Δn at these two wavelengths found by Zankel et al. (220) is now explained by the sizable contribution of scattering which "dilutes" the difference in absorption.

Absorption by carotene is not irrelevant, as shown by a comparison of *car-5* and *carR21*. Although they scatter the same amount of light, *carR21* absorbs as much as it scatters. Correspondingly, Δn of *carR21* exceeds that of *car-5* by about a factor of two.

It follows that the receptor pigment must be

[5] The refractive index of an aqueous solution at 22 C, relative to air, at the Na wavelength, $\lambda = 590$ nm, is $n = 1.333 + \alpha c$, where c = concentration of solute (in g/100 ml); $\alpha = 0.0018$ for proteins, 0.0015 for sucrose, and 0.0017 for NaCl, and very similar values for other solutes. The values measured by Castle (50) ranged from 1.35 to 1.40, with an average of 1.38. This value was used by Castle and others in model calculations. The model calculations were made before the measurements cited in Table 17-1 became available. At that time, 1.35 appeared to be the best guess for the refractive index of the cytoplasm.

located at least in part peripheral to the scattering and absorbing material. How much lies peripheral we do not know. The computed "path length" or the "lever arm" predictions for Δn are much too small and/or the *effective* attenuation is much less than the optically measured one, possibly because some of it is peripheral to the receptor pigment.

18. Phototropic neutrality at appropriate wavelength

Phototropic neutrality, i.e., a balance between the focusing advantage and the attenuation disadvantage, can be obtained without immersing the sporangiophore in any medium. One simply changes the wavelength until f and a balance.

For most strains, the neutral wavelength is close to 300 nm; for the lycopene-rich mutant *carR21*, it is close to 310 nm (100 M.D.). Since this is a difficult region for transmission spectrophotometry, no detailed analysis has been made, although it might be helpful for locating the relative positions of the screen (gallic acid) and the receptor pigment. Determination of the neutral wavelength may also be a quick way to identify mutants or growth conditions giving sporangiophores with unusual amounts of gallic acid.

19. Detailed analysis of the growing zone (longitudinal autonomy versus integration)

The growing zone occupies a fixed position in the sporangiophore, extending from 0.1 to at least 2 mm below the sporangium. It continuously produces cell wall and other materials necessary for the elongation of the sporangiophore. To be effective, the sensory input must occur here. Bending responses are limited to the growing zone and occur only in conjunction with growth. Whether the level of adaptation can change in the absence of growth has not been settled.

Several investigators have studied the behavior and properties of the growing zone. Cohen and Delbrück (64) and Castle (56) labeled the cell wall at various places in the growing zone with small starch grains and followed their displacement relative to the sporangium or relative to the base of the growing zone. Any marker in the growing zone moves away from the base of the sporangiophore *and* away from the sporangium until it reaches the lower edge of the growing zone. Any element dx of the cell wall (defined by its distance x from the sporangium) has associated with it a specific elongation rate and a specific twist rate. These rates, considered as functions of x, are the elongation (or twist) functions. They have a single maximum located slightly above the middle of the growing zone.

The light responses of the growing zone elements have been compared with the elongation and twist functions. Castle (56) measured the elongation distribution under steady-state conditions. In contrast, Cohen and Delbrück, stimulating every 5 min, found that the responsive zone is only half the size of the growing zone and that the upper 0.5 mm does not participate at all in the growth response. The explanation for this discrepancy may be the different stimulus programs used. Support for this idea is Castle's (59) observation that during a phototropic reaction the bending speed in the upper part of the growing zone is initially very high, but decreases after a few minutes. Castle suggested that the lack of response in the upper part of the growing zone, in the experiments with periodic light programs, is due to a shortage of a cell wall precursor supplied from below. This suggestion is ruled out because the time-averaged elongation function is still normal. In other words, the upper part still elongates as much as in the unstimulated case.

In further experiments, Cohen and Delbrück (65) and Delbrück and Varjú (73) used narrow horizontal line stimuli to produce phototropic responses. They found that a bend initiated in the upper part of the growing zone travels down the growing zone with a speed close to the growth speed of the sporangiophore. Correspondingly, the bend has maximum sharpness if the line stimulus is kept at a constant distance from the sporangiophore base. Castle (59) made a similar observation when he measured the bending rates of the growing zone elements at different times after the onset of unilateral irradiation. The upper parts of the growing zone stop bending after a while and the bend maximum migrates downward. At present we do not understand how the bending response travels down with the speed of growth.

Recently, a mutant (*mad-54*) has been discovered which under constant unilateral irradiation produces a constant bend in the growing zone, but none below (Section 29). The elongation and twist distributions of this mutant have not yet been studied.

Another part of the light response system, the adaptation, has been studied in its relation to the elongation distribution (73). A boundary between a bright and a dark ultraviolet (UV) field was projected onto a rotating sporangiophore at a certain distance from the sporangium. Ten minutes later the position and sharpness of

the corresponding boundary between light- and dark-adapted regions was assessed by using narrow UV line tropic stimuli. The adaptational boundary stays for at least 10 min at the growing zone element at which it was placed originally and does not blur during that time. The sharpness has been tested down to 0.1 mm. This finding should be compared with Castle's theory of the tropic response (60; Section 15). In this theory, adaptation is required to spread out azimuthally, a distance comparable to the one tested in the longitudinal spreading test.

Also, the width of the line stimulus in the growing zone was compared to the magnitude of response measured. A graded relation over a reasonably wide range was found, indicating that the stimulation is not an all-or-none process (73).

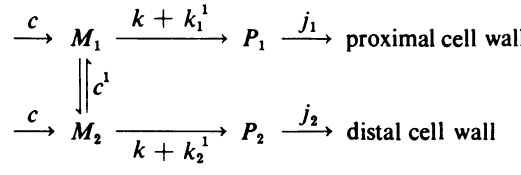
In summary, the alternative autonomy or integration does not seem very appropriate if applied to the whole stimulus-response system. One might rather say: reception is locally autonomous; response is integrated.

20. Temporary inversions of tropic responses

Reichardt and Varjú (157) discovered that during unilateral irradiation a step-up in light intensity causes a temporary inversion of the steady phototropic reaction. Castle (58) reinvestigated this phenomenon and demonstrated that a big step-up in light intensity from any direction causes inversion during a steady-state tropic response and that the inversion occurs always in the plane of the original bend. This implies different reactivities of the growing zone elements on the proximal and distal sides during a steady-state tropic response. Castle proposed that the distal side has a lower reactivity because it had been growing faster and was therefore short of supplies. Three further inversion phenomena were subsequently described by Dennison: (i) inversion occurs not only after a step *up* but also after a step *down* in light intensity (79); (ii) a step-up in symmetric light intensity during a steady-state avoidance response (tropostat) causes inversion (100 D.S.D.); and (iii) similarly a step-up of the symmetric light intensity during a geotropic bending response causes inversion. In this case, the inversion has a 20-min latency in wild type similar to the latency of the geotropic response itself. Corresponding experiments with the mutant *car-10* (Sections 25 and 29), which has a short geotropic latency, are in progress.

The second and third observations support Castle's idea that the difference in reactivity between distal and proximal sides can be attributed to the "transducer output" (e.g., growth regulation) rather than to the "transducer input" (photoreceptor).

Castle (61) formulated a model to account for the inversion phenomena on the basis of a "limited supply" hypothesis. In this model the level of adaptation of the sporangiophore is expressed as the pool size (M) of a cell wall precursor which is supplied at a constant rate (c). The rate constants k can be thought of as valves regulating the outflow from pools M. There are constant valves k and additional valves k^1 whose opening is controlled by light. In complete darkness the pool is full; if the sporangiophore is adapted to a high light level (k^1 large), the pool is nearly empty, having provided its material for the additional growth which occurred after the step-up in light intensity. To account for the steady-state bending situation, Castle postulates a continuous exchange, c^1 (diffusion), of material between the pools on the proximal and distal side, preventing complete adaptation of either side.



The virtue of this model is that it explains very elegantly all the inversion effects. But it has to make assumptions about the mechanisms of adaptation which are contradicted by experiments. The kinetics of the light growth response require that, in Castle's model, the precursor pool (M) cannot last for much more than one full-sized growth response. However, the "sunrise" experiment (Section 14) shows that the growth speed can be kept 50% above the steady-state level for about 20 min. Furthermore, Shropshire (Section 21) observed that a sudden transition to a very high light intensity causes the growth speed to stay at the high level for several hours. Probably, the regulation of growth speed in the sporangiophore is more complicated than the "limited supply" theory indicates.

Recently, a Phycomyces mutant (*mad-59*) has been isolated which might contribute to this problem; its sporangiophores grow 40% faster than those of wild type and show almost no light growth response or phototropic response (Section 29).

21. Effects of light in the high-intensity range

The high-intensity range for responses of Phycomyces sporangiophores is defined as any intensity greater than 20 μw cm^{-2} ($\log_2 I = +1$) of tungsten light filtered through a blue filter (Corning 5-61). We present here some unpublished findings[6] concerning this range.

The findings are of two kinds. The first kind we will designate as *indifference to light*, the second kind as *loss of growth rate regulation*.

(a) **Indifference to light.** *Phototropic indifference*: If rotating sporangiophores are adapted to a unilateral blue stimulus impinging at 90° to the long axis, and rotation is stopped, within 3 to 4 min a phototropic response begins. In the normal range ($\log_2 I = -8$ to $+1$), bending continues at an average rate of 5 to 7° per min for 15 to 20 min. In the high range, with increasing intensities the latency remains the same but the bending rate decreases (157); for intensities greater than +6 (approximately 1,000 μw cm^{-2}), there is little or no phototropic response even after many hours of exposure (Fig. 21-1 and 21-2).

This failure to bend under continuous irradiation of high intensity was discovered by Oltmanns (135) and designated as "phototropic indifference." Castle (47) used the term "phototropic indifference" to describe a different effect: the failure of sporangiophores to give tropic responses to brief light pulses which are intense compared to the adapting light but still in the normal range. Castle's "phototropic indifference" is associated with a strong growth response and the "indifference" is probably due to saturation of the growth response on both sides of the sporangiophore; it is quite distinct from the phototropic indifference in the high range.

Growth response indifference: For transient increases in intensity, a transient light growth response occurs (Section 13). In the high range, no light growth responses are observed for sporangiophores adapted to intensities of $\log_2 I = +5$ or greater. For adapting intensities of +1 to +4, growth responses occur with the same time course as in the normal range, but with diminishing amplitude.

Action spectra: Action spectra were determined for the appearance of phototropic indifference,

[6] The high-intensity source in these experiments was a modified Withrow interference filter monochromator (216) in which energy from a 1,500-w projector lamp passed through appropriate condensing lenses, 10 cm of aqueous copper sulfate solution, and a Schott UG-1 heat-absorbing filter. A nearly parallel beam of light then passed through appropriate broad-band filters or Baird Atomic or Bausch & Lomb interference filters. Intensities were measured with a calibrated thermopile at the sporangiophore position.

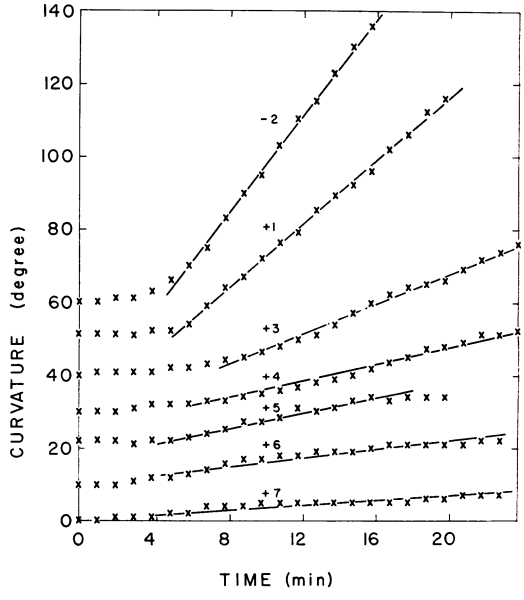


FIG. 21-1. *Phototropic responses of adapted sporangiophores at various high-intensity levels. Bend angle as a function of time after stopping rotation. The curves are offset by 10° at time zero to avoid overlap.*

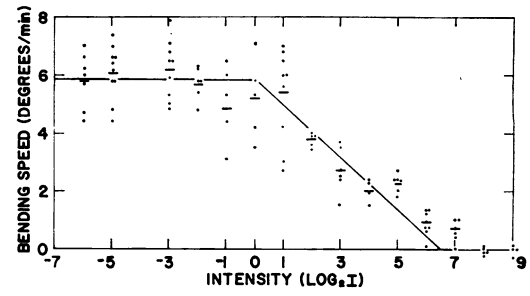


FIG. 21-2. *Phototropic rate as a function of intensity. The rates plotted here are the slopes indicated in Fig. 21-1. The dots represent individual experiments, the horizontal lines averages.*

and for light growth responses at $\log_2 I = +3$. The intensity required for indifference was determined by constructing a plot as in Fig. 21-2 for each wavelength measured between 360 and 510 nm. The precision for determining the intensity for indifference is very poor (±40%) as indicated by the scattering of points in Fig. 21-3. The growth action spectrum was determined by a null balancing method (72; Section 26) in which a standard intensity of $\log_2 I = +3$ broad band blue was used. The precision for the growth action spectrum is somewhat better (±20%). These high-intensity action spectra indicate only that the same pigment system is operating in the

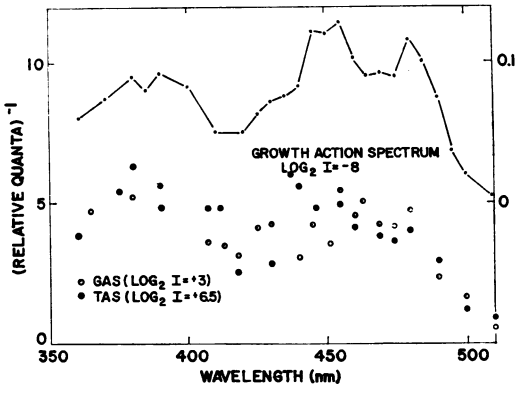


FIG. 21-3. *Growth action spectrum (GAS) and tropic action spectrum (TAS) in the high-intensity range. The GAS was measured by alternating 5 min of blue light at* $\log_2 I = +3$ *with 5 min of test light of variable wavelength and adjusting the latter so as to zero the growth response. The TAS was measured by constructing a plot as in Fig. 21–2 for each wavelength. The GAS in the normal range is plotted for comparison.*

high range as in the normal range. The scatter in the points is such that no significant differences between the growth and tropic action spectra in the high range can be discerned. The relative height of the high-range action spectra in the region between 360 and 400 nm compared to the visible region appears to be greater than for the normal-range action spectra. The reason for this is not known and must await resolution of the exact shape of the high-intensity action spectra.

The most straightforward interpretation of these findings is to assume that each absorption act of a receptor pigment molecule results in its temporary inability to function because of a chemical change either in the pigment ("bleaching") or in an acceptor of the energy. Let us assume that the restitution rate of the receptor pigment is a first-order dark reaction with a fixed rate and that at $\log_2 I = +1$ the bleaching rate begins to be comparable with the restitution rate. Then at $\log_2 I = +6$ bleaching is fast compared to restitution. Under steady irradiation the concentration of functional receptor pigment would drop from near 100% at $\log_2 I = +1$ to a few per cent at $\log_2 I = +6$. The specimens thus would become indifferent because of lack of functional receptor pigment. Judging from the intensity range in which bleaching is presumed to match restitution (5×10^{14} quanta cm^{-2} sec^{-1}) and a likely capture cross-section, $q = 1.5 \times 10^{-16}$ cm^2 (Section 24), it may be inferred that the restitution time constant is of the order of 10 sec.

This interpretation of the high-intensity effects has an important implication: the inferred time constant of restitution is considerably shorter than the time constant of dark adaptation in the normal range and the latencies of growth and tropic responses. It follows that at high intensities the product rule should break down for stimulus durations comparable to this new time constant. Much of the stimulus will then be "wasted" on nonfunctional pigment, whereas for longer stimuli the restitution process would replenish the pigment concurrently. To test the product rule, higher intensities than those available in conventional sources are needed.

(b) **Loss of growth rate regulation.** If sporangiophores are adapted to $\log_2 I = -3$ and are then given a step-up to $\log_2 I = +9$, a large transient growth response is observed within 10 min. Then the growth rate decreases from a maximum of 1.5 v_0 to about 1.2 v_0 at 15 min, and gradually climbs to 1.6 to 2.0 v_0 where it is maintained for many hours. Figure 21-4 gives the average growth rate for 5-min intervals taken at 30-min sample points, over a period of 4 hr after such a step-up. In about 40% of such step-ups, the growth rate locks in immediately at the high values without any drop after the first increase. Upon reduction of the intensity to $\log_2 I = -3$, whether gradual or sudden, normal growth rates are resumed within 30 min and are comparable with the rate observed for sporangiophores if they had been maintained for an equal time at an intensity of $\log_2 I = -3$. Thus, the sporangiophore has the capacity for persistent growth at twice the normal rate. Several sporangiophores have been observed growing at rates of 7.5 mm hr^{-1} for at least 2 hr. The persistent high growth rates occur only in response to sudden step-ups,

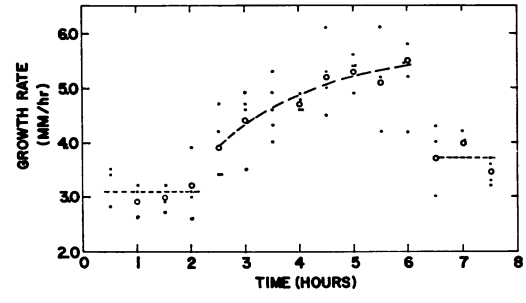


FIG. 21-4. *Loss of growth rate regulation. At time* $t = 2$ *hr, the intensity was stepped up from* $\log_2 I = -3$ *to* $+9$ *and maintained at this level for 4 hr. The growth rate was measured every 30 min during a 5-min sample interval. The dots are individual measurements, the circles averages. Note that the initial response which follows a few minutes after the step-up is not included among the sample intervals.*

whereas in sunrise experiments the sporangiophore returns to normal growth rates shortly after an intensity of $\log_2 I = +9$ is reached. Thus, there are two stable levels of growth rate depending upon the time course by which the high intensity is reached. Such high growth rates are observed most frequently when the relative humidity is maintained at greater than 70% and care is taken to exclude all wavelengths greater than 700 nm.

Occasionally, negative curvatures of 10 to 15° are observed at very high intensities but these could always be shown to be due to infrared present due to failure of heat-absorbing filters (KG-1 Schott). Castle (48) described this slow thermotropic response, but it has never been investigated in detail. Sporangiophores grown under $\log_2 I = +9$ or higher for several hours may be damaged since frequently, when left in the dark overnight, these sporangiophores cease elongating and produce side branches.

The observations reported here are probably related to those in Section 21a since they occur at the same intensities. Since the *speed* with which the high intensity is reached matters, it becomes important to find out which one of the time constants is relevant: that of adaptation or that of restitution of the receptor pigment.

22. Tropic hunting

If irradiated by two light beams of equal intensity and wavelength, 120° apart, the sporangiophore is in a stable equilibrium and grows in the direction which bisects this angle (75). At any angle above 120°, the sporangiophore grows straight in an arbitrary direction close to the bisectant of the angle (Section 13). At any angle below 120°, the sporangiophore grows in the direction bisecting this angle but it performs oscillations around this direction which have a variable amplitude (maximum, 30°). The "wavelength" produced by this hunting is remarkably constant (2 mm) because the period (30 to 60 min) is inversely dependent upon the growth speed of the sporangiophore (Fig. 22-1). Specimens which do not show "hunting" under these conditions can be triggered to do so by giving them a short bending stimulus after which the oscillations are self-sustaining.

The mutant *car-10* has a high steady-state phototropic bending rate, at least 1.5 times faster than that of wild type. In addition, it shows oscillations even if irradiated by two

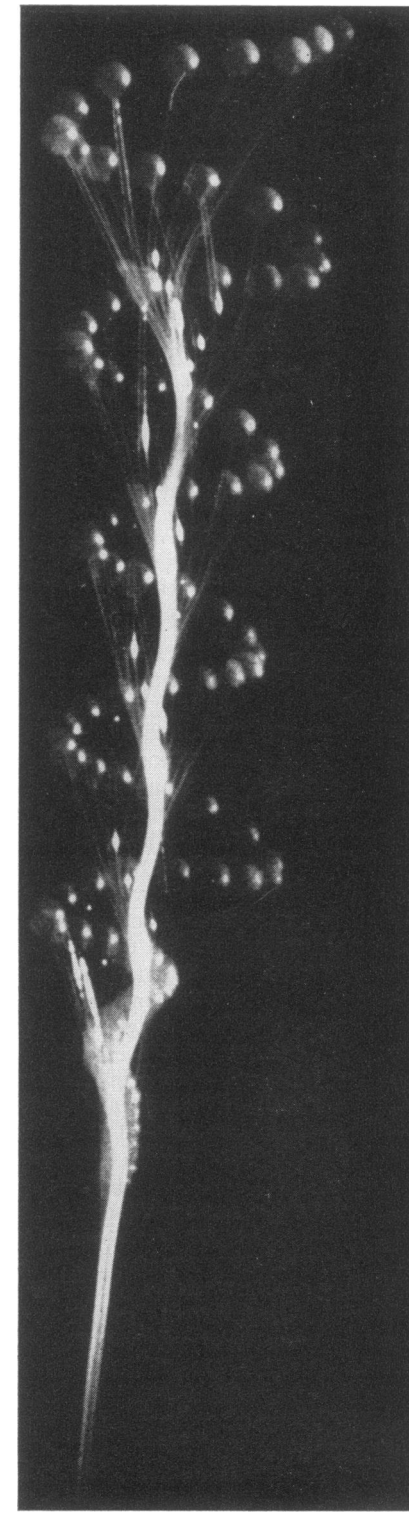


FIG. 22-1

FIG. 22-1. *Phototropic hunting. A sporangiophore was illuminated from above by two light sources 60° apart. Photographs taken every 5 min. Amplitude of hunting about ± 30°, period about 60 min. (Photo D. S. Dennison.)*

beams 120° apart, and under favorable conditions (growing away from a UV source) the oscillations have an amplitude larger than 90°. For other properties of this mutant, *see* Sections 17, 24, and 29.

Another type of oscillation with an amplitude of 2° and a period of 5 to 7.5 min has been described by Dennison (Ph.D. Thesis, California Institute of Technology, Pasadena). It can occur superimposed upon any irradiation program but disappears completely in darkness.

RECEPTOR PIGMENT

23. Action spectra

(a) **General remarks about action spectra.** Action spectra are measured to obtain information about the absorption spectrum of the receptor pigment. Action spectra can be misleading because of a number of distorting factors. The principal distorting factors are a dependence of the quantum yield on the wavelength, and screening pigments.

Ignoring the possible variability of the quantum yield is rationalized by the faith that nature would use a pigment with maximum quantum yield throughout the relevant portion of the spectrum, i.e., with quantum yield 1.

Basically, one is dealing with chemoreception, the chemical in question being a photochemical product. Therefore, one should aim at expressing the stimulus in terms of the amount of primary photoproduct. For different wavelengths the amount of photoproduct is then proportional to the number of incident quanta and to the capture cross-section for these quanta. This latter quantity, the capture cross-section, is directly proportional to the molecular extinction coefficient (Section 24). Since the relation between primary photoproduct and ultimate output (growth or tropic response, or change of adaptation) is in general nonlinear, the most straightforward way to determine the absorption spectrum of the receptor pigment is to measure the light flux producing *equal effect* and not to measure the magnitude of the effect at *constant flux* for different wavelengths. Thus, the standard procedure should be to measure the flux of quanta (not the energy flux) at each wavelength which gives a standard effect. If the effect can be made to assume positive or negative values in a symmetric way, then it is optimal to use the neutrality point as the standard effect. This point may be interpolated from measurements close to it.

(b) **Action spectrum of growth response.** Using these principles, Delbrück and Shropshire (72; Fig. 38-2) measured the action spectra of the growth and tropic responses. To measure the growth response, a standard light and a test light were alternated at 5-min intervals, giving a periodic growth output with a positive or negative amplitude, depending on the relative effectiveness of standard and test light. This action spectrum shows clear maxima at 485, 455, 385, and 280 nm and a sharp drop-off above 485 nm. At 515 nm the effectiveness is about $\frac{1}{100}$th of the peak at 485 nm (100 M.D.). In the visible region this action spectrum is not grossly distorted by screening pigments. At 280 nm it is distorted by at least a factor of 2 since we know that light of this wavelength is strongly absorbed in its passage through the sporangiophore. Whether it is additionally distorted by screening external to the receptor pigment on the proximal side is unknown.

(c) **Action spectrum of tropic response.** To measure the action spectrum of the tropic response, a standard light from the right is balanced by a test light from the left (66, 72). We have stated in Section 15 our reasons for believing that light adaptation can be considered uniform over the cross section. Tropic balance then means the balancing of two asymmetric light distributions which the plant evaluates as equal and opposite in effect. Here the discussion of distorting effects is more difficult. Internal screening below 300 nm in fact reverses the sign of the tropic response and increases its magnitude by a large factor. In the visible it is significant that the action spectrum of the tropic response closely matches that of the growth response. This is an argument for believing that a portion of the screening by β-carotene occurs peripheral to the receptor pigment.

(d) **Unity of receptor pigment.** The four peaks of the action spectra may not all belong to one pigment. Perhaps we are dealing with a situation similar to the well-known one in photosynthesis in which a variety of pigments feed the energy they absorb into a common reaction center. The suspicion that such a multiplicity of receptors might be present is strengthened by the discovery of "night blind" mutants (Section 29). This finding has raised the possibility that there are separate pigment systems employed by the plant for the high and for the low range (as in vertebrates) and that these pigment systems have different spectra. If so, it is of interest to measure action spectra separately in the high and in the low range. The action spectra measured by Delbrück and Shropshire (72) may represent a mixture of the action spectra for the high range and the low range.

Thus, it is important to make a new attack on the action spectra which should incorporate: (i) measurement at various light levels; (ii) use

of a monochromatic source for the standard light rather than broad band blue; (iii) comparison of various mutants with wild type, especially the color mutants and the night blind ones; and (iv) greater precision.

This program will have to await the development of automated equipment.

(e) **Orientation of receptor pigment.** It is likely that the receptor pigment is dichroic, i.e., that for a given orientation of the molecule and direction of the light the extinction coefficient is different for different directions of linear polarization. *If* the receptor molecules in the growing zone are nonrandomly oriented, then one might find an effectiveness which depends on the direction of polarization of the stimulating light.

The history of this idea begins with a paper by Castle (51). He balanced a standard light coming from one side with a light polarized either transversely or longitudinally coming from the other side. The transverse light was about 15% more effective. Since by Fresnel's formula the transverse component is less reflected, he interpreted this as a reflection effect.

Jaffe (116) found that the direction of polarization affected the direction in which the germ tubes sprouted out of the eggs of the alga *Fucus*. He interpreted this effect as due to dichroic chromophores oriented in the cell wall and suggested that the same might be true for Phycomyces.

Shropshire (186) tried to settle this point by measuring the growth response rather than the tropic response of Phycomyces. He tested the effectiveness of the two directions of polarization at two wavelengths (450 and 380 nm) and in two media (air and a fluorochemical with a refractive index of 1.29). He found that in air at both wavelengths the transverse light was about 20% more effective than the longitudinal light and that in the immersion fluid the two lights were equally effective within the error limits. These findings seemed to settle the argument in favor of reflection and against dichroism.

Jaffe (117), however, in a purely theoretical paper showed that Shropshire had not proved his point. He argued that the dichroic effect could well be similar for the two absorption bands Shropshire had tested, and that reflection could not give an effect of the magnitude found. He showed this by taking into account not only the reflection at the first surface but also the internal reflection at the distal surface and subsequent internal reflections.

He proved a theorem which shows that in the ideal case of zero absorption and scattering the internal reflections compensate quantitatively for the reflection losses suffered at the first surface. Since in the real case scattering and absorption by screening pigments are not zero, he also computed the case of 100% absorption and then interpolated to the real situation. This interpolation is of dubious value. We cannot make a precise calculation of the effects of internal absorption since we know too little of the radial distribution of the receptor pigment and of the screening pigment.

Thus, the matter has not been resolved, but a new opportunity to clarify it has arisen with the discovery of albino mutants. For these, Jaffe's theorem should hold with a high degree of approximation and there should be little effect of the direction of polarization of the light due to reflection when the growth response is studied. Therefore, if for an albino strain one finds a similar difference between the effects of the two directions of polarization this would be a strong argument for a visual pigment of preferential orientation. Such a finding would give one a handle in the search for its localization.

Experiments of this kind have been performed by A. J. Jesaitis (100). The growth responses of rotating sporangiophores of wild type and of the colorless mutant *car-10* were measured under a program in which the direction of polarization alternated every 5 min between horizontal and vertical. In each case, 100 cycles were measured. The average growth rates during the "horizontal" and "vertical" exposure periods were compared. For both strains, 4% higher growth rates were found during the "vertical" period. Since there is a delay of the response of about 5 min, the "horizontal light" is more effective. A calibration of this effect, using unpolarized light alternating between two intensities, shows that this growth response corresponds to about 20% difference in effective intensity. This finding argues in favor of dichroic, oriented receptor molecules.

(f) **Comparison with other blue action spectra.** A variety of photobiological effects in plants have action spectra which are similar, if not identical, to those of Phycomyces. Action spectra have been determined for: (i) phototropism in Avena coleoptiles (67, 190); (ii) initiation of the synthesis of colored carotenes in *Neurospora crassa* (219); (iii) similarly in *Fusarium aquaeductuum*, an ascomycete (156); (iv) similarly in *Mycobacterium* (159); (v) oxygen uptake in *Chlorella* (124, 142); and (vi) phototropism in sporangiophores of *Pilobolus kleinii*, a zygomycete (139).

In Fig. 23-1 these action spectra are compared. Possibly, we are dealing with a photosensitive mechanism of ancient lineage and multifarious application in higher and lower plants and bacteria. If this is so, then the identification of the receptor pigment and its mode of operation in

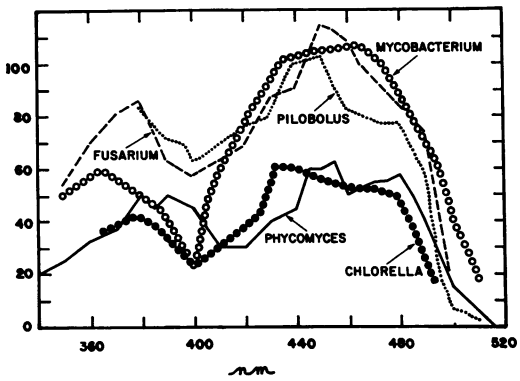


FIG. 23-1. *Action spectra of processes sensitive to blue light in various organisms: light growth response in Phycomyces (72); phototropism in stage I of the zygomycete Pilobolus kleinii (139); carotene synthesis in the ascomycete Fusarium (156); carotene synthesis in a Mycobacterium (159); oxygen uptake in Chlorella (142). These action spectra should be compared only with respect to the approximate location of maxima, minima, and cut-off, since they were determined by different procedures (some by the "equal action," some by the "equal dose" procedure; some in terms of quantum flux, some in terms of energy flux).*

any one of these organisms would be a great help in the study of the others.

(g) **Receptor pigment in mycelium?** The widespread occurrence of "blue light effects" has suggested a search for it in the mycelium of Phycomyces.

Wild type and several mutants were grown in racing tubes, using various media and light programs, and observations were made on the growth rate of the mycelial front and on the pattern of sporangiophore initiation relative to this front (100 G.M. and K.B.). Results to date may be summarized as follows. (i) The growth rate of the mycelium is not influenced by light under any conditions. (ii) Sporangiophore initiation deteriorates progressively over several days after replacing white light by complete darkness or by red light. (iii) Under constant light (white or blue), the pattern of sporangiophore initiation is constant. (iv) Under a 12 hr light-12 hr dark cycle, the pattern of sporangiophore initiation shows a 24-hr cycle, with new sporangiophores visible near the mycelial front after the first third of the light period.

These findings demonstrate that the mycelium is sensitive to light and encourage a much more detailed study of the effect.

24. Transmission spectrophotometry, estimates of upper and lower limits of the concentration of receptor pigment

The transmission of light through small regions of the individual sporangiophores has been studied in the visible range by using a Cary recording spectrophotometer with microscope attachment (220). In the UV, scattering becomes very troublesome. Transmission can still be measured by direct shadowgraphing (72). It shows a very sharp rise in extinction beginning at about 305 nm.

The transmission of albino mutants (*car-5* and *car-10*) shows a gradual drop in transmission with shortening wavelength, undoubtedly due to scattering (Fig. 24-1). The transmission loss measured here must be due to large-angle scattering and back scattering, since light scattered by less than 60° is still collected by the optical system used. This large-angle scattering loss is considerably larger for *car-5* than for *car-10*. Around 450 nm it amounts to an optical density (OD) value of 0.08 for *car-5* and 0.045 for *car-10*.

In wild type (Fig. 24-1) there is superimposed upon this scattering background the absorption spectrum of β-carotene, contributing an OD of between 0.08 and 0.10 at the λ_{max} for β-carotene. In vivo, the maxima for β-carotene are at 492 and 462 nm.

The mutant *carR21*, which contains lycopene instead of β-carotene, has in vivo absorption maxima at 517, 483, 455, and 425 nm (Fig. 24-1).

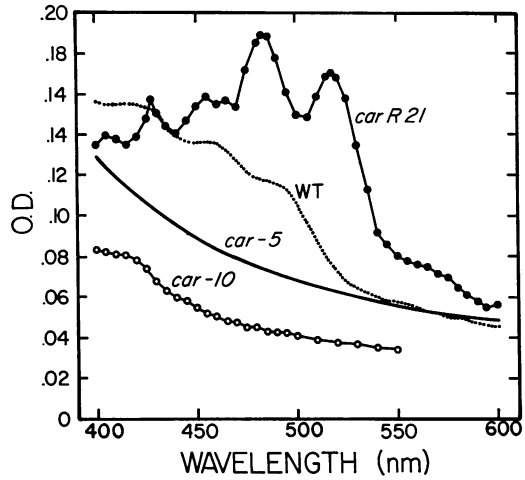


FIG. 24-1. *Absorption spectra of wild type (WT) and three mutants. The exit slit of a Cary model 15 spectrophotometer is imaged on the axis of a sporangiophore. Image size, 20 by 200 μm. Angle of convergence at image, 0.4 radians. The transmitted and much of the scattered light is collected with a 90×, oil immersion, apochromat and measured by the PM tube. The cultures had been grown on potato-dextrose-agar medium, at* $\log_2 I = -1$. *The spectra are averages of several specimens, as follows. Number of specimens: WT, 7; carR21, 1; car-5, 4; car-10, 3. Mean diameter, WT, 100; carR21, 68; car-5, 103; car-10, 81. Mean cytoplasm thickness: WT, 27, carR21, 17; car-5, 26; car-10, 21.*

Its OD at the maximum (0.12) is higher than that of wild type.

In the albinos, this carotene absorption is missing and one might have hoped that the removal of the interfering pigment would make the receptor pigment visible. However, except for a slight peak around 425 nm, undoubtedly due to the cytochromes, there are no discernible peaks in the region where the action spectrum has its maxima. We may thus estimate an upper limit for the OD contributed by the receptor pigment at 485 nm to be 0.002.

We may also estimate a lower limit for this OD by the following argument. Experimentally, we know that sporangiophore will respond phototropically to a light intensity of about 10^6 quanta cm^{-2} sec^{-1} applied for a period of about 10^3 sec, i.e., to a total flux of about $N = 10^9$ quanta cm^{-2}.

Let us assume that the receptor pigment has a molecular extinction coefficient $\epsilon = 4 \times 10^4$, like that of the receptor pigments of animal vision and close to the upper theoretical limit for any chromophore showing a broad absorption band. We first convert this molecular extinction coefficient to a capture cross section by the relation.

$$q = 0.38 \times 10^{-20}\epsilon \text{ cm}^2 \qquad (24\text{-}1)$$

Thus, we arrive for our receptor pigment at a capture cross section of

$$q = 1.5 \times 10^{-16} \text{ cm}^2 \qquad (24\text{-}2)$$

With the incident flux given above, we obtain as the number of excitations during 1,000 sec at the threshold

$$Nq = 1.5 \times 10^{-7} \text{ excitations/receptor molecule} \qquad (24\text{-}3)$$

The cell must be able to distinguish proximal from distal with respect to the light source by means of the lens effect, i.e., by a significant concentration of the excitations in the distal focusing band. A rough guess as to the number of excitations needed for the determination of this asymmetry in a statistically significant manner would be a total of 500 excitations, or about 3×10^9 receptor molecules/growing zone.

This leads to an estimate of 10^{-4} for the lower limit of the OD contributed by the receptor pigment, about a factor 20 below the present resolving power of the spectrophotometric arrangement. It is likely that by pushing the experimental design the resolving power could be made to reach this limit.

The calculated lower limit can also be expressed as a molarity of the receptor pigment and leads to 3×10^{-7} M. The amount of the pigment isolatable from a crop of sporangiophores depends on whether the pigment is limited to the growing zone or is distributed throughout the thallus, the sporangiophore, and the mycelium.

25. Receptor pigment

The receptor pigment of Phycomyces is not known. Studies to find it have been based on the action spectra, since no other assay to recognize the pigment is known as yet. It has been suggested that the receptor pigment might be β-carotene, retinal attached to a protein, or a flavoprotein. These compounds exhibit absorption spectra resembling the action spectra.

β-Carotene is the principal pigment absorbing in the 400 to 500 nm region as shown by in vivo measurements through the growing zone of single sporangiophores (220; Fig. 24-1). Its absorption spectrum in vivo is similar to the visible part of the action spectra, though displaced 5 to 10 nm to the red. Centrifugation of single sporangiophores demonstrates that the bulk of β-carotene (more than 95%) is bound to lipid particles which move centripetally upon centrifugation (Section 12). The absorption spectra of other bands, for example those containing mitochondria and nuclei, show only traces of β-carotene. The discovery of several pigment mutants, which contain less than 1/1,000th of the β-carotene of wild type and which are still fully photosensitive, clearly demonstrates that the bulk of β-carotene is not the effective pigment (131). These β-carotene-deficient mutants still contain about 10^{10} molecules of β-carotene/sporangiophore. We estimated a lower limit of 3×10^9 receptor molecules/growing zone (Section 24). Thus, it is not possible to rule out a special form of β-carotene as part of the receptor pigment.

Wild type contains very small quantities of retinal, between 10^9 and 10^{10} molecules of retinal/sporangiophore, about 1/10,000th that of its content of β-carotene (131). However, attempts to find retinal protein complexes which liberate retinal upon exposure to light gave negative results. No retinal was found in the β-carotene-deficient mutant *car*-5. The retinal reported in wild type is probably a spurious oxidation product of β-carotene and not involved in the processes mediating light sensitivity.

Flavoproteins have been found with an OD of about 0.1 at 450 nm in 1 g (dry weight) of sporangiophore (100 G.M.). About 65% of these proteins are present in the soluble fraction of cell extracts. The proteins of this fraction were partially purified by gel filtration and ammonium

sulfate fractionation. Three water-soluble flavoproteins with molecular weights between 50,000 and 100,000 seem to be present, having flavine adenine dinucleotide and riboflavine 5'-phosphate as prosthetic groups. The absorption spectra of some of these fractions closely resemble the visible part of the action spectra. These preliminary results suggest that a flavoprotein may be the receptor pigment of Phycomyces.

SENSORY PHYSIOLOGY (OTHER)

26. Stretch

(a) **Mechanical properties of the sporangiophore.** *Stretching by pressure (iron lung experiments).* Roelofsen (168) applied internal pressures varying up to 2 atm to plucked sporangiophores in an "iron lung" and measured the resulting elongations. Typically, 2 atm caused an elongation of about 0.2 mm (7%) in the top 3 mm of a stage IVb cell. Sometimes the first elongation had a small irreversible (plastic) component. The elongation versus pressure diagrams were generally nonlinear, with the extensibility (elongation per unit of pressure) greater at lower pressures. Markers (Lycopodium spores) attached to the cell indicated that the elongation per unit length drops steeply in the upper 1 mm, from a maximum probably at 0.1 mm or higher. C. R. Hamilton (100) reported elongations of the order of 0.8 mm (8 mm long cell) for 1.7 atm. About three-fourths of this elongation was plastic on the first application of pressure, a much greater plastic component than reported by Roelofsen. Neither author reported any growth response to the elongations caused by the iron lung. The pressures used probably were much too high, causing distension of the columella, severe displacement of protoplasm, and transient arrest of growth.

Stretching by external load; asymmetric stretch (lateral force). Most of these observations are from the centrifuge experiments of Dennison (77, 78). A photographic analysis of the curvature of a sporangiophore bent passively by a lateral centrifugal acceleration of $6.7 \times g$ showed in one instance that a total curvature of 25° was approximately uniform over the upper 3.5 mm, but increased slightly in the top 1.0 mm. When the variation in lever arm is taken into consideration, the curvature per unit length per unit of torque rises steeply towards the top of the growing zone, resembling Roelofsen's extensibility curves. This curvature corresponds to only about 1% elongation of the distal side.

Blue light has no apparent effect on the amount of passive bending observed in centrifuge experiments (78).

Stretching by external load; symmetric stretch (longitudinal force). In this case the cell is stretched longitudinally, but, instead of using changes in internal pressure to provide the force, the sporangiophore is inverted and a weight (up to 40 mg) is hung from the sporangium (80). Growth of the cell continues at nearly the normal rate with the load applied. Stretching the cell in the iron lung halts growth for up to 2 hr (100 C. R. Hamilton).

The relative amount of the plastic component of elongation is much less when the cell is stretched by a weight than when it is stretched by the iron lung. A load of 8 mg causes a typical elongation of 0.2 mm in a 30-mm cell, and about half of this elongation, 0.1 mm, occurs in the upper 1.5 mm (7% elongation). This elongation is 93% elastic. No difference was noted between the first and subsequent applications of load.

The pattern of stresses set up in the cell wall by internal pressure is quite different from that caused by the external load. Increased pressure causes increased cell wall tension both longitudinally and circumferentially, whereas the external load causes increased longitudinal tension while the circumferential tension might actually decrease. The effect of load on pressure has not been investigated. It is remarkable that a load of only 8 mg causes approximately the same amount of elongation as a pressure increase of 2 atm. A pressure of only about 0.1 atm exerts a force equivalent to 8 mg when acting on an area equal to the sporangiophore cross section.

The load versus elongation curve is nonlinear in the range from 1 to 8 mg, the cell being more extensible at the low end of the range. This curve also changes as the sporangiophore ages. For example, over a period of 8 hr the extensibility (initial slope of the elongation versus load curve) of one cell increased by a factor of 35, while during the same interval the growth rate increased by a factor of 10, from 0.2 to 2 mm/hr.

(b) **Responses to stretch.** *Asymmetric stretch (lateral force).* When a lateral force 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 (sometimes sooner), a rapid tropic reaction begins [originally called "transient geotropic response" (77)]. 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.

The distribution of this response along the growing zone is different from that of the passive

bend and shows a definite maximum in the region 1 to 2 mm below the sporangium (77). This distribution resembles that of the phototropic responses measured by Cohen and Delbrück (65). These similarities indicate that this response to a unilateral force shares its "effector system" with the phototropic and light growth responses and perhaps a portion of its sensory system as well.

Blue light (unilateral flashes given alternately from opposite sides) reduces the average magnitude of this response to about 35% of its value in red light (78). The variability of this inhibition is very high, however, and for individual specimens light may have a negligible effect or it may completely inhibit the response.

The mutant *car-10* shows responses to asymmetric or symmetric stretch which are identical to those of the wild type (100 D.S.D.).

Symmetric stretch (longitudinal force). The response to loading the cell with a longitudinal force is a negative growth response. The growth rate drops within 2 min after the stimulus and remains below normal for about 5 min. After a saturating stimulus, the growth rate typically drops to about one-half its prestimulus value. The response to removal of a load is a positive growth response with similar latency and duration and with a growth rate reaching a maximum of twice the prestimulus value (saturating response). Thus, the stretch response mechanism is symmetric since the cell responds to an elongation (load added) with slower growth and to a shortening (load removed) with faster growth.

This symmetric property of the stretch response mechanism satisfactorily accounts for the tropic response to a lateral force if it is assumed that, in a cell curved by a lateral force on the sporangium, the convex flank is stretched and the concave flank is relaxed. If these two areas show local growth responses corresponding to those seen in the longitudinal stretch experiments, then there should follow a transient tropic response opposite to the original curvature, as found experimentally.

There seems to be a relative refractory period following a stretch response. Average response size is clearly less if the interval between stimuli is 20 min than if it is 60 min. Recovery may not be complete after 60 min, since even greater responses are obtained from previously unstretched cells.

In a systematic study, irradiation with blue light failed to produce more than a 10% difference between response magnitude in darkness (red light) and under blue irradiation, contrary to preliminary findings reported by C. C. Roth (M.A. Thesis, Dartmouth College, Hanover, N.H., 1966).

The size of the negative growth response is clearly correlated with the magnitude of the applied load (80). The stimulus versus response curve is strongly nonlinear in the stimulus range from 0.3 to 8 mg, with a fairly sharp threshold between 0.5 and 1 mg. At 0.5 mg and below, no response is detectable, and above 1 mg the response is large and increases only slowly with further increases in load. The effective stimulus is more likely related to the amount of elongation of the growing zone than to the weight applied. The response and elongation curves diverge sharply at the load threshold: at 0.3 and 0.5 mg, there is no response but still a finite amount of elongation; at higher loads, elongation and response run parallel.

The rate of stretching was not very well controlled in the stretch experiments. The application of load was made by dropping the platform that supported the weight hanging from the sporangium. The viscous properties of the cell could be such that the same load might elicit varying responses, depending on how rapidly it is applied.

27. Gravity

(a) **Geotropism in the absence of blue light.** If a sporangiophore is placed horizontally, it bends upwards until the direction of growth is vertical (negative geotropism). After being placed horizontally, a stage IVb cell begins bending after a variable latency of 30 to 180 min. The bending rate for wild type may be as high as 0.3°/min initially, when the angle between gravity and the growing zone is 90°, but drops to about 0.17°/min when this angle is 45° and about 0.08°/min when this angle is 20° (77). Starting from horizontal, about 12 hr is required for the sporangiophore to reach vertical. There is considerable variability in the time delay and the initial bending speed, which may easily be lower than 0.1°/min. This variability probably reflects the sensitivity of geotropism to such factors as sporangiophore maturity, culture age, and possibly humidity.

Pilet (143) found that dark-grown sporangiophores showed relatively high geotropic bending speeds in early stage I (0.45°/min), which gradually decrease with increasing maturity, reaching 0.07°/min for 16-mm stage IV cells. Cells exposed to white light for 30 min before horizontal placement showed a much slower bending rate, 0.07°/min for 2-mm cells (presumably stage I) and 0.05°/min for 8-mm cells (presumably stage IV). That Pilet's study is confined to dark-

grown cultures makes the results not directly comparable with other work, most of which used light-grown cultures.

The geotropic response of *car-10* to gravity and to centrifugation up to 4 × *g* has been measured (100 L. Steinman). In contrast to wild type, the *car-10* geotropic latent period is less than 10 min. The initial geotropic bending speed of *car-10* is two to three times greater than wild type.

When vertical sporangiophores are centrifuged about a vertical axis, the geotropic response is more rapid than with natural gravity and continues until the direction of growth is parallel to the resultant of the gravitational and centrifugal vectors (77). At any specific angle, the geotropic bending speed increases with increased centrifugal force in the range 1 to 4.3 × *g*. For example, at 4.3 × *g* the initial geotropic bending speed is 0.6 degree/min, and about 5 hr is required for a 90° bend. Reproducibility is improved by preadapting the specimens in the humidified chamber of the centrifuge for 2 hr in darkness and by strictly controlling the culture age and sporangiophore height. Some sporangiophores show large deviations from the radial plane, making angle measurements misleading and sometimes leading to the collapse of the cell. A time delay of about 30 min occurs between the start of the centrifuge and the onset of the geotropic response. This delay is not affected by variations in centrifugal force in the range 1 to 4.3 × *g*.

The geotropic response at 4.3 × *g* is substantially unaffected by the total immersion of the sporangiophore in fluorochemical FC-43 (density, 1.87) during centrifugation. Since the cell is less dense than the surrounding medium, the externally acting stresses are reversed and, as discussed in Section 26, the stretch response is reversed. The geotropic response in FC-43 is not reversed, indicating that the geotropic sensory mechanism is internal, having to do with the internal rearrangement of particles or liquid phases.

There are several possibilities for the primary gravity receptor. Sedimentation of the small particles or lipid droplets is barely possible,[7] but the rapid streaming would be expected to disturb any concentration gradients induced by gravity. No redistribution of such particles in the cell has been seen in response to placing the cell horizontally. Shifting of the position of the large central vacuole is also possible, as is the redistribution of vesicular systems, such as found by Sievers (191) in *Chara*.

(b) **Effects of blue light on geotropism.** *Phototropically neutral light.* If a beam of parallel light is directed along the diameter of the centrifuge, the sporangiophores receive equal flashes from alternate sides and thus are kept in a state of phototropic neutrality (78). The effect of such irradiation on the geotropic bending speed is an enhancement at low mean intensities ($\log_2 I = -11.5$ to -4.5) and a complete inhibition at a slightly higher mean intensity ($\log_2 I = -3$).

The intensity needed for the inhibition of geotropism is far less than that causing phototropic indifference to unilateral irradiation, given by Shropshire as $\log_2 I = +6$ (Section 21).

The inhibition caused by light is difficult to interpret because no inhibition is seen in sporangiophores from 4-day-old cultures (the "first crop"), only in those from cultures 5 days old or more. The 4-day sporangiophores do not seem different in any other respect.

The possibility that the light inhibition may be acting through a mechanism separate from that of the light growth and phototropic response systems is suggested by its long time delay. If a sporangiophore is centrifuged at an inhibitory light intensity for about 1 hr and the intensity lowered to an enhancing level, the geotropic response begins abruptly 20 min later. Further, an increase in light intensity (both intensities below the inhibitory level) during the course of geotropic bending causes a geotropic reversal, beginning 20 min after the intensity increase and persisting for another 15 min. Although superficially similar to the phototropic inversion studied by Reichardt and Varjú (157) and Castle (58; Section 20), it has too long a latent period to be due to a light growth response.

Further evidence that these effects of light on geotropism may not be linked to the normal photoresponse system comes from the observation that the inhibition is lacking in *car-10*, which still has the photoresponse system (100 L. Steinman).

[7] A particle gravity receptor must fulfill two conditions. Its "decay height" at equilibrium must be smaller than the cell diameter, and it must sediment through at least this distance during the time the stimulus is presented. A suspension of particles which is subject to Brownian motion and to gravity does not settle on the bottom side of the cell but tends to an equilibrium distribution in which its concentration decreases exponentially with a decay height, $h_s = kT/V(\rho - \rho_0)g$, where V and ρ are volume and density of the particle, ρ_0 the density of the medium, k is Boltzmann's constant, T the absolute temperature (taken to be 300 K). A particle will sediment through h_s in a time, $t_s = h_s/u_s$, where the velocity of sedimentation is $u_s = V(\rho - \rho_0)g/6\pi\eta a$, where η is the coefficient of viscosity and a is the radius of the particle.

For a large mitochondrion, lipid droplet, or protein crystal in a sporangiophore, we might take the values $a = 5 \times 10^{-5}$ cm, $\rho - \rho_0 = 0.1$ g cm^{-3}, $\eta = 10^{-2}$ dyne sec cm^{-2}, $kT = 4 \times 10^{-14}$ erg, $g = 10^3$ cm sec^{-2}. We then obtain $u_s = 5.5 \times 10^{-6}$ cm sec^{-1}; $h_s = 8 \times 10^{-4}$ cm; and $t_s = 145$ sec.

Phototropic-geotropic equilibrium. When irradiated by a horizontal beam of blue light in the normal intensity range, a sporangiophore takes up an equilibrium direction of growth 20 to 30° above horizontal, often hunting about this equilibrium. This deviation from horizontal is due to negative geotropism.

The equilibrium growth direction deviates less from the irradiation direction as the irradiation moves from horizontal towards vertical. The quantitative dependence of this deviation on the angle of irradiation fits a simple model in which the geotropic and phototropic contributions are simple trigonometric functions and a correction is made for the shading of the growing zone by the sporangium (75).

The light-gravity equilibrium position was found by Dennison (Ph.D. Thesis) to be essentially independent of light intensity, varying between 30 and 20° over the intensity range $\log_2 I = -20$ to $+5$. As the intensity is decreased below $\log_2 I = -20$, the phototropic threshold is reached and the growth direction approaches vertical. Likewise, as the intensity is raised above $\log_2 I = +5$, the phototropic system fails and the growth direction also approaches vertical.

The relative constancy of the light-gravity balance over an enormous range of intensities may be partly understood in terms of the adaptation system. Since only one light source is involved, the relative intensity distribution within the cell is not a function of source intensity and should cause a constant phototropic component, provided the intensity maxima and minima are within the adaptive range of the system. At both intensity extremes, the limits of the adaptive range are exceeded and the light-gravity balance becomes intensity-dependent. This intensity dependence allows the determination of action spectra at threshold (211) and at saturation (Section 21).

The light-gravity equilibrium angle of 20 to 30° is also connected to the light distribution within the growing zone, because in either paraffin oil or at wavelengths below 300 nm the light-gravity equilibrium is strictly horizontal in the normal intensity range (211). The phototropic effectiveness of blue light in air falls off with the angle between the light and the cell axis. Dennison's "tropostat" studies (79) showed that at 30° incidence the steady-state phototropic bending speed is 60% of that at 60°, and at 20° it falls to 20% of that of 60°. Thus, in the range of 20 to 30° there is a sharp decline in phototropic effectiveness. At 14° the phototropic bending speed becomes essentially zero. Thus, the sporangiophore cannot reach a horizontal equilibrium for blue light in air because the phototropic stimulus becomes too weak for angles less than 20°. Faint stray light from the apparatus or the glass box surrounding the specimen can change this angle by as much as 10° (Dennison, Ph.D. Thesis), indicating the feebleness of the phototropic stimulus at these acute angles.

No complete inhibition of geotropism has been noted under conditions of light-gravity equilibrium with a single blue light in air. This is difficult to reconcile with the light inhibition of geotropism in the centrifuge. It is possible that this difference has to do with the differences in the optical situation. In the centrifuge experiments the sporangiophores are irradiated in effect by two opposed light beams (initially normal to the cell axis), whereas in the light-gravity equilibrium the irradiation is a single light beam at an acute angle. The inhibitory effect of light on geotropism appears to depend on the optical situation and hence on the intracellular intensity distribution. Geotropism also vanishes under blue light irradiation when a sporangiophore is irradiated in paraffin oil (211) or when it is irradiated by two blue lights whose directions make an angle of 120° with each other and both are in the horizontal plane (Dennison, Ph.D. Thesis). In paraffin oil a geotropic component appears when the light intensity is lowered to a point near the phototropic threshold. In the second case the observations were all made at $\log_2 I = -4$, which is close to the mean intensity causing geotropic inhibition on the centrifuge.

The dependence of the light-gravity equilibrium angle on intensity has also been studied at 2.3, 4.2, and 8.4 $\times$ g (100 D.S.D.). At each of the three centrifuge speeds, a parallel beam of blue light irradiated the centrifuged sporangiophores in a direction 90° from the resultant acceleration vector. The results of the experiment at 2.3 $\times$ g are shown in Fig. 27-1, along with the previous measurements at 1.0 $\times$ g (Dennison, Ph.D. Thesis). The angle given is that between the mean direction of sporangiophore growth and the direction of irradiation.

Increased gravity makes the equilibrium angle a function of intensity over the entire intensity range. At the upper end of the normal range, the curves intersect and the increased gravity has little effect on the equilibrium. The phototropic threshold is only slightly affected, as shown by the extrapolation of the curves to 90°. The curves for 4.2 and 8.4 $\times$ g are similar to that for 2.3 $\times$ g, but shifted slightly to the right.

We conclude that the constancy of the equilibrium at 1.0 $\times$ g is not only due to adaptation, but also to the drastic weakening of the phototropic stimulus for the angles below 30°. Thus,

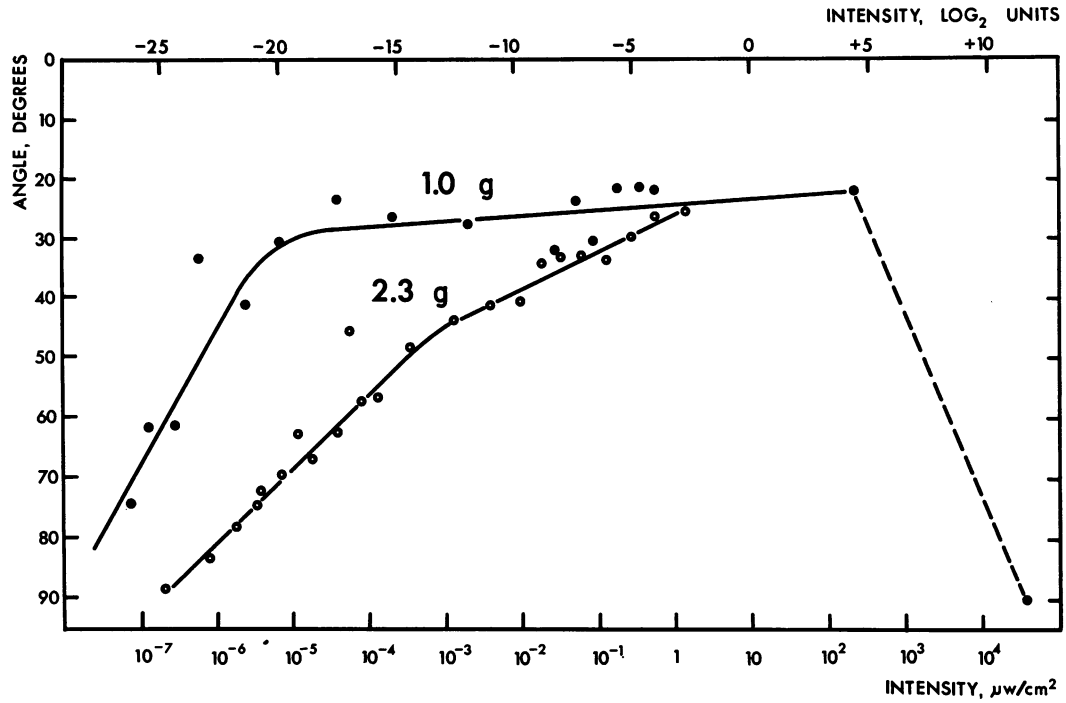


FIG. 27-1. *Light-gravity equilibrium as a function of light intensity and gravity. At 1.0 × g, the illumination is horizontal; at 2.3 × g, it is 90° from the resultant of the centrifugal and gravitational vectors. The angle plotted is that between the equilibrium growth direction and the illumination direction. The intensity is the mean intensity of illumination, with a tungsten source and a Corning 5-61 blue filter (Fig. 38-1C).*

the "best" that phototropism can do is bring the cell within 20 to 30° of the light source when acting against a weak geotropism at 1.0 × *g*. Phototropism is no match for the stronger geotropism at 2.3 × *g* at the lower intensities, however, and can bring the cell to its normal limiting angle of 30° only at high intensities. The slope of the curve at 2.3 × *g* would then be a result of the increased phototropic effectiveness at higher intensities, corresponding to the proportionality between phototropic bending speed and intensity observed in the range $\log_2 I = -20$ to -8 by Reichardt and Varjú (157).

28. Avoidance response

The least understood of the sensory properties of Phycomyces is the avoidance response. It was first described by Elfving (82) and extensively studied by him and by Steyer, Errera, Jost, Slotte, and others [reviewed by Elfving (83)]. It was rediscovered by Shropshire (187), and studied during summer workshops at Cold Spring Harbor, 1965–1968. A sporangiophore placed close to a solid barrier grows away from it. The response begins about 3 min after placing the barrier 2 to 3 mm from the sporangiophore. The rate of response in the steady state varies with the distance, about 1°/min at 2 to 3 mm, about 2°/min at 1 mm. Total angle of bend in both cases is about 50°. If the barrier is present for 3 min and then removed, the response begins at the end of the presentation time and continues for about 5 min. In the tropostat (79), the response can be kept up indefinitely. How the sporangiophore senses the barrier we do not know. 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 growth response. (ii) The avoidance response occurs in complete darkness. (iii) It occurs at 100% humidity. (iv) Seemingly, 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 vertical glass rod (diameter, 150 μm; 187), by a copper wire mesh, by a single horizontal copper wire (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.

These observations exclude as the stimulus reflections of infrared radiation emitted by the sporangiophore or electrostatic forces producing a stretch response.

Speculations have centered around the idea that a gas evaporates from the growing zone (e.g., water, CO_2, organic molecule) which develops a higher concentration on the side of the sporangiophore proximal to the barrier than on the distal side. A concentration gradient across the growing zone might then cause bending, and a step-up in concentration (with bilateral barriers), a transient growth response.

The magnitude of such a "reflection gradient" cannot be estimated since the micro-environment of the growing zone is not known. Improvised experiments have given only conflicting results so far; barriers absorbing water or organic compounds were used, and the gas between growing zone and barrier was sucked or blown away. However, the complicated interplay among transpiration, temperature, wind, and possibly other effects at the growing zone requires experiments with a higher degree of sophistication.

The effect occurs in the presence of 100% relative humidity. This finding does not rule out differential transpiration of water as the mediator of the effect since transpiration continues to occur under these circumstances. The vapor pressure, p, of water increases with decreasing radius of curvature of the water surface:

$$(\Delta p)/p = 10^{-7} r$$

where r is the radius of curvature in centimeters (1). Thus, water squeezed out through very small hydrophobic pores in the wall of the sporangiophore evaporates even at 100% relative humidity.

Thimann and Gruen (203) discovered and described in detail a negative tropic response of the sporangiophore to the local application of a small drop of distilled water. The mean size of the drops used in these experiments was 15 nliters (diameter, 0.3 mm). The experiments were carried out at 100% humidity and the drops did not change in volume appreciably over 2 hr.

The bend occurs with a latency of a few minutes, quickly reaches a steady rate of several degrees per minute, and continues for 15 min or longer, depending on the point where the drop has been applied. During the response, the drop moves out of the growing zone, as any applied marker would do. Addition of sodium azide (10^{-2} M) to the drop causes slowing of the growth rate and bending in the opposite direction (towards the drop). Addition of indoleacetic acid to the drop in amounts equal to or greater than those found in the growing zone (Section 37) has little or no effect on the response.

The relation of this interesting tropic response to the avoidance response is uncertain.

GENETICS

29. Asexual genetics

The asexual cycle starts with a multinucleate spore and proceeds through the development of mycelium, sporangiophores, and sporangia to the next generation of spores. The mycelium can also be propagated by transplantation of a small piece to new growth medium.

Here our goal is to describe the genetic constitution of Phycomyces in different phases of its life cycle and to study the laws that determine the genetic constitution of the asexual offspring of any given individual.

(a) **Mutants.** Burgeff (31, 32, 36) made a detailed search for variant forms spontaneously present in laboratory strains. He described several morphological mutants and used them in genetic crosses.

Phycomyces was one of the organisms used in early attempts of artificial mutagenesis (81), but very scant results were obtained until 1966, when E. T. Young (100) and S. H. Goodgal and Lois Edgar (100) applied nitrosoguanidine as a mutagen. Heat-shocked spores, suspended in a solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.1 to 1 mg/ml) in buffer (acetate or tris(hydroxymethyl)aminomethane-maleic, *p*H 5 to 7) for 15 to 80 min, washed in distilled water, and plated out, give a high yield of mutants and a survival rate sufficiently low to produce few heterokaryons. Under these conditions, nitrosoguanidine may induce many base changes, which tend to be clustered in the regions of the deoxyribonucleic acid (DNA) replicating at the time of the treatment (62).

The recommendations of Demerec et al. (74) will form the basis of the genetic nomenclature used in this review. More detailed rules are being worked out in consultation with other workers in this field.

A class of color mutants (100 M.H.) which differ from wild type in the synthesis of β-carotene are designated by the abbreviation *car* and their isolation number. The albino mutants have an

early block in the synthetic pathway; they can be classed into different groups by chemical analysis (131) or by genetic complementation. The mutants *car-1*, *car-5*, *car-10*, and *car-12* have been referred to in previous reports (131, 107) under the names alb 1, etc. The red mutants have a block in the cyclization steps of the synthesis (Fig. 34-2) and accumulate lycopene; the mutant *carR21* has been designated previously as r1. Other pigment mutants present different shades of yellow color. The *car* mutants form few or no zygospores, possibly due to the relation of the carotenes to the gamones (Section 5).

Nonphototropic mutants may be screened by seeding agar plates with mutagenized spores and illuminating them from below in a glass-bottom box. The sporangiophores of wild type grow down over the rim of the plate or wind along the surface of the agar; those of the mutants grow up and their spores can be collected (100 M.H.).

A frequent type of mutant selected by this procedure is distended in the growing zone, just below the sporangium. This type was described by Burgeff (31) and named piloboloides (*pil*).

The mutants which appear nonphototropic in the glass-bottom box but present normal gross morphology will be named *mad*. About 10 of those isolated so far react to bright light, but not to dim light. They are "night blind." The behavior of two of them, *mad-35* and *mad-51*, has been analyzed in some detail (100 M.H., K.B., D.S.D.). Their lower threshold corresponds to an intensity 10^5 to 10^6 times greater than in wild type; their latency period is slightly longer and their bending speed slower; geotropism and avoidance are normal, except for the slower bending. They behave as single locus mutations in crosses and their nonphototropic descendants show about the same threshold as the parents.

An otherwise heterogeneous group of more than 12 *mad* mutants have in common a reduced phototropic response and a lack of avoidance response. Their defects appear to be close to the growth output. They are "stiff" mutants. Generally, they react slightly throughout the entire normal intensity range of light. The mutant *mad-59* grows one-third faster than wild type; *mad-102* twists slower than wild type; *mad-107*, previously (131) called Ph 107, accumulates large amounts of β-carotene and may turn out to be a double mutant. The growth and tropic responses of *mad-54* differ from those of wild type in interesting ways. In this mutant, a large step-up in light intensity produces, after a normal latency, a very brief and slight positive response, followed by a longer and shallower negative response (Fig. 29-1A). Its phototropic response is even more peculiar and is illustrated in Fig. 29-1B: instead of bending progressively away from a UV light source, the mutant develops a kink in the growing zone, directed away from the light source, but this kink remains in a fixed position relative to the sporangium; i.e., it constantly forms anew at the top and reverts at the bottom. This mutant is also disturbed in the formation of the sporangia, often forming long stage I sporangiophores with several bulges along the stalk (100 M.H.).

Mutants totally lacking reactions to light have not yet been found.

A number of stable auxotrophic mutants have been isolated and their nutritional requirements (amino acids, vitamins, adenine) have been determined (100 S. H. Goodgal). Many auxotrophic isolates are unstable, reverting to prototrophy.

Several mutants resistant to canavanine (*can*) at 200 μg/ml have been isolated; 1 μg of this arginine analogue per ml completely inhibits the growth of the wild type.

Morphological mutants frequently found include piloboloides (*pil*), arbusculus (*arb*), a type of colonial, and imberbis (*imb*), which forms normal mycelia but does not develop sporangiophores. Of special interest are the temperature-sensitive *imb* mutants that are unable to form sporangiophores at 26 C but able to do so at 16 C (100 E.C.-O.).

(b) **Heterokaryons.** The mutants described and, presumably, the laboratory "wild" types are homokaryotic; i.e., all the nuclei of each individual contain the same genetic information.

Unlike the vegetative hyphae of Neurospora

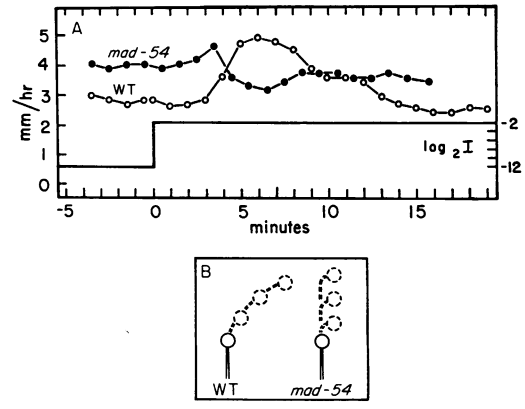


FIG. 29-1. *Growth and tropic responses to light of the "stiff mutant" mad-54 compared to wild type. (A) Growth response of mutant and wild type after a step-up of the light intensity from* $\log_2 I = -12$ *to* -2. *(B) Tropic responses of mutant and wild type (diagrammatic).*

and Aspergillus, those of Phycomyces stop growing shortly before they meet and do not anastomose with each other. Heterokaryosis, or simultaneous presence of different types of nuclei in the same cytoplasm, cannot be achieved simply by growing together two different strains. Heterokaryons of Phycomyces can be prepared artificially by a technique based on the regeneration of a mixture of cytoplasm obtained from sporangiophores of different genetic constitution (32, 107, 214). Heterokaryons may also arise naturally from the sexual cycle (17, 32, 36, 138) or from mutations occurring in a mycelium. Some of Burgeff's spontaneous mutants were in fact heterokaryons.

(c) **Karyology of the asexual cycle.** The spores contain several nuclei. Studies on the distribution of nuclei per spore in several strains have been reported by Johannes (120), H. Harm (100), and Heisenberg and Cerdá-Olmedo (107). Table 29-1 gives the frequencies $f(n)$ of spores with n nuclei in strain NRRL 1555, a strain of Phycomyces commonly used in biophysical studies.

The nuclei start dividing before the formation of the germ tubes (119). Descriptions of nuclear division are given by Baird (5) and Robinow (166). In the hyphae the nuclei divide frequently and move with the cytoplasmic stream. The hyphae are extensively branched and do not contain septa except in special cases that are not of genetic significance (Section 3).

The sporangiophores and sporangia contain many nuclei, but no divisions have been observed (100 Ute Wagenmann). There are some indications (Section 4b) that the nuclei in the sporangiophore and sporangium may be taken directly from the mycelium during stage 1. Many nuclei accumulate in the peripheral part of the young sporangium and a columella (an interior cell wall) differentiates towards the center, separating sporangium and sporangiophore. The nucleated region contains many vacuoles, which grow and fuse with each other, forming furrows that separate portions of the cytoplasm with several nuclei in each. These masses of cytoplasm round up, sheathe themselves in thick walls, and form spores. No nuclear divisions occur during this process (201).

TABLE 29-1. *Distribution of nuclei per spore in Phycomyces blakesleeanus strain NRRL 1555*

No. of nuclei, (n)	Proportion of spores, $f(n)$
1	0.003
2	0.090
3	0.420
4	0·410
5	0.074
6	0.003
$\geqslant 7$	<0.001

(d) **Quantitative segregation.** The preceding information and some new assumptions can be integrated into a theory of nuclear distribution in the asexual cycle, as follows. (i) The mycelium of Phycomyces is a wholly randomized coenocyte, containing everywhere the same proportions of the different types of nuclei that may be present. (ii) The nuclei of the sporangiophore and sporangium derive from a large sample of mycelial nuclei, thus preserving the same nuclear composition. (iii) Nuclear divisions do not occur after the beginning of spore formation. (iv) The nuclei included in the spores represent random samples taken from the general population of nuclei.

This model predicts that the spores of a heterokaryon which contains a proportion p of type A nuclei and $1 - p$ of type B nuclei can be classified into three groups:

type A homokaryons, whose frequency is

$$\sum_{n=1}^{\infty} p^n f(n);$$

type B homokaryons, whose frequency is

$$\sum_{n=1}^{\infty} (1 - p)^n f(n);$$

heterokaryons, the remainder.

Figure 29-2 depicts the frequencies of the three possible phenotypes as a function of p, assuming a distribution of nuclei per spore as in Table 29-1. This theory has been confirmed (107). The

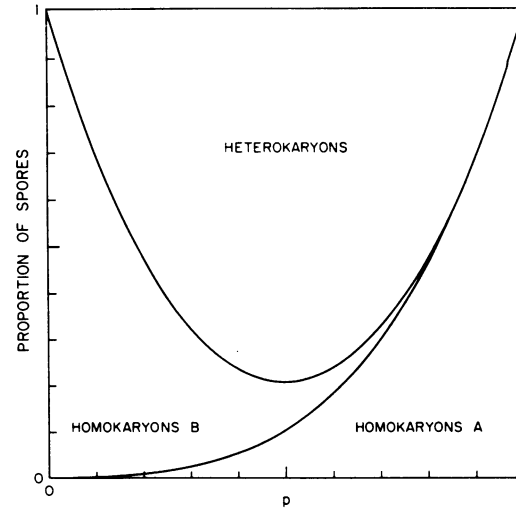


FIG. 29-2. *Proportion of heterokaryotic and of homokaryotic spores as a function of the proportion P of nuclei of one of the two types. Calculations based on the observed frequencies of spores with specified numbers of nuclei (Table 29–1).*

nuclear proportions remain constant also after transfer of mycelial pieces to new growth media. In contrast to other fungi (69), Phycomyces appears to follow closely the hypothesis of a completely random distribution of nuclei during mycelial growth, during mycelial transfer, and during spore formation.

In the absence of selection, the proportion of heterokaryons in a population of Phycomyces will diminish rapidly because only a fraction of the spores produced are heterokaryotic. Selection in favor of the heterokaryon may maintain a stable polymorphism. The selective pressure needed to maintain heterokaryosis depends on the distribution of the number of nuclei per spore, known to be different in different strains. Thus, Phycomyces may be an interesting organism for the study of some problems of population genetics.

If heterokaryotic spores are subjected to an agent that inactivates nuclei, some of the survivors will be homokaryotic. This effect permits one to test whether a particular agent does indeed inactivate nuclei. The proportion of heterokaryons is plotted against survival in a spore suspension derived from a single, well-balanced, heterokaryotic mycelium. The best approximation to the decrease in the proportion of heterokaryons expected from nuclear inactivation is found for X rays, followed by UV and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; heat (60 C) does not inactivate nuclei since the proportion of heterokaryons remains constant (E. C.-O., *in preparation*).

30. Sexual genetics

(a) **Sex determination.** The genetic and physiological behavior of the sexual determinants (+ and −) could be due to a single pair of allelomorphs with pleiotropic action and great mutational stability. More likely, these determinants consist of several genes functioning as a single unit, as "supergenes" (127) or as a differentiated segment of a chromosome (215).

Usually, the mycelium is homokaryotic for sex; i.e., all its nuclei carry determinants of the same sign (32). Heterokaryons containing the two sexual determinants in the same cytoplasm may be found among the descendants of a cross (17, 33, 138) or they may result from mycelial germination of the zygospore (33, 138), or from rare hyphal fusion and growth just below the zygospore (34). They can be prepared by the same surgical methods as other heterokaryons (Section 29) or by transplantation of the hyphae at the base of the zygospore to a new medium (100 S. H. Goodgal). The mycelium of sex heterokaryons sometimes has been called homothallic.

The morphology and behavior of sex heterokaryons is varied. Some produce deep-yellow mycelia with many pseudophores (Section 5) and develop, after a considerable delay, a few normal sporangiophores (17, 32, 34, 138). During mycelial growth, sex heterokaryons may be less stable than others, sometimes segregating mycelial regions with different sexual properties (32, 138, 214). Some of these heterokaryons react sexually with one of the homokaryotic sex types; others give a more or less abnormal reaction with both. Morphology and behavior of sex heterokaryons are influenced by environmental factors (138), but they depend mainly on the nuclear ratio of the two components of the heterokaryon, the more unbalanced types resembling closely the corresponding homokaryon and the best balanced showing the extreme morphology described above. The vegetative spores are produced in the same way as in other heterokaryons and give rise to mycelia of both homokaryotic types and to heterokaryons of all possible nuclear ratios (17; 100 E.C.-O.).

(b) **Karyology of the sexual cycle.** The behavior of the nuclei during the sexual cycle has received considerable attention, but their small size, their inconspicuous mode of division, and some technical difficulties have left many uncertainties and contradictions. A final decision on some important points will require more cytological and genetic work.

The number of nuclei increases rapidly by active division in the conjugating regions both before the formation of the gametangia (68, 121) and during the formation of the zygospore (5, 129), so that the zygospore receives thousands of nuclei of each sign. Initially, these nuclei are distributed uniformly over the young zygospore, but soon divisions cease and the nuclei become distributed in small groups, of around a dozen nuclei each, situated in the periphery of the zygospore (5, 68, 121, 129).

In the zygospore, many nuclei fuse in pairs, others remain apogamic, and others degenerate. The different authors do not agree on the timing of this important process. Some contend that it occurs in the young zygospore, soon after the nuclei arrange themselves in the periphery (121, 129, 132); others claim that it happens shortly before the germination of the zygospore, after the long period of dormancy (33, 36, 68). One observer did not see any fusions or degenerations during the entire sexual cycle (5). In any event, at some moment the zygospore seems to contain both diploid and haploid nuclei.

The germ tube that is formed at the end of the dormancy becomes a sporangiophore. It contains

many nuclei of different sizes and shapes, which one can guess to be diploid or haploid, dividing or resting. No meiotic divisions have been described. Possibly, meiosis occurs in the germinating zygospore, and the reduced nuclei divide actively and migrate towards the tip of the sporangiophore; together with diploid, resting nuclei abundant in the sporangiophore they enter into the spores (33, 36, 192). Or, both diploid and haploid nuclei divide actively and participate in spore formation (68). In the case of mycelial germination of the zygospore, it has been proposed that meiosis takes place after some growth has occurred, so that diploid and haploid parts of the mycelium are obtained (33).

At the time of spore formation, three types of nuclei might be present: apogamic, that come directly from the parents; diploid, that result from fusion; and haploid, that have undergone meiosis. The larger, supposedly diploid, nuclei do not appear in mycelia derived from the spores, possibly because they impose sterility on the spore, or because they degenerate, or because they undergo meiosis (36, 68).

In the asexual sporangium, the spores enclose random samples of nuclei taken from a preexisting pool and contain from the beginning the definitive number of nuclei per spore (Section 29). Despite some contrary assertions (68), the formation of the germsporangiospores, or germspores, is very different: usually a single nucleus is enclosed in each spore initially and then divides to form several identical nuclei, insuring the homokaryosis of the spore (33). At a certain moment, more than half of the spore initials contain a single nucleus, and the nuclei seem to be actively dividing (100 Patricia Reau and E. C.-O.). Asexual sporangia usually contain more spores (about 10^5) than do germsporangia (2×10^4).

(c) **Crosses.** Burgeff (33, 36), using morphological mutants, showed that the germspores may contain the parental types and their mendelian combinations. In two-factor crosses he obtained the expected recombinants of several markers, including the sex character and the mutants *arb* and *pil*. In three-factor crosses he did not recover all eight possible combinations from a given zygospore, but at most four; this crucial result is based on meager data from two zygospores involving sex, *arb* and *pil*, and a few more involving somewhat dubious markers. The frequencies of germspores of each type are very irregular, and often expected types are not found. Burgeff concluded that the germspores are the descendants of the meiotic products of a *single diploid nucleus*; this imposes a maximum limit of four different types per germsporangium. The irregular frequencies and the occasional lack of some expected types may be due to loss of a meiotic product or to variable mitotic rates intervening between meiosis and the formation of germ spores.

Helga Harm (100) tried to repeat this work but found that the markers *arb* and *pil* received from Burgeff behaved as closely linked, so that her crosses were in fact two-factor crosses that could not resolve the problem.

A new approach to this problem is to cross a heterokaryon of a white and a red mutant with the normal yellow strains

car-5 (−)**carR21* (−) × (+), and

car-10 (−)**carR21* (−) × *mad-54* (+),

where the asterisk separates the components of the heterokaryon and (−) and (+) indicate the sex character. If all the germspores of any one zygospore are derived from a single diploid nucleus, the mycelia obtained from these spores should exhibit only two different colors (presumably yellow and white or yellow and red); if they are derived from more than one diploid nucleus or if they include apogamic nuclei, the three colors, yellow, red, and white, should be represented in at least some of the germsporangia. Of some 20 germsporangia studied in some detail, 3 contained the three colors and the others only yellow and white. The second cross indicated above is a three-factor cross for any given pair of nuclei; about half of the zygospores analyzed so far and giving rise to yellow and white descendants contained more than four different types of nuclei among their germ spores (E. C.-O., M. H., and Patricia Reau, *in preparation*). Consequently, the predictions based on a single meiosis have not been substantiated, but the number of diploid nuclei involved, if not always one, is probably small.

The frequency of heterokaryotic germ spores has been generally underestimated because only a fraction of the sex heterokaryons show the characteristic extreme morphology. In our studies the proportion of heterokaryons is very variable, usually near zero but sometimes up to about one-half. It may be explained by the packaging of more than one nucleus into a germ spore initial.

It is unlikely that diploid nuclei pass to the descendants of a cross. Mycelia containing such diploid nuclei would be sexual heterozygotes and should not segregate at the time of asexual spore formation. Strains of this type were looked for but were not found. Apogamic nuclei are unlikely, too. They should yield parental types in the off-spring, but many germsporangia yield recombinants only and lack the parental types.

BIOCHEMISTRY

31. Nutrient requirements

Minimal medium. Phycomyces grows well on a synthetic medium containing salts, glucose, asparagine (GA medium), and thiamine (vitamin B_1) at a concentration of 0.05 μg/ml (161, 181). The thiamine requirement is not absolute. The whole molecule can be dispensed with if both its parts—pyrimidine and thiazole—are supplied in equimolar amounts (19, 20).

The best study of the requirements for a minimal medium leading to normal growth is that of Ødegård (133). The nitrogen source may be either inorganic (ammonium tartrate, ammonium citrate) or organic (asparagine), although the latter gives generally better growth.

As micronutrients Phycomyces requires Ca^{2+} (80 mg/liter), Fe^{3+} (0.3 mg/liter), and Zn^{2+} (0.4 mg/liter). Omission of any one of these reduces growth to 25 to 75% of normal and leads to aberrant sporangiophore development. The other components of the minimal medium are usually sufficiently impure to make addition of these micronutrients unnecessary.

Complete medium. Phycomyces grows well on numerous types of media. For large-scale preparations of sporangiophores, commercial powdered mashed potatoes yield a convenient, reproducible, and inexpensive medium. This medium gives about 400 g (wet weight) of sporangiophores per m^2.

Difco Potato Dextrose Agar (PDA) is a medium which yields only a few sporangiophores per cm^2. It is useful for biophysical studies on single sporangiophores or for obtaining plucked sporangiophores (Section 7).

For zygospore production, conditions are much more critical than for vegetative growth. The upper temperature limit for a good sexual reaction is 22 C, the optimum 17 C (110, 170, 182). The warmer temperatures cause rapid acidification, unsuitable for the sexual reactions (165). Phloroglucin inhibits the sexual reactions but does not inhibit normal growth (170).

Moderate light intensities are optimal for zygospore formation (110).

Plempel (144) concluded that a high ratio of organic nitrogen to carbon permits better sexual reactions. For the isolation of gamones he used an extremely rich medium (Sabourod's medium, containing peptone, meat extract, sugar, salt, malt extract).

Robbins and Kavanagh (162) found a stimulating effect of hypoxanthine or guanine on sexual reproduction. We have found optimum zygospore formation with potato-dextrose-agar and hypoxanthine (100 E.C.-O.).

32. Base composition of nucleic acids; incorporation of precursors into nucleic acids, proteins, and ferritin

Information on nucleic acids in Phycomyces is limited to a few data on base composition in mycelial extracts. These data are summarized in Table 32-1.

Attempts have been made to measure the incorporation of ^{14}C- and ^{3}H-labeled precursors into nucleic acids and proteins. E. T. Young and M. Heisenberg (100) grew mycelium from spores for 2 to 3 days in minimal medium containing uracil, uridine, thymine, thymidine, or bromouracil labeled with ^{3}H or ^{14}C; 90 to 95% of the label in the mycelium was extractable with methanol-chloroform, suggesting its incorporation into lipid. Of the radioactivity remaining in the water phase, about 20% was tentatively identified as ribonucleic acid (RNA) by digestion with ribonuclease.

S. Heinemann (100) grew 7-day-old mycelia in ^{14}C-valine for 4 hr. Only 3% of the radioactivity incorporated into the mycelium was recovered in

TABLE 32-1. *Nucleic acid composition of Phycomyces*[a]

Nucleic acid	Strain	G	A	C	T	(G + C)	Method	Reference
		%	%	%	%	%		
DNA	(−)	19.2	30.4	19.4	31.0	38.6	Chromatography	208
DNA	(+)	19.3	30.4	19.5	30.8	38.8	Chromatography	208
DNA	(−)					43	Buoyant density	199
DNA	(−)					44	"Melting point"	199
					U			
RNA	(−)	29.7	28.2	21.1	21.0	50.8	Chromatography	208
RNA	(+)	29.9	27.4	21.2	21.5	51.1	Chromatography	208
RNA		27.8	27.8	20.8	23.6	48.6	Chromatography	198

[a] G, guanosine; A, adenosine; C, cytosine; T, thymidine; U, uridine.

the protein fraction. After hydrolysis of this fraction, the radioactivity was found to be still mainly in valine. This experiment proves that externally supplied amino acids can be used by the mycelium for protein synthesis. David (100) demonstrated that in Phycomyces the uptake of ^{3}H-leucine into 3-day-old mycelia is very slow compared to *Neurospora crassa* under similar conditions.

Ferritin (Section 33) can be easily and specifically labeled with ^{59}Fe added to the growth medium in the form of $^{59}FeCl_3$. Although ferric chloride precipitates at the physiological *p*H of the growth medium, it is taken up efficiently and incorporated into ferritin.

33. Ferritin

A large iron-containing protein, found in the mycelium, sporangiophore, and spores of Phycomyces, has been characterized by David and Easterbrook (*submitted for publication*). Because of its similarity to ferritin in mammalian tissues (95, 104) and higher plants (112), it has been called Phycomyces ferritin. Phycomyces ferritin is bound to large lipid droplets in the cytoplasm, often forming ordered two-dimensional crystalline patterns over the surface of the droplets, conspicuous in electron micrographs (141, 179). The ferritin-lipid complex can be isolated from sporangiophore homogenates or the ferritin can be purified directly by a simple butanol extraction procedure.

Phycomyces ferritin has been obtained completely pure as judged by gel electrophoresis. Because of its high iron content it absorbs strongly in the UV (at 260 nm, OD = 77 per mg of ferritin iron per ml), sediments rapidly ($S_{20,w}$ = 55), and has a high density (ρ = 1.82 g cm^{-3}) in CsCl equilibrium density gradients. In electron micrographs, Phycomyces ferritin appears in unstained preparations as an electron-dense iron core (diameter, 5.0 to 6.0 nm); negative staining reveals a protein shell (diameter, 12 nm) surrounding the iron core.

The level of ferritin in Phycomyces is regulated by the level of iron in the growth medium. Addition of 15 μg of Fe per ml to an "iron-free" medium results in a 30- to 50-fold increase in ferritin iron (0.02 μg/sporangiophore). Whether apoferritin synthesis is regulated by the iron levels has not been demonstrated.

With labeled ion (^{59}Fe), the distribution of ferritin in sporangiophores has been studied. Of the ^{59}Fe in sporangiophores, 70 to 80% is found in spores; of this fraction, at least 50% is ferritin. (The remaining 50% was not extracted by the procedure used.)

Because of its selective presence in spores, ferritin probably serves as an iron storage compound for use following spore germination. Indeed, under growth conditions low in iron, ferritin is degraded following spore germination, yielding its iron to a "soluble" pool. The mechanism of this degradation is an all-or-none process. Species of ferritin molecules which have lost only part of their iron complement were not found under conditions in which most of the extractable ferritin in spores was degraded (C.N.D., *in preparation*).

34. Lipids

(a) **Glycerides and phospholipids.** Four principal classes of lipids have been found in Phycomyces: glycerides, phospholipids, sterols, and carotenes. Biosynthesis of lipids, specifically of unsaturated fatty acids and carotenes, has received considerable attention from numerous investigators, but little work has been done on their metabolism and function. Almost all of the studies were done with mycelium, hardly any with sporangiophores.

Phycomyces is capable of accumulating large amounts of lipid; globules of oil are visible in mycelium and sporangiophores. Lipids amount to 20% of dry weight of mycelium, and glycerides, phospholipids, and ergosterol are the principal constituents (11). Lecithins and cephalins have been found in the phospholipid fraction (197). As major fatty acids of the total lipid, Stoffel and Wiese (197) found palmitic acid (21%), stearic acid (4.5%), oleic acid (27%), linoleic acid (15%), and γ-linolenic acid (23%).

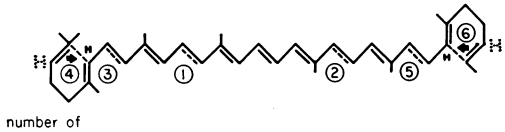
Slightly different results for the fatty acid composition have been reported by Shaw (184) and by Bernhard et al. (9). The presence of γ-linolenic and the absence of α-linolenic, the single octadecatrienoic acid in Ascomycetes and Basidiomycetes, have been confirmed by all investigators. γ-Linolenic acid is characteristic for zygomycetes and oomycetes (86, 184, 185). Both saturated and unsaturated fatty acids arise from acetyl-coenzyme A (CoA). Exogenously supplied labeled stearic, oleic, and linoleic acids are converted to the more highly unsaturated fatty acids, showing that the saturated carbon chain is formed first and that synthesis of the unsaturated acids follows by introduction of *cis* olefinic double bonds (197). The second possibility, the anaerobic pathway, has been excluded.

The growth effect of lipid has been studied for mycelium grown in a basal glucose-potassium acetate-ammonium sulfate medium. The addition of glycerol trioleate to such a medium increases glucose utilization, growth, and carotenogenesis. The added lipid seems to increase the rate of glucose uptake rather than forming a carbon and energy source (128).

(b) **Sterols and carotenes.** Phycomyces has been extensively used to study in vivo and in vitro

biosynthesis of sterols and carotenes. This work has been reviewed by Porter and Anderson (153). Condensation of isopentenyl pyrophosphate, the biological isoprene unit, yields the typical C_5 skeleton of these compounds. Reductive coupling of two C_{15} units leads to squalene, the basic C_{30} precursor of sterols. The corresponding formation of phytoene, the first carotene, from two C_{20} units occurs without a change in the oxidation state of the system. Phytoene, consequently, in contrast to squalene, contains a central double bond (*cis*). The absence of the double bond in squalene allows extensive folding and cyclization, leading to the formation of lanosterol. This compound is converted in a complicated way to ergosterol, the principal sterol in Phycomyces. Some of these last steps have been investigated in Phycomyces. In cultures grown in the presence of ^{14}C-methionine, 24-methylenelanosterol and possibly 14-demethyl-24-methylenelanosterol but not lanosterol were labeled (94). These results indicate that in the biosynthesis of ergosterol transmethylation takes place at the lanosterol level. Thin-layer chromatography of unsaponified lipid extracts shows that ergosterol is partly esterified (115). No details have been published.

β-Carotene accounts for over 95% of the colored carotenes (90). The pathway of β-carotene starting with phytoene is shown in Fig. 34-1. Steps 1, 2, 3, and 5 symbolize dehydrogenations, steps 4 and 6 cyclizations. Evidence for this pathway includes: inhibition of β-carotene synthesis and accumulation of the more saturated C_{40} precursors after addition of diphenylamine (90), and studies of carotene mutants (131). Similar to other organisms, three main mutant blocks have been located, causing, respectively, (i) a decrease in the production of phytoene, resulting in a low overall concentration of carotenes; (ii) a failure of the dehydrogenation enzymes, yielding large amounts of phytoene but low concentrations of the less-saturated successors; and (iii) a defect of the cyclization step, causing a replacement of β-carotene by lycopene.



number of conjugated double bonds

3 PHYTOENE
↓ ①
5 PHYTOFLUENE
↓ ②
7 ζ-CAROTENE
↓ ③ ④
9 NEUROSPORENE → β-ZEACAROTENE
↓⑤ ↓⑤ ⑥
11 LYCOPENE γ-CAROTENE → β-CAROTENE

FIG. 34-1. *Pathway of β-carotene synthesis. Starting with phytoene, successive dehydrogenations increase the length of the system of conjugated double bonds. Reaction steps 4 and 6 cyclise the terminal segments. These cyclisations fail to occur in the lycopene-producing mutant carR21.*

Depending on the growth conditions, the concentration of β-carotene varies, averaging around 0.05% of dry weight (91). The growth medium most often used contains glucose (carbon source) and asparagine (nitrogen source) as the main components. Replacement of glucose by acetate reduces carotenogenesis; that of asparagine by leucine causes an increase. Leucine seems to be readily converted to β-hydroxy-β-methyl glutaryl-CoA, one of the presurcors of terpene synthesis. In addition, *p*H, temperature, and light influence terpene biosynthesis.

β-Ionone stimulates β-carotene and ergosterol biosynthesis in Phycomyces without being significantly incorporated into either of them. It seems to act catalytically (158).

Diphenylamine is a moderately specific inhibitor for β-carotene synthesis. Ergosterol and lipid biosynthesis are only slightly influenced by diphenylamine concentrations which strongly throttle β-carotene synthesis in Phycomyces (134). The decrease in β-carotene synthesis is accompanied by an accumulation of the more saturated C_{40} precursors, specifically phytoene and phytofluene.

β-Carotene is located mainly in the oil globules of mycelium and sporangiophores. Because of its intense yellow color it is one of the most conspicuous compounds. Its unlikely role as receptor pigment in sporangiophores has been discussed (Section 25). The possible relation of β-carotene synthesis to the early stages of zygote formation has been discussed in Section 5.

35. Phenolic acids

Phycomyces is a convenient material for studying the biosynthesis of phenolic acids (24–27, 106). When grown on glucose-asparagine medium, the mycelium of Phycomyces produces large amounts of gallic acid and protocatechuic acid (10). Pyrogallol, catechol, shikimic acid, and 5-dehydroshikimic acid are present in low concentrations (106). About 90% of the phenolic acids are secreted into the culture medium.

In sporangiophores (4 cm long) a concentration of 10 mg of gallic acid/ml has been reported by Dennison (76).

In Phycomyces three biosynthetic routes have been detected for the formation of the phenolic acids: (i) the shikimic pathway, with glucose as the carbon source and leading predominantly to

protocatechuic acid; (ii) the conversion of glycine, asparagine, and alanine to gallic acid via an unknown route; and (iii) the phenylpropane pathway.

Hashem and Brucker (105) found that ^{14}C-glucose (in the presence of unlabeled glycine) is incorporated into protocatechuic acid and shikimic acid with a high specific activity. Gallic acid shows a low specific activity under these conditions. Similarly, when ^{14}C-shikimic acid was added to the growth medium protocatechuic acid had a high and gallic acid a low specific activity. Figure 35-1 shows the probable biosynthetic pathway of protocatechuic acid. The last step, the direct conversion of dehydroshikimic acid into protocatechuic acid, has been demonstrated in a mutant strain of Neurospora which accumulated dehydroshikimic acid (98). Shikimic acid does not seem to be a direct intermediate in the biosynthesis of the two phenolic acids.

In higher plants, gallic acid may be formed via the shikimic route. Haslam et al. (106) therefore considered two possible pathways for the formation of gallic acid in Phycomyces, both via the shikimic route: the oxidation of protocatechuic acid and the dehydrogenation of dehydroshikimic acid. Replacement studies showed that unlabeled 5-dehydroshikimic acid stimulated the formation of gallic acid, being superior to shikimic acid, 5-dehydroquinic acid, and quinic acid. ^{14}C-protocatechuic acid was not converted to gallic acid. Although these experiments suggest that gallic acid can be formed directly from dehydroshikimic acid, the above-mentioned results by Hashem and Brucker (105) demonstrate that in Phycomyces this route is normally unimportant for gallic acid formation.

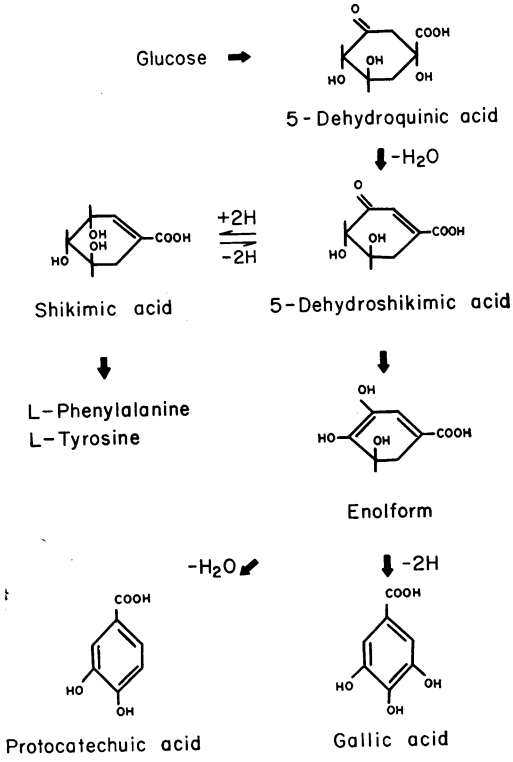


FIG. 35-1. *Biosynthetic pathways of phenolic acids.*

Labeled glycine, asparagine, and alanine (in the presence of glucose) were preferentially incorporated into gallic acid. Protocatechuic acid had a low specific activity in these experiments (26). This indicates that gallic acid is a product of amino acid metabolism and that it is not formed via the shikimic pathway. However, no details have been established about its way of formation.

Tyrosine is an excellent source for phenolic acid formation; i.e., phenolic acids may also arise by way of phenylpropane metabolism (24). This pathway includes deamination of tyrosine, β-oxidation of the side chain, and hydroxylation of the ring. The exact sequence of these steps is not known. The concentrations of gallic acid and of protocatechuic acid were not separately determined in these experiments.

A specific function of the phenolic acids for Phycomyces has not been reported. They seem to be metabolic end products which are excreted by the mycelium into the culture medium and by the sporangiophore into the vacuole. Some of the phenolic acids probably polymerize, giving the cell wall of sporangiophores its green color.

36. Cell wall of sporangiophores

Sporangiophores of Phycomyces are surrounded by a cytomembrane of standard thickness (7 nm) and a cell wall of about 600 nm thickness. The wall has been studied almost exclusively by physical methods. Polarization microscopy shows birefringence of native cell walls (53, 137). Both in stage I and stage IV the birefringence is negative in the growing zone and positive in the nongrowing part. The two zones are divided by a small zone of isotropic behavior. This birefringe is predominantly form birefringence. The intrinsic birefringence of chitin polymers is weak and its sign is uncertain (52). Electron microscopy confirmed the assumptions that the optical anisotropy is the result of the arrangement of chitin chains (87, 169). Alkaline extraction removes a thin layer of waxy cuticle from the outside of the wall and amorphous substances of similar electron density from within the wall, and reveals a network of microfibrils

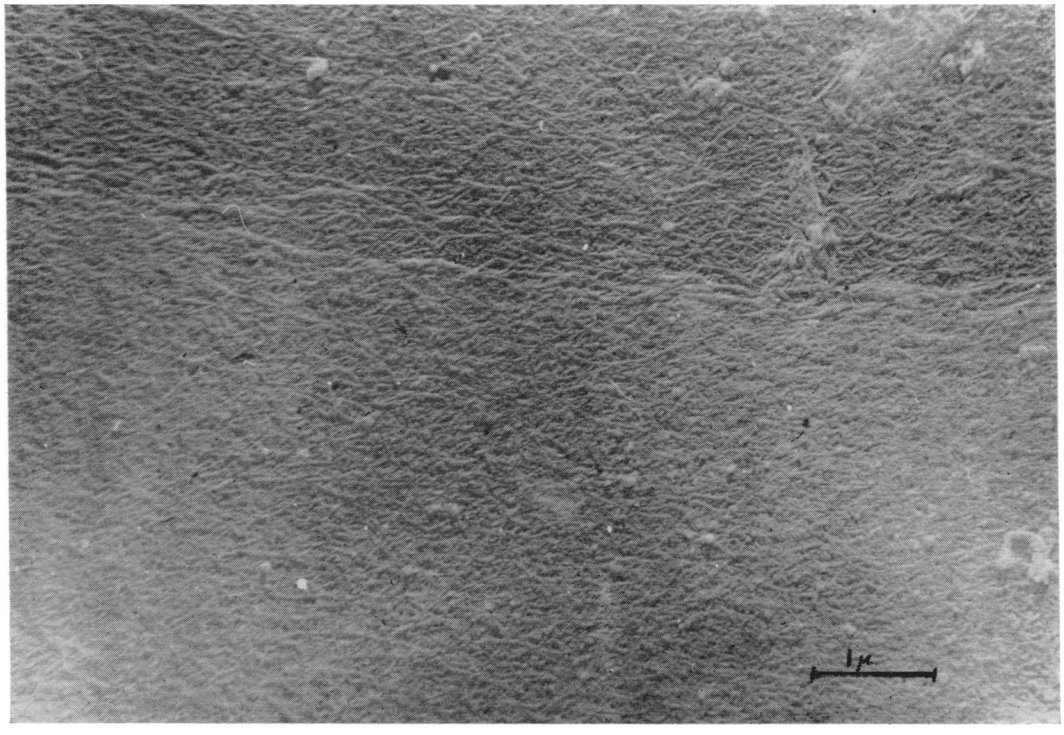


FIG. 36–1. *Electron micrograph of a cell wall fragment from the growing zone of a stage I sporangiophore. The cell wall was cleaned with alkali and macerated. The bottom portion shows the outer layer, consisting of randomly oriented chitin fibrils. In the top portion, the inner layer is exposed. The outer layer has been removed by the mechanical treatment. In this layer, the fibrils are predominantly transversely oriented [Fig. 3 of Frey-Wyssling and Mühlethaler (87)].*

with a thickness of 15 to 25 nm. Van Iterson et al. (210) found, by X-ray diffraction, that this non-alkali-extractable material is chitin. Chitin is a linear polymeric substance, and crystalline chitin fibrils 15 to 20 nm thickness are found in the cell walls of all fungi (except oomycetes), and in the exoskeletons of crustaceans and insects. The polymer resembles cellulose in its structure (i.e., 1,4′-β-glucoside linkages) except that glucose is replaced by *N*-acetylglucosamine. The arrangement of the polymeric chains in the fibrils is similar in chitin and cellulose, but the dimensions of the unit cell are sufficiently different to make these two structures clearly distinguishable in fiber diagrams.

Figure 36-1 is an electron micrograph of the pretreated wall in the growing zone of stage I. An outer layer of randomly oriented fibrils and an inner layer of predominantly transversely oriented fibrils are visible. This inner layer causes the negative optical birefringence of the growing zone. Additional layers with longitudinally oriented fibrils are present below the growing zone, leading to a reversion of the sign of birefringence. Although the nongrowing wall contains much more fibrous material than the wall of the growing zone, there is not much difference in thickness of the cell wall throughout intact sporangiophores (100 M.Z.). This indicates that the growing zone contains predominantly less organized material, easily extractable with alkali. Observations like the ones in Phycomyces form the basis of the multinet theory of cell wall growth (154).

Only a few experiments have been done to determine the chemical composition of the cell wall. Using X-ray diffraction, Kreger (125) found about 25% chitin in the cell wall. Chitosan, a deacetylated form of chitin, was also detected. Its concentration is not known. Another 25% of the wall substances are extractable with $CHCl_3$ and seem to be waxy or fatty compounds (125). The rest of the wall, consisting mostly of substances extractable with alkali, has not yet been characterized.

37. Indoleacetic acid (IAA)

After earlier qualitative work by Heyn (109) and by Kögl and Verkaaik (123) had given sug-

gestive evidence for the presence of IAA; Gruen (101) demonstrated unambiguously the identity of the auxin found as IAA and determined the quantities obtainable by extracting sporangiophores, mycelium, and culture medium (a glucose-asparagine medium). Mycelium and culture fluid contained small amounts, sporangiophores about 20 times more per unit wet volume. The amount found in sporangiophores was about 2×10^{-7} M, quite comparable to the concentrations of transported IAA found in coleoptiles of higher plants (193).

Attempts to show evidence of physiological effects of IAA on Phycomyces by Banbury (6), by Maass (130), and by Thimann and Gruen (203) gave negative results. These attempts were all modeled on one of the coleoptile tests, namely, the production of asymmetric growth by asymmetric application of IAA. These tests may have given negative results because of limited penetration, or because of excessive endogenous amounts. Perhaps detailed tests with IAA antagonists (111) should be tried. Previous work along these lines has been reviewed by Gruen (99*a*).

APPENDIX

38. Intensity units (conversion factors)

Various intensity units and scales have been used in Phycomyces research: meter candles, foot candles, erg cm^{-2} sec^{-1}, μw cm^{-2}, quanta cm^{-2} sec^{-1}, and a logarithmic scale, with logarithms to base 2. The scales used by different authors often can only be crudely related to each other since details on the spectral quality of the source and of the filters are not given, and the action spectra were not known at the time.

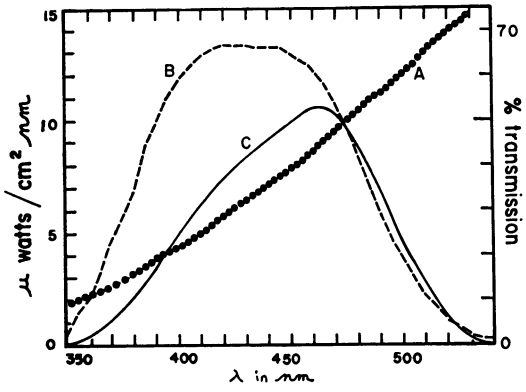


FIG. 38–1. *Spectral distribution of incandescent light source filtered through a blue filter. (A) Emission of a quartz-iodine-tungsten filament lamp (150-w General Electric FCS lamp), in $\mu w/cm^2$ nm, at 24 v, distance 20 cm. (B) Transmission of Corning 5-61 filter, 5 mm thick. (C) Spectral distribution of transmitted light (product of A and B).*

Much recent work has been done with incandescent sources and a particular type of filter, Corning 5-61, 5 to 8 mm thick. Figure 38–1 shows (A) the transmission curve of such a filter, together with (B) the emission curve of a calibrated quartz-iodine-tungsten lamp, used for calibration of photometers, and (C) the product of these two curves, i.e., the spectral distribution of the transmitted light. Figure 38–2 shows (A) this transmitted light, (B) the action spectrum of the growth response, and (C) the product of these two functions, i.e., the relative emphasis which different wavelengths receive with this type of light source. This curve is dependent on the temperature of the source used, and this correction (which is slight) will be relevant in experiments in which the light intensity is controlled by the lamp voltage.

Delbrück and Reichardt (71) used this kind of source and introduced a logarithmic intensity scale with a unit intensity ($\log_2 I = 0$) equal to 10 μw cm^{-2} (5 μw cm^{-2} from each side in cases of bilateral irradiation). The logarithms are taken to the base 2 so that each unit on this scale corresponds to doubling of the intensity. The normal range ($\log_2 I = -8$ to $+1$) corresponds to 0.04 to 20 μw cm^{-2}, the total range in which Phycomyces responds ($\log_2 I = -25$ to $+6$) corresponds to 3×10^{-7} to 640 μw cm^{-2}.

For comparison with work with monochromatic light, 450 nm may be considered an appropriate average wavelength of the broad-band blue sources illustrated in Fig. 38–1 and 38–2. A rough correspondence of the intensity scales used by various authors is as follows. The intensity called $\log_2 I = 0$ by Delbrück and Reichardt (71) corresponds to a total incident flux of: 100 erg cm^{-2} sec^{-1}; 10 μw cm^{-2}; 2.3×10^{13} quanta cm^{-2} sec^{-1}; 3.8×10^{-11} einstein cm^{-2} sec^{-1}; 2,500 meter-candles; 250 foot-candles. With meter-candles and foot-candles, we have tried to relate them to the other scales with respect to biological effectiveness, not with respect to energy flux.

In future work it is recommended that einsteins

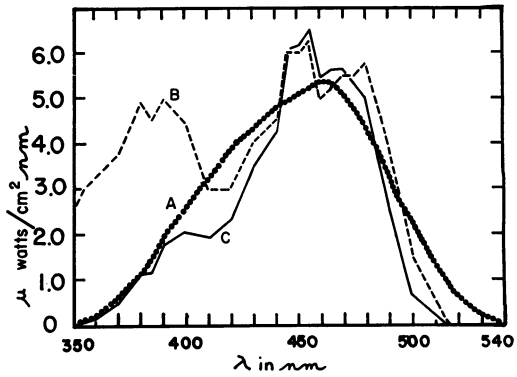


FIG. 38–2. *Effective spectral distribution when stimulating with blue light. (A) Spectral distribution of incident light (see curve C of Fig. 38-1). (B) Action spectrum of light growth response. (C) Effective spectral distribution (product of A and B).*

cm^{-2} sec^{-1} be used for monochromatic light and that in such cases the spectral quality should be characterized as to band width and stray light. For broad-band blue light, we recommend the $\log_2 I$ scale.

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