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Invited Review

Photophysics and Multifunctionality of Hypericin-Like Pigments in Heterotrich Ciliates: A Phylogenetic Perspective

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ABSTRACT

In this paper, we review the literature and present some new data to examine the occurrence and photophysics of the diverse hypericin-like chromophores in heterotrichs, the photoresponses of the cells, the various roles of the pigments and the taxa that might be studied to advance our understanding of these pigments. Hypericin-like chromophores are known chemically and spectrally so far only from the stentorids and *Fabrea*, the latter now seen to be sister to stentorids in the phylogenetic tree. For three hypericin-like pigments, the structures are known but these probably do not account for all the colors seen in stentorids. At least eight physiological groups of *Stentor* exist depending on pigment color and presence/absence of zoochlorellae, and some species can be bleached, leading to many opportunities for comparison of pigment chemistry and cell behavior. Several different responses to light are exhibited among heterotrichs, sometimes by the same cell; in particular, cells with algal symbionts are photophilic in contrast to the well-studied sciaphilous (shade-loving) species. Hypericin-like pigments are involved in some well-known photophobic reactions but other pigments (rhodopsin and flavins) are also involved in photoresponses in heterotrichs and other protists. The best characterized role of hypericin-like pigments in heterotrichs is in photoresponses and they have at least twice evolved a role as photoreceptors. However, hypericin and hypericin-like pigments in diverse organisms more commonly serve as predator defense and the pigments are multifunctional in heterotrichs. A direct role for the pigments in UV protection is possible but evidence is equivocal. New observations are presented on a folliculinid from deep water, including physical characterization of its hypericin-like pigment and its phylogenetic position based on SSU rRNA sequences. The photophysics of hypericin and hypericin-like pigments is reviewed. Particular attention is given to how their excited-state properties are modified by the environment. Dramatic changes in excited-state behavior are observed as hypericin is moved from the homogeneous environment of organic solvents to the much more structured surroundings provided by the complexes it forms with proteins. Among these complexes, it is useful to consider the differences between environments where hypericin is not found naturally and those where it is, notably, for example, in heterotrichs. It is clear that interaction with a protein modifies the photophysics of hypericin and understanding the molecular basis of this interaction is one of the outstanding problems in elucidating the function of hypericin and hypericin-like chromophores.

INTRODUCTION

Hypericin (Fig. 1) is a widely occurring natural perylene quinone pigment, best known from flowering plants in the genus *Hypericum*, with a broad spectrum of light-induced biological activities (1–5). Hypericin-like compounds (Fig. 1) occur in diverse taxa and include stentorin, the blepharismins and maristentorin in heterotrich ciliates (6–10), fringelites in fossil crinoids (11), brominated gymnochromes in modern crinoids (11–14) and fagopyrin in *Fagopyrum* (buckwheat) (5). Among the more striking aspects of hypericin and the hypericin-like pigments are their absorption and emission spectra. Figure 2 presents these for hypericin. The absorption spectrum extends from the red (~610 nm) to the UV with no gaps of zero absorption. Equally striking are the mirror image symmetry of the fluorescence with the low-energy absorption spectrum and the relatively intense fluorescence of the free chromophore. In the class of broadly and intensely absorbing, naturally occurring pigments, the hypericins, which include the perylene quinones discussed here and the hypocrellins (2,15,16), are rivaled only by the porphyrins in their richness of photophysical behavior and the breadth of their biological activity. The ability of the hypericins to execute a wide variety of excited-state photochemistry (intramolecular hydrogen atom transfer [17–19], intermolecular proton transfer [20–26], oxidation and reduction [27–29] and generation of reactive oxygen species [1–3,30–33]) clearly contributes to their range of biological activities. For example, hypericin in *Hypericum* spp. (including St. John's Wort) causes sometimes-fatal toxicity (hypericism) in domestic grazing animals but its bioactivity makes it valuable for photodynamic therapy of tumors and other medicinal uses in

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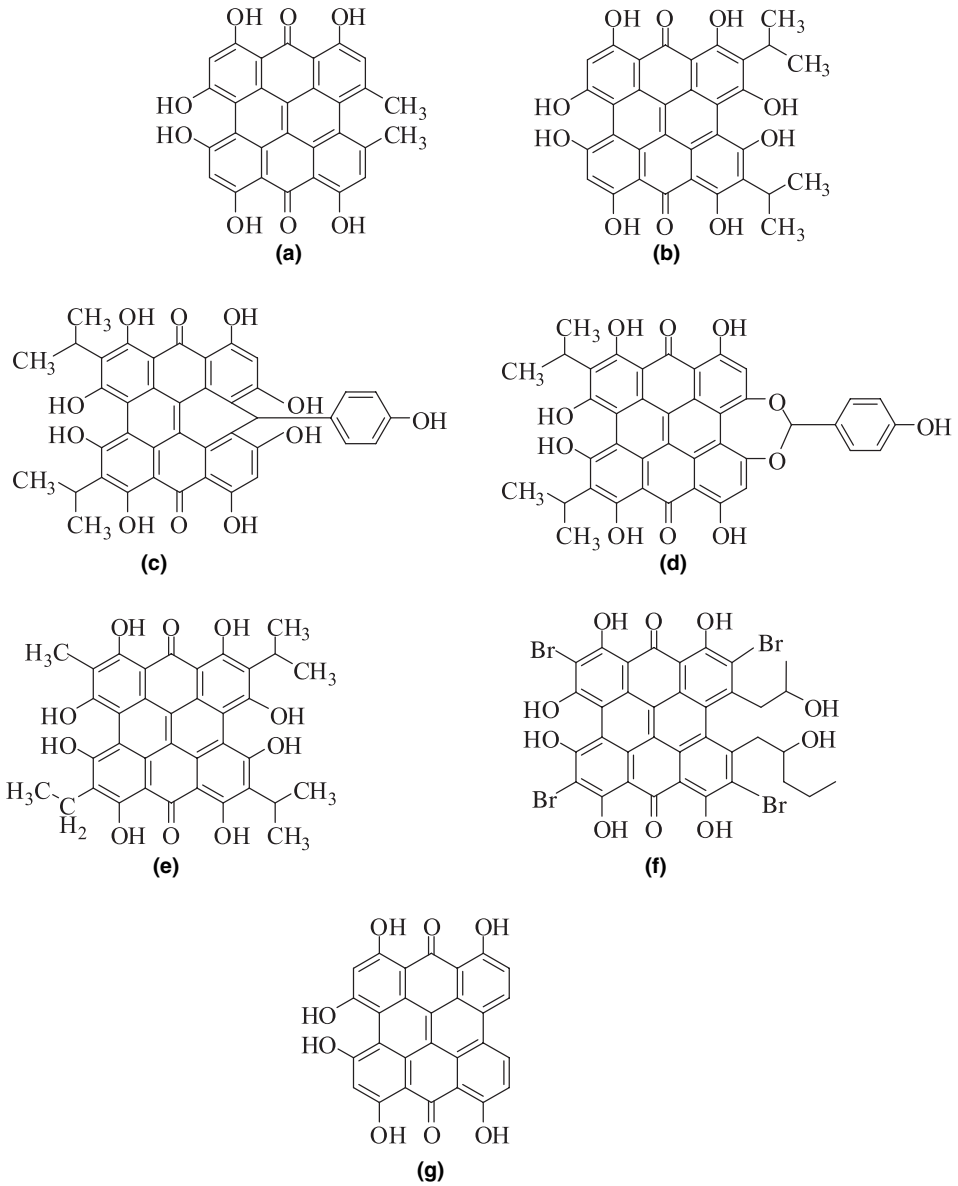

Figure 1. Structures of hypericin and some of its naturally occurring analogs: (a) hypericin, (b) stentorin, (c) blepharismin C, (d) oxyblepharismin C, (e) maristentorin, (f) gymnochrome A and (g) fringelite F.

humans (34). Hypericin is the most thoroughly studied of all the hypericin-like chromophores, and in the course of our discussion, it will often be the chromophore against which others are compared.

In this paper, we review the literature and present some new data to explore the photophysics and multifunctionality of hypericin-like chromophores in heterotrichs, with reference to better-studied hypericin, to advance our understanding of these chromophores, using a phylogenetic framework. We place these studies in the contexts of (1) occurrence of such pigments in ciliates and other organisms; (2) photoreceptor molecules in protists; and (3) chemical defense in ciliates. We do not include the biochemical pathways of sensory transduction, which were recently reviewed by Sobierajska *et al.* (35).

Two roles for hypericin-like photosensitizing pigments have been identified in heterotrichs (Ciliophora: Heterotrichea). First, in *Stentor coeruleus* (Fig. 3) and *Blepharisma japonicum* these pigments are the photoreceptors for abrupt light-avoidance responses. This role is well established, but there are few details of the signaling cascades leading to ciliary stroke reversal (35–40). These species are, however, susceptible to light in the presence of oxygen and must stay in dim light or darkness to survive endogenous toxicity of their own pigment (37,41,42). Yet the heterotrichs include several photophilic species, *i.e.*, that live in moderate to bright light. Some, including some *Stentor* spp., have endosymbiotic algae (green zoochlorellae in the freshwater species or brown zooxanthellae in the marine *Maristentor dinoferus*) (43–45) (Fig. 3). These species depend on light for photosynthesis, which produces oxygen; some have cortical pigment, but others do not. How do species cope with both photosynthesizers and potentially phototoxic pigments? In addition, there are other, almost colorless species, such as *Fabrea salina,* that are positively phototactic. There has been little work on pigments in such

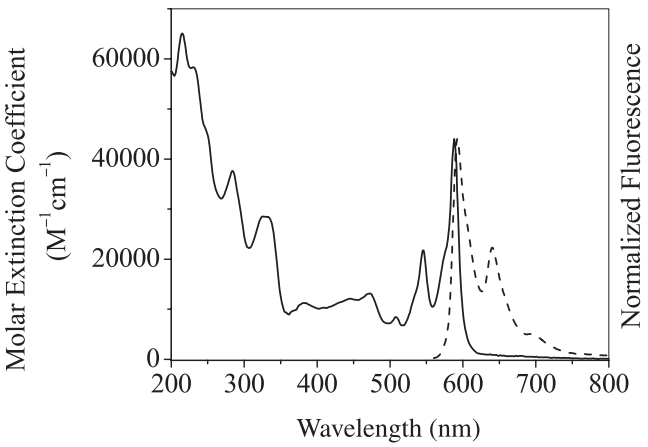

Figure 2. Normalized steady state absorption (solid line) and emission (dashed line) spectra of hypericin in methanol. The excitation wavelength for the emission spectrum is 550 nm.

photophilic heterotrichs (8,46), but some results indicate that hypericin-like chromophores are not involved in some photoresponses (47).

Second, these molecules act as exogenous photosensitizers and have been shown to act in predator defense in these species and others (48–52), a role that overlaps with that of colorless climacostol (1,3-dihydroxy-5-[(Z)-2′-nonenyl]benzene) in the cortical granules of the related species *Climacostomum virens* (53).

Two additional roles have been proposed for hypericin-like pigments in heterotrichs. Paradoxically, the same pigments that cause photosensitivity may contribute to protection against UV-B radiation damage (41,54), a function to which mycosporine-like amino acids (MAAs) may also contribute in some species (55,164). Mukherjee *et al*. (8) speculated that maristentorin, which is strongly fluorescent *in vivo* (Fig. 3a and b), could aid photosynthesis by the algal symbionts of *M. dinoferus* in low light (by analogy with deep-water corals [56]).

In the following sections, we review the heterotrich taxa and the occurrence of hypericin-like pigments, including a new folliculinid, the photobehavior of heterotrichs and the photophysics of hypericin and hypericin-like pigments. Then, after brief consideration of other relevant photoreceptors, we turn to the other demonstrated or potential roles of hypericin-like pigments in heterotrichs. Except in the case of the folliculinids, T580 and T731, materials and experimental procedures are not discussed but can be found in the references cited.

HETEROTRICHS, STENTORIDS AND OCCURRENCE OF PIGMENTS

Taxa involved

The class Heterotrichea is a distinct group within a relatively small subphylum of ciliates, the Postciliodesmatophora (Table 1, Fig. 4) (57). Within the order Heterotrichida, which includes all but one genus of the Class, a cluster of families known informally as stentorids has been recognized. These families include the Stentoridae, Blepharismidae, Folliculinidae and Maristentoridae. However, there are still few taxa in the molecular phylogenies, so these trees are likely to be revised as additional species are added from these and other families. (For example, *Fabrea* and *Climacostomum* are currently in one family, Climacostomidae [Table 1], but appear separated in the molecular phylogeny [Fig. 4].) Heterotrichida are noted for colored taxa (58); colored and/or toxic compounds are widespread in the order (Table 2) and occur in granules, but not all are hypericin like. In addition, karyorelictids, the other class within Postciliodesmatophora, also contain at least two species of *Loxodes* with a toxic pigment linked to photomovements (52,59), which may be instructive by comparison.

Cortical granules

The pigment granules in heterotrichs are membrane-bound organelles usually 0.3–0.6 μm diameter, arranged in stripes between the rows of cilia (Fig. 3c). There is some indication of membrane structures inside the granules of several species (37,41,46,60), but the structure of the granules has been worked out in detail only for *B. japonicum* (61). In that species the granule *per se*, inside a surrounding membrane, consists of a "honeycomb-like structure" of highly folded membranes, perpendicular to the cell surface, in which are embedded 200 kDa proteins with blepharismin chromophores. In addition, blepharismin accumulates in the spaces between the membranes. Such an organized structure is expected for photoreceptor organelles (61) but would not be necessary for defensive extrusomes. Sgarbossa *et al*. (37) estimated the volume of *Blepharisma* cortical granules at 0.5×10^{-15} L and the number of oxyblepharismin molecules in each at 3×10^{6}. Although pigment granules occur primarily just below the membrane, and are therefore often called cortical granules (sometimes subpellicular granules), they also occur deeper in the cytoplasm, especially around the macronucleus of *S. coeruleus* (62), *Folliculina ampulla* (63), *Blepharisma* spp. (41) and *M. dinoferus* (44,45) and around the micronuclei of *Stentor amethystinus* (43); they are not fixed in place. The term pigmentosome has been used for them, but they are evidently part of a class of organelles that includes colorless, toxic extrusomes (see Toxicity, UV-protection and other possible roles for hypericin-like pigments in heterotrichs, below). These in turn represent a type of mucocyst (64) or, preferably "secretion granule" (65) as they discharge faster than most mucocysts (51) and do not appear to contain mucous substances (65) except in folliculinids (66). There does not appear to be a term to describe accurately all these bodies wherever they are in a cell, and whether they are known to secrete or not, so we will use cortical granule, with the caveat that they are not strictly confined to the cortex.

Chemically characterized pigments

The pigments of *S. coeruleus* and *B. japonicum* have been chemically characterized (see Characteristics of hypericin-like chromophores and primary photoprocesses, below). Other *Stentor* species have evident pigment of various colors, while still others (*e.g. Stentor polymorphus*) have colorless cortical granules (Table 2). There are at least four color groups of granules in *Stentor* spp. (43,62): (1) bluish to green as in *S. coeruleus* and some others; (2) yellow/brown in *Stentor niger* and *Stentor fuliginosus* (in *S. niger* there is a red-violet pigment, with red fluorescence, named stentorol, and a brown

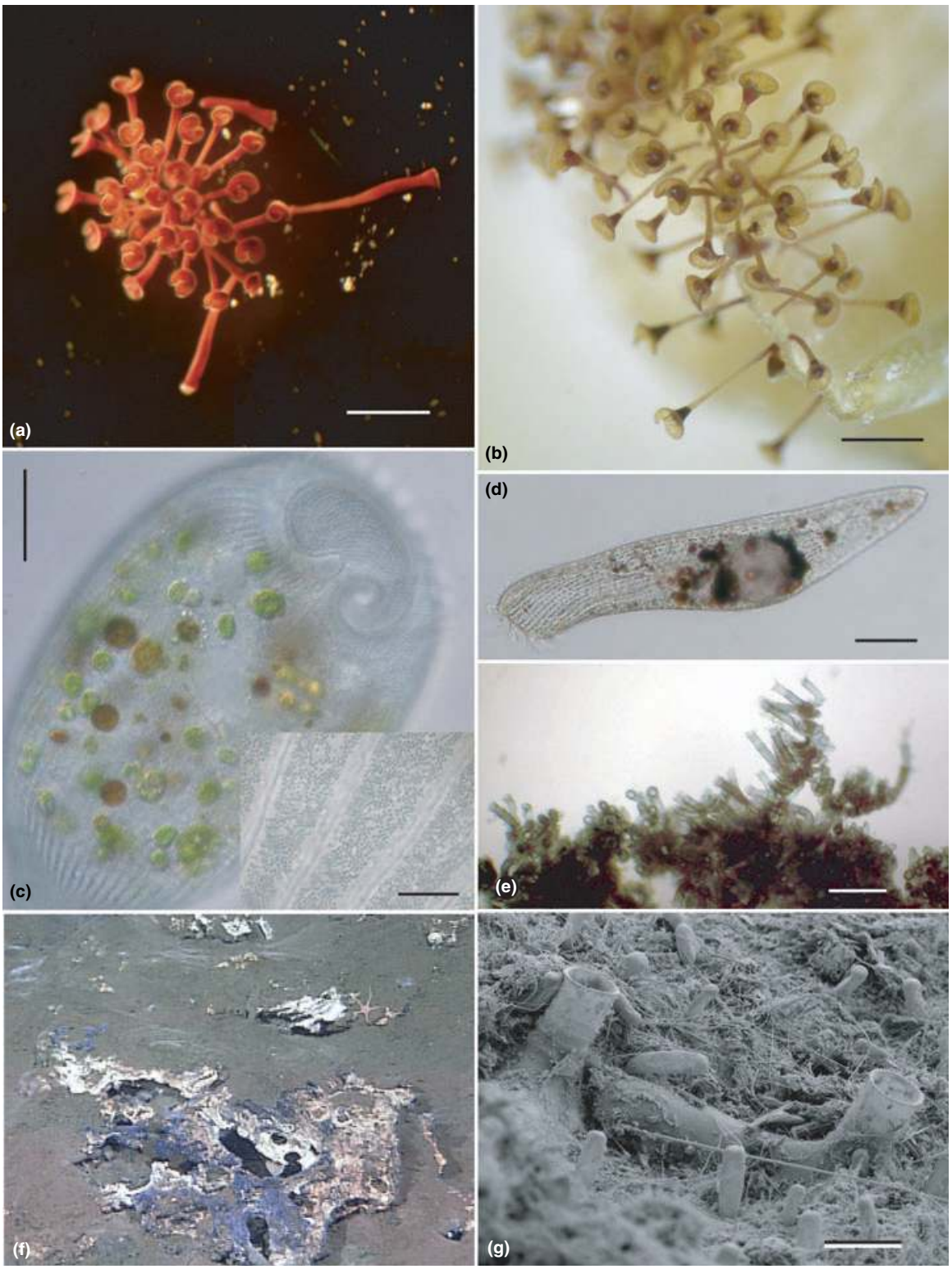

Figure 3. Heterotrichs and their pigments. (a) *Maristentor dinoferus* cluster at night, photographed with oblique lighting; fluorescence from the pigment is distributed all over the cells; three cells can be seen gliding around the cluster of attached cells. (b) *M. dinoferus* cluster during daytime; zooxanthellae can be seen in the stalk and cap, while the pigment is concentrated below the cap, around the nucleus. In some cells fluorescence of the cortical granules is evident, in others the dense pigment appears black. (c) *Stentor coeruleus* whole cell (the algae in the cell are recently ingested euglenoids, not symbiotic zoochlorellae); inset detail of cortical granules. (d) *Condylostoma* sp. from a Guam reef, showing rows of cortical granules. (e) T580 folliculinid, clump of loricas in light microscope showing color. (f) T580: photograph of indigo mat *in situ* at 1567 m, photo courtesy of MBARI. (g) T580: scanning electron microscope view of loricas showing folliculinid characteristics. Scale bars: a, b and e = 500 μm; g = 50 μm; inset in c = 10 μm.

Table 1. Current classification of heterotrichs and other genera of Phylum Ciliophora mentioned in this review (after Lynn and Small [58,167]).

SUBPHYLUM POSTCILIODESMATOPHORA
- Class Karyorelictea
  - Order Protostomatida
  - Order Loxodida
    - Loxodidae: *Loxodes*
- Class Heterotrichea
  - Order Licnophorida
  - Order Heterotrichida
    - Blepharismidae: *Blepharisma*
    - Climacostomidae: *Climacostomum*, *Fabrea*
    - Condylostomatidae: *Condylostoma*
    - Folliculinidae: *Eufolliculina, Folliculina, Halofolliculina*
    - Maristentoridae: *Maristentor*
    - Peritromidae: *Peritromus*
    - Spirostomidae: *Spirostomum*, *Gruberia*
    - Stentoridae: *Stentor*, ?*Heterostentor*

SUBPHYLUM INTRAMACRONUCLEATA
- Class Spirotrichea
- Subclass Hypotrichia
  - Order Sporadotrichina
    - Trachelostylidae: *Terricirra*
- Class Litostomatea
- Subclass Haptoria
  - Order Haptorida
    - Tracheliidae: *Dileptus*
- Class Oligohymenophorea
- Subclass Peniculia
  - Order Peniculida
    - Parameciidae: *Paramecium*

pigment [67]); (3) reddish in *Stentor igneus* and violet to purple-red in *S. amethystinus*; (4) the colorless species include *S. polymorphus* and *Stentor roeselii*. In each color group there are species with zoochlorellae and species without, making eight potentially different physiological combinations (Table 2). In addition, there are strains of *S. coeruleus* that are fluorescent *in vivo* and phototoxic, and others that are nonfluorescent and nontoxic (68). *Stentor loricatus* has dark green pigment granules (69) that Foissner and Wölfl (43) compared with the larger (1.0 μm diameter), sparser green cortical granules in species of the unrelated hypotrich ciliates in the genus *Terricirra* (Tables 1 and 2) (70). The pigment in *S. niger* was soluble in chloroform and ether but not in ethanol (67). (Although Barbier *et al.* [67] identified their specimens *S. niger*, they described separating algal pigments from the ciliate pigment. If these algal cells were endosymbionts and not just associated plankton or ingested prey [cf. Fig. 3c]—it is not clear from their text—then the species should properly be called *S. fuliginosus*, according to criteria in Foissner and Wölfl [43].) Thus it is likely that there are several pigments in different species of *Stentor*.

Blepharisma spp. are usually red (if strongly pigmented) or pink, turning blue in dim light when blepharismins are photooxidized to oxyblepharismins (41) (Table 2). Structures for these compounds were proposed by Checcucci *et al.* (7), Maeda *et al.* (9) and Spitzner *et al.* (10), respectively. Some species were described as colorless but Giese (41) cautioned that the effect of light on the pigment means that the color of

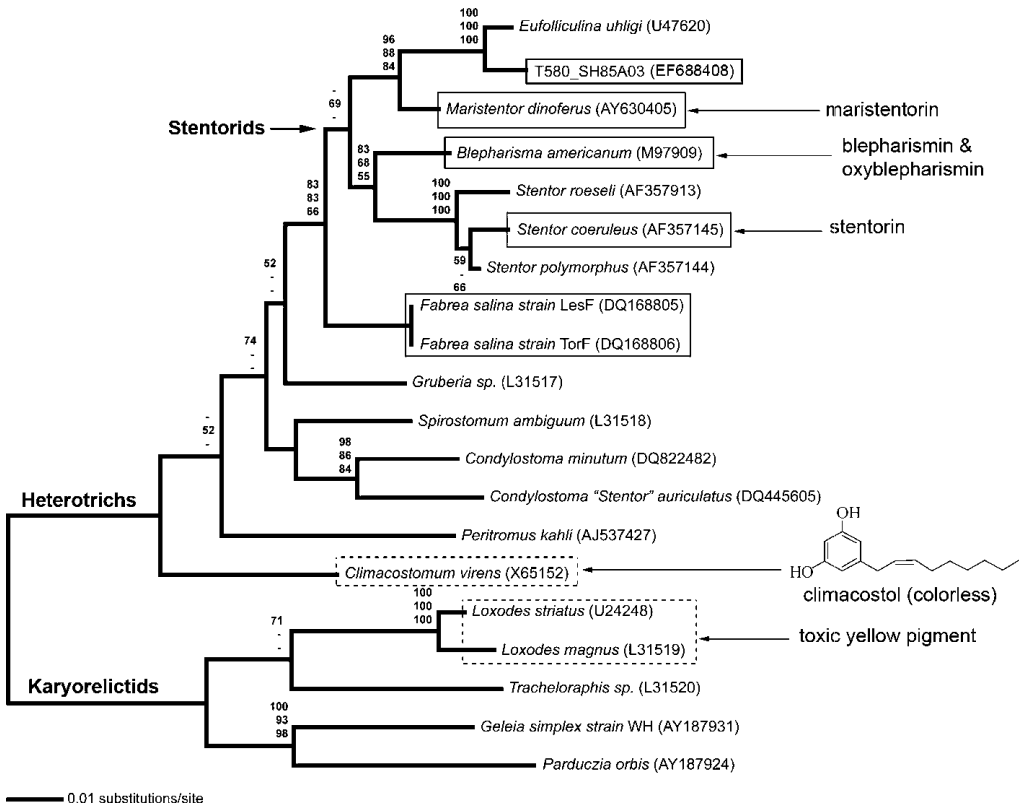

Figure 4. Phylogenetic tree of all heterotrichs and karyorelictids sequenced to date, showing molecular identity of deep-water folliculinid samples and species with pigments or colorless toxins. Numbers in parentheses are the GenBank accession numbers of the sequences used. Solid lines around taxa indicate hypericin-like compounds known chemically or spectrally (blepharismin identified from *Blepharisma japonicum* but gene sequence from *Blepharisma americanum*), dashed lines indicate toxic compounds that are not hypericin like.

Table 2. Taxa of heterotrichs, plus some other ciliates, that have pigmented and/or toxic cortical granules, algal symbionts or both.

Class/Family	Genus, species	Pigment (if any); color of cortical granules	Toxic (predator defense) (if known)	Symbiotic algae	References for pigment, symbiont, toxin
HETEROTRICHEA					
Stentoridae blue/green group	*Stentor araucanus* Foissner & Wölfl, 1994	Yes: blue-green		Yes	(43)
	Stentor coeruleus (Pallas, 1776) Ehrenberg, 1831	Stentorin: blue-green	Yes: stentorin	No	(43,49)
	Stentor introversus Tartar, 1958	Yes: blue-green		No	(43)
	Stentor baicalius Foissner & Wölfl, 1994	Yes: sea green		No	(43)
	Stentor multiformis (O.F. Müller, 1786) Ehrenberg, 1838	Yes: azure to sea green		No	(43)
	Stentor loricatus Bary, 1950	Yes: dark green		No	(43,69)
Stentoridae reddish group	*Stentor amethystinus* Leidy, 1880	Yes: lilac, amethystine or purplish red	Yes	Yes	(43)
	Stentor igneus Ehrenberg, 1838 (*S. roseus*)	Yes: pink or brick red		No	(43,62)
	Stentor tartari Narayana Murthy & Kasturi Bai, 1974	Yes: purplish red		Yes	(43)
Stentoridae brownish group	*Stentor fuliginosus* Forbes, 1891	Yes: yellowish brown, brown or red-orange		Yes	(43)
	Stentor niger (O.F. Müller, 1773) Ehrenberg, 1831	Yes: rusty-brown to brownish; "stentorol" red to red-violet; plus a brown pigment		No	(43,67)
Stentoridae colorless	*Stentor polymorphus* (O.F. Müller, 1773) Ehrenberg, 1838	No	Yes (strain without zoochlorellae)	Yes	(43,50)
	Stentor roeselii Ehrenberg, 1835	No	Yes	No	(43,50)
Family uncertain	*Heterostentor coeruleus* Song & Wilbert, 2002	Yes: blue		No	(78)
Blepharismidae	*Blepharisma japonicum* (Suzuki, 1954) Giese, 1973	Blepharismins: red (oxidizing to blue oxyblepharismins in dim light)	Yes: blepharismin(s)	No	(7,41,51)
	Blepharisma japonicum	Yellow (minor additional pigment)			(72,73)
	Blepharisma coeruleum Gajevskaja, 1927	Light gray, gray-blue or greenish blue		No	(41)
Folliculinidae (examples; the family is characterized by blue or green color)	*Eufolliculina uhligi* Mulisch & Patterson, 1983	Yes: cell deep green-blue, lorica blue-green		No	(76)
	Eufolliculina moebiusi (Kahl, 1932) Hadži, 1951	Yes: cell dark green or dark blue, lorica yellowish		No	(76)
	Folliculina "baltica" (undescribed species)	Yes: cells and lorica bluish green: stentorin 2 (spectrophotomet.)		No	(75)
	Folliculuina ampulla (O.F. Müller, 1786) Lamarck, 1816	Yes: blue		No	(63)
	Folliculina simplex (Dons, 1917)	Yes: blue-grey to blue-greenish		No	(168)
	Folliculinopsis andrewsi (Hadži, 1938) Das, 1949	Yes: green-blue		No	(74,77)
	Folliculinopsis producta (Wright, 1859) Fraué-Fremiet, 1936	Yes: green to dark green		No	(169)
	T580	Yes: blue		No	this paper
Maristentoridae	*Maristentor dinoferus* Lobban *et al.*, 2002	Maristentorin: bluish		Yes	(8)
Climacostomidae	*Climacostomum virens* (Ehrenberg, 1833) Stein, 1859	No	Yes: climacostol	Yes (but toxicity studies performed on bleached cultures)	(53)
	Fabrea salina Henneguy, 1890	Yes: but not readily visible; black "fabrein" in some strains?		No	(41,161)
Condylostomatidae	*Condylostoma* sp.	Weakly		No	this paper
Spirostomidae	*Spirostomum teres* Claparède & Lachmann, 1859	No	Yes	No	(48)

Table 2. (*Continued*).

Class/Family	Genus, species	Pigment (if any); color of cortical granules	Toxic (predator defense) (if known)	Symbiotic algae	References for pigment, symbiont, toxin
Peritromidae	only one genus, *Peritromus*, not known to have pigment or symbiotic algae				
KARYORELICTEA	*Loxodes striatus* (Engelmann, 1862) Penard, 1917 and *Loxodes magnus* Stokes	Weakly; yellow-brown	Yes	No	(52,59)
HYPOTRICHIA	*Terricirra* spp. incl. *Terricirra viridis* (Foissner, 1982) Berger & Foissner, 2002 and *Terricirra matsusakai* Berger & Foissner, 2002	Yes; green to blue, Dark green		No	(70)

field collections may not be the normal color. *B. japonicum* can be bleached by extrusion of pigment granules induced by cold shock (71). There is a green-blue species, *Blepharisma coeruleum,* which "is almost always found on the alga *Tetraspora*" (41), *i.e.*, in a lighted environment. Probably this color represents some combination of oxyblepharismins, but could it be stentorin? The amount of material needed and the effort required for chemical characterization has so far deterred exploration of the range of pigments in *Stentor* and *Blepharisma*.

Besides the major pigments in these two genera, there is a minor yellow pigment in *B. japonicum*, which has similar solubility but a spectrum quite unlike the blepharismins (72,73); this was also noted by Giese (41) who suggested it was just an oxidation product of blepharismin. We note that were there more than one pigment or pigment form occurring (*e.g.*, stentorin-1 *versus* stentorin-2), it would be difficult to detect whether they were in the same granules or partitioned into different ones. Conceivably, the major pigments could be partitioned among different granules such that toxins are in true extrusomes and the photoreceptor pigments are in granules that do not discharge.

The only other hypericin-like pigment in heterotrichs for which the structure has been determined is maristentorin, which we characterized from *M. dinoferus* (see section Characteristics of hypericin-like chromophores and primary photoprocesses, below) (8). This species is more closely related to folliculinids than to *Stentor* and *Blepharisma* (Fig. 4) (57). *M. dinoferus* is a benthic marine species on coral reefs and, like corals, has zooxanthellae (44,45).

Other cortical pigments

Other heterotrichs have been studied at least briefly for toxic or pigmented compounds in the cortical granules (Table 2). One can easily detect species that are strongly pigmented, but for most there is little or no evidence for the structure of the pigment beyond spectral similarity to hypericin (or not). The Folliculinidae are characteristically blue, green-blue or green (74). For example, both currently recognized species of *Eufolliculina* are green-blue, and the pigment is transferred to the lorica (a loose external covering, see Fig. 3g) during development, while chitin fibrils and mucopolysaccharides—also in the cortical granules—are extruded and shaped (66). Sjögren (75) claimed to have spectral evidence for "stentorin II" in "*Eufolliculina baltica*" (*nomen nudum* according to Mulisch and Patterson [76]). *Folliculinopsis andrewsi* (74) (studied as *Semifolliculina producta*) cells and newly secreted loricas were green-blue and showed red fluorescence (77). The recently discovered Antarctic species *Heterostentor coeruleus* (78) is a brilliant blue, but its relationship to *S. coeruleus* (notwithstanding the name) is unclear and one should not assume *a priori* that the pigment in this or the folliculinids is stentorin. On the other hand, strains of *F. salina* studied by Marangoni *et al.* (46,79) appeared colorless but proved to have low numbers of cortical granules with a hypericin-like pigment, judged from absorption spectra. Earlier studies (41,80) described strains of *F. salina* colored with black pigment. It would be worth exploring other apparently colorless species for traces of hypericin-like pigments, especially in organisms-like *C. virens* and *S. polymorphus* that have zoochlorellae (both also have toxic cortical granule contents). We note that all hypericin-like pigments so far spectrally or chemically identified are in the stentorids and *Fabrea*. *Fabrea* is now seen to be sister to the stentorids (Fig. 4) and might therefore eventually be included in that group, but is currently in Climacostomidae (Table 1).

Two very deep-water folliculinids were collected by researchers at the Monterey Bay Aquarium Research Institute (MBARI) and documented by S.H.: "T580" from 1567 m depth in cold hydrocarbon seeps on the seafloor in the Gulf of California and "T731" from 2300 m depth in the vicinity of hydrothermal chimneys in the Juan de Fuca Ridge system. T580 has been identified on the basis of both general morphology and 18S ribosomal DNA gene sequencing (Figs. 3 and 4), while T731 has only been indentified on the basis of general morphology. Neither T580 nor T731 have been the subject of detailed taxonomic characterization. Like many other folliculinids, they are blue and initial analysis of their pigment by P.M. and J.W.P. shows that it is hypericin-like (see section Characteristics of hypericin-like chromophores and primary photoprocesses, below). Both T580 and T731 live in

deep marine habitats far below the maximal depth to which sunlight penetrates. However, in the case of T731, we cannot rule out the possiblilty that blackbody radiation derived from superheated vent fluids could produce enough light to evoke photoresponses at or near the seafloor, as in the case of "eyeless" shrimp, which have no image-forming optics (81,82). Work by White et al. (171) using a special camera at selected vent sites seems to confirm this. For example, her website provides data where light is seen at wavelengths greater than 700 nm, matching the spectrum of an ideal blackbody at similar temperatures—in this case ~350°C (171).

A *Condylostoma* sp. that is common on Guam reefs has low numbers of colored cortical granules (Fig. 3d). The identity of the species and the characteristics of the pigment have yet to be determined. This species does not have symbiotic algae but two temperate marine relatives, *Condylostoma wangi* and *Condylostoma tenuis,* have zoochlorellae (43). If this pigment or the pigment in *Terricirra* turn out to be hypericin-like, that would indicate that such compounds are more widely found in ciliates than just in stentorids/*Fabrea*.

The karyorelictids *Loxodes magnus* and *Loxodes striatus* have a faint yellow-brown color overall; their pigment granules form stripes between the ciliary rows on the right (ciliated) side of the cell and looser rows on the left (largely unciliated) side, but there are concentrations of granules around the oral aperture and pharynx, and along the path of the stomatogenic kinety (59). The pigment spectrum has increasing absorbance below 500 nm, rising to a peak at 210 nm, with shoulders near 275, 360 and 435 nm (52,59) and is thus not hypericin like.

PHOTORESPONSES IN HETEROTRICHS

Many protists respond positively or negatively to light, to acquire food (including *via* both predation and photosynthesis), to avoid predators or to avoid direct harmful effects of irradiation. Flagellates (*e.g.*, *Chlamydomonas* and *Euglena*) often have an eyespot consisting of layered carotenoid pigment and a photoreceptor in the underlying membrane, which in *Chlamydomonas*, at least, is rhodopsin (83–85). However, *Euglena* has a flavoprotein receptor (86). Ciliates, in contrast, have both locomotor organelles (cilia) and photoreceptors distributed all over the cell surface. Phototaxis is movement oriented to the direction of light and can be positive or negative. Phototaxis and other behavioral responses to light can result in photoaccumulation in bright light or photodispersal into dim light or darkness. Photokinesis is a positive or negative change in the swimming speed of a cell in response to irradiance. A photophobic reaction is an abrupt stop, reversal and turn in response to a rapid increase (step-up) or decrease (step-down) in the light flux (37,87,88). Although often depicted as occurring when a cell swims from darkness into a light spot (as seen under a microscope), the response more generally occurs when the down-welling light changes, as in a passing sun fleck or disturbance of overlying shade materials. Some species that have photophobic reactions may also be negatively phototactic and are overall sciaphilous (shade-loving) species, *i.e.*, their net movement is away from light (*e.g.*, *B. japonicum*, some strains of *S. coeruleus* [41,62]), but others are nevertheless photophilic species, living in moderate to bright light but reacting to abrupt changes in intensity (*e.g.*, the photosynthetic flagellates *Chlamydomonas reinhardtii* and *Euglena gracilis*). In ciliates, step-up photophobic responses are seen in *S. coeruleus* and *Blepharisma* spp., a step-down photophobic response in *Fabrea*. On the basis of the current phylogenetic tree (Fig. 4), it appears that these responses involving hypericin-like pigments arose independently in these three genera.

Stentor coeruleus and *Blepharisma japonicum*

The photophobic response of *S. coeruleus* is described as follows (35,36,38,39,87). When cells experience a sudden increase in irradiance flux they first stop. If the change in irradiance is small (*i.e.*, the stimulus is weak), they then continue on their original path, but if it is larger they back up briefly along a curved path, then resume swimming in a new direction. There is also photodispersal of *S. coeruleus* into shade or darkness (see below). The chain of events proposed for the photophobic reaction (39) is that some initial photoprocess (perhaps proton release, see next section) starts a signaling cascade leading to a reversal of the ciliary stroke.

The photophobic behavior of *B. japonicum* is similar to that of *S. coeruleus* and the photoreceptor is blepharismin (73,89). However, rather than going into reverse, the cell pivots around the adoral side, making a 180° turn before moving forward (40,72). While Kraml and Marwan (72) concluded that *B. japonicum* lacked the negative phototaxis seen in *S. coeruleus,* Matsuoka (89) at the same time presented evidence for its occurrence. Both studies found positive photokinesis when *B. japonicum* was illuminated and Matsuoka (89) noted that the cells elongated when in brighter light. The light perception for both photophobic and phototactic responses was localized in the anterior of the cell, and not all over, which is interesting, considering that the pigment granules are distributed over the whole surface. Kraml and Marwan (72) presented evidence for a minor yellow pigment being the photoreceptor, and although this view is no longer accepted (73,87), it does suggest how a localized photoreceptor could be hidden within a cell that has strong uniform color.

Photodispersal of *S. coeruleus* in a gradual light gradient may have a phototactic element but appears to result chiefly from a difference in the frequency of reorientation when cells were swimming up or down a gradient, *i.e.*, a biased random walk (also called klinokinesis) (90). Swimming cells displayed ciliary reversals and "smooth turns" (distinct from the photophobic reaction). The turns were usually much greater than needed to direct them away from light, which would not be expected in phototaxis. However, when swimming toward light they reoriented more often and the net effect was to disperse away from light. Song *et al.* (91) had concluded that there was negative phototaxis by observing cells in a convergent light beam, where cells swimming away from the light accumulated in higher irradiance. However, while 19% of the turns Menzies *et al.* (90) tracked could be attributed to gradual phototaxis, they did not result in the cells becoming oriented in the photodispersal direction and therefore phototaxis did not appear to contribute significantly to photodispersal.

Photoresponses of other ciliates

Photophobic and phototactic behavior of *Fabrea* result in its photoaccumulation. This species lives in high-irradiance shallow pools, in marked contrast to the habitat of *Blepharisma* (79). Its photophobic reaction is to stepped-down irradiance but, like the step-up reaction of *Stentor* and *Blepharisma*, it results in a tumbling and change in direction. This reaction has an action spectrum matching hypericin-like pigments, and small amounts of these do occur in the cortical granules. However, the phototactic reaction is driven instead by rhodopsin, which is distributed all over the cell membrane (47).

Although *Loxodes* is a karyorelictid and not a heterotrich, the close relationship of the groups and the presence of a pigment that is both a photoreceptor and a defense chemical, make the analogy worth considering. However, the responses of *Loxodes* to light are complex, including phobic, kinetic and tactic reactions, which, in these microaerophilic species are also linked to responses to oxygen (92). There is even a component of positive geotaxis in movements of *Loxodes* (93). Fenchel and Finlay (92) concluded that the light sensitivity is closely related to oxygen toxicity and demonstrated that the pigment is probably a flavin and produces superoxide when illuminated in the presence of oxygen.

The movements of other heterotrichs are less well known. The yellow-pigmented *S. niger* photoaccumulated and could be ''trapped'' in a sharply defined light spot; cells also showed an abrupt reaction to illumination with a narrow and bright light spot, apparently similar to the photophobic response of *S. coeruleus* (94). There is evidence that some of the many species of folliculinids are sciaphilous: some species react to strong light and seek out cryptic and sometimes specialized, dark habitats (77,95,96). Other folliculinids live in bright environments, such as the recently described *Halofolliculina corallasia*, which settles on and erodes coral surfaces (97). The links between pigments, movements, and habitats in folliculinids have not been studied, nor is there any indication of whether the apparently hypericin-like pigments are toxic either to the folliculinids or to potential predators.

Comparisons within species of movements of cells with and without algal symbionts have not been made in heterotrichs. In the unrelated ciliate *Paramecium bursaria* (Table 1), there is a circadian rhythm of photoaccumulation, but photoaccumulation occurs only when zoochlorellae are present (98) and is stopped with 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea, an inhibitor of photosynthetic electron transport, implying that the stimulus is detected by the algae and communicated to the host ciliate (99). The opportunity exists to carry out similar studies in some heterotrich species that have already been cultured without their symbionts: *S. polymorphus* and *C. virens* (50).

Other pigments that may regulate photoresponses in heterotrichs

In exploring the occurrence and roles of hypericin-like pigments in heterotrichs, it is worth pausing briefly to consider the range of chromophores that act as light detectors in microorganisms. They comprise carotenoids including retinal, pterins, flavins, as well as the dianthrones that are the focus of this paper (85,87). Two compounds of particular relevance are retinal and flavins.

Retinal, the characteristic animal eye pigment (rhodopsin = retinal chromophore + opsin protein), is also the functional chromophore in the green flagellate *Chlamydomonas* (Chlorophyceae) (83,100); and it mediates both phototactic and photophobic responses of *Chlamydomonas* (84). Rhodopsin was detected in the membrane of *F. salina* by immunofluorescence and shown to be involved in the positive phototactic response of that heterotrich (47). On the other hand, the almost-undetectable quantity of hypericin-like pigment in that strain of *F. salina* was involved in the tumbling response to stepped-down irradiance. The most important point for our review is that we should not exclude the possibility of the presence of retinal just because another pigment is present. No one has yet looked for rhodopsin in *Maristentor* and other positively phototactic heterotrichs.

Flavins are blue-absorbing compounds that have been shown to be involved in microbial photoresponses. A flavoprotein was recently shown to be the photoreceptor for the photophobic reaction of *E. gracilis* (86). It is especially worth keeping flavins in mind with heterotrichs because of the flavin-like yellow pigment in *Loxodes* (59) and the presence of small amounts of a spectrally similar pigment in *B. japonicum* (72,73).

CHARACTERISTICS OF HYPERICIN-LIKE CHROMOPHORES AND PRIMARY PHOTOPROCESSES

There is a strong influence of the environment on the photophysics of hypericin and its analogs. Dramatic changes in excited-state behavior are observed as hypericin is moved from the homogeneous environment of organic solvents to the much more structured surroundings provided by the complexes it forms with proteins. Among these complexes, it is useful to consider the differences between environments where hypericin is not found naturally and those where it is. It is clear that interaction with a protein modifies the photophysics of hypericin and understanding the molecular basis of this interaction is one of the outstanding problems in elucidating the function of hypericin and hypericin-like chromophores.

Table 3 summarizes many of the salient points of this discussion by demonstrating how fluorescence lifetimes and fluorescence quantum yields are influenced by moving hypericin-like chromophores from bulk organic solvents to complexes with proteins. We briefly define the parameters tabulated. When a molecule absorbs a photon, it is promoted from the ground electronic state, S_0 to an excited electronic state. The lowest-energy excited electronic state is referred to as S_1, and it is predominantly from this state that fluorescence originates. The behavior of this state provides useful information about how the chromophore interacts with its environment, and this behavior can be quantified by measuring its fluorescence lifetime and its fluorescence quantum yield. The energy deposited into S_1 (Fig. 5) by photon absorption can be lost either by reemitting the photon radiatively (for which the rate is denoted k_R) or nonradiatively (by executing photochemical processes or through interactions with its surroundings, for which these nonradiative processes are denoted k_{NRi}). The duration that the molecule stays in S_1 is quantified by its fluorescence lifetime, $\tau_F = (k_R + \Sigma k_{NRi})^{-1}$. Ideally, the decay of S_1 is described by a single exponential: $F(t) = a \exp(-t/\tau)$.

Table 3. Fluorescence properties of hypericin-like chromophores.

Species	τ_{fast} (ns)	a_{fast}	$\langle\tau\rangle$ (ns)*	Φ_F†
Hypericin/DMSO (8,101)	5.6 ± 0.2	1.00	5.6	0.35
Hypericin/HSA (101,134)	2.8	0.51	3.9	0.17
Hypericin/GSTA1-1 (134)	0.30	0.34	3.2	0.04
Hypericin/GSTP1-1 (134)	0.17	0.24	3.5	0.19
Stentorin (28)	5.5	1.00	5.5	0.35‡
Stentorin I (170)	2.3	0.49	3.8	0.24‡
Stentorin II (170)	0.04	0.59	0.18	0.01‡
Oxyblepharismin/EtOH (40)	1.8	0.98	1.9	0.12‡
Oxyblepharismin/protein (40)	0.5	0.37	2.1	0.13‡, 0.03 (136)
Maristentorin/DMSO (8)	5.5 ± 0.2	1.00	5.5	0.35‡
T580/acetone§	–	–	3.6	0.23‡
T731/acetone§	–	–	3.9	0.24‡

*Fluorescence decays are fit to sums of decaying exponentials: $F(t) = \sum_i a_i \exp(-t/\tau_i)$, where the preexponential factors are normalized to sum to unity; and the average fluorescence lifetime is computed as $\langle\tau\rangle = \sum_i a_i\tau_i$. Since some lifetimes are sums of more than two exponentials, to present a simpler table, only the shortest lifetime and its weight are reported. If the decay is single exponential, that lifetime is given. †The fluorescence quantum yield. Unless otherwise indicated, the result reported is obtained from a direct measurement. ‡The average fluorescence lifetime, $\langle\tau\rangle$ is directly proportional to the fluorescence quantum yield, Φ_F. In cases where a fluorescence quantum yield was not directly obtained, the value reported for a compound, *i*, is given by $\Phi_{F_i} = \frac{\langle\tau\rangle_i}{\langle\tau\rangle_{hyp}}\Phi_{F_{hyp}}$, where the subscript, hyp, denotes the quantities for hypericin in organic solvents such as DMSO. The directly and indirectly determined fluorescence quantum yield measurements do not agree for the oxyblepharismin/protein complex, which suggests that short lived lifetime components are not being detected in the measurement in reference (40). §See Figure 10 for more detail.

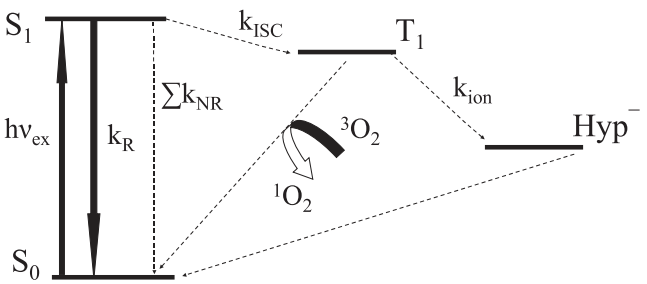

Figure 5. Electronic energy levels of hypericin and their deactivation processes. k_R is the radiative rate of the first excited singlet and Σk_{NR} is the sum of all the nonradiative processes deactivating this state except for k_{ISC}, the intersystem crossing rate, which is specifically indicated, taking the singlet to the triplet. At room temperature, we suggest that the triplet state decays predominantly by deprotonation to form the anion, k_{ion} or by interaction with ground-state oxygen to form singlet oxygen, $k[^3O_2]$.

In complex, heterogenous environments, such as a protein, however, $F(t)$ is more often described by a sum of decaying exponentials. In this latter case, Table 3 reports the amplitude, a, and the lifetime, τ, of the most rapidly decaying component. In this way, the table quantifies the ability of the protein environment to induce nonradiative processes in the hypericin-like chromophore. The table also reports a related quantity called the fluorescence quantum yield, $\Phi_F = k_R/(k_R + \Sigma k_{NRi})$.

Hypericin in bulk organic solvents

Owing to this light-induced biological activity, which is diverse and widespread, we have devoted considerable effort to the study of the early time photophysics of hypericin and its analogs in organic solvents (101–114). By means of H/D substitution, investigation of methoxy analogs and complementary studies using both transient absorption and fluorescence upconversion spectroscopies (Fig. 6), we have argued that the major primary photophysical process of hypericin in organic solvents is excited-state intramolecular hydrogen atom transfer. The experimental techniques employed are described in detail elsewhere (112,113,115–118). Our argument was based upon the interpretation of a rising component in the kinetic traces of stimulated emission or fluorescence (see below) and it has not been without controversy, as we discuss elsewhere (17,114). Most recently, Plaza *et al.* (119) have suggested that it may be an artifact arising from depositing too much excitation energy. We note, however, that this component is still present when we use nanojoule excitation energies (114), but recognize that these experiments are, nonetheless, performed with rather high-energy photons (414 nm) and that a definitive response must await performing the experiment with excitation into the O–O band, *i.e.* at ~600 nm.

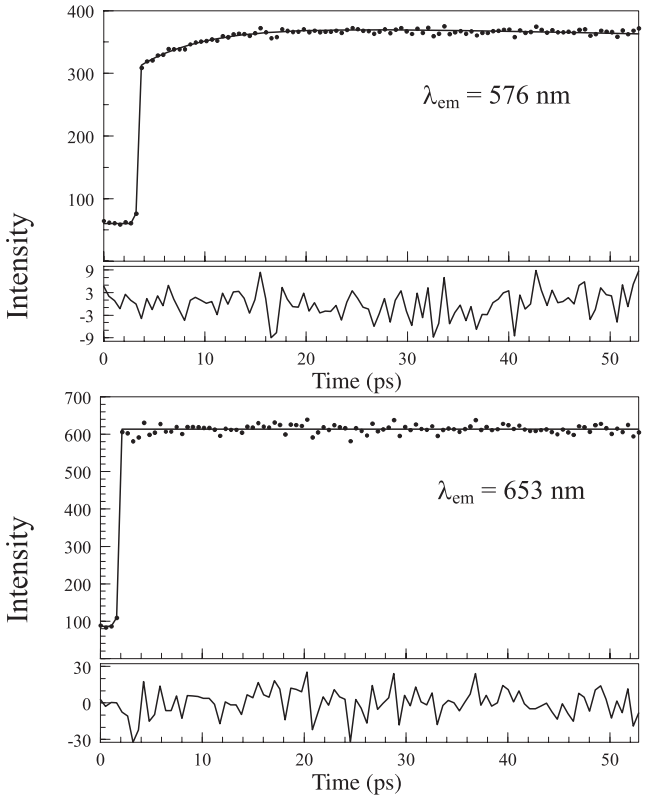

Figure 6. Upper panel: Fluorescence upconversion transient for hypericin in DMSO at λ_{em} = 576 nm. The fit curve is described by the following equation (with background subtracted): $F(t) = -0.21 \exp(-t/6.5\text{ ps}) + 1.00 \exp(-t/\infty)$. Lower panel: Fluorescence upconversion transient for hypericin in DMSO at λ_{em} = 653 nm. The fit curve is described by the following equation (with background subtracted): $F(t) = 1.00 \exp(-t/\infty)$. The excitation wavelength was the second harmonic of our unamplified Ti:sapphire oscillator, 414 nm.

Subsequent to this primary event, there is an *intermolecular* proton transfer to the solvent (20,22,120). This event is a reversible process *in vitro*, as demonstrated by the conservation of the sample optical density over the course of several cycles of pH measurement. We have suggested that these photogenerated protons may be important for understanding the light-induced biological activity of hypericin (4). Although numerous studies dealing with the biological and photophysical properties of hypericin have been performed, the mechanism and site of action of hypericin in a biological context is still unclear.

Nonnaturally occurring complexes of hypericin with proteins

Human serum albumin. Human serum albumin (HSA) is a transport protein in the blood plasma. It binds a wide variety of substances, such as metals, fatty acids, amino acids, hormones and a large number of therapeutic drugs (121). Because of its clinical and pharmaceutical importance, the interaction of HSA with a variety of ligands has been studied (122,123). HSA consists of six helical subdomains and its polypeptide backbone is formed by 585 amino acids. HSA has two major binding sites denoted IIA and IIIA according to the subdomains where they occur. The interaction of hypericin with HSA has been studied by various groups (124–127). Hypericin in aqueous physiological solution is aggregated (125,128), and binding with albumin helps to solubilize it in monomeric form, which is believed to be important for virucidal action. For example, Burel and Jardon have noted that the photodynamic properties of hypericin are greatly diminished when it is aggregated (129).

Hypericin has a single-exponential fluorescence decay of ~5.5 ns in all nonaqueous pure solvents. Bound to HSA, however, its fluorescence decay is best described by a double exponential with a short component of ~2.5 ns (Table 3). To understand what induces the nonexponential decay, hypericin was also studied in Brij-35 micelles and in complex with the protein myoglobin. In both cases, the resulting fluorescence decay is nonexponential. In the case of myoglobin, where hypericin can bind only to the surface of the protein, the decay is best described by a triple exponential. In HSA, the presence of this short component may arise from new nonradiative decay processes for a subset of hypericin molecules in the binding pocket, protruding into the solvent or bound to the surface of the protein or both.

In pump-probe transient absorption experiments, stimulated emission can be induced from the excited state of hypericin in the region of 600–660 nm. This stimulated emission grows with a time constant of 6–10 ps in all solvents, except sulfuric acid, where it appears within the duration of our laser pulses. We have argued that this transient is a signature of excited-state intramolecular proton (or hydrogen atom) transfer between the hydroxyl groups *peri* to the carbonyl. This interpretation has been supported in numerous experiments involving hypericin analogs and by the fluorescence upconversion technique (see above). Figure 7 presents the stimulated emission transients of hypericin in sulfuric acid, DMSO and in ~5:1 and 1:1 [hypericin]:[HSA] complexes. In the 1:1 complex, the rising component disappears, indicating that the intramolecular proton transfer process has been impeded.

Because of the possibility that the rising component was obscured in the 1:1 complex by the presence of other absorbing species or because the signal-to-noise ratio was insufficient, we searched for the rising component with the fluorescence upconversion technique, which measures only emission. Figure 8 presents the fluorescence upconversion trace of the 1:1 hypericin/HSA complex at 606 nm. On the shortest time

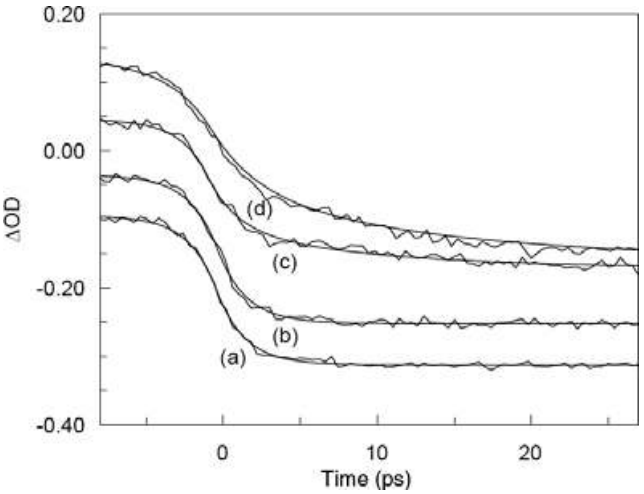

Figure 7. Transient absorption traces of hypericin in different environments. The traces are normalized to the maximum absorption in the figure. λ_{pump} = 588 nm; λ_{probe} = 600 nm. (a) Sulfuric acid: $\Delta A(t)/\Delta A_{max} = -1.0 \exp(-t/\infty)$; (b) HSA: $\Delta A(t)/\Delta A_{max} = -1.0 \exp(-t/\infty)$; 1:1 complex, [HSA] = [hyp] = 7×10^{-5} M; (c) HSA: $\Delta A(t)/\Delta A_{max} = -0.36 [\exp(-t/6.0 \text{ ps} - 1] - 0.64$; ~1:5 complex; [HSA] = 1.5×10^{-5} M; [hyp] = 7×10^{-5} M; (d) DMSO: $\Delta A(t)/\Delta A_{max} = -0.65 [\exp(-t/6.0 \text{ ps} - 1] - 0.35$.

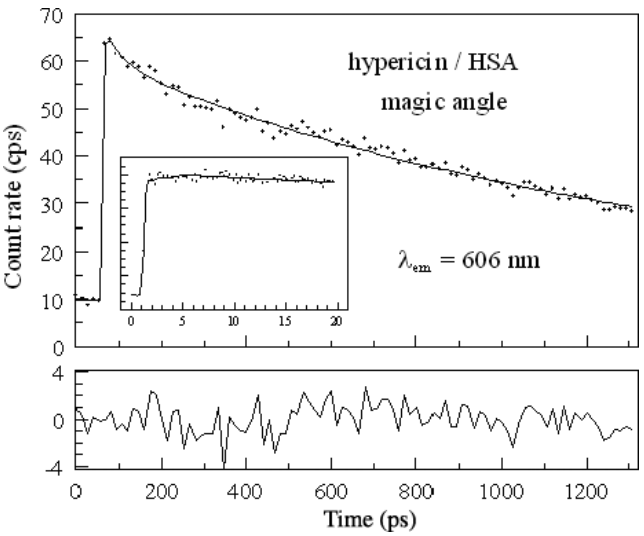

Figure 8. Fluorescence upconversion at the "magic angle" of 1:1 hypericin:HSA complex, [HSA] = 1×10^{-4} M, λ_{ex} = 414 nm, λ_{em} = 606 nm. A fit to double exponential yields: $F(t) = 0.12 \exp(-t/50 \text{ ps}) + 0.88 \exp(-t/1320 \text{ ps})$. The 50 ps component is not observed with the limited resolution afforded by time correlated single photon counting measurements. The insert shows results obtained on a 20 ps time scale.

scales investigated (20 ps full scale), there was no evidence for a rising component in the fluorescence signal, confirming the conclusions obtained from the transient absorption measurements that intramolecular proton transfer is impeded in the 1:1 hypericin/HSA complex.

An important problem raised by this study is understanding the nature of the nonradiative processes induced upon binding hypericin to proteins, DNA or micelles. In all the model systems to which hypericin is bound, nonexponential fluorescence decay is induced (101). This question has been raised by Song and coworkers (6,130) in the context of another system that is very similar to that of hypericin, namely the stentorin chromophore (Fig. 1). Stentorin serves as the primary photosensor in the single cell ciliate, *S. coeruleus*. Proton transfer has been suggested as a possible primary photoprocess in triggering the light signal transduction chain in *S. coeruleus*. The stentorin chromophore is linked to an ~50 kDa apoprotein. Bound to the protein, in water, this chromophore exhibits a very short-lived nonexponential fluorescence decay that is dominated (~95%) by an 8 ps component. Song and coworkers have shown that the long-lived hypericin fluorescence can be efficiently quenched by electron acceptors, such as benzoquinone (28,131) and they have proposed that excited-state electron transfer of the stentorin chromophore to disulfide bonds in the protein result in photooxidation of the stentorin chromophore (HO–ST–OH) to oxystentorin (O=ST=O) and the loss of two protons by the following mechanism, which is reversible (130,131):

$$\begin{aligned}\mathrm{HO{-}ST{-}OH} + h\nu &\rightarrow \mathrm{HO{-}ST{-}OH^{*}} \\ &\rightarrow \mathrm{HO{-}ST{-}OH^{\bullet +}} \rightarrow \mathrm{O{=}ST{=}O} + 2\mathrm{H^{+}}\end{aligned}$$

Glutathione S-transferases. Glutathione *S*-transferases (GSTs) are biologically significant because they catalyze the conjugation of reduced glutathione (GSH) to a variety of electrophiles and thereby detoxify carcinogens, pesticides and reactive products generated under oxidative stress. The conjugation of GSH with a foreign compound generally results in the formation of a nontoxic product that can be readily eliminated (132). In addition to their catalytic function, GSTs also serve as nonenzymatic-binding proteins, known as ligandins, which interact with various lipophilic compounds that include steroid and thyroid hormones. Finally, reactive oxygen species can trigger apoptosis, programmed cell death. The production of GSTs, by helping to protect the cell from oxidative damage caused by reactive oxygen species, assists in obviating the induction of apoptosis (132). Our hypericin/HSA results discussed above serve as a baseline reference in the following discussion.

Glutathione *S*-transferase binds hypericin very tightly (133); we have studied two GST isoforms referred to as A1-1 and P1-1: A1-1 with K_D = 0.65 μM; P1-1 with K_D = 0.51 μM. Binding of hypericin to GSTs also inhibits their catalytic activity (133). The submicromolar affinity with which hypericin binds to GST is the first case of high affinity binding of hypericin to a biologically important molecule that has been documented. An intriguing observation is that the photophysical properties of hypericin can be altered upon binding to GSTs. In particular, when hypericin binds to A1-1 (or HSA), light-induced oxidative damage is produced, as evidenced by the mass spectrometry data obtained after 30 min of optical irradiation. On the other hand, no such oxidative damage is observed for P1-1 (133).

As noted above, we have argued that one of the primary processes deactivating the excited-state singlet of hypericin in bulk organic solvent is intramolecular hydrogen atom transfer. The signature of this process is the rise time in the kinetics, as indicated in Fig. 6 for hypericin in DMSO. This process is not, however, observed when hypericin is bound to HSA (101). We interpreted this result in terms of hypericin binding to a tryptophan residue in HSA that inhibited its H-atom transfer. Given that intramolecular H-atom transfer is impeded in hypericin/HSA and that light-induced protein oxidation of hypericin/HSA is more similar to that of hypericin/A1-1 than to hypericin/P1-1, it was our original expectation that the photophysics of hypericin/A1-1 should more nearly resemble that of hypericin/HSA than that of hypericin/P1-1. This expectation was not, however, borne out uniformly by subsequent experiment: the singlet kinetics and fluorescence intensities of hypericin/P1-1 more closely resemble those of hypericin/HSA (Table 3). On the other hand, the long-time kinetics indicate that the population of hypericin radical ion in A1-1 is essentially identical to that generated in complex with HSA and about a factor of two less than that obtained in complex with P1-1.

The unanticipated behavior of the singlet kinetics and the fluorescence quantum yields originally proved quite puzzling in light of the hypericin activity. They, however, can be rationalized by invoking a different branching ratio from the triplet, which can either decay into the radical ion or by collision with oxygen. When this is carried out, it is found that:

$$20 = \frac{\Phi_{\Delta}^{\mathrm{A1-1}}}{\Phi_{\Delta}^{\mathrm{P1-1}}} \cdot \frac{\Phi_{\mathrm{ion}}^{\mathrm{P1-1}}}{\Phi_{\mathrm{ion}}^{\mathrm{A1-1}}} = \frac{k_{\mathrm{ion}}^{\mathrm{P1-1}}}{k_{\mathrm{ion}}^{\mathrm{A1-1}}},$$

where the Φ represent the quantum yields for production of singlet oxygen or ion formation and the k are rate constants for ion formation. In other words, because the triplet state of hypericin is much more susceptible to decay into the anion when it is bound to P1-1 than to A1-1 or HSA (134), it generates considerably less singlet oxygen when complexed to P1-1 than to the two latter proteins. The origin of this difference can only be attributed to the amino acids surrounding hypericin in the various proteins and their effect on the excited-state photophysics of hypericin. An energy level diagram summarizing these processes is given in Fig. 5.

Naturally occurring hypericin-like chromophores and their complexes with proteins

Stentorin. The first class of naturally occurring hypericin-like chromophores studied in complex with their proteins was that of the stentorins (39). As indicated above, the stentorins are membrane-bound chromoproteins found in pigment granules of the blue-green ciliate, *S. coeruleus*. The structure of stentorin is given in Fig. 1. Isolated from the protein, it has a 5.5 ns fluorescent lifetime, as does hypericin in bulk organic solvent (Table 3). Using pump-probe absorption spectroscopy, Savikhin *et al*. (135) found that when isolated or when bound

to the protein (*i.e.*, stentorin I and stentorin II), stentorin, stentorin I and hypericin all provided similar excited state spectra and kinetics. On the other hand, they found that stentorin II rapidly develops spectral features that are different from the other three systems.

It is significant that upon binding to protein, the fluorescence quantum yield of stentorin is reduced. This reduction is most pronounced in the chromoprotein, stentorin II, *i.e.*, a change from 0.35 to 0.01 (Table 3).

Blepharismin. Plaza and coworkers (136,137) have recently extended studies of these naturally occurring chromoprotein complexes to oxyblepharismin (Fig. 1) and its chromoprotein, the oxyblepharismin-binding protein, which are obtained from light-adapted cells of *B. japonicum*. These workers identify two classes of chromoprotein. The ''reactive'' species possesses a 680 nm band that is described by biexponential decay with time constants of 4 and 56 ps. They also identify a species to which they identify as ''nonreactive'' because it behaves similarly to free oxyblepharismin. They suggest that electron transfer could be involved in the primary photoprocesses of the reactive chromoprotein and hence trigger the sensory transduction chain of *B. japonicum*. Finally, they investigated the nonnatural complex of oxyblepharismin with HSA and observed that its excited-state photophysics are much more similar to that of free oxyblepharismin than to that of the reactive chromoprotein. These results suggest strongly that there is a specific interaction between the chromophore and its protein that leads to the photoreceptor response of the ciliate.

Maristentorin and the T580 and T731 pigments. Maristentorin: Recently, we have proposed a structure for the photoreceptor pigment of the heterotrich ciliate, *M. dinoferus* (8). It is similar to those of *S coeruleus* and *B. japonicum* but differs significantly in that it bears no aromatic hydrogens (Fig. 1). It is fluorescent *in vivo* (Fig. 3a and b). The structure of maristentorin is based upon the hypericin skeleton and its spectra are nearly identical to those of hypericin but shifted toward the red. Within experimental error, its fluorescence lifetime is identical to that of hypericin, ~5.5 ns in DMSO (Table 3). It is remarkable that while the pigments are structurally similar in *S. coeruleus* and *M. dinoferus*, the photoresponses are opposite (see section Photoresponses in heterotrichs, above).

Folliculinid pigments: As indicated in the section Heterotrichs, stentorids and occurrence of pigments, above, S.H. at MBARI collected two folliculinids T580 and T731; and his group and that of J.W.P. have submitted them and their pigments to analysis.

Folliculinid Sample Collection, DNA extraction and purification. Carbonate rock fragments encrusted with indigo mat were collected with the aid of the ROV *Tiburon* deployed from the R/V *Western Flyer* during dive T580, LAT: 27.596, LON: −111.486, at a depth of ~1567 m, ambient temperature 2.965°C, salinity = 34.53 PPT and dissolved oxygen = 0.47 mL/L. Mat samples were scraped from selected carbonate surfaces aboard ship, processed for microscopy and DNA extraction and subsequently frozen below −20°C for land-based analyses. Selected mat samples were dissected, fixed in 2% gluteraldehdye (EM grade; Sigma) diluted in 0.2 μM filtered seawater and stored at 4°C until processed for TEM. Following fixation, samples were postfixed, dehydrated infiltrated and polymerized using the microwave techniques of Giberson *et al.* (138). Thin sections were cut, stained with lead citrate and placed on formar coated grids for viewing in either JOEL 100B or 1200EX TEM. Genomic DNA was extracted from 0.25 g of mat sample, using the Qiagen DNA isolation kit according to manufacturer's instructions (Qiagen Inc., Valencia, CA). DNA was eluted with 200 μL TE pH 7.8 and stored at −20°C prior to polymerase chain reaction (PCR) amplification.

Eukaryotic and SSU rRNA gene amplification. Eukaryotic SSU ribosomal RNA (rRNA) sequences were PCR amplified using kingdom specific-primers: (EukF 5′-AACCTGGTT GATCCTGCCAGT and EukR 5′-TGATCCTTCTG CAGGTTCACCTAC). Fifty microliter amplification reaction mixtures contained 1 μL template DNA, 41.5 μL 1X buffer, 1 mL each 10 mM forward and reverse primer, 2.5 U TaqPlus Precision polymerase (Stratagene, La Jolla, CA) and 5 mL 10 mM stock dNTP mixture. Amplifications were carried out using the following profile: 94°C/3 min., X36 cycles 94°C/40 s, 55°C/1.5 min and 72°C/2 min, followed by a final extension at 72°C/10 min.

Clone library construction and sequencing. SSU rRNA amplicons were visualized on 1% agarose gels in 1X TBE and purified directly using Qiaquick PCR purification kit (Qiagen Inc.). Purified amplicons were cloned into pCR4-TOPO vector using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA) and transformed by chemical transformation into TOP10 one shot cells according to the manufacturer's instructions. Transformants were transferred to 96 well plates containing 180 μL Lbkan50 and 7% glycerol and stored at −80°C. Plasmid DNA was purified from glycerol stocks using the Montage Plasmid Miniprep96 kit (Millipore, Bedford, MA) following the manufacturer's protocol and stored at −20°C. Plasmid insert sequence data were collected on an ABI Prism 3100 DNA sequencer (Applied Biosystems Inc, Foster, CA) using Big DyeTM chemistry (PE Biosystems, Foster, CA) according to manufacturers' instructions. Plasmids were sequenced bidirectionally with M13F and M13R primers. Sequences were edited manually from traces using Sequencher software V4.1.2 (Gene Codes Corporation, Ann Arbor, MI). The sequence of T580 has been submitted to GenBank under accession number EF688408.

Phylogenetic analysis. SSU rRNA phylogenetic analyses were performed on sequences recovered from the indigo mat in conjunction with related and described species groups. SSU rRNA sequence data were compiled with ARB software (http://www.arb-home.de) and aligned with sequences from the Genbank database using the FastAligner program. Aligned sequences were visually inspected for conservation of secondary structure features and manually edited when necessary. The final masked alignment contained 1171 nucleotides for comparison. A ciliate SSU rRNA tree based on the tree reported by Miao *et al.* (57) was generated from this alignent using distance, parsimony and maximum likelihood methods implemented in PAUP* version 4.0b10 (139). Bootstrapping for all three methods was accomplished with 1000 replicates per tree using heuristic search methods. The phylogenetic tree was updated using the following recent GenBank entries *F. salina* (two strains), *Condylostoma minutum,* two species of *Loxodes* and ''*Stentor auriculatus*.'' Gong *et al.* (140) showed that the last of these is not a *Stentor*; its last formal

reassignment was to *Condylostentor* (141); but following Foissner and Wölfl (43), we refer it to *Condylostoma*, a view that is supported by the analysis.

The spectroscopic characterization of the pigments is summarized in Figs. 9 and 10 and in Table 3. There is considerable similarity between their emission and absorption spectra with those of hypericin, and it is reasonable to suggest that they are hypericin-like. As noted above, hypericin bound to proteins (HSA and GST) exhibits nonexponential decay kinetics. We observed biexponential fluorescence decay in T580 and T731 pigments dissolved in acetone. The average fluorescence lifetime for these two pigments is ~3.8 ns, which is less than that for hypericin in pure organic solvents (~5.5 ns). The decrease in fluorescence lifetime and deviation from single exponential decay kinetics might indicate that these pigments are still associated with proteins or that other nonradiative rates are operative.

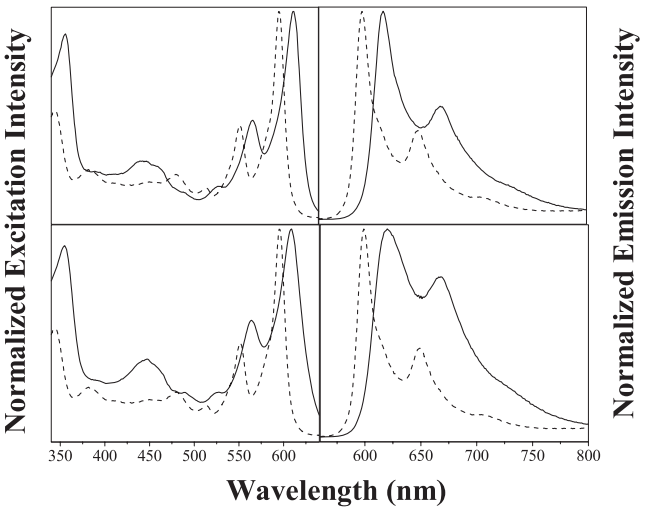

Figure 9. Normalized steady state excitation and emission spectra of pigments T580 (upper panel, solid) and T731 (lower panel, solid) in acetone. For comparison the corresponding spectra of hypericin dissolved in acetone is also included (dashed). The excitation wavelength for the emission spectra is 550 nm. For the excitation spectra, the emission monochromator is set to 650 nm. The spectra for both pigments are nearly identical with those of hypericin but shifted toward the red. The spectra suggest that the pigments are hypericin-like. Note that the relative intensities of the ~600 and ~650 nm bands differ in the two pigments. Figure 10 presents the fluorescence decay of the two pigments in acetone.

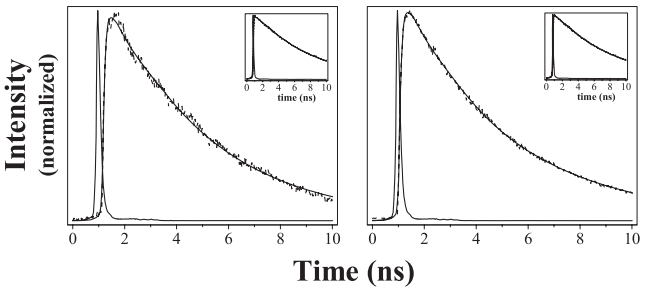

Figure 10. Fluorescence decay of pigments T580 (left panel) and T731 (right panel) in acetone. The samples are excited at 407 nm and emission is collected after 570 nm using cut-off filters. Both fluorescence decays are well described by a sum of two exponentially decaying components. For T580, $F(t) = 0.07 \exp(-t/0.5\text{ ns}) + 0.93 \exp(-t/3.8\text{ ns})$ and for T731, $F(t) = 0.24 \exp(-t/1.7\text{ ns}) + 0.76 \exp(-t/4.6\text{ ns})$. For comparison, the fluorescence decay of hypericin in acetone is shown (inset). The decay kinetics for both pigments are well described by biexponential behavior having average fluorescence lifetime of ~3.8 ns.

TOXICITY, UV-PROTECTION AND OTHER POSSIBLE ROLES FOR HYPERICIN-LIKE PIGMENTS IN HETEROTRICHS

Stentorin and blepharismin are well known to be both endogenous photosensitizers, capable of damaging the cells that produce them, and exogenous photosensitizers, damaging other cells, such as *Paramecium* and predators. As noted at the outset, there is clear evidence for a role of several hypericin-like pigments in predator defense and some evidence for a role in UV protection. These roles may be instead of or in addition to any role in photoreception, and we should not consider whether any role is "primary," as all roles are presumably important to the well-being of the organism (or symbiotic association as the case may be). Chemical defenses can serve multiple roles in marine invertebrates, as well; for instance, Stachowicz and Lindquist (142) found that grazer-deterrent secondary metabolites in a hydroid also protected it from UV stress; in such cases, the quantities needed for one role may be much more than would suffice for another. We consider endogenous and exogenous toxicity first, then the potential role of hypericin-like pigments in the broader context of chemical protection from UV wavelengths.

Endogenous toxicity

It seems evolutionarily odd, as Häder and Häder (143) noted that an organism produces a photodynamically active compound that is harmful to its survival in high irradiance yet uses that same compound to avoid high irradiance. Looking over the range of organisms with hypericin-like compounds, a striking commonality is that virtually all live in lighted environments. Some, such as *Hypericum*, sequester the toxins in special glands; do cortical granules of heterotrichs serve the same role? And if they do, why are *Blepharisma* spp. and *S. coeruleus* photosensitized? *B. japonicum* became insensitive to light when its pigment was extruded by cold shock and regained sensitivity when it regenerated its pigment (71). Perhaps, rather than asking, how do photophilic organisms cope with photosensitizers but rather, how did *S. coeruleus* and *B. japonicum* become sensitive to their own defense compounds? What ecological differences are there between fluorescent, phototoxic strains of *S. coeruleus* and nonfluorescent, nontoxic strains? (68) Modenutti *et al.* (54) thought it significant that the photophilic species *Stentor araucanus* is not fluorescent *in vivo*.

Toxicity of blepharismin and stentorin chromophores in the light comes about primarily because they generate singlet molecular oxygen ($^1O_2$) and/or other reactive oxygen species, both *in situ* and in the medium (see section Characteristics of hypericin-like chromophores and primary photoprocesses, above) (39,40,49,51). Surprisingly, some blepharismins and oxyblepharismins are also exogenously toxic *in the dark* to the predatory haptorid ciliate *Dileptus margaritifer* (42); this must be due to some other mechanism. When *Blepharisma* is

exposed to weak light it changes color from red to blue because of phototransformation of blepharismins to oxyblepharismins and also reduces the amount of pigment; the changes decrease the cell's defensive ability but increase resistance to endogenous phototoxicity.

How does symbiosis interact with endogenous toxicity? Perhaps, the ability to enter symbiosis with algae results from development of tolerance to reactive oxygen, as suggested by Kawano *et al*. (144) for green *P. bursaria*. If that is the case in heterotrichs, might it also affect their ability to contain large amounts of toxic pigments and live in light? That is, species such as *S. coeruleus*, without algae, and perhaps without tolerance to oxygen radicals, survive because they seek darkness and have adapted the pigment to help them avoid light, whereas symbiotic species need oxygen resistance to cope with algal photosynthesis, and this also protects them from harmful effects of the pigment in light. This argument would be strengthened if it turned out that pigmented heterotrichs without algae are generally sciaphilous.

While Miyake *et al*. (50) used "white" (zoochlorella free) *C. virens*, obtained by culturing originally green cells in darkness, they noted from preliminary experiments that symbiotic algae reduced the vulnerability to attack by *Dileptus*. Unlike other heterotrichs, *C. virens* is facultatively symbiotic with zoochlorellae. Repak (145) in his revision of this species made no mention of algae and described it as a bottom-dwelling predator. However, the algal symbiont of this species was studied by Reisser and Kurmeier (146). Colorless *C. virens* can harbor endosymbiotic bacterial cells (147) but loses them if zoochlorellae reestablish, leading Karadzhian and Vishiakov (148) to conclude that *C. virens* cannot support both at the same time. The potential for bacteria to be involved with defense compounds can be seen from the many recent works on the role of bacterial symbionts in marine invertebrate toxicity (149). More studies on colorless strains of *C. virens*—or of bleached *Stentor* spp.—might lead to deeper understanding of the role(s) of the algae in the pigment/toxin/behavior relationships. How do green and colorless cells respond to light gradients? What are the trade-offs for *C. virens* to take up (or be "infected by?") zoochlorellae in light *versus* living in dim/dark environments with (or without?) symbiotic bacteria? The relationship between the symbionts is likely to be driven to a large extent by growth considerations, as Stabell *et al*. (150) found in green *versus* aposymbiotic *Coleps* (a ciliate in the Class Prostomatea, Order Prorodontida), but the role of algae and/or bacteria in chemical defense or offense may prove critical to success in different environments.

Exogenous toxicity: predator defense

Several heterotrich ciliates are chemically defended against predatory ciliates and multicellular predators by the contents of cortical granules (Table 2). The pigments of *S. coeruleus* (49), *B. japonicum* (48,51,151), *L. striatus* (52) and the colorless toxin in *C. virens* (50) can be extruded upon attack and repel the ciliate *Dileptus*. This predator is both physically damaged and behaviorally disturbed by the toxin and will die if trapped in toxin solutions. As a common predator on heterotrichs, it has become a convenient assay organism for toxins in extrusomes (42,50,51). Miyake *et al*. (50) also conducted preliminary experiments on several other heterotrichs (Table 2). From these observations, it seems probable that cortical granule contents in heterotrichs are generally extrusible and toxic, whatever other roles they may have. However, while the toxins may be broadly effective as feeding deterrents—*Loxodes*' pigment defends it against the [mesograzer] catenulid turbellarian (flatworm) *Stenostomum sphagnetorum* as well as predatory ciliates (52)—they are not universal. *B. japonicum* is defended by its pigment against *Amoeba proteus* but not *C. virens* (151) and Giese (41) noted that blepharismas in general are "readily eaten" by some other protists and small crustaceans.

All these compounds are hypothetical derivatives of the polyketide (acetogenin or acetate-malonate) biosynthetic pathway that is ubiquitous in eukaryotes (152). The pathway for hypericin biosynthesis is thought to be from acetyl coenzyme A *via* the intermediate emodin anthrone, and then oxidative dimerization to hypericin, but the pathway has not yet been demonstrated unequivocally (153). Masaki *et al*. (53) saw a "close relation between the biosynthesis of climacostol and that of stentorin" coming from C16 polyketide. This may prove interesting as more of the phylogenetic picture is filled in, but with only one colorless toxin identified so far, the phylogenetic significance is not clear.

In other organisms such as crinoids (11) and *Hypericum* (154,155), the defense role of hypericin or hypericin-like compounds is more obvious. Moreover, such compounds (fringelites [Fig. 1] and even hypericin itself) already occurred in crinoids in the Middle Triassic, some 225 million years ago (11). Gymnochromes (Fig. 1) are brominated hypericins that occur in living and fossil crinoids (12,153). Brominated acetogenins are well known and common secondary metabolites in marine organisms (156); their toxicity does not depend on photochemistry.

There is likely to be a wealth of colorless toxins, such as climacostol, among the heterotrichs. However, bioprospectors might note that the occurrence of saxitoxin in dinoflagellates does not follow phylogenetic lines, but has spread to disparate taxa (157). There is even some evidence that saxitoxin might be produced by endosymbiotic bacteria (158).

UV protection

Most organisms must be able to deal with damage from UV light, especially UV-B (280–320 nm). UV-A (320–400 nm) can be both harmful and beneficial. UV-B causes direct damage to nucleic acids and proteins, as well as oxidative stress through the formation of oxygen radicals. Shallow water does not protect organisms from UV; for example, UV-B at 308 and 320 nm penetrated 9 and 27 m, respectively, into coastal Antarctic waters and UV-A penetrates much deeper (159,160). Some organisms avoid the stress by living in or moving to protected areas, others tolerate the stress through chemical coping mechanisms.

Avoidance of UV damage implies the ability to detect and respond to these wavelengths. UV-B has been shown experimentally to affect the motility of several heterotrichs, all lacking algae, but including the photophilic, almost-colorless species *F. salina*, which has high tolerance to UV (41,161), and sciaphilous species with hypericin-like pigments, *S. coeruleus* (143) and *B. japonicum* (87,162). Motility and phototaxis of

F. salina were not affected by UV-A irradiation but UV-B caused an increase in cell speed after a few minutes and loss of phototaxis after 60 min (161). In *S. coeruleus* after 4 min in unfiltered sunlight, all cells had irreversibly stopped swimming; filtering out UV-B and UV-A increased the period of motility but only for a few minutes. Evidently, all three components of sunlight affect motility. Exposure to light *reduced* speed of cells in this species (143) and in *B. japonicum*. In the latter species, also, UV decreased swimming speed and photoresponsiveness (see below) (87).

Among the most widespread means of coping is the presence in many aquatic organisms of suites of MAAs that act as sunscreens. These compounds are formed from the shikimic acid pathway in bacteria and algae and acquired by animals through diet or symbiosis (160,163). Some MAAs, mainly shinorine, palythenic acid and palythine, were identified in *Maristentor* (55), the first report of these compounds in an alga-bearing protist, but MAAs have also been found in some *Chlorella*-bearing freshwater ciliates (164). Several mixotrophic heterotrichs, including *S. amethystinus* and *Stentor araucaria,* dominate lake plankton blooms (165,166) and at least *S. araucaria* is evidently resistant to UV (54), implying the presence of defense compounds. Both these *Stentor* spp. are strongly pigmented (Table 2) and Modenutti *et al*. (54) proposed that stentorin serves this role.

The potential for hypericin-like compounds to act in UV protection derives from the relatively large absorbance through UV-A and UV-B wavelengths (Fig. 2), and the hypothesis was promoted by Giese (41) for *Blepharisma* (before MAAs were known). One might think that an abundance of pigment in cortical granules would provide a strong sunscreen. However, experiments on *B. japonicum* (87) showed that UV-B harmed both red and blue cells by impacting some step(s) in the transduction chain, and the pigments, which were not altered by UV, apparently did not shield cells. UV-B irradiance altered cell shape and reduced motility and the photophobic response, so that cells essentially became ''trapped'' in harmful irradiance; within an hour they were barely motile and totally unresponsive to stepped-up visible irradiance. However, Lenci's group (162) subsequently showed that *B. japonicum* could detect and avoid UV-B and that blepharismin was the photoreceptor. Thus in this species, the pigment does protect the cells from UV-B, but *via* the cells' behavioral reaction rather than by physical screening. The implication for photophilic species from these results is that their cortical pigments probably do not supplement or replace the role of MAAs, but because many photophilic species have algal symbionts it is likely that they have MAAs.

It seems doubtful that hypericin-like compounds have any direct role in protecting cells from UV irradiance. However, the various roles of pigments, MAAs, behavior, *etc*., need to be understood in the integrated ecology of the ciliate or both partners in a symbiosis. While we have only hints so far of the complexity in *Maristentor*, the fact that this species alters the distribution of both cortical granules (Fig. 3a and b) and zooxanthellae from day to night (44,45) suggests that different roles might be optimized in different circumstances.

These considerations allow us to speculate on the possible role of the hypericin-like pigment in the deep-water folliculinids. The most plausible hypothesis is that it is a defense compound that, like the pigments of *B. japonicum* (42), is toxic in darkness. It is also possible that the pigment in this species (and other heterotrichs) fills some as yet unimagined role. It is even conceivable that the pigment in this deep water form is ''vestigial'' and has no present function.

CONCLUSIONS AND HYPOTHESES

Although little is known of the protein–pigment complexes in ciliates, the diversity of structure and physiology offer many opportunities, for comparison and perhaps new models to address questions, such as the nature of nonradiative processes induced in protein-bound chromophores. It might be particularly interesting to compare structure and properties of molecules from photophilic and/or symbiotic species with those from photophobic species but care must be taken to establish that the photoreceptor is in the ciliate and not (as, apparently, in green *Paramecium*) in the alga.

Hypericin-like chromophores are known so far only in the stentorids and their sister group *Fabrea*. The most likely sources of novel pigments are in the species of *Stentor* that are not bluish like *S. coeruleus*—*S. amethystinus*, *S. niger*, *S. fuliginosus, S. igneus* and *S. loricatus*—and in the diverse genera of folliculinids, which are phylogenetically separated from *Stentor* and *Blepharisma*. Evidence on the extent and origin of hypericin-like pigments in heterotrichs, however, can best be sought in nonstentorids, such as *Condylostoma*, and other taxa that may, like *F. salina*, appear colorless. We do not yet know enough about the yellow and brown pigments from *Stentor* and *Loxodes* to form any phylogenetic hypothesis.

The diversity of colors among *Stentor* spp. contrasts with the comparative uniformity in *Blepharisma* spp. and the several genera of folliculinids. The chemistry of the pigments may make a useful contribution to understanding phylogeny within *Stentor*.

Hypericin-like pigments and other contents of cortical granules are evidently multifunctional and play key roles in the behavior and ecology of heterotrich species. The roles are likely to be affected by whether the ciliate contains endosymbiotic algae or not and could also be adaptable to different conditions of light, predation, *etc*. Adaptation of different functions for these molecules and replacement of the functions by other compounds may have occurred on several occasions.

A reasonable generalization (as a starting point) is that heterotrich cortical granules function in predator defense; the toxin(s) may be pigmented and/or not. Colorless toxic compounds could be present in cortical granules that are pigmented. It will be interesting to compare the toxic compound(s) in the (apparently) colorless *S. polymorphus* and *S. roeselii* with climacostol.

Hypericin-like pigments may not be involved in photoresponses, even when an organism responds to light, and other photoreceptors could be overlooked when bulk pigments are present; it is especially likely that true phototactic behavior is mediated by other pigments, such as rhodopsin.

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