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# Identification of chlorophyll *b* in extracts of prokaryotic algae by fluorescence spectroscopy

(chlorophyll *a*:*b* ratio/photosynthetic membranes/ultrastructure)

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ABSTRACT **Solvent extracts of three different prokaryotic algae from three species of didemnid ascidians contained pigments identified, on the basis of their fluorescence excitation (E) and fluorescence emission (F) spectral maxima (measured in nm) at 77 K, as chlorophyll *a* (E 449, F 678) and chlorophyll *b* (E 478, F 658). The release of algae on cutting or freezing *Diplosoma virens* was accompanied by a strong unidentified acid that converted these pigments to pheophytins. This unexpected finding provided further confirmation of the identity of the chlorophylls on the basis of the fluorescence spectra at 77 K of pheophytin *a* (E 415, F 669) and pheophytin *b* (E 439, F 655). There was no evidence for the presence of the fluorescent bilin pigments found in other prokaryotic blue-green algae. Chlorophyll *a*/*b* ratios ranged from 2.6 to 12.0 in algae from different ascidians. The photosynthetic membranes were not organized into appressed thylakoids or grana in the algae from any of the three species of ascidians. The relationship between these observations and those in higher eukaryotic organisms is discussed.**

Several species of colonial ascidians in the family Didemnidae (Phylum Chordata, Subphylum Urochordata) from the Great Barrier Reef of northeastern Australia harbor bright green, unicellular algae in their cloacal cavities. Earlier studies of these algae showed that they are prokaryotic, with an ultrastructure resembling that of blue-green algae, but that they apparently lack the phycobilin pigments and the phycobilosomes characteristic of the blue-green algae (1). Similar prokaryotic algae have been reported growing on the surface of an unidentified didemnid ascidian from Baja California, and the presence of chlorophylls *a* and *b* in these algae was provisionally established on the basis of chromatography and room temperature absorbance spectra (2, 3). The published spectra for chlorophyll *b* do not coincide with authentic chlorophyll *b* and indicate either contamination, or breakdown, or the presence of an atypical chlorophyll *b*.

We have used low-temperature (77 K), narrow-band fluorescence excitation and fluorescence emission spectroscopy to analyze the fluorescent pigments of these unusual photosynthetic organisms. These techniques have the advantages of high sensitivity and high specificity, and may be applied directly to mixtures of pigments, thus avoiding artifacts and breakdown products which may arise during pigment separation. Using such methods, we have established, unequivocally, the presence of chlorophyll *b*, and the absence of phycobilins, in algae associated with three species of ascidians from the Great Barrier Reef. These observations firmly establish the existence of a unique anomaly wherein the members of a group of algae that are prokaryotic in structure possess the complement of chlorophyll pigments heretofore known to exist only in eukaryotic cells.

## MATERIALS AND METHODS

**Collection of Ascidians and Algae.** The ascidians were collected on the reef at Heron Island (23° 27′ S, 151° 55′ E) during two visits in late July and early September 1976. *Diplosoma virens* (Hartmeyer), *Trididemnum cyclops* (Michaelsen), and juvenile colonies of *Didemnum ternatanum* (Gottschaldt) were collected at low tide from coral rocks immediately behind the reef front. Larger, older colonies of *D. ternatanum* were found attached to branches of staghorn coral in deeper water along the front edge of the reef. The specimens collected in July were identified at the Heron Island Research Station laboratory by Patricia Kott Mather. Samples were embedded as previously described (4) for later electron microscopic study.

Differences were noted in the ease with which the algae are removed from their ascidian hosts. Those from *Diplosoma virens* were released readily by applying pressure to the ascidian colony after it had been cut in two. The algae from this ascidian were either expressed into cold, filtered sea water, or into cold 0.1 M Tris·HCl buffer at pH 8.3, and then sedimented by centrifugation. Algae were much more difficult to extract from the other two ascidians, and consequently were isolated in relatively small quantities from these species. In *Didemnum ternatanum* their release is accompanied by the breakage of many of the algal cells and by embedment of the remainder in copious amounts of an extraordinarily cohesive mucilaginous material. In each species the separated algae, as well as intact colonies, were placed in sealed vials and frozen in a canister of liquid nitrogen for transport back to Canberra.

**Extraction and Chromatography of Pigments.** Pigments were extracted from freshly harvested algae at Heron Island or from frozen algae in the laboratory. At Heron Island the sedimented algae were immediately suspended in cold 100% acetone and extracted by several passages in a Ten-Broek glass homogenizer. The extract was centrifuged and the acetone extract was sealed in a vial and stored in liquid nitrogen. In the laboratory the frozen algal pellets or the intact colonies were thawed and the algae were harvested by centrifugation and extracted into acetone in the same way. The residue from acetone extractions was practically colorless. Any remaining water-soluble pigments were extracted from this residue with methanol containing 5% (vol/vol) HCl. Water-soluble pigments were also extracted by repeated freezing and thawing of algal suspensions in 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer at pH 6.5.

For chromatography the acetone extract was treated with an equal volume of diethyl ether and with 20 volumes of cold 10% NaCl. Pigments were extracted into the ether layer at 5°C and the ether fraction was dried with solid NaCl before evaporation in a stream of dry nitrogen at room temperature. Pigments were separated on ready-prepared, cellulose powder thin-layer plates (MN-300) using a two-dimensional system described by Jeffrey (5). An extract of spinach leaves was used as a reference source of chlorophyll *a* and *b*.

**Fluorescence Spectroscopy.** The mixture of extracted pig-

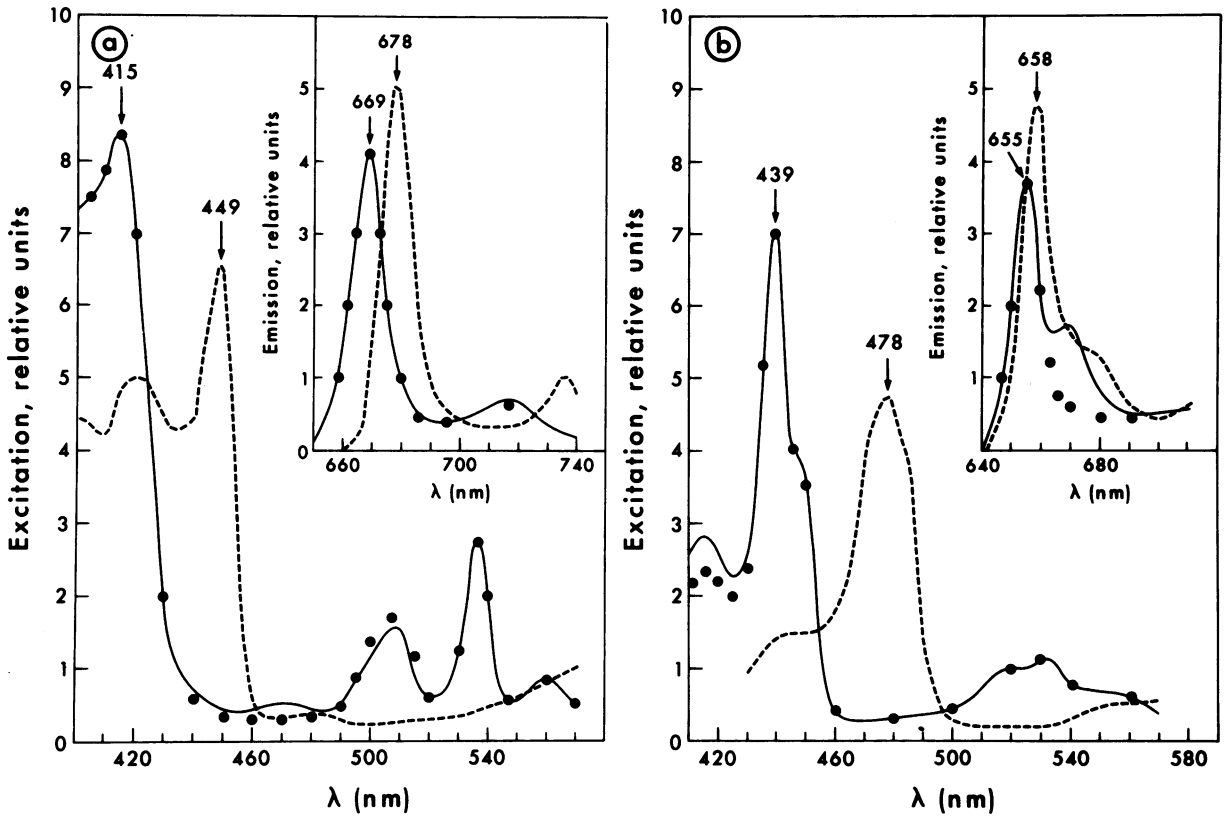


FIG. 1. Relative narrow band fluorescence excitation and fluorescence emission spectra in ethanol at 77 K of the photoactive pigments extracted from the unicellular algae associated with the didemnid ascidian *Diplosoma virens* (procedures given in *Materials and Methods*). (a) Spectra of "a" type pigments. (b) Spectra of "b" type pigments. In both parts of figure spectra shown by broken lines are of pigments extracted in acetone from algae isolated in buffer at pH 8.3. These spectra are identical with those of chlorophyll *a* (a) and chlorophyll *b* (b) (6). Spectra shown by solid lines were obtained with pigments extracted in acetone from algae isolated from frozen and thawed colonies of *Diplosoma virens*. These spectra are identical with those of pheophytin *a* (a) and pheophytin *b* (b) as shown by the overlay of points (●) taken from spectra of authentic compounds.

ment was diluted 100-fold into ethanol (absorbance <0.05 at 660 nm) and was analyzed by narrow band fluorescence spectroscopy at 77 K. At this temperature, the excitation and emission spectra are considerably sharpened and enhanced, whilst ethanol provides a clear, frozen, transparent medium. Also, as has been shown previously (6), advantageous shifts in relative peak positions of both chlorophylls and pheophytins occur at 77 K, allowing greater wavelength separation of the mixed pigment peak positions and giving up to a 10-fold increase in both sensitivity and selectivity over measurements at 20°C.

Fluorescence emission and excitation spectra were recorded on a fluorescence spectrometer incorporating automatic corrections for photomultiplier and monochromator responses with wavelength, and variation in the output of the light source (7, 8). The fluorescence excitation band width was 1.5 nm with a fluorescence emission band width of 1 nm. The excitation spectra were recorded by scanning a wavelength range on the excitation monochromator with the fluorescence emission monochromator fixed on an emission peak, or fluorescence emission spectra were recorded by scanning a wavelength range on the fluorescence emission monochromator with the excitation monochromator fixed on an excitation peak. A comparative method of analysis was used for the identification of the unknown pigments in these algae. The fluorescence excitation and emission spectra were compared with a library of established spectra of known pigments purified by previously described methods (6, 9, 10).

The state of esterification of the chlorophyll-type pigments was determined by solvent phase partition between acetone/water/ammonia (specific gravity 0.91) mixed 44/5/1 (vol/vol) and petroleum ether (boiling point 40–60°C) as previously described (11). The pigment content of the separated phases was determined by means of fluorescence spectroscopy at 20°C. The ratio of chlorophyll *a* to chlorophyll *b* was estimated from 77 K fluorescence spectra as detailed previously (6).

## RESULTS AND DISCUSSION

Chromatography of the pigments extracted from algae separated from all three species of didemnid ascidians revealed two green pigments, which had approximately the same $R_F$ in the two-dimensional solvent system as the two green pigments of spinach leaves. The extracts of all species also contained substantial amounts of three other pigments, which were pale-yellow, yellow, and orange. The identity of these compounds, presumably carotenoids and xanthophylls, was not further examined.

Spectroscopic analysis of extracts from *Diplosoma virens* showed at least four fluorescent pigments and it was evident that the proportion of each depended on the method of preparation and extraction used. Fig. 1a and b shows the 77 K fluorescence excitation and fluorescence emission spectra for extracts from *Diplosoma virens* measured under conditions detailed in *Materials and Methods*. These spectra establish the presence in these extracts of both chlorophyll *a* and *b* and of pheophytin *a* and *b*, derived from the chlorophylls. When the algae were expressed from the cloacal cavity of *Diplosoma virens* directly into buffer at pH 8.3, and the sedimented algae

Table 1. Ratio of chlorophyll *a*/*b* in acetone extracts of algae isolated from colonial ascidians and the extent of esterification of the chlorophyll pigments

| Source of algae | Chlorophyll *a*/*b* | Esterification (%) |
|---|---|---|
| *Diplosoma virens* | 2.7, 2.6, 2.7, (2.8)* | 98, 99 |
| *T. cyclops* | 5.5, 7.0 | 98 |
| *Didemnum ternatanum*, | | |
| intertidal colonies | 6.7 | 85† |
| reef front colonies | 11.5, 12.0 | 99, 99 |

* Computed from extinction coefficients for pheophytin *a*, *b*.
† Phase separation hindered by mucilaginous substances.

were immediately extracted into cold acetone, analysis by fluorescence spectroscopy in ethanol dilutions showed that only chlorophyll *a* and *b* were present. These may be characterized by the fluorescence excitation peaks E and fluorescence emission peaks F shown in Fig. 1a for chlorophyll *a* (E 449, F 678) and in Fig. 1b for chlorophyll *b* (E 478, F 658), which are identical to those described previously for the authentic purified pigments (6).

When the algae were released from colonies of *Diplosoma virens* that had been frozen for transport to the laboratory, brownish-green pigments were extracted into acetone. The fluorescence emission and the fluorescence excitation spectra of these ethanol-diluted pigments showed them to correspond to pheophytin *a* and pheophytin *b* of Fig. 1a and b, which may be characterized by the peaks for authentic pheophytin *a* (E 415, F 669) and pheophytin *b* (E 439, F 665). The fluorescence emission and excitation spectra show an almost exact correspondence with the spectra of pheophytin *a* and *b* prepared from purified chlorophyll *a* and *b* by strong acid treatment. In Fig. 1b the fluorescence emission spectra of the pigments from *Diplosoma virens* vary from those of the authentic pheophytin *b* or chlorophyll *b* by showing shoulders at 669 nm and 678 nm. These shoulders are indicative of pheophytin *a* and chlorophyll *a*, respectively, which break through with the mixed pigment extracts when pheophytin *b* or chlorophyll *b* are excited preferentially. The breakthrough appears here because the "b" type pigments are present at lower concentration and have a lower fluorescence quantum efficiency than the "a" type pigments.

If the algae of *Diplosoma virens* were expressed directly into sea water, rather than buffer, and were immediately extracted into cold acetone or frozen for transport to the laboratory, spectra were obtained that indicated mixtures of fluorescing pigments with peaks corresponding to chlorophylls *a* and *b*, as well as pheophytins *a* and *b*. Subsequent checks showed that colonies of *Diplosoma virens* released a strong acid when cut or thawed after freezing, and this unidentified acid was presumably responsible for the formation of pheophytins. This unexpected phenomenon, which resulted in the formation of pheophytins, provided an independent check on the identity of the chlorophylls.

Although algae were more difficult to isolate from the other ascidians, they were evidently not exposed to acidic conditions, because acetone extracts prepared from algae of the other species showed no evidence for the formation of pheophytins. The fluorescence emission and excitation spectra of ethanol dilutions were identical to those obtained for purified chlorophylls *a* and *b*. A further fluorescent pigment was identified in extracts of *Diplosoma virens* at a level of about 1% of the total chlorophyll pigments. At 77 K this pigment gave an emission

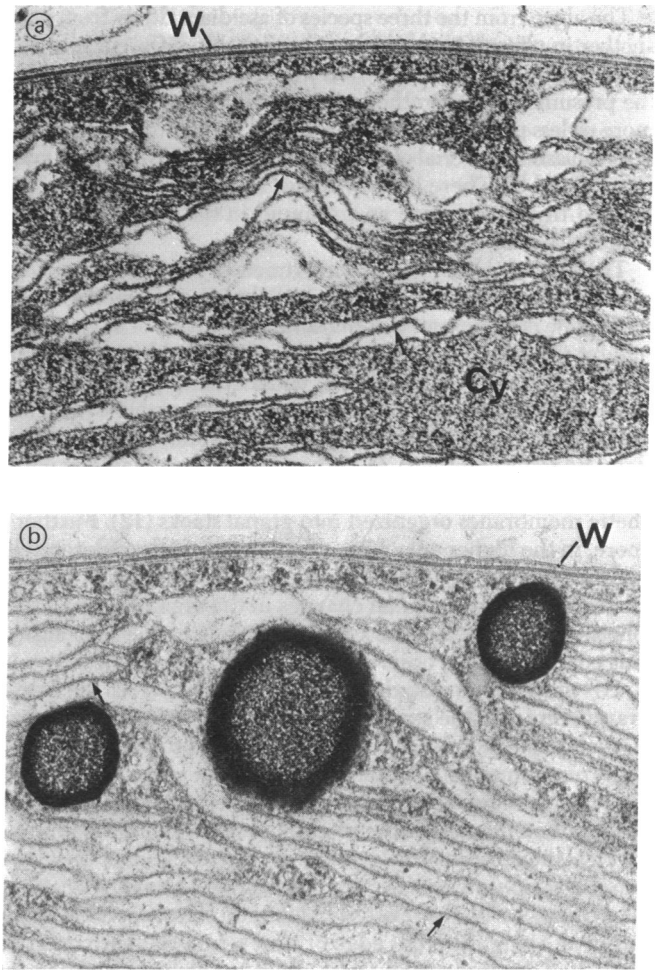


FIG. 2. Electron micrograph of part of a section of an alga in the cloacal cavity of the ascidians *Diplosoma virens* (a) and *Didemnum ternatanum* (b) showing numerous presumptive photosynthetic lamellae (arrows). The lamellae are oriented generally parallel to the cell wall (W) in the outer region of the cell and are separated by cytoplasmic areas (Cy) of variable thickness. In neither alga is there evidence of phycobilisome-like granules on the membrane surfaces. Fixation was in glutaraldehyde followed by osmium tetroxide. Micrographs taken by Thomas D. Pugh. ×50,000.

peak at 635 nm and an excitation peak at 453 nm in the blue-violet region. Under similar experimental conditions chlorophyll *c* appears as E 462, F 641, and protochlorophyll as E 440, F 635, all having excitation spectra of similar shape. Unlike the chlorophylls and pheophytins of these algae, which were more than 98% esterified as indicated by the phase separation technique (Table 1), this minor fluorescent pigment remained in the aqueous phase, suggesting that it is not esterified. It is tentatively identified as protochlorophyllide.

In none of the extracts of these algae was there spectral evidence for either of the strongly fluorescent phycocyanin or phycoerythrin pigments normally associated with the blue-green algae. Freeze/thawing of the algae in buffer failed to release colored material and, aside from minute traces of a fluorescent compound resembling riboflavin, there was no evidence for fluorescent pigments. Furthermore, the tissue residues remaining after acetone extraction did not yield fluorescent pigments on further extraction with 5% HCl in methanol. This evidence corroborates the evidence for the absence of phycobilisome-like granules in electron micrographs of these algae (ref. 4 and Fig. 2).

The algae from the three species of ascidians differ from one another in ultrastructure, and are presumably different species. In the algae of *Diplosoma virens* and *Didemnum ternatanum* the presumed photosynthetic thylakoids are found orientated more or less parallel to the cell wall and are separated by areas of cytoplasmic material of various thicknesses (refs. 1 and 4, and Fig. 2a and b). There is no evidence for the formation of appressed thylakoids, such as are found in the Euglenoids and Chlorophytes.

The ratio of chlorophyll *a*/*b* in extracts of the algae associated with these ascidians varies substantially between species and, in the case of *Didemnum ternatanum*, with age of colony and habitat (Table 1). Although the presence of chlorophyll *b* in these prokaryotic algae is itself of considerable significance, the ratio chlorophyll *a*/*b* and its relationship to the photosynthetic membrane structure and to the light environment of the algae demand some comment. In higher eukaryotic organisms with chlorophyll *a*/*b* ratios less than 3 it is usual to find photosynthetic membranes organized into granal stacks (12). Furthermore, in the higher eukaryotes, differences in the chlorophyll *a*/*b* ratio of the magnitude shown in Table 1 are invariably associated with substantial changes in the organization of the photosynthetic membrane systems. In these prokaryotic algae, this condition is not observed (compare Fig. 2, Table 1).

Of the ascidians studied, *Diplosoma virens* is the most transparent and the specimens used in the investigation were growing on dead pieces of staghorn coral in the most exposed light environments. *T. cyclops* and the intertidal colonies of *Didemnum ternatanum* were generally found in more shaded locations on the undersides of coral rocks. Of considerable interest is the difference in the chlorophyll *a*/*b* ratios of the algae from *Didemnum ternatanum* depending on colony location. The colonies with the relatively high ratio chlorophyll *a*/*b* were growing along the front edge of the reef on staghorn coral at depths of 1–4 m, varying with the tide. These colonies were much larger and presumably older than the intertidal forms, and had a greater development of spicules and of a brown pigment localized in the surface layers of the test. Thus their algae were evidently exposed to low light intensities of somewhat different spectral composition compared with those of the intertidal colonies of the same species and with those of *Diplosoma virens* and *T. cyclops*. Higher eukaryotic photoautotrophs show a decrease in chlorophyll *a*/*b* ratio in response to low light intensities, ratios of less than 3.0 being characteristic of plants from extremely low light environments (12). The data in Table 1 suggest that this relationship is exactly the opposite in these prokaryotic algae, which, together with an absence of correlation between chlorophyll *a*/*b* ratio and membrane structure, may point to a significant difference from eukaryotes in the organization of the chlorophyll pigments in photosynthetic membranes.

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