

# Phycoerythrin: Release from Cryptophycean Algae and Bilin Storage by the Primitive Metazoon *Trichoplax adhaerens* (Placozoa)

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Animal species that store bilins from external sources are very rare. A new example is described here. – Since the primitive marine metazoon *Trichoplax adhaerens* stains crimson-red when feeding on a phycoerythrin-containing alga, i.e. *Pyrenomonas* sp. (Cryptophyceae), the question arose whether an algal pigment can be identified as the staining matter. Thin layer chromatography and visible light absorption spectrography of aqueous *Trichoplax* extract disclosed several bilin components representing chromophores of phycoerythrin, a photosynthetic antenna protein that occurs only in certain algae and cyanobacteria. – Additional experiments showed that a cell-free *Trichoplax* extract kills and incompletely digests *Pyrenomonas* algae releasing phycoerythrin into the medium. These digestive faculties of *Trichoplax* tissue components, probably enzymes, contribute to the animal's natural feeding mechanisms that proceed extra- as well as intracorporally. While the large apoprotein component of phycoerythrin is metabolized as a nutrient, the remaining chromophore bilins, strikingly coloured tetrapyrroles, are stored within *Trichoplax*' distinct cellular inclusions, staining the animal crimson.

## Introduction

*Trichoplax adhaerens* F.E. Schulze (Placozoa) is a small plate-like metazoon (diameter 0.2–3 mm; Fig. 1) living in the coastal waters of tropical and subtropical seas. The primitive organism consists of only three layers (Grell and Benwitz, 1971, 1981; Grell and Ruthmann, 1991). A flat dorsal epithelium, a ventral epithelium of cylindrical cells, both of them flagellated, and an intermediate layer of contractile “fibre cells” with long extensions and phagocytotic abilities (Fig. 2). Many cells bear osmiophilic globular inclusions probably storing waste material from metabolism but likewise retaining dyes offered from without. *Trichoplax* predominantly feeds on unicellular algae, for example on flagellates of the cryptophycean or chlorophycean classes. The animal digests food particles either extracorporally by secreted enzymes (Grell and Benwitz, 1971) or intracorporally after transepithelial phagocytosis by the fibre cells (Wenderoth, 1986). During studies of the feeding behaviour of *Trichoplax* potentially related observations were made: First, when grazing on a brown

*Pyrenomonas* sp. (Cryptophyceae) carpet *Trichoplax* leaves behind a track of dead algae, now green; second, pale *Trichoplax* that have fed on *Dunaliella* sp. (Chlorophyceae) stain reddish soon after the animal's transfer to a *Pyrenomonas* suspension. This raised the possibility that a red pigment was taken up by *Trichoplax* from *Pyrenomonas* and stimulated the present investigation aiming at clarifying the reason for the colour change and looking for connections between one of *Pyrenomonas*' photosynthetic pigments, phycoerythrin, and the matter that stains the animal. Supplementary experiments were carried out to demonstrate the digestive potency of *Trichoplax* tissue extracts.

## Materials and Methods

*Trichoplax* has been kept for years in this laboratory and feeds either on *Pyrenomonas* sp. (Cryptophyceae), or on *Dunaliella* sp. (Chlorophyceae), both cultured in Erd-Schreiber medium at 20 °C. Photographs were taken of the whole live *Trichoplax* as well as of its dorsal cell layer, perpendicular sections through the embedded animal were used for TEM. As described previously (Ruthmann and Wenderoth, 1975), squash preparations

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of *Trichoplax* were made and Giemsa-stained. To obtain a pigment solution, about 10 µl of wet *Trichoplax* bodies, comprising 800–1200 individuals grown on *Pyrenomonas* and packed by centrifugation, were osmotically disrupted in 0.3 ml of distilled water containing 0.01 M phosphate buffer, pH 7.0, or in 0.5 ml glycerol, and homogenized by sonication, all handling taking place at 4 °C in dim light. The homogenate was cleared by centrifugation, and the filtrate stored at –16 °C under N<sub>2</sub> or under liquid paraffin, if not used immediately. After vacuum concentration, a portion of the red solution served for thin-layer chromatography on “Kieselgel 60” plastic foil (Merck, Darmstadt, Germany) using 30% 2-propanol in water as a solvent. The *Trichoplax* extract was also used for SDS polyacrylamide gel electrophoresis in the same way as a *Pyrenomonas* pigment extract that was produced as follows: The algae, collected by centrifugation, were suspended in distilled water with 0.01 M phosphate buffer, pH 7.0, and sonicated. The lipophilic matter was removed by several washings with diethylether, the remains of which evaporated in the vacuum. After addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (final concentration 70%), the algal phycoerythrin precipitated within 10 h. The precipitate was redissolved in phosphate buffer and dialyzed against the same buffer. The UV-VIS absorption spectra of the pigments present in the *Trichoplax* and *Pyrenomonas* extracts were recorded with a Hitachi U 3210 spectrophotometer.

To obtain an isotonic *Trichoplax* extract for testing digestive tissue properties, about 10 µl of washed and packed pale animals cultured previously on *Dunaliella*, were brought into 0.3 ml of sterile seawater, pH 7.4, at 4 °C. To disrupt the animals, they were freeze-thawed thrice and either sonicated or ground with glass beads in a china mortar. After filtering through cotton wool and centrifugation, 0.1 ml of the supernatant was mixed with an equal volume of a *Pyrenomonas* or *Dunaliella* suspension each containing about 500 algae/µl. The resulting morphological changes of the algal cells were photographed while being observed in a glass chamber at 20 °C. Samples of the disintegrating algae were also routinely prepared for DNA reaction after Feulgen and for TEM. *Trichoplax* extracts, modified as follows, served as controls: 1. addition of the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) in

2-propanol to a final concentration of 0.001 M PMSF and 5% 2-propanol; 2. addition of 2-propanol, final concentration 5%; 3. heating to 95 °C for 15 min; 4. membrane filtration (Centrisart I, Sartorius, Göttingen, Germany) to exclude any substance with a molecular weight above ≈1 kDa. KCl (final concentration 0.1–1.0%) was added to a *Pyrenomonas* suspension in seawater to test effects of K<sup>+</sup> ions possibly stemming from destroyed *Trichoplax* tissues in the reacting mixtures. – To test for photobleaching, pigment-laden *Trichoplax* in seawater were positioned 10 cm below a commercial fluorescent lamp (Fluora L 18 W/77, Osram, Germany) up to 12 h (control: animals kept in dim light).

## Results

Fig. 1\* shows a *Trichoplax* specimen in its algal environment. Fig. 2 exemplifies the animal's three tissue layers, stressing the large phagocytosing fibre cells. The thin-layer chromatogram of *Trichoplax* extract shows three closely spaced red bands, slightly different in colour shade (Fig. 3). SDS electrophoresis of the algal extract gave the bands of the α- and β-apoproteins, probably dimers, at about 30 and 50 kDa with positive fluorescence that was markedly enhanced by ZnSO<sub>4</sub> solution, confirming the presence of phycobiliproteins (O'Carra and O'hEocha, 1976). In the *Trichoplax* extract, pigments did not move electrophoretically nor did they fluoresce after addition of ZnSO<sub>4</sub>. Aqueous as well as glycerol extracts from *Trichoplax* showed light absorption maxima at 495 and 540 nm with a shoulder at 560 nm. The longer the animals were separated from *Pyrenomonas* the more prominent was the 495 nm component. In contrast to the aqueous extract, the glycerol preparation is remarkably resistant to light, elevated temperature, and atmospheric O<sub>2</sub>. The absorption maxima obtained from the *Pyrenomonas* extract were similar to those from *Trichoplax*, 500 (rather weak), 545 and 560 nm.

The pigment uptake by *Trichoplax* cell inclusions is microscopically documented in Fig. 4–6. – When *Trichoplax* seawater extract was mixed with equal parts of a *Pyrenomonas* suspension, striking changes in the algae occurred (Fig. 7–11): After

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\* Fig. 1–15 see plate on page 461.

a few minutes the cells were immobile and soon became globular. After about 10 min from the beginning of the experiment most algae protruded one to several transparent vesicles that grew rapidly, filled with red fluid, and eventually burst, releasing their coloured content into the medium. The algae did not break down synchronously but all of them ended in shrunken remnants, often containing a green lipid droplet. The presence of starch granules could be demonstrated by the iodine reaction, sometimes even in the late phases of algal decomposition. In these final states, nuclear DNA could no longer be identified. Transmission electron micrographs (Fig. 12 and 13) show the progressive changes of the *Pyrenomonas* algae and the vesicles enclosed by single unit membranes resembling disentangled thylakoid structures (Fig. 14). In contrast to the effects on *Pyrenomonas*, *Trichoplax* extract did not alter the motility and morphology of the chlorophycean *Dunaliella* sp. within several hours. Such an algal specimen is shown engulfed by a fibre cell (Fig. 15). Although more than one day has elapsed

since phagocytosis took place, the alga is relatively well preserved.

In the controls, addition of PMSF (1 mM) completely stopped, and heating of the extract to 95 °C prior to addition to *Pyrenomonas* partially inhibited the digestive activity of the *Trichoplax* extract. The extract became inactive after membrane filtration (1 kDa exclusion limit). 5% propanol or KCl in the concentrations mentioned did not impair the efficacy of the mixture nor did it influence the viability of the algae. *Pyrenomonas*-grown *Trichoplax* bleached within 8–12 h when irradiated near a fluorescent lamp while animals under dim light kept their colour.

## Discussion

Earlier findings already suggested that the water-soluble pigments obtained from *Pyrenomonas* and from *Trichoplax* were identical or closely related (Wenderoth, 1990), but the proof was still lacking. In spite of the small pigment quantities that could be extracted from *Trichoplax* and the

Fig. 1. *Trichoplax adhaerens* grazing on a *Pyrenomonas* substratum. By flagellar action, the animal has collected many unicellular algae on its dorsal surface (dark areas). Bar 0.2 mm.

Fig. 2. Perpendicular section through *Trichoplax*. Two large fibre cells (arrow-heads) with osmiophilic residues of phagocytosed algae. D, dorsal; V, ventral epithelium. Bar 5 µm.

Fig. 3. Thin layer chromatogram of the water-soluble pigments extracted from *Trichoplax* fed on *Pyrenomonas*. Bar 1 cm.

Fig. 4. Live *Trichoplax*, *Dunaliella* fed, in transparent light, focussed just below the dorsal surface. Pale inclusions barely visible. Bar 10 µm.

Fig. 5. Live *Trichoplax* after feeding on *Pyrenomonas*, viewed as in Fig. 4, dark inclusions stained by phycoerythrin-derived pigment. Bar 10 µm.

Fig. 6. *Trichoplax* squash preparation, Giemsa-stained. Inclusions (arrow-heads) in many cells. Bar 10 µm.

Fig. 7. *Pyrenomonas* alga, immobilized after 2 min of exposure to *Trichoplax* tissue extract. Phase contrast, bar 10 µm.

Fig. 8. *Pyrenomonas*, cell body rounded off after 4 min in *Trichoplax* extract. Bar 10 µm.

Fig. 9. After 7 min, *Pyrenomonas* extrudes vesicles. Starch grains stained with iodine. Large lipid droplet (L). Bar 10 µm.

Fig. 10. *Pyrenomonas* cell residues with several vesicles, 12 min after exposure to *Trichoplax* extract. Bar 10 µm.

Fig. 11. After 15 min, the vesicles have bursted, a granulated "shadow" of the destroyed alga remains. Bar 10 µm.

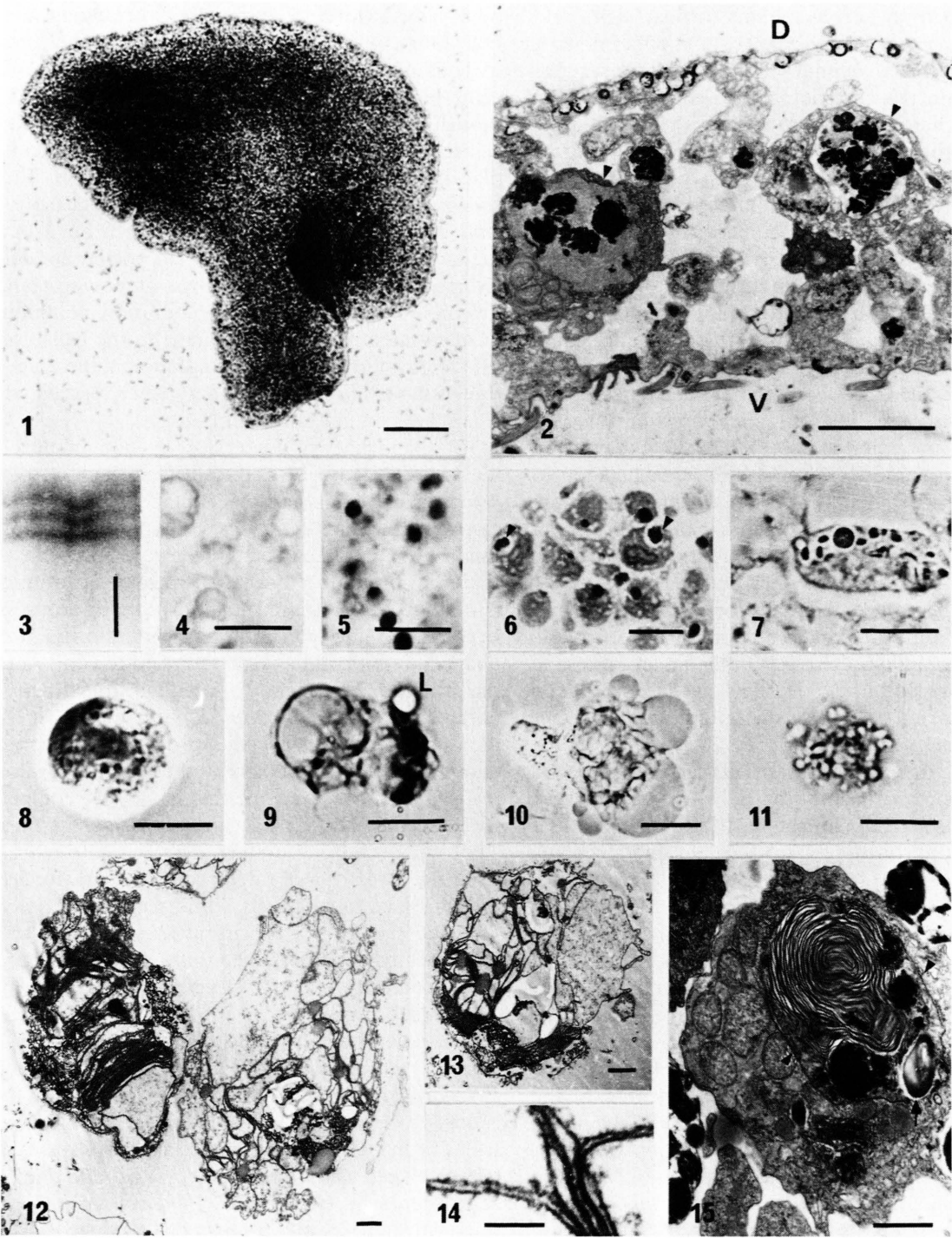
Fig. 12. TEM picture of *Pyrenomonas* algae exposed to *Trichoplax* extract for 10 min. Loosened and detached thylakoid membranes, preserved starch granules, vesicles shrunken in the course of preparation. Bar 1 µm.

Fig. 13. Later stage of *Pyrenomonas* disintegration. Cytoplasmic compartments formed by decomposed chloroplast portions. Bar 1 µm.

Fig. 14. Section of isolated thylakoid membranes from digested *Pyrenomonas*. Bar 0.1 µm.

Fig. 15. A chlorophycean alga (*Dunaliella* sp.) phagocytosed at least 30 h ago by a *Trichoplax* fiber cell. Cell membrane (arrow-heads), thylakoid structure, and starch (arrow) still preserved. Bar 1 µm.





high sensitivity of phycobilins to heat, light, and O<sub>2</sub> (O'Carra, 1970; Glazer, 1981), it could now be shown that the animal's crimson stain as rediscovered in the chromatogram is due to the bilin chromophores (prosthetic groups) of the apo-protein-chromophore complex phycoerythrin, a genuine photosynthetic antenna pigment of the Cryptophyceae that served *Trichoplax* as food. As in the experiments reported here, the light absorption values of the isolated tetrapyrrole pigments and of their protein-bound algal counterparts do not precisely correspond (Killilea *et al.*, 1980; Alberte *et al.*, 1984). Shifts in the absorption maxima can also occur by conformation changes of the free bilins (Falk, 1989). Since *Trichoplax* pigments neither move in the electric field nor fluoresce and are not precipitated by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, they must have lost their attachments to the specific apoproteins (Scheer, 1986). The fact that phycourobilin (495 nm), an established member of the bilin family in Cryptophyceae, is more prominent in the *Trichoplax* extract may be due to its relatively greater resistance than phycoerythrobilin to environmental influences, or to its formation (Cole *et al.*, 1967) from the other bilins taken up by the animal. A multitude of bilins has recently been demonstrated in various cryptophycean species (Wedemayer *et al.*, 1991, 1992; Wemmer *et al.*, 1993). Up to three different bilins can be attached by one or two bonds to the same polypeptide, either an  $\alpha$ - or  $\beta$ -apoprotein. It also emerged that a distinct absorption value does not unequivocally characterize a structurally defined bilin. Therefore, "the identity of the bilin prosthetic groups on cryptophycean phycobiliproteins cannot be unambiguously inferred from simple inspection of the visible absorption spectra" (Wedemayer *et al.*, 1992). Thus the phycoerythrin-derived bilins that are retained in small quantities by *Trichoplax* can not exactly be designated as yet. However, the three pigment components that appeared in the chromatogram are likely to represent the three maxima in the UV-VIS light absorption spectra.

Among the rare examples of animals that store algal bilin pigments, the sea hare *Aplysia* sp. is known best. The defense secretion of this opisthobranchian gastropod contains a pigment the shade of which depends on the algal species devoured (Borradaile *et al.*, 1941). The coloured matter in the secretion turned out to be a modified phyco-

bilin, aplysiocyanin (Rüdiger, 1967; Chapman and Fox, 1969). A few other molluscs, *i.e.* archaeogastropods of the genus *Haliotis* (abalone), deposit algal pigments in their shells (Fox, 1979). Although the causal connection between the food ingested and the bilin compounds retained seems plausible, there remains a possible objection: The staining matter, linear tetrapyrroles, might possibly stem not from an external source but from the animal's own production of porphyrin derivatives, *i.e.* from the breakdown of hemoglobin, myoglobin, *etc.* This pigment generation, common in many metazoan phyla, cannot be the cause of *Trichoplax*' tetrapyrrole accumulation in the quantities found, since it occurs only after feeding on algae that contain phycobiliproteins.

The significance of the food-derived pigment retention can plainly be deduced from the fact that *Trichoplax* thrives on unicellular algae. If the phycoerythrin-containing *Pyrenomonas* serves as food, it is reasonable that the pigment complex, representing up to 60% of the total algal protein (Bogorad, 1975), is used for nutritional purposes. In the case of intracorporal digestion following the phagocytosis of whole algae the need to cope with the protein-bound pigment is evident. In extracorporal digestion by *Trichoplax*' secreted enzymes, the resorption of the phycobiliprotein or its degradation products is demonstrated by the loss of the red pigment in those algae that came into contact with the animal's ventral surface. In contrast to chlorophyll, the phycobilins are not phototoxic and therefore are well tolerated by the *Trichoplax* tissues. Since the isolated bilins, comprising only ~5% of the phycoerythrin mass, probably are nutritionally worthless, they are intermittently stored as a waste product, either to be metabolized or extruded.

The experiments showed that aqueous *Trichoplax* extracts are able to kill and partly destroy *Pyrenomonas* but not *Dunaliella* algae. The former is only covered by its cell membrane and the thin scales of the periplast while the latter is protected by a thick cell wall. Even phagocytosed *Dunaliella* cells often seen enclosed within *Trichoplax* fibre cells, withstand the digestion for days (Fig. 15) as do the chitin-walled yeast specimens (Wenderoth, 1986). Obviously, the surface structure of *Pyrenomonas* allows active *Trichoplax* substances, probably enzymes, to enter the algal cell and destroy it.

Since cryptophycean phycobiliproteins are not confined to membrane-associated phycobilisomes, the water-soluble pigment diffuses easily into the vesicles observed in the dying alga. Proteinase activities of the *Trichoplax* extract may explain their inhibition by PMSF or heat, as well as the inactivity of extracts devoid of proteins. Since starch, lipids, and chlorophyll persist in the disintegrating alga, the respective degrading enzymes apparently do not exist or are very weak in the *Trichoplax* extract.

The investigations described here should contribute to our understanding of the uptake of algal tetrapyrrole pigments by a primitive metazoan

and their metabolism within it. Experiments with *Trichoplax* feeding on algae that contain the blue pigment phycocyanin are in progress. The results shall be published separately.

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