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 ß-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 sporangiophores 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, Phycomyces 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 function 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. Microspectrophotometry 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 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

Washing with buffer

Crystals

Supernatant above 50% sucrose (discarded)

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

Centrifugation (500 g for 10 min)

Pellet

Supernatant (discarded)

FIGURE 1 Procedure for isolation of crystals from Phycomyces sporangiophores.

albunin 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 µm². 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 × 10⁴ µW/cm² 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 #5343. 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 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-

![Figure 3](image3.png) Crystals in situ, stage IV sporangiophore. ×760.

![Figure 4](image4.png) Octahedral crystals isolated from stage I sporangiophore. (a) Side view, (b) top view, showing equilateral triangular crystal faces. ×2,300.

**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.

228 The Journal of Cell Biology • Volume 57, 1973
TABLE II
Number of Crystals in Wild Type at Different Stages of Growth

| Stage of sporangiophore | Light conditions | Crystal number/single sporangiophore (× 10²) | Crystal number/fresh weight (mg) of sporangiophores (× 10²) |
|-------------------------|-----------------|---------------------------------------------|-------------------------------------------------------------|
|                         |                 | Darkness (10 µW/cm²) | 640 µW/cm² | Darkness (10 µW/cm²) | 640 µW/cm² |
| I                       |                 | 97 ± 4 (100) | 55 ± 6 (50) | 34 ± 4 (210) | 31 ± 1 (120) |
| II–III                  |                 | 497 ± 21 (50)* | 55 ± 6 (50) | 212 ± 33 (110) | 81 ± 6 (50) |
| IVa                     |                 | 398 ± 51 (25)* | 55 ± 6 (50) | 334 ± 31 (70) | 198 ± 17 (50) |
| IVb (old)               |                 | 169 ± 47 (80) | 55 ± 6 (50) | 334 ± 31 (70) | 198 ± 17 (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/cm², respectively, 0.5 cm, 2.5–3.5 cm, 3–5 cm, and 12–15 cm; under 640 µW/cm², 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 X 10².

and the light-grown (640 µW/cm²) 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 columnel (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/cm²), 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/cm² of white light. Arrows show the direction of the centrifugal force. X 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/cm² 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 X 10⁻² N. No effect was
found in urea (6 M). In acids, concentrated 
H$_2$SO$_4$ immediately dissolved the crystals; in
concentrations of 1 N, 1 × 10$^{-2}$ and 1 × 10$^{-3}$ 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 shrink-
age occurring, but could be solubilized at con-
centrations of 1 × 10$^{-2}$ N, 1 × 10$^{-3}$ 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).

**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. How-
ever, 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-
Fig. 9a. 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.

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

Fig. 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 $5 \times 10^4 \mu W/cm^2$ of white light for 15 s, (3) after 10 min in darkness.

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 a loss 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
photo-reduced 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.

We would like to acknowledge the helpful advice of Dr. A. J. Schultz, Jr., and the technical assistance of O. J. Bashor, Jr. and A. J. Wolken. We would also like to thank Professor Max Delbrück for reviewing the text.

T. Ootaki was supported by the Scaife Family Charitable Trust Fellowship to the Biophysical Research Laboratory, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213.

Received for publication 3 May 1972, and in revised form 27 December 1972.

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. Bacterial. 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. Nature (Lond.). 180:202.

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 photo-biology 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.

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

CERRY, G. M., and H. E. GRUEN. 1939. Action spectra for the positive and negative phototropism of Phycomyces sporangiophores. Proc. Natl. Acad. Sci. U.S.A. 45:797.

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

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

LILY, V. B., H. L. BARNETT, and R. F. KRAUSE. 1960. The production of carotene by Phycomyces blakesleeanus. W. Ya. Agric. Exp. Sta. Bull. 441 T5.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 154:265.

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

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

OBERTERHELT, 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. BANSURY. 1967. Ultrastructure, protoplasmic streaming, growth and tropisms of Phycomyces sporangiophores. New Physiol. 66:475.

REINERT, J. 1952. Über 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. 44:384.

THORNTON, R. M., and K. V. THIMANN. 1964. On a crystal-containing body in cells of the oat coleoptile. J. Cell Biol. 29:345.

VAN TIEGHEN, 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 microspectrophotometry. 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.