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Crystalloids of Phycomyces Sporangiophores: Nature and Photosensitive Accumulation[1]

Robert M. Thornton[2]
Division of Natural Sciences, University of California, Santa Cruz, California

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Abstract. **Crystalloids accumulate in the vacuoles of the giant sporangiophores of *Phycomyces blakesleeanus* Burgeff during growth. On the basis of solubility in alkaline solutions, cytochemical staining reactions, trypsin sensitivity, optical absorption and response in the Lowry protein test, the crystalloids have been judged to consist principally of an acidic protein. In assays by Lowry test and by reference to optical absorption at 280 mμ, dark-grown sporangiophores were consistently found to contain from 2 to 4 times as much crystalloid material as light-grown counterparts. Concurrent assays of soluble phenolic materials revealed no significant effect of culture illumination, while carotene content of sporangiophores and mycelium was found to be raised from 2 to 4-fold by illumination during growth.**

The growth rate of giant sporangiophores of *Phycomyces blakesleeanus* is transiently responsive to changes in illumination, but under steady bright illumination the growth rate is the same as in total darkness (2, 6). Such adaptation is not evident in all facets of the photoresponses of this fungus; it has long been known that light triggers an increase in the rate of accumulation of carotene that persists after a return to darkness (4, 10). Kinetic analyses have likewise pointed to the possibility that adaptation of the growth rate involves relatively large changes in metabolic pool levels (3, 7). Thus it seems that a study of constitutional changes in response to light may yield insights into the still elusive early steps in the photoresponses of this fungus. Toward that end, the present report describes light-induced changes in the accumulation of crystalloids in the sporangiophore.

The occurrence of octahedral crystalloids in the sporangiophores of mucoraceous fungi has been known since 1872 (12, 18). The crystalloids of *P. blakesleeanus* have been found by electron microscopic examination to consist of approximately isodiametric subunits in a cubic space lattice with spacings in the vicinity of 120Å (18, 23). In the same studies, crystalloids were found in sporangiophores that were as small as 1 % of the terminal size, but not in vegetative hyphae or storage vesicles. Crystalloids occur both free in the vacuole and in membrane-bounded vesicles in the cytoplasm. The origin of the crystalloids remains to be elucidated but the direction of the movement seems to be from the cytoplasm in the sporangiophore growing tip to the vacuole (20, 21). The present report further characterizes the crystalloids and, in fractionation studies, compares crystalloid photophysiology with that of carotene and phenolic materials in the sporangiophores. The results have been reported in brief elsewhere (22).

Materials and Methods

The strain *Phycomyces blakesleeanus* G5(+), provided by Dr. H. E. Gruen, was cultured at room temperature on petri dishes containing 25 g of sterile medium or in 50 ml plastic centrifuge tubes containing 5 g of medium. In experiment II of table I (below) the nutrient medium consisted of 1.5 g purified Difco agar, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 1.43 × 10^{-3} mg thiamin, and 1.3 mg asparagine per 100 ml of medium. In all other cases potato-dextrose-agar (11) was used.

For staining and examining individual crystalloids, cytoplasmic smears were obtained by squeezing droplets of sporangiophore sap onto microscope slides. The smears were fixed either by gentle flame heating or by brief immersion in 5 % aq. acrolein, and were treated with reagents (*cf.* Results) by perfusion under a cover glass during observation at 400 diameters with Zeiss and Wild phase and light-field microscopes.

For the isolation of crystalloid material, masses of sporangiophores in centrifuge-tube cultures were grasped with forceps, withdrawn and excised just above the nutrient medium. The cell contents were then expressed by finger pressure into mineral oil.

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[2] Present address: Botany Department, University of California, Davis, California 95616.

The culture tubes before and after excision of sporangiophores, and the pressed sporangiophore walls, were weighed on a Sartorius microanalytical electronic balance. These figures afforded measures of cell sap weights for standardization of results. The expressed sap was diluted with water and separated into crystalloid sediment, aqueous and floating oil phases by centrifugation at 500*g* for 5 min in a Servall benchtop centrifuge. The crystalloid sediment was washed several times with water and finally was dissolved in 0.1 M phosphate buffer at pH 9. The crystalloid solution was then separated from debris and spores by a final centrifugation and was examined for optical absorption with a Cary spectrophotometer and subjected to the Lowry phenol test for protein (13). During fractionation 0.01 ml aliquots of the various phases were removed, deposited on microscope slides, stained with Hg-bromphenol blue (14), and examined for crystalloid content. Both the original aqueous fraction after removal of crystalloids and the water washes of crystalloid fractions were checked for phenolic content by absorption spectrum determinations in a Cary spectrophotometer (single absorption peak, 266 mμ) and by test for blue-black or green color reactions in the presence of 6 N $FeCl_3$ (8). Carotene was collected from both the oil and aqueous phases of centrifuged sporangiophore sap and from mycelial mats by repeated extraction with ethyl ether. The ether extracts were brought to standard volume and assayed by reference to optical absorption at 454 mμ.

For tests of physiological light effects, centrifuge-tube cultures were incubated in both chambers of a divided box that was provided with continuous circulation of water-saturated air. One chamber was equipped with a top window covered with transparent colorless plastic to permit continuous illumination from ordinary laboratory fluorescent lamps. The other chamber was masked to exclude light, and the box as a whole was encased in plastic sheeting to equalize aeration conditions between the chambers. Sporangiophores were harvested after growth periods sufficient to permit maturation of nearly all fruiting structures (*cf.* table I); individual cultures were handled alternately from light- and dark-grown conditions to exclude systematic differences during collection of cell sap. In general, each figure for crystalloid, phenolic and carotene content was gained from a pooled set of 7 cultures.

Results and Discussion

The crystalloids of *P. blakesleeanus* reach dimensions of *ca.* 15 μ and therefore could be examined in protoplasmic smears at 400 diameters magnification during perfusion with cytochemical reagents as described by Tandler (17) in the case of *Acetabularia* crystalloids.

Calcium oxalate, a common component of plant and fungal cells, is decomposed in the presence of acidic 1 % K permanganate with the release of CO_2. This response was checked using known oxalate. Under similar treatment, the fixed *Phycomyces* crystalloids puff and immediately shrink to a stable size slightly smaller than before treatment. Thus while the crystalloids are subject to modification by permanganate, they are not composed of oxalate.

Gallic acid, abundant in the sporangiophore (1, 8), gives a black color reaction in the presence of 6 N $FeCl_3$ (8). With this reagent the crystalloids gave no color reaction, either in smears or in treatment of milligram quantities of isolated crystalloid material.

The polyindole crystalloids of *Acetabularia* stain with Salkowski and Ehrlich reagents (17). *Phycomyces* crystalloids were colorless under similar treatment, and therefore are not comparable to those of *Acetabularia*.

The presence of protein in the crystalloids was suggested by the development of blue color with Hg-bromphenol blue; red color in 1 % acid fuchsin (14); and red color in smears that were fixed with 5 % aq. acolein for a few min, washed, and stained with Schiff reagent (9). Schiff reagent alone gave no color reaction.

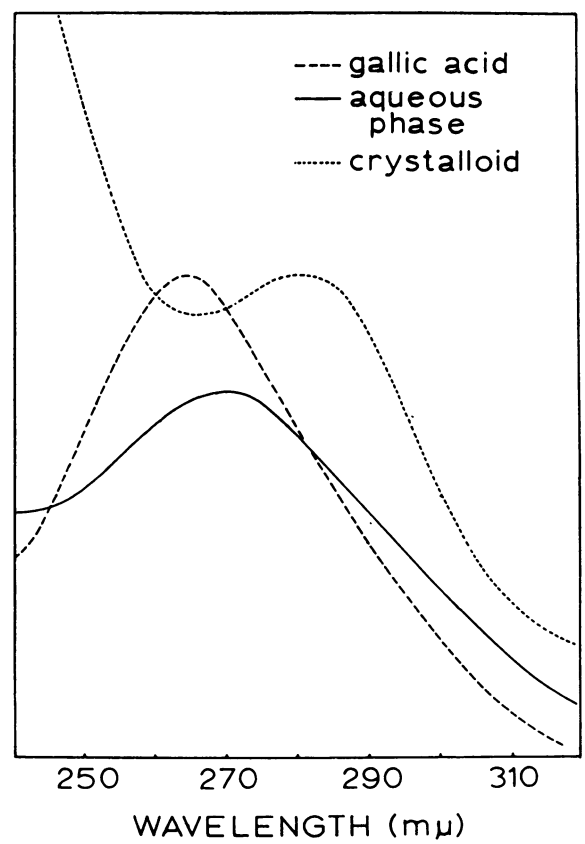

FIG. 1. Absorption spectra of pure gallic acid in water, crystalloid material in pH 9 phosphate buffer, and the aqueous phase of fractionated sporangiophore contents after removal of crystalloids and lipid (floating) phase.

The crystalloids dissolve immediately in media of pH above 7 but are stable in 1 N HCl. Crystalloids that were first fixed in 5 % acrolein and then incubated at 30° in pH 9 phosphate buffer were stable for at least 24 hr. The addition of 1 % trypsin to the buffer led to complete dissolution of the crystalloids within an hr.

The absence of carbohydrate was indicated by negative staining reactions to I_2-KI and PAS and by the absence of metachromatic staining with 1 % toluidine blue O. If lipids are present they must be sequestered or absent from structurally important positions, since the crystals were found to be stable in *n*-hexane, carbon tetrachloride, carbon disulfide, ethanol, and acetone.

For further characterization, crystalloids were collected from masses of sporangiophores by centrifugation of sporangiophore sap. Dissolved in phosphate buffer at pH 9, the crystalloid material was found to have no appreciable absorption above *ca.* 320 mμ, and exhibited the ultraviolet absorption spectrum in Fig. 1. This pattern, with a peak near 280 mμ and high end absorption below 240 mμ, is typical of protein in general. Aliquots of dissolved crystalloid material were also assayed for protein by the Lowry technique (13). When protein content was judged by the Lowry test against bovine serum albumin, comparison with the optical density of crystalloid solutions at 280 mμ indicated an absorption of about 1 OD unit per mg/ml of crystalloid material. This figure is typical of protein (C. N. David, personal communication).

These results indicate that the crystalloids contain appreciable amounts of a protein which has acidic groups in positions that are important to the integrity of the crystal. Since the sporangiophores are known to accumulate large amounts of ferritin, a further test for iron content was performed through the courtesy of Dr. C. N. David, yielding a protein: iron ratio of about 400 for the crystalloid material. Purified ferritin from *P. blakesleeanus* exhibits a protein:iron ratio of about 5. In addition, the crystalloid material does not show the 325 to 350 mμ absorption typical of ferritin (5; C. N. David, personal communication). Thus the crystalloids do not appear to consist of ferritin, though these observations do not rule out the protein moiety of ferritin as a crystalloid component.

The effect of illumination during growth on the crystalloid content of mature sporangiophores was checked by comparing crystalloid fractions from cultures that had been grown either in complete darkness or in continuous light. It is already known that the dry weight yield of *P. blakesleeanus* cultures is not greatly affected by conditions of illumination (10); nevertheless, individual cultures vary in yield. Thus before comparing Lowry assays and 280 mμ OD measures, the assay figures were normalized on a per unit cell sap basis. As shown in table I, illumination consistently reduced the crystalloid content of sporangiophores, resulting in a 2 to 3-fold difference between dark- and light-grown cultures on the average.

It has long been known that *P. blakesleeanus* accumulates large amounts of soluble phenolic compounds—principally gallic and protocatechuic acids—during growth (1, 8). Since these materials may participate in protein tanning reactions and may interfere with protein determinations, it was deemed necessary to ascertain whether the apparent photoresponsiveness of crystalloid content might not be a secondary effect that arose from photoresponsive phenolic metabolism. Thus in fractionation experiments the optical absorption arising from phenolic materials was monitored (Fig. 1). The phenolic materials were found to remain in the aqueous phase when crystalloids and oil droplets were removed from the sporangiophore sap. This conclusion was verified by similar confinement of the $FeCl_3$ color reaction to the aqueous fraction. The absorption spectrum shown in Fig. 1 represents a complex pattern with components from materials other than soluble phenolic compounds. However, the major absorption of the sporangiophore contents in the

Table I *Crystalloid Content of Sporangiophores in Light- and Dark-grown Cultures*

Results are given as percent of highest figure in each experiment after correction for differences in sporangiophore fresh weight.

Expt	Growth period	Crystalloid content as assessed by: Lowry test			285 mμ absorption		
		Dark	Light	Dark: light	Dark	Light	Dark: light
	days			*ratio*			*ratio*
I a	6				31	11	2.8
b					24	20	1.2
c					100	26	3.9
d					64	32	2.0
II a	7	64	33	1.9	98	55	1.8
b		100	42	2.4	100	62	1.6
III a	8	68	27	2.5			
		100	21	4.6			
Avg		83	31	2.7	70	34	2.1

266 mμ region is known to be associated with gallic acid (8, 16), presumably in a combined form (*cf.* comparison with pure gallic acid in Fig. 1); hence even in such impure extracts any sizeable photoresponse of soluble phenolic content should be apparent.

Table II shows the relationship between light- and dark-grown cultures with respect to absorption at 266 mμ. The differences were not found to be significant when checked by t-test of the difference between means. Thus there is no basis for holding that the accumulation of phenolic compounds is photoresponsive. The large effect of light on assayed crystalloid content must therefore be assigned to an effect on crystalloid metabolism.

Finally, ether extracts of sporangiophore contents were checked for carotene by reference to optical absorption at 454 mμ. Similar measures were taken of the mycelial mats for comparison with the published data of Garton *et al.* (10). As indicated in table II, growth in the light promotes carotene accumulation in both the mycelium and the sporangiophores; the average light/dark ratios are 3.7-fold and 2.8-fold respectively. These differences are somewhat larger than the reports of Garton *et al.* (10), but smaller than those of Schopfer, who reported no carotene at all in dark-grown cultures (15). Apparently the size of the photoeffect on carotene content varies with the culture conditions and the strain of *P. blakesleeanus*.

In sum, the present account indicates that the crystalloids of *P. blakesleeanus* consist of a protein, not identical with ferritin, which contains acidic groups in structurally important positions; and that the provision of light during growth reduces the crystalloid accumulation by a factor of 2 to 3. By contrast, light stimulates carotene accumulation by a similar factor. These opposed effects of light on crystalloid and carotene metabolism may be heuristically connected by suggesting that all the visible-light photoresponses of the fungus derive from effects of light on a single photoreceptive system, but further comments must at present be restricted to the general observation that carotene metabolism (4) as well as fruiting development (19) are responsive in a complex way to both light and nitrogen metabolism. The widespread occurrence of crystalloids in plant (23) and fungal (12) materials, together with the elusiveness of the early mechanism of light reactions in fungi, prompt additional studies of photoeffects in crystalloid metabolism.

Table II. *Carotene and Soluble Phenolic Content of Sporangiophores and Mycelium of Light- and Dark-grown Cultures*

Results are given as OD units per gram sporangiophores and per 7 mycelial mats.

Material	Sporangiophores			Mycelium		
	Light	Dark	Light : dark	Light	Dark	Light : dark
			ratio			*ratio*
Carotene	0.125	0.030	4.1	1.48	0.34	4.4
(OD 454 mμ)	0.032	0.012	2.6	1.30	0.41	3.2
	0.043	0.025	1.7			
Average	0.067	0.022	2.8	1.39	0.37	3.7
Phenolic materials	0.45	0.49				
(OD 266 mμ)	0.42	0.46				
Average	0.44	0.48	0.91			

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