OCTAHEDRAL CRYSTALS IN *PHYCOMYCES*. II

T. OOTAKI and J. J. WOLKEN

From the Biophysical Research Laboratory, Carnegie-Mellon University, Pittsburgh,
Pennsylvania 15213. Dr. Ootaki's present address is the Department of Biology, Yamagata
University, Yamagata, Japan.

ABSTRACT

Phycomyces blakesleeanus sporangiophores contain octahedral crystals throughout their
cytoplasm and vacuole. More octahedral crystals were found in the wild-type strain G5
(+) than in the β-carotene-deficient mutant C5 (−), and much more than in the mutant
C141 (−), which is sensitive to only high light intensity. In the wild type, the number of
crystals per sporangiophore increased until the sporangiophore reached stage IV, and then
decreased. Stage I contained the most crystals per unit volume. Cultures grown in darkness
had the maximum number of crystals. Under high light intensity, there was an overall
reduction of crystals. The crystals are regular octahedrons. The crystals were isolated from
the sporangiophores by a method of sucrose density-gradient centrifugation. They contain
nearly 95% protein, are stable in organic solvents, but can be solubilized in buffer solution
above pH 9.5 and below 2.5. The crystals weakly fluoresce with an emission peak at 540
nm, which is affected by irradiation with white light. Absorption spectra of freshly prepared
crystals show absorption maxima around 265–285 nm, 350–380 nm, and 450–470 nm.
These absorption peaks for the crystals are close to those of the phototropic and light-
growth action spectra. These data suggest that the crystals may contain a flavoprotein
which may be the photoreceptor pigment of *Phycomyces*.

INTRODUCTION

The fungus *Phycomyces blakesleeanus* sporangio-
phores are large single cells, centimeters in
length, which are sensitive to light and to other
physical stimuli (Shropshire, 1963; Bergman et al.,
1969). Because of their sensitivity to light, *Phy-
comyces* is of considerable interest as a model cell for
photosensory studies. To date, no photoreceptor
structure has been identified, and it is not known
what pigment or pigment system is responsible for
these light responses. Action spectra for *Phycomyces*
phototropism and light-growth response show
absorption around 280, 370–385, 445–455, and
470–485 nm (Curry and Gruen, 1959; Delbrück
and Shropshire, 1960).

Crystals in *Phycomyces* sporangiophores were
first observed by van Tieghem (1875). More
recently, they have been investigated by Thornton

and Thimann (1964), Thornton (1969), Wolken
(1969), and Zalokar (1969). However, the func-
tion of these crystals in *Phycomyces* is not known. A
hypothesis which we have pursued is that the
crystals which are located in the growth zone of
the sporangiophores are connected in some way
with the photoreceptor process. Microspectro-
photometry of these crystals *in situ* suggested that
these crystals might contain a flavine (Wolken,
1969, 1972).

In the following experiments we set out to
establish the culture conditions which produced
the maximum yield of crystals, to determine
where in the cell the crystals are located, and then
to isolate the crystals from the sporangiophores.
The isolated crystals' chemical nature and their

properties as a possible photoreceptor structure were then explored.

MATERIALS AND METHODS

Cultures

Phycomyces blakesleeanus wild strain G5 (+) and β-carotene-deficient mutants, strain C5 (−) which is still fully photosensitive, and strain C141 (−) showing no phototropic response to dim light (Bergman et al., 1969), were used. They were cultured under sterile conditions on 6.5% Sabouraud Dextrose Agar (Fisher Scientific Co., Pittsburgh, Pa., no. J-1113-C). A spore suspension was used as inoculum. To obtain thick sporangiophores, inocula of about 3 spores/cm² were used. The Petri dish was placed in a larger Petri dish (10 cm in diameter) and covered by an inverted 400 ml Pyrex beaker of 12.5 cm height without spout. The inoculated spores were cultured at 23 ± 1°C under continuous overhead illumination from a white fluorescent lamp (Westinghouse, F 40 CW Cool White). The light intensities were adjusted at the dish level with a radiometer (model 65, Yellow Springs Instrument Co., Yellow Springs, Ohio).

Developmental Stages of Sporangiophores

Sporangiophore development followed from stage I to IV as described by Castle (1942). We have designated those sporangiophores bearing yellow sporangia (white in the β-carotene-deficient mutants), regardless of the sporangium size, as stage II-III, and those with brown to black sporangia (gray in the mutants) as stage IV.

Estimating the Number of Crystals

To estimate the total number of crystals in a sporangiophore, 10–50 sporangiophores at each developmental stage was harvested and segmented with microscissors. The cell contents were squeezed into 0.1 ml of 0.1 M phosphate buffer (pH 6.0) in a watch glass, and after mixing, 1 drop of the suspension of the cell contents was placed under the cover slip of a hemocytometer. Under microscope observations the number of crystals were counted. This was repeated at least three times for each of five different groups of sporangiophores.

To determine the number of crystals *in situ*, the sporangiophores were fixed in Carnoy's solution (3 ethyl alcohol:1 acetic acid) for 15 min at room temperature. They were then transferred to xylene through an alcohol-xylene series, which made the sporangiophores transparent, and then mounted in Canada balsam on slides. Under a microscope, the number of crystals per unit volume of sporangiophore was counted along the sporangiophore axis.

Centrifugation of Sporangiophores

To stratify the cell contents in a sporangiophore according to their densities, single sporangiophores were centrifuged in the following way. A plucked sporangiophore was inversely immersed in a cellulose nitrate centrifuge tube (1.3 cm in diameter and 5 cm in height), filled with distilled water. To prevent the sporangiophore from collapsing during centrifugation, the base of the sporangiophore was tied with a piece of sewing thread, the other end of which was fixed with glue on a slightly incised edge of the tube. The length of the thread was adjusted so that the sporangiophore tip could not touch the bottom of the tube. In stage IV sporangiophore, the spore mass covering the columella was removed before plucking the sporangiophore from the mycelia, because the spore mass prevented the microscope observations of the cell contents which were stratified in the columella after centrifugation. To do so, the outer sporangial wall was broken with fine forceps and then the spores were removed by wiping the surface of the columella. No turgor loss was found, unless the columella was wounded. Stage I and II-III sporangiophores were centrifuged at 100,000 g for 30 min, and stage IV at 50,000 g for 30 min. For microscope observations, the successfully centrifuged sporangiophores were mounted in water under a long cover slip supported on a piece of thread.

Crystal Isolation

To isolate the crystals, only stage I and II–III sporangiophores were harvested. The procedures which were followed are shown in Fig. 1.

Protein Determination

For identification of protein in the crystals, two qualitative tests were applied. (a) The biuret reaction: the isolated crystals were dissolved in 1 N NaOH and then 1% CuSo4 was added, and a color change of the solution to reddish-violet or to bluish purple was observed; (b) The xanthoproteic reaction: the crystals were boiled in concentrated HNO₃ and, after cooling, NH₃ was added. The solution was allowed to develop to an orange color.

For quantitative estimation of protein, the isolated crystals were washed with distilled water and weighed after drying first in a vacuum desiccator at 4°C and then under a spotlight lamp. The dried crystals were then dissolved in 0.1 N NaOH. The method of Lowry et al. (1951) was used to determine the protein concentration in the solution. Bovine
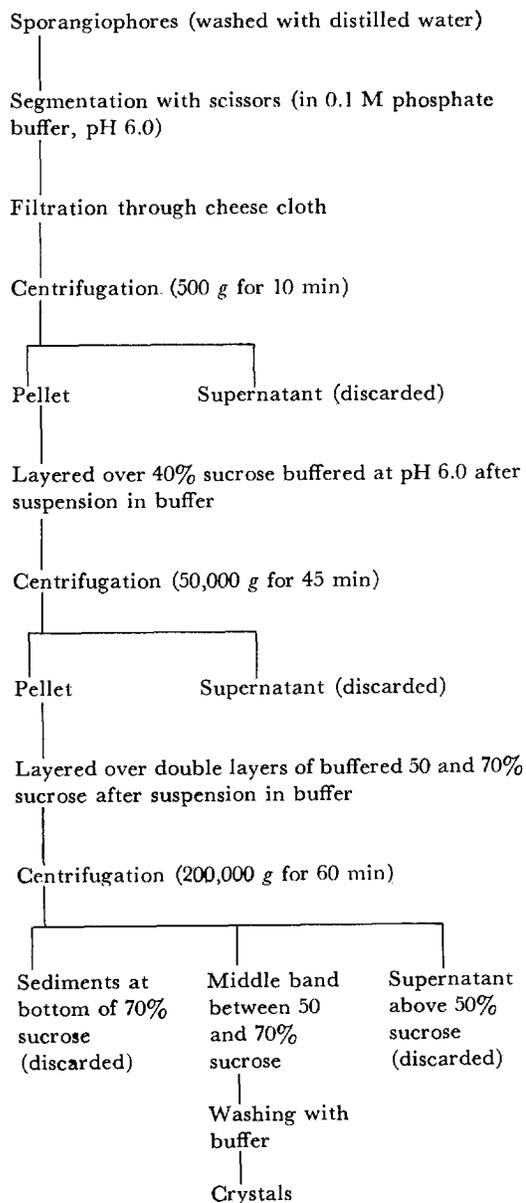
Sporangiophores (washed with distilled water)

Segmentation with scissors (in 0.1 M phosphate buffer, pH 6.0)

Filtration through cheese cloth

Centrifugation (500 g for 10 min)

Pellet   Supernatant (discarded)

Layered over 40% sucrose buffered at pH 6.0 after suspension in buffer

Centrifugation (50,000 g for 45 min)

Pellet   Supernatant (discarded)

Layered over double layers of buffered 50 and 70% sucrose after suspension in buffer

Centrifugation (200,000 g for 60 min)

Sediments at bottom of 70% sucrose (discarded)   Middle band between 50 and 70% sucrose   Supernatant above 50% sucrose (discarded)

Washing with buffer

Crystals

FIGURE 1 Procedure for isolation of crystals from *Phycomyces* sporangiophores.

albumin fraction V (Sigma Chemical Co., St. Louis, Mo.) was used for a standard.

Spectrophotometry

To obtain an absorption spectrum of a single crystal, the crystals were isolated as shown in Fig. 1, or squeezed out of either stage I or II-III sporangiophores under red light. They were mounted between quartz slide and quartz cover slip in 0.1 M phosphate buffer, pH 6.0. The cover slip was lightly pressed with the tips of forceps to obtain a monolayer of crystals. Relatively large crystals were chosen under a microscope by use of red background illumination. The area of the light spot on a crystal for absorption spectrum was 1.8 μm^2. The spectrum was obtained with a recording microspectrophotometer M-5 (Wolken et al., 1968; Wolken, 1969).

For the absorption spectrum from a mass of crystals, special cells were constructed. Fig. 2 A, shows the cell components and Fig. 2 B, the assembled cell. A piece of parafilm (b, Fig. 2) was sandwiched by two quartz slides (a, Fig. 2) to cancel the scattering effect of the crystals. Onto this, three U-shaped plastic pieces (each 0.2 mm thick; c, Fig. 2) and quartz cover slips (d, Fig. 2) were alternately glued with silicon rubber cement. The cells were completely dried for more than 48 h at room temperature after washing. The crystals in 0.1 M phosphate buffer (pH 6.0) were then transferred in three layers of space of the sample cell with a micropipette under red light. A slight sedimentation of crystals occurred in this cell during the measurement. The reference cell contained only the buffer solution. Absorption spectra were obtained with a Cary Recording Spectrophotometer, Model 14, with a 0-0.2 slidewire. To see whether the spectrum changed after light illumination, the samples were irradiated with white light of 5×10^4 $\mu W/cm_2$ for 30 s and the absorption spectrum was compared with that of the dark-adapted crystals.

Fluorescence

To determine the fluorescence from the crystals in a single sporangiophore, the sporangiophores were centrifuged at different stages of development by the method described for stratifying the cell contents. After centrifugation, the sporangiophores were mounted on a slide in either 0.1 M phosphate buffer (pH 6.0) or distilled water. Fluorescence was measured with a Zeiss fluorescence microscope adapted with a high pressure mercury bulb, HBO 200 W. The exciting light had a range from 392 to 504 nm with a peak at 455 nm. This was possible by a combination of the built-in exciter filter BG 38 (300-700 nm), the Plexiglas filter #2424, and the Corning glass filter #5543. The fluorescence emission was detected above 530 nm.

To obtain a fluorescence spectrum, a suspension of the isolated crystals in buffer solution (pH 6.0) was transferred into a glass capillary (inner diameter of 0.9-1.1 mm and 2.5 cm length, Kimax #34502). One end of the capillary had been tapered and sealed. The capillary was hung in a centrifuge tube, the sealed end down. The method was similar to that used for centrifuging a single sporangiophore;
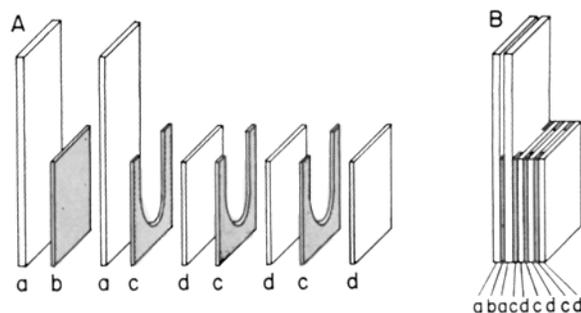
FIGURE 2 Schematic drawing of cell chamber constructed for the Cary-14 Spectrophotometer to obtain the absorption spectrum of the isolated crystals. (A) Arrangement of parts. a, quartz slides; b, parafilm sheet; c, cell well: plastic sheets 0.2 mm thick, with a U shape cut 18 mm deep and 10 mm wide; d, quartz cover slips. (B) Assembled cell chamber.

the centrifugal force applied was 500 g for 10 min. After centrifugation, the capillary was laid in immersion oil on a slide and the sedimented crystals were brought into focus under the microscope of a microfluorometer. The microfluorometer used to obtain the fluorescence spectra was constructed in our laboratory with an EMI 9558B photomultiplier tube, Zeiss fluorescence microscope, two Bausch and Lomb grating monochromators, a Hewlett-Packard voltmeter (model 412A) and a xenon lamp. All preparations of the samples after harvesting were performed under red light.

RESULTS

Observations of Phycomyces Growth

Changes in light intensity had no effect on the growth rate of the mycelia, but greatly affected the sporangiophore initiation. The density of spore seeding also affected the sporangiophore initiation. The more spores used for inoculation, the faster the sporangiophore emerged, but this also accelerated dwarf sporangiophore formation. Therefore, in these experiments, 3 spores/cm² were used for inoculation. Under these conditions, the first crop of sporangiophores appeared after 47 h from inoculation under 10 μW/cm². The length of stage II-III sporangiophores greatly depended on the light intensity: At 10 μW/cm² it was 3 cm, and from 160 to 640 μW/cm² it was 0.5 cm.

When Phycomyces is cultured in darkness, it was found that more than 72 h were necessary before the appearance of the sporangiophores on the mycelia. Many more sporangiophores were initiated in darkness than in the light. These sporangiophores were very wavy and thin, and

many dwarf sporangiophores appeared. Sporangium formation was also delayed and did not appear until the sporangiophore length was about 5.7 cm. The mycelia, sporangiophores, and sporangia were very pale yellow compared with the bright yellow of the light-grown cultures (Garton et al., 1951; Mackinney, 1952; Chichester et al., 1954; Lilly et al., 1960).

In the mutants, strains C5 and C141, sporangiophore formation was greatly inhibited and dwarf sporangiophores often appeared at intensities below 80 μW/cm² of white light. Sometimes no sporangiophores appeared in darkness, even after 144 h. In the light, above 160 μW/cm², sporangiophores emerged which increased in length and diameter to 180 μm. The length of stage II-III sporangiophores above 160 μW/cm² was similar to that of the wild strain. The sporangiophores as well as the mycelia were white in both mutant strains, since they are deficient in β-carotene (Bergman et al., 1969).

Observations of Crystals

Crystals are randomly oriented in sporangiophores from stages I through IV in all strains (Fig. 3). Several crystals were found even in the sporangiophores less than 0.2 mm in length. They were not observed in the vegetative hyphae, the storage vesicles, and the spores.

These crystals are translucent and octahedral. Each of the crystal faces is an equilateral triangle (Figs. 4 a, b). Although the size of the crystals varies, they averaged about 8 μm in a crystallographic axis in stage I and II-III.

Table I shows the intracellular localization of crystals in 5-mm long stage I sporangiophores of

the wild type and the mutants, grown under the same light condition (160 μW/cm²). In none of the stages were crystals detected within 100 μm from the tip. The first recognizable crystals were found in the cytoplasm at the region of 100–200
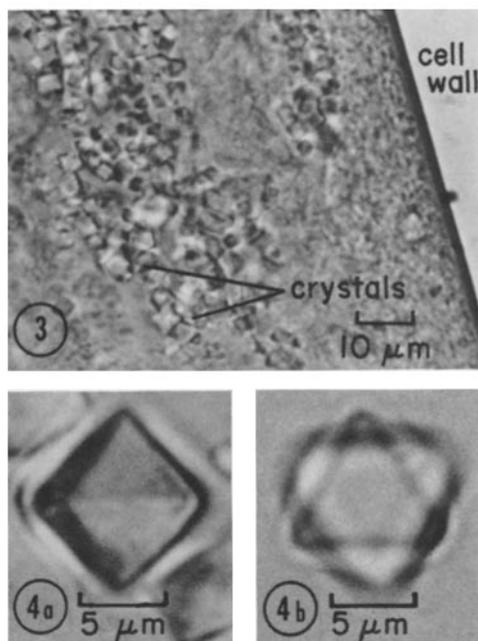

FIGURE 3 Crystals *in situ*, stage IV sporangiophore. × 760.

FIGURE 4 Octahedral crystals isolated from stage I sporangiophore. (a) Side view, (b) top view, showing equilateral triangular crystal faces. × 2,300.

μm from the tip, before the central vacuole. The crystals were almost uniformly spread throughout the rest of the cell, except the extreme base. The crystals were mainly in the central vacuole near the tonoplast membrane, but a few crystals were also observed in the cytoplasm surrounding the vacuole. In stage II-III and IV sporangiophores, their distribution was similar to that observed in stage I. Several crystals were found in the yellow or white sporangia of stage II-III sporangiophores and in the columella of stage IV, but no specific accumulation was observed in the swelling sporangia or in the growing zone immediately below the black sporangia. Thus, no differences between the wild strain and the mutants were recognizable regarding the intracellular distribution of crystals through stages I–IV. The wild-type sporangiophores, however, contained about twice as many crystals as the C5 and six times as many as the mutant C141 sporangiophores. In stage IV, the crystals were much smaller.

Light Effects on Crystal Formation

Since it was found that wild type produced more crystals than the β-carotene-deficient mutants, only the wild type was used in the following experiments. In white light, the total number of crystals per sporangiophore consistently increased until early stage IV and then gradually decreased (Table II). In darkness, no significant difference was found between young stage IV sporangiophores and stage II-III. Irradiation with white light reduced the number of crystals in the sporangiophores at all stages of growth. A nearly twofold difference was found between the dark-

TABLE I

Intracellular Location of Crystals, Stage I (5-mm Long Sporangiophores)

Regions of sporangiophore: (distance from sporangiophore tip in mm)	Crystal number/unit volume of sporangiophore (5 × 10⁵ μm³)		
	Wild type	C5	C141
0.0–0.1	0	0	0
0.1–0.2	10.0 ± 2.0	3.0 ± 0.8	1.0 ± 0.7
1.0–1.1	14.2 ± 1.7	6.5 ± 1.4	3.1 ± 0.3
2.0–2.1	13.0 ± 1.1	4.8 ± 1.3	2.2 ± 0.2
3.0–3.1	13.0 ± 1.4	7.9 ± 0.8	2.0 ± 0.5
4.0–4.1	13.3 ± 1.9	8.3 ± 1.9	5.1 ± 1.0
4.9–5.0	0	0	0

Sporangiophores were cultured under 160 μW/cm² of white light. Number of individuals, six for the wild type and the C141, and five for the C5. Variation is given as the standard error of the mean.

Table II

Number of Crystals in Wild Type at Different Stages of Growth

| | Light conditions | | |
Stage of sporangiophore	Darkness	10 μW/cm^2	640 μW/cm^2
Crystal number/single sporangiophore ($\times$ 10^2)			
I	97 $\pm$ 4 (100)	34 $\pm$ 4 (210)	31 $\pm$ 1 (120)
II–III	497 $\pm$ 21 (50)*	212 $\pm$ 33 (110)	81 $\pm$ 6 (50)
IVa	398 $\pm$ 51 (25)*	334 $\pm$ 31 (70)	198 $\pm$ 17 (50)
IVb (old)	169 $\pm$ 47 (80)	55 $\pm$ 6 (50)	47 $\pm$ 7 (50)
Crystal number/fresh weight (mg) of sporangiophores ($\times$ 10^2)			
I	438 $\pm$ 29 (100)	267 $\pm$ 12 (210)	218 $\pm$ 8 (120)
II–III	350 $\pm$ 15 (50)	201 $\pm$ 3 (110)	188 $\pm$ 5 (50)
IVa	203 $\pm$ 21 (25)	167 $\pm$ 15 (70)	115 $\pm$ 6 (50)
IVb (old)	77 $\pm$ 16 (80)	35 $\pm$ 4 (50)	32 $\pm$ 2 (50)

The length of sporangiophores; in darkness, 0.1 cm for stage I, 4–6 cm for stage II–III, 5–7 cm for stage IVa, and 13–15 cm for stage IVb old; under 10 μW/cm^2, respectively, 0.5 cm, 2.5–3.5 cm, 3–5 cm, and 12–15 cm; under 640 μW/cm^2, 0.5 cm, 0.5–1.0 cm, 3–5 cm, and 12–15 cm.

Variation is given as the standard error of the mean. The number of individuals is in parentheses.

*Difference between means at $P = 0.05$ was not significant. Least significant difference = 117 $\times$ 10^2.

and the light-grown (640 μW/cm^2) cultures (Table II). The crystal density was highest in stage I sporangiophores for all the light conditions used.

Stratified Crystals in Sporangiophore

At all developmental stages the crystals could be concentrated into a layer by high speed centrifugation. The separation of the organelles in a single stage I sporangiophore (Fig. 5 a, b) was similar to that described by Zalokar (1969). The major layer of crystals occupied a position around 0.8 mm from the tip, just before the mitochondrial layer. The crystal layer usually consisted of two sublayers. Some crystals were also found at the centrifugal tip of the sporangiophore. In a stage II-III sporangiophore the main crystal layer also consisted of two sublayers, and appeared in the sporangium, at the position of 300 μm from the tip (Fig. 5 c). A few crystals also sedimented to the sporangium tip. The volume of the main crystal layer was much larger in the stage II-III sporangiophore than in stage I, supporting the observation that the total number of crystals per sporangiophore was greater in stage II-III

(Table II). In stage IV, the crystal layer appeared in the base of the columella (Fig. 5 d).

Isolated Crystals and their Properties

Young stage IV sporangiophores grown in darkness contain the maximum concentration of crystals. However, these sporangiophores are not suitable for crystal isolation, because we found it difficult to separate the crystals from the contaminating spores. In addition, the formation of dwarf sporangiophores, which favors spore contamination, is accelerated in darkness. As a consequence these crystals were isolated only from stage I and II-III sporangiophores. To increase the crystal yield, Phycomyces were cultured under dim white light ($<$10 μW/cm^2), in which long but still quite thick sporangiophores were obtained.

Crystals obtained by the method of sucrose density-gradient centrifugation are shown in Fig. 6. In the final step of the isolation, some crystals sedimented to the bottom of 70% sucrose, although the main crystal fraction appeared between 50 and 70% sucrose as a white band. These heavier crystals may correspond to the crystals which sedimented to the centrifugal ends
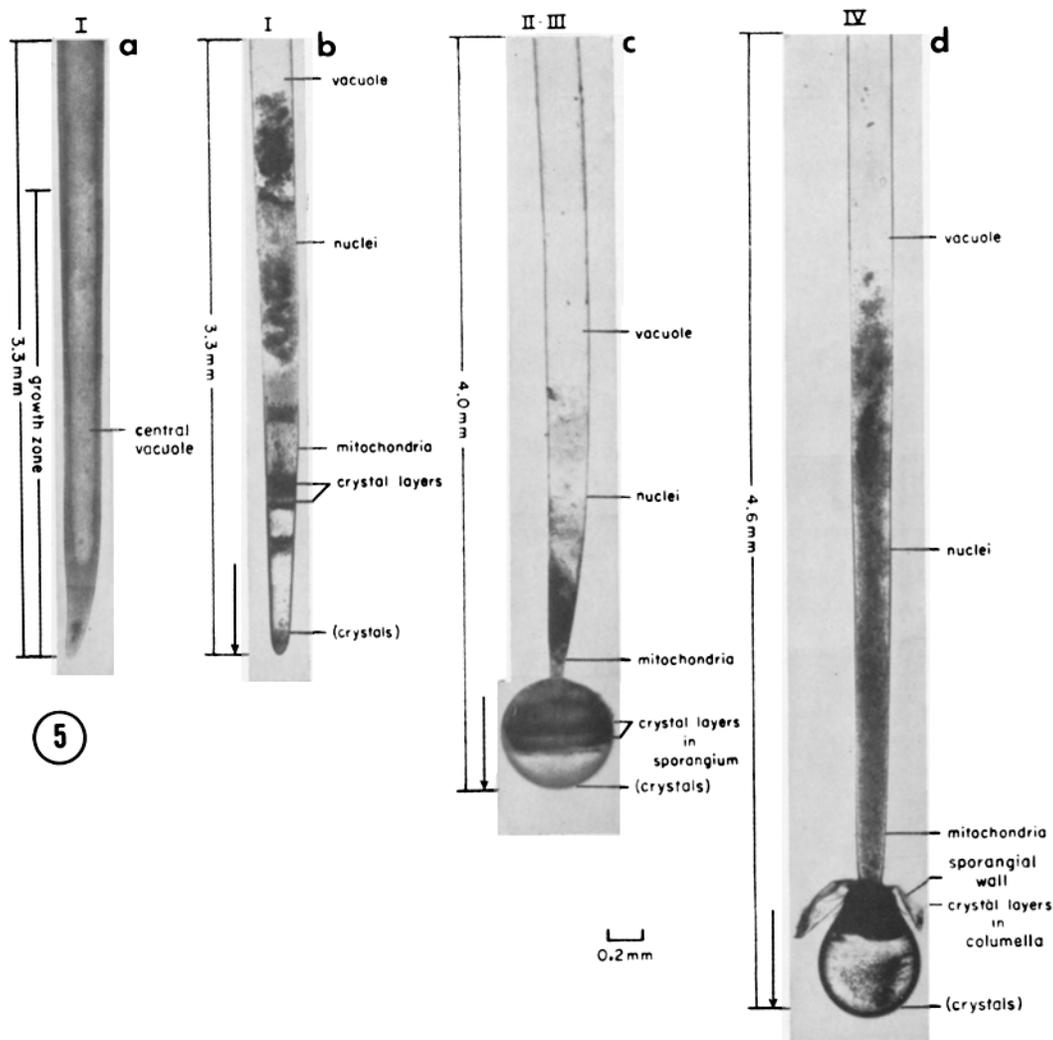
FIGURE 5 (a) Wild-type sporangiophore stage I, apical region, before centrifugation. Shown inversely to compare with the centrifuged sporangiophores. (b) Stage I sporangiophore centrifuged at 100,000 g for 30 min. The crystal band consists of two layers at a distance of about 0.8 mm from the tip, and crystals sedimented to the tip. (c) Stage II–III sporangiophore centrifuged at 100,000 g for 30 min. (d) Stage IV sporangiophore centrifuged at 50,000 g for 30 min, all crystals sedimented into sporangium. Cells cultured under 10 μW/cm^2 of white light. Arrows show the direction of the centrifugal force. $\times$ 23.

of the centrifuged sporangiophores. No obvious morphological differences were recognizable between these two crystals which sedimented at different densities. Stages I and II-III cultured under 100 μW/cm^2 of white light yielded about 8 mg wet crystals/g sporangiophores.

The isolated crystals were positive for both biuret and xanthoproteic reactions. The protein content was estimated to be about 95%, using the method of Lowry et al. (1951). Lipids were also

indicated by the stain reaction with Sudan black B. The crystals were insoluble in ethyl alcohol, methyl alcohol, ether, petroleum ether, and acetone. The crystals could be solubilized in buffer solutions above pH 9.5 or below 2.5. No clear "salting-in" effect was found, i.e., an addition of low concentration (0.02 M) of neutral salt (NaCl) was ineffective in increasing the solubility. The crystals were very soluble in NaOH of higher concentration than 1×10^{-3} N. No effect was
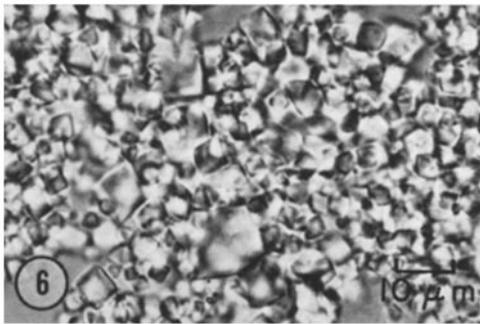
FIGURE 6 Octahedral crystals isolated from stage I and II–III sporangiophores by sucrose density-gradient centrifugation. × 720.

found in urea (6 M). In acids, concentrated H₂SO₄ immediately dissolved the crystals; in concentrations of 1 N, 1 × 10⁻¹ and 1 × 10⁻² N, a marked shrinkage occurred. The crystals were soluble in 10% acetic acid, but stable in 100%. The crystals were stable in 1 N HCl, with shrinkage occurring, but could be solubilized at concentrations of 1 × 10⁻¹ N, 1 × 10⁻² N, and 5 × 10⁻² N. Therefore, the solubility of these crystals in acids was irregular, probably because of protein denaturation. Digitonin (1% in 0.1 M phosphate buffer at pH 8.0 or 9.0) was not effective, but in trypsin (0.5 or 1.0% in 0.1 M phosphate buffer at pH 8.0, 30°C) the crystals dissolved within 2–3 h.

Polarization of Crystals

Crystals in the sporangiophore, freely floating as well as those moving in the sporangiophore vacuole, showed no birefringence, nor did the isolated crystals.

Absorption Spectra

Absorption spectra of *in situ* octahedral crystals obtained by microspectrophotometry showed absorption maxima around 271–284 nm, 330–370 nm, and near 460 nm. A single crystal isolated from a stage I sporangiophore showed absorption peaks about 276, 350, and 470 nm (Fig. 7). No differences in absorption spectra were found for crystals from stage I and from stage II-III sporangiophores.

Absorption spectra from isolated crystals which were dark adapted indicated absorption peaks around 275–285 nm, 350–360 nm, and 450–470 nm (Fig. 8 *a*). Irradiation of these crystals with white light bleached the visible peaks (Fig. 8 *b*).
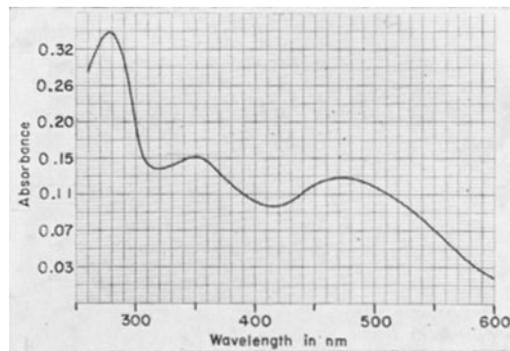

FIGURE 7 Absorption spectrum of a single crystal squeezed out of a stage I sporangiophore. Obtained by microspectrophotometry, spot size 1.5 μm × 1.25 μm. (Traced from original spectrum.)
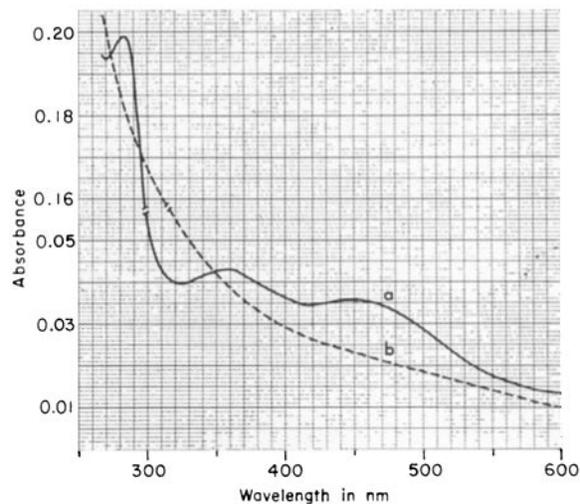

FIGURE 8 Absorption spectrum of a mass of crystals isolated from stage I and II–III sporangiophores. (*a*) Dark adapted, (*b*) after photobleaching with 5 × 10⁴ μW/cm² of white light for 30 s. Spectra obtained using cell chamber, Fig. 2, and Cary-14 Spectrophotometer. (Traced from original spectra.)

Fluorescence of Crystals

The sporangiophores through stages I to IV showed an overall intense green fluorescence on the tonoplast membrane or in the cell sap. However, no detectable fluorescence was observed for an *in situ* crystal or for an isolated crystal.

In the centrifuged sporangiophores, stages I to IV, the main crystal layer consisting of two layers exhibited a moderate amount of green fluo-
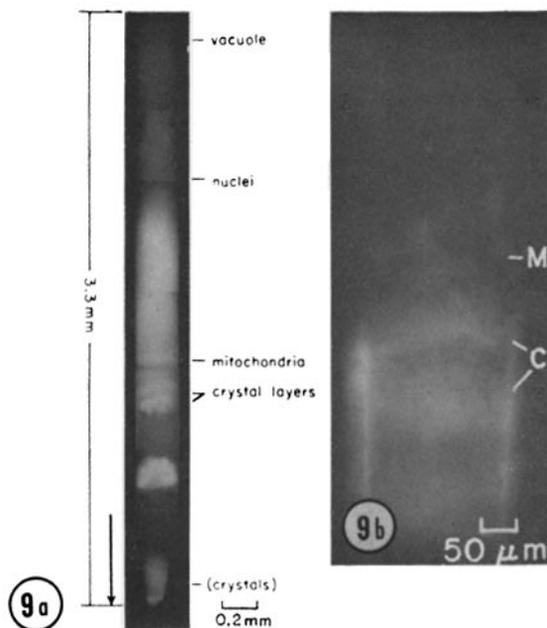
FIGURE 9 a Centrifuged stage I sporangiophore fluorescence of the stratified cell contents. Excitation at 455 nm (range 392–504 nm). Emission detected above 530 nm. Arrow shows the direction of centrifugal force. × 23.

FIGURE 9 b Enlargement of Fig. 9 a showing fluorescence of the crystal layers. Region of mitochondria stained with nitro-blue-tetrazolium to eliminate mitochondria fluorescence. C, crystal layers. M, mitochondria layer (dark region). × 100. Photomicrographs (Fig. 9 a, b) obtained with Zeiss fluorescent microscope.

rescence. Fig. 9 a shows the fluorescence of a centrifuged stage I sporangiophore. The mitochondria, stratified adjacent on the centripetal side, showed an intense fluorescence. Therefore, it was suspected that the fluorescence of the crystal layers might originate from mitochondria mixed with the crystal layers. To determine whether this was so, the mitochondria of the centrifuged sporangiophores were stained blue-black with nitro-blue-tetrazolium (Pearse, 1960). The stained mitochondria remained dark under the fluorescence microscope without interfering with the fluorescence of the crystal layers. Under these conditions, the crystals still emitted a moderate green fluorescence (Fig. 9 b).

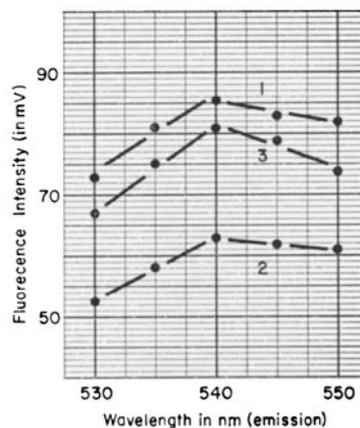Since the intensity of fluorescence of the crystal

FIGURE 10 Fluorescence spectra of crystals isolated from stage I and II–III sporangiophores. Excitation at 465 nm, emission spectra of (1) dark-adapted crystals, (2) immediately after irradiation with 5×10^4 μW/cm^2 of white light for 15 s. (3) After 10 min in darkness.

layers in the centrifuged sporangiophores was still too weak to measure by the microfluorometer, abundant isolated crystals were packed in a glass capillary tube to produce measurable fluorescence. The fluorescence emission peak appeared at 540 nm (Fig. 10). Irradiation of the crystals with 5×10^4 μW/cm^2 of white light for 15 s clearly decreased the fluorescence intensity, followed by a gradual recovery in darkness. However, the fluorescence intensity often increased when the crystals were irradiated for more than 1 min.

DISCUSSION

Crystals are found in *Phycomyces* sporangiophores. Previously, we described birefringent rod crystals which appear to be aligned along the plasma membrane or vacuole in the growth zone of stage IVb sporangiophores (Wolken, 1969). The rod crystals are about 1 μm in thickness and range from 2 to 10 μm in length. Microspectrophotometry of the *in situ* rods indicated that their absorption spectrum was similar to that of crystallized riboflavin. Also, in the growth zone and throughout the sporangiophore are octahedral-shaped crystals which are not birefringent. These crystals are similar in structure to those described by Thornton (1969) and Zalokar (1969) and were the object of this investigation.

Thornton (1969) found by cytochemical tests that these crystals were proteinaceous and did not contain calcium oxalate, gallic acid, polyindol, or

carbohydrate, which confirmed van Tieghem's (1875) early description. In addition, Thornton (1969) observed that the number of these crystals decreased with time in the light, which we confirmed.

We established that these crystals are octahedrals. First, they showed no birefringence under crossed nicols and passed the polarized light unchanged. They can be placed in the isometric system having three equal crystallographic axes which cross at right angles to each other. This information together with our microscope observation of the crystal structure (Fig. 4) confirmed that the crystal was a regular octahedron with 24 symmetry elements (Fig. 11).

The axial ratio (X:Y:Z) of the crystal is 1:1:1, and each crystal face is an equilateral triangle. Establishing the crystal structure made it possible to compute the angles of cut crystal sections observed in our electron micrographs. It was also important to know the angles of cuts of the crystal sections, in order to optically diffract the electron micrographs. Based on the computation of the angles of cut of the sections and on the optical diffraction pattern, a model for the unit cell was proposed (Wolken, 1972). The mol wt estimated from this model was of the order of 450,000.

The hypothesis that the crystals might be associated with the photoreceptor system (Thornton and Thimann, 1964; Wolken, 1969, 1972) must now be examined. The absorption spectrum of the crystal shows peaks around 270–285, 350–380, and 450–465 nm in the visible part of the spectrum (Figs. 7 and 8). These absorption maxima are close to the peaks 280, 370–385, 445–455, and 470–485 nm, found in the action spectra for phototropism and light-growth response of the sporangiophores (Curry and Gruen, 1959; Delbrück and Shropshire, 1960). Together with the absorption spectrum, the photobleaching of the visible peak near 465 nm (Fig. 8) and the emission of weak fluorescence (Fig. 9 b) would suggest that these crystals contain a flavine or flavoprotein and could participate in the photoprocess. Flavoproteins generally show very weak fluorescence and their absorption spectra maxima lie near 280, 380, and 450 nm, indicating that they could be the photoreceptor molecule in Phycomyces (Reinert, 1952; Carlile, 1957, 1962, 1965; Delbrück and Shropshire, 1960; Berns and Vaughn, 1970). If we compare the spectrum of the photobleaching of the visual pigment rhodopsin, there is a similarity between them. That is, there is loss

$$6C_4 \cdot 3C_2 = 3C_4^2$$

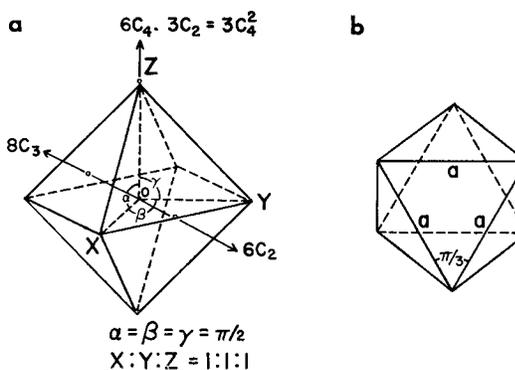$$a = \beta = \gamma = \pi/2$$
$$X:Y:Z = 1:1:1$$

FIGURE 11 The *Phycomyces* regular octahedral crystal. (a) A drawing showing the rotation symmetries, and (b) the equilateral crystal faces. Compare with Fig. 4 a, b.

in absorbance of the major peak in the visible region of the spectrum around 450–500 nm. Although it was thought for some time that only animal cells could synthesize retinal from its precursor β-carotene, it was recently found that a rhodopsin-like pigment complex could be extracted from the membranes of a bacterium, *Halobacterium halobium* (Oesterhelt and Stoeckenius, 1971). In the wild type *Phycomyces* sporangiophores small quantities of retinal were detected (Meissner and Delbrück, 1968). This suggested that a rhodopsin-like pigment was also the photoreceptor molecule in *Phycomyces*. However, this possibility for the present can be ruled out, for it was then found that in the β-carotene-deficient mutant which was still fully photosensitive, no retinal could be detected.

Although crystals can be found throughout the sporangiophore, their major concentration is in the growth zone (Table I). Therefore, it is quite possible that the crystals located in the growth zone are the functionally active ones. In many fungal sporangiophores and hyphae, the apical or growth zone differs from the subapical region with respect to the localization and activity of various organelles and enzymes (Zalokar, 1965). In *Phycomyces* sporangiophores, the mitochondria in the growth zone have well-developed cristae compared with those in the subapical region, suggesting a higher metabolic activity in the growth zone (Peat and Banbury, 1967). This structural observation for the mitochondria in the growth zone could reflect the state of its cytochrome. If the crystals in the growth zone are

photoreduced and oxidized via a cytochrome, a photoreversible receptor system then exists.

In the absence of finding a structure which could be identified as *Phycomyces* photoreceptor, it is worth considering that the photoreceptor structure is a crystal. A photopigment in or associated with a crystalline structure would serve as an efficient device for light capture, since it brings molecules close together for interaction and, therefore, for energy transfer. But we cannot specifically say that the octahedral crystal is the photoreceptor. We have recently isolated, in addition to the octahedral crystals, needle-, rod-, and rhomboid-shaped crystals. The rod and octahedral crystals contain a flavin or flavoprotein which becomes photoreduced to a lumichrome or semiquinone, and therefore a flavin could function in these crystals as the photoreceptor molecule.

REFERENCES

BERGMAN, K., P. V. BURKE, E. CEDRA-OLMEDO, C. N. DAVID, M. DELBRÜCK, K. W. FOSTER, E. W. GOODELL, M. HEISENBERG, G. MEISSNER, M. ZALOKAR, D. S. DENNISON, and W. SHROPSHIRE, JR. 1969. *Phycomyces. Bacteriol. Rev.* 33:99.

BERNS, D. S., and J. R. VAUGHN. 1970. Studies on the photopigment system in *Phycomyces. Biochem. Biophys. Res. Commun.* 39:1094.

CARLILE, M. J. 1957. Phototropism of *Phycomyces* sporangiophores. *Nature (Lond.).* 180:202.

CARLILE, M. J. 1962. Evidence for a flavoprotein photoreceptor in *Phycomyces. J. Gen. Microbiol.* 28:161.

CARLILE, M. J. 1965. The photobiology of fungi. *Annu. Rev. Plant Physiol.* 16:175.

CASTLE, E. S. 1942. Spiral growth and reversal of spiraling in *Phycomyces*, and their bearing on primary wall structure. *Am. J. Bot.* 29:664.

CHICHESTER, C. O., P. S. WONG, and G. MACKINNEY. 1954. On the biosynthesis of carotenoids. *Plant Physiol.* 29:238.

CURRY, G. M., and H. E. GRUEN. 1959. Action spectra for the positive and negative phototropism of *Phycomyces* sporangiophores. *Proc. Natl. Acad. Sci. U.S.A.* 45:797.

DELBRÜCK, M., and W. SHROPSHIRE, JR. 1960. Action of transmission spectra of *Phycomyces. Plant Physiol.* 35:194.

GARTON, G. A., T. W. GOODWIN, and W. LIJINSKY. 1951. Studies in carotogenesis. I. General conditions governing β-carotene synthesis by the fungus *Phycomyces blakesleeanus* Burgeff. *Biochem. J.* 48:154.

LILLY, V. B., H. L. BARNETT, and R. F. KRAUSE. 1960. The production of carotene by *Phycomyces blakesleeanus. W. Va. Agric. Exp. Stn. Bull.* 441 T:5.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.

MACKINNEY, G. 1952. Carotenoids. *Annu. Rev. Biochem.* 21:473.

MEISSNER, G., and M. DELBRÜCK. 1968. Carotenes and retinal in *Phycomyces* mutants. *Plant Physiol.* 43:1279.

OESTERHELT, D., and W. STOECKENIUS. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium. Nature (Lond.).* 233:149.

PEARSE, A. G. E. 1960. Histochemistry, Theoretical and Applied. J. and A. Churchill Ltd., London.

PEAT, A., and G. H. BANBURY. 1967. Ultrastructure, protoplasmic streaming, growth and tropisms of *Phycomyces* sporangiophores. *New Phytol.* 66:475.

REINERT, J. 1952. Uber die Bedeutung von Carotin und Riboflavin für die Lichtreizaufnahme bei Pflanzen. *Naturwissenschaften.* 39:47.

SHROPSHIRE, W., JR. 1963. Photoresponses of the fungus *Phycomyces. Physiol. Rev.* 43:38.

THORNTON, R. M. 1969. Crystalloids of *Phycomyces* sporangiophores. Nature and photosensitive accumulation. *Plant Physiol.* 44:861.

THORNTON, R. M., and K. V. THIMANN. 1964. On a crystal-containing body in cells of the oat coleoptile. *J. Cell Biol.* 20:345.

VAN TIEGHEM, M. P. 1875. Nouvelles recherches sur les mucorinees. *Ann. Sci. Nat. Bot.* 1:5.

WOLKEN, J. J. 1969. Microspectrophotometry and the photoreceptor of *Phycomyces.* I. *J. Cell Biol.* 43:354.

WOLKEN, J. J. 1972. *Phycomyces.* A model photosensory cell. *Int. J. Neurosci.* 3:135.

WOLKEN, J. J., R. FORSBERG, G. J. GALLIK, and R. G. FLORIDA. 1968. Rapid recording microspectrophotometer. *Rev. Sci. Instrum.* 39:1734.

WOLKEN, J. J., and C. S. NAKAGAWA. 1973. Photosensitivity and crystals in *Phycomyces* sporangiophores. *Biophys. Soc. Annu. Meet. Abstr.* 13: 233a.

ZALOKAR, M. 1965. Integration of cellular metabolism. *In* The Fungi. G. C. Ainsworth and A. S. Sussman, editors. Academic Press Inc., New York. 1:377.

ZALOKAR, M. 1969. Intracellular centrifugal separation of organelles in *Phycomyces. J. Cell Biol.* 41:494.