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Role of Bromine in the Formation of the Refractile Inclusions of the Vesicle Cells of the *Bonnemaisoniaceae* (*Rhodophyta*)

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Summary. Refractile inclusions characteristic of the vesicle cells of members of the *Bonnemaisoniaceae* fail to form if bromide is omitted from the culture medium. Electron microprobe analysis shows the localization of bromine in these cells. When grown in the presence of bromide, but not when grown in its absence, these organisms oxidize iodide to iodine; in *Trailliella*, this oxidation occurs at the vesicle cells.

Introduction

Thalli of members of the family *Bonnemaisoniaceae* of red algae contain so-called vesicle cells. These cells are characterized at maturity by the presence of a refractile inclusion and by the absence of photosynthetic pigments. Molecular iodine is found to be released by certain of these cells (Golenkin, 1894; Kylin, 1916). Since the presence of iodide ion in adjacent cells has been indicated by staining reactions (Kylin, 1915, 1928; Sauvageau, 1925) the possibility has existed that the vesicle cells, rather than being rich in an iodine-containing substance, can oxidize iodide to iodine (Kylin, 1928).

In this note, the dependence of the formation of the refractile inclusions and the oxidation of iodide on the presence of bromide ion in the medium, and the direct demonstration of bromine in the vesicle cells will be described.

Materials and Methods

Bonnemaisonia nootkana (Esper) Silva was obtained by scuba divers from a depth of 70—80 feet in the Carmel, California, submarine canyon. *Trailliella*-stage cultures were derived from *B. nootkana* carpospores. In addition, *T. intricata* Batters was obtained from Dr. Dieter Müller, and *Asparagopsis armata* Harv. and the *Falkenbergia*-stage culture from which it was derived were obtained from Prof. H. A. von Stosch.

All of these organisms have been routinely cultured in Fries' (1963) medium ASP6F, modified as follows (cf. v. Stosch, 1962): $NaNO_3$ was reduced to 0.59 mmol/l, KI was increased to 5 μmol/l, phosphate was incorporated as a combination of Na_2glycerophosphate·$5^1/_2H_2O$ and $Na_2HPO_4 \cdot 12H_4O$, each at 6 μmol/l, and KBr — at the molar concentration of bromide in sea water — was replaced by (J. T. Baker reagent grade) NaBr at the same molar concentration. In certain cases,

the organisms were cultured in media identical to the above except that NaBr was present in decreased concentration, or was omitted. By means of treatment with antibiotics, and by "dipping and dragging" (Tatewaki and Provasoli, 1964), *Bonnemaisonia* and its derivate *Trailliella's* were all brought into unialgal culture, and the microflora accompanying all of the algae greatly decreased. However, attempts to grow the algae in the absence of bacteria have thus far been unsuccessful.

All but *B. nootkana* have been cultured at 15° C, 16 hours light per day, in a modified Sherer-Gillette GM4-4 growth chamber. Cultures were illuminated by a single 30-W, cool-white fluorescent lamp which yielded an intensity of ca. 60 ft.-c. (Weston model 197a illumination meter, type 1A) at the position of the cultures. *B. nootkana* gametophytes were grown at 11° under conditions otherwise similar but for a green cellophane filter between the light source and the cultures. Cultures were grown in Erlenmeyer flasks, for periods of $1^1/_2$ to 3 months.

For electron microprobe analysis, small pieces of thallus were drained of medium and affixed to a quartz slide by means of a thin layer of OCT cutting compound (Fisher Scientific Co.). The samples were examined on a model AMX electron microprobe X-ray analyzer (Applied Research Laboratory, Dearborn, Michigan). Acceleration voltage was 25 kv. Resolution was approximately 1 μ.

Results

It was found with *B. nootkana* ♂ and ♀, the *Trailliella* stage of *B. nootkana*, *T. intricata*, and *A. armata* and its *Falkenbergia* stage that the refractile inclusions characteristic of vesicle cells failed to form when the algae were cultured in the absence of bromide ion in their medium (cf. Fig. 1). Where vesicle cells formed, in the *Trailliella* strains, a small non-refractile body (cf. Fig. 1b) was found in place of the large refractile inclusion. Acridine orange (Hooker and Summanwar, 1963) and, less clearly, Feulgen staining show this body to be the cell nucleus. In *Asparagopsis*, where vesicle cells are usually most conspicuous in positions along the central axis of the determinate laterals, and in the other algae, the presence of the cells in the absence of bromine was less easily observed. In these organisms, vesicle cells may have decreased in frequency when bromide ion was eliminated from the growth medium. However, at least some such cells still formed — although without a refractile inclusion — in all of the algae. Preliminary observations indicate that vestigial refractile granules still form when bromide ion is present at 0.1 (*Trailliella*) to 0.3 (*Asparagopsis*) its concentration in sea water. When cultures repeatedly subcultured in bromide-free media

Fig. 1. Vesicle cell development in *Trailliella* and *Asparagopsis* as influenced by the bromide content of the culture medium. a *Trailliella* stage of *Bonnemaisonia nootkana* grown in bromide-containing medium. Vesicle cells (*v*) have large refractile inclusions. b The same organism grown in bromide-free medium. Vesicle cells (*v*) lack refractile inclusions. Small, non-refractile bodies, such as may be seen in the upper right-hand vesicle cell, stain as nuclei. c *Asparagopsis armata* grown in the presence of bromide ion. Vesicle cells (at *v*) have conspicuous refractile inclusions. d The same organism grown in the absence of bromide. Refractile inclusions are not observed

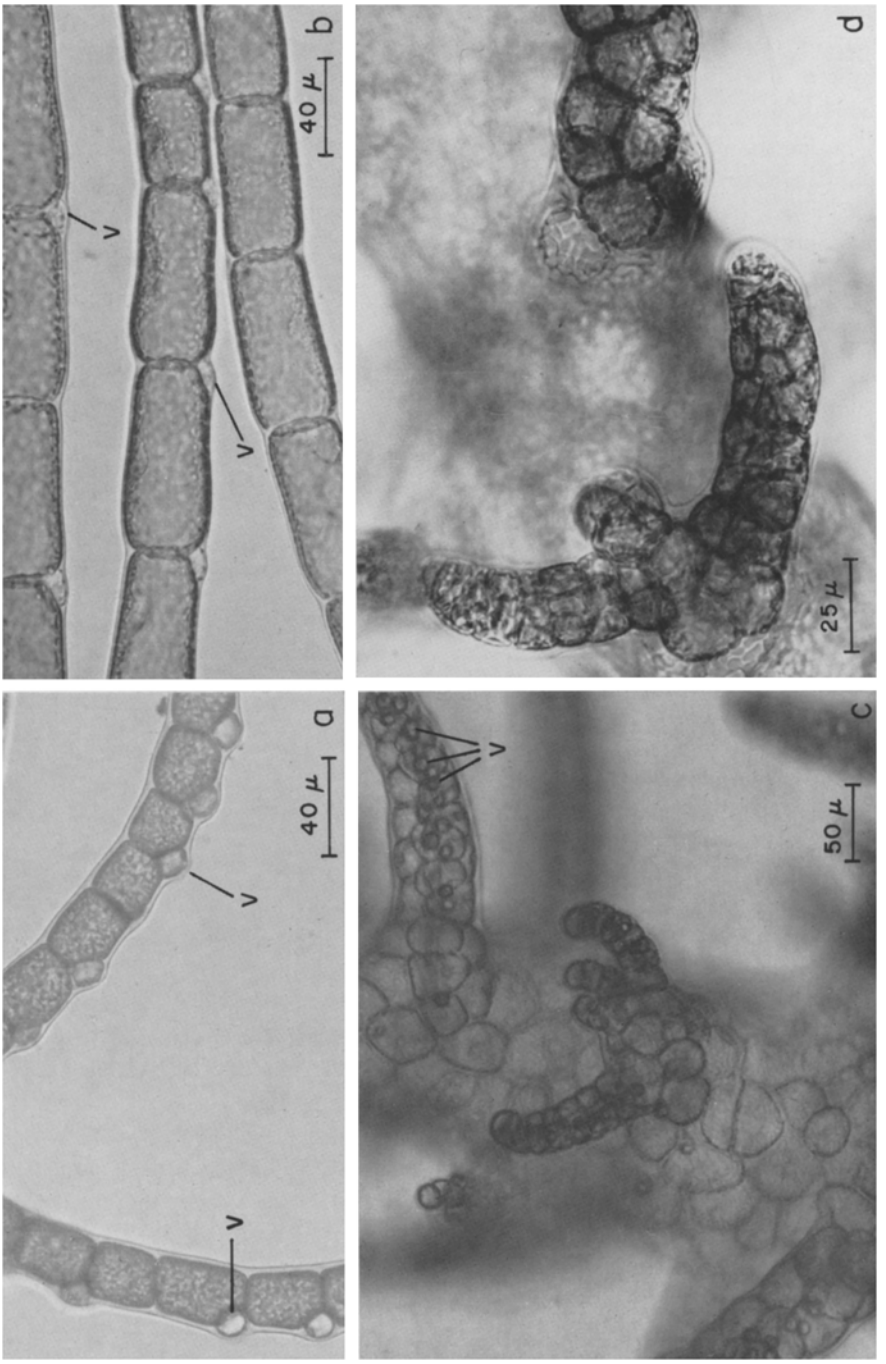

Fig. 1 a—d (for legend see p. 372)

were transferred to bromide-containing media the refractile inclusions again formed; controls transferred to bromide-free media failed to form the inclusions.

Repeated subculture in bromide-free media led to no progressive diminution of growth relative to parallel cultures in bromide-containing media. Growth has generally been little affected by the absence of bromide [Fries (1966) has, in fact, reported increased growth of *T. intricata* in the absence of bromine].

The effect of the bromide ion on the appearance of the refractile inclusions of vesicle cells could be an indirect effect, mediated by bacteria. If the effect is a direct one, it could be that the refractile substance contains no bromine, but that its biosynthesis is somehow dependent on bromine. Finally, it could be that bromine — although known to be present in members of the *Bonnemaisoniaceae* (Kylin, 1929) — is a heretofore unsuspected component of the refractile material. In order to test these possibilities directly, and also to determine whether or not iodine is indeed present in the vesicle cells, recourse was had to the electron microprobe. In this instrument, a sample bombarded by electrons is excited to emit X-rays of wavelengths characteristic of the elements present. Detection and analysis of X-rays emitted make possible qualitative analysis at microscopic dimensions.

Fig. 2 shows a back-scattered electron image of the end of a filament of the *Trailliella* stage of *B. nootkana*, grown in bromide-containing medium. The translucent areas correspond to vesicle cells seen concurrently in a light microscope. Traces along a line through several of these areas and through adjacent cells, with the X-ray analyzer tuned to the iodine (Fig. 2a) and bromine (Fig. 2b) L_α wavelengths, showed peaks corresponding to these areas. Additional evidence of discrete localization in these cells was provided by 1. an X-ray photograph at the bromine L_α wavelength, which provided a two-dimensional image of the localization of bromine; and 2. by bromine scans through the same cells but displaced laterally from the scan shown. Evidence was also obtained of discrete localization of bromine within the determinate laterals of *Asparagopsis*. Bromine was not detected in pieces of *Trailliella* and *Asparagopsis* thalli grown and subcultured in the absence of bromide.

The back-scattered electron image of vesicle cells of *Trailliella* is often in the form of a translucent annulus. This indicates that some electron-dense substance(s) is present, and concentrated, not uniformly throughout the cell, but rather around some central region. Acridine orange staining (Hooker and Summanwar, 1963) indicates that this latter region is the cell nucleus (compare the easy visualization of the nucleus in *Trailliella* vesicle cells formed in the absence of bromide).

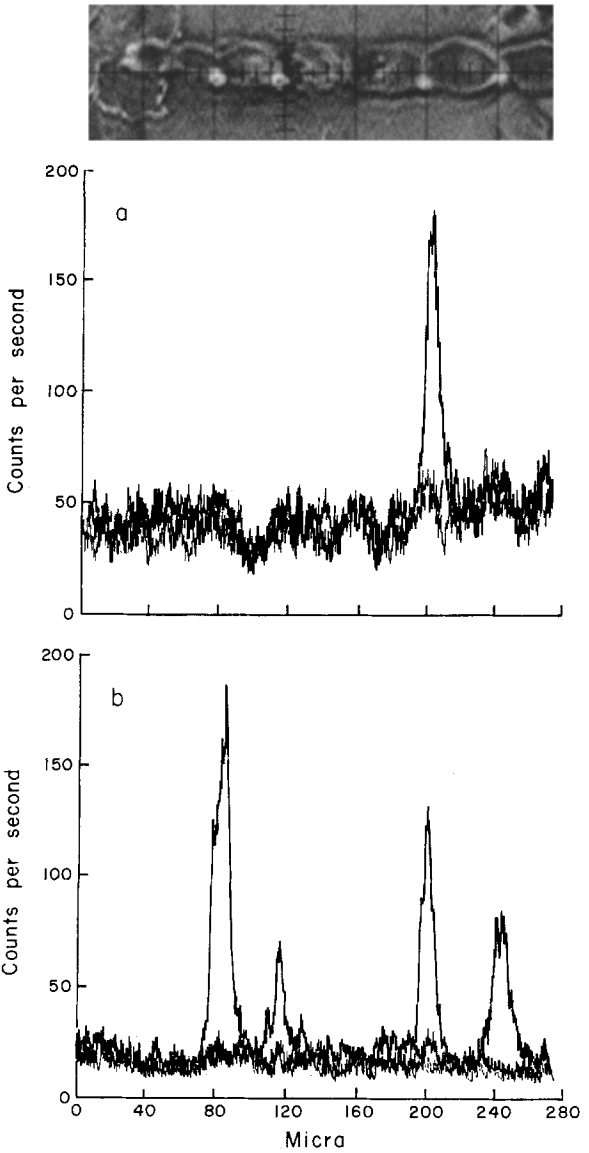

Fig. 2. Localization of bromine and iodine in vesicle cells of the *Trailliella* stage of *Bonnemaisonia nootkana*. The photograph is the back-scattered electron image of the end of a filament. Tracings a and b are along a line, through the four vesicle cells, slightly oblique to the photographic midline. a Iodine L_α radiation (thick line) and background scans (two thinner lines) at the L_α wavelength ± 0.075 Å. Iodine is extensively concentrated in only one of the vesicle cells. b Bromine L_α radiation (thick line) and background scans (two thinner lines) at the L_α wavelength $+0.23$ Å and -0.12 Å. Bromine is localized in all four of the vesicle cells

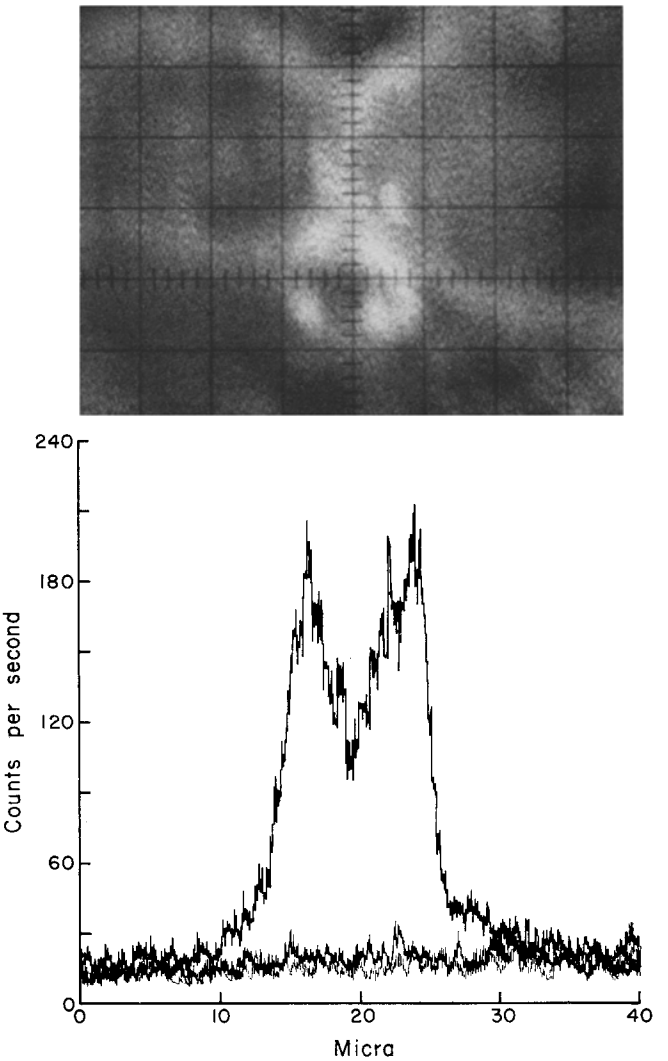

Fig. 3. Bromine distribution within a vesicle cell with an annular back-scattered electron image. Bromine L_α radiation trace (thick line) and the background scans (two thinner lines) at the L_α wavelength +0.23 Å and —0.12 Å, are along a line parallel to, and 2 mm below, the cross-hatched horizontal line in the photograph. Bromine appears to be distributed not uniformly, but around a central region

Line scans (Fig. 3) suggest that the localization of bromine, when present, parallels that of the electron-dense material.

The second vesicle cell from the tip of the filament of Fig. 2 was examined for various elements, and was compared with a point more or

less centrally located in an adjacent vegetative cell. The results are presented in the table, as counts per second above background. Absolute concentrations were not determined. Bromine is concentrated in the vesicle cell; iodine may be. Chlorine is apparently present in the vesicle cell in lower concentration than in the contiguous vegetative cell. Sodium, phosphorus and sulfur show little preferential localization. Potassium, apparently present in low supply at both positions, gave peaks approaching those of sodium in a different pair of cells. Small calcium and magnesium peaks were obtained from both cell types. Specimens were not examined for elements of atomic number lower than that of chlorine.

Table. *Electron microprobe measurements, in counts per second above background, of various elements at the position of the bromine peak of a vesicle cell, and in an adjacent cell*

Element	Counts per second	
	At Br peak	In neighboring cell
Br	99.8 ± 2.4	3.6 ± 0.9
I	10.9 ± 2.9	3.9 ± 2.4
Cl	748.3 ± 6.3	1538.5 ± 8.8
Na	118.4 ± 2.7	101.4 ± 2.5
P	13.7 ± 1.5	16.9 ± 1.5
S	90.7 ± 2.6	69.1 ± 2.2
K	-0.2 ± 1.8	2.2 ± 1.6

Pieces of *Asparagopsis* and of the *Trailliella* stage of *B. nootkana* were immersed in solutions of 1% starch ± 1% KI, in sea water (use of distilled water in lieu of sea water did not change the results). Pieces grown in bromide-free media produced no staining reaction. Pieces grown in the presence of bromide gave no staining reactions in the iodide-free starch solution, but did stain the starch solution when iodide was present. The staining reaction was found around the determinate laterals of *Asparagopsis*, whereas *Trailliella* stained only at the periphery of vesicle cells, primarily vesicle cells in terminal branches.

Discussion

Refractile inclusions appeared in vesicle cells when and only when bromide ion was present in the medium. In these cultures, far more sodium chloride than sodium bromide was present. Iodine was present at a concentration optimal for growth. Moreover, the effect of iodine starvation on *Asparagopsis* (v. STOSCH, 1962) is very different from the effect of bromide starvation. For these reasons it seems clear that the effects of sodium bromide were due neither to contamination, by these other halides, of the bromide salt used, nor to the sodium ion. The demonstrable localization of bromine in the young vesicle cells which contain refractile inclusions further supports the possibility that the refractile material initially formed contains bromine.

Dependence of an iodine starch-stain on the presence of iodide in the reaction mixture implies that instead of releasing molecular iodine directly,

these algae oxidize iodide to the iodine observed. The additional observation, that iodide oxidation is dependent on the presence of bromide in the culture medium, can be interpreted in at least two ways: 1. a bromide-activated iodide oxidase may be present, or 2. molecular bromine may be formed.

The microprobe was used primarily for analysis of *Trailliella*, and provided corroboratory evidence with *Asparagopsis*. Nevertheless, the significance of the conclusions derived from its use are probably valid for the entire *Bonnemaisoniaceae*, since in all strains that were tested, refractile inclusions formed only when bromine was incorporated in the growth medium.

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