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Pigment Cells Representing Polychromatic Colony Color in *Botrylloides simodensis* (Ascidiacea, Urochordata): Cell Morphology and Pigment Substances

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ABSTRACT—The colonial ascidian *Botrylloides simodensis* displays multiple body colors —yellow, orange-red, violet, black and white— in a clonal colony. The colors are due to pigmented blood cells that exist particularly around a branchial siphon or on an atrial languet of individual zooids. These pigment cells are distributed in mesenchymal space or vascular lumen, and many of them are loosely bound to the epithelium. In the space, there are also colorless blood cells that are circulating with blood. When the colorless blood cells are isolated and cultured, some of the cells produce colored substances and change to pigment cells. Therefore, it is presumed that the pigment cells are derived from colorless blood cells. The pigment cells in this ascidian have a spherical shape with no dendrites and contain many granules. Electron microscopic observation showed that there are several different types of granules, and all types of granules are similarly packed in a large vacuole in the cytoplasm. Chemical analysis disclosed that the pigmentary tissues contain carotenoids, pteridine, and purines that are known pigments in vertebrates. The main components of black and violet pigment cells are still unidentified. The former may be a melanin-like substance, but a significant dose of eumelanin or phaeomelanin was not obtained. Although ascidian pigment cells show a certain extent of similarity to vertebrates in their pigments, their cell structure is quite different from that in vertebrates. Because of the phylogenetic relationship between ascidians and vertebrates, it is assumed that ascidian pigment cells might be a primitive type of those in chordates, although these ascidian cells seem to have a unique origin and function.

INTRODUCTION

Ascidians, members of primitive chordates, are thought to originate from a common ancestor with vertebrates. Several ascidian species exhibit various body colors. In particular, colonial ascidians often display polychromatism in a single colony. The colorations are due to various tissues, such as blood cells (reviewed in Wright, 1981), tunic cells (Endean, 1961), epithelial cells (Ishii *et al*., 1993), tunic spicules, and symbionts (cf. Monniot *et al*., 1991). Because an ascidian colony is usually a clone that is asexually reproduced from a single oozooid, all zooids in a colony have the same genetic background; however, a colony can display an irregular, multicolored pattern. Adjacent zooids sometimes have different colors and even a single zooid can be multicolored.

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Pigment cells in poikilotherms are classified as mesenchymal cells derived from the neural crest, and many of them are distributed beneath the epidermis. For example, fish have several types of chromatophores, such as melanophores, xanthophores, erythrophores, leucophores, and iridophores (Fujii, 1993). The major pigment substances in these cells are melanin (melanophores), carotenoid (xanthophores, erythrophores) , pteridine (xanthophores, erythrophores) or purine (leucophores, iridophores). Although these pigments have been studied in many invertebrate species (Kennedy, 1979), there are few phylogenetic attempts that describe both pigment cell morphology and pigment substances. Recently, the structure of the tyrosinase (a key enzyme in melanin production) gene was determined in the solitary ascidian *Halocynthia roretzi* and its' product is 36–39% identical in amino acid sequence to the vertebrate tyrosinase gene product (Sato *et al*., 1997). In the embryonic stages of ascidian *H. roretzi*, this gene is specifically expressed in the pigment cells

of the otolith and photolith. Melanin is a principal and ubiquitous pigment in many animals. There are a few reports on pigment substances in ascidians, and the existences of melanin, carotenoids and purines have been individually reported in different species (reviewed in Wright, 1981). In this study, we present morphological, cytochemical, and biochemical data on pigment cells in a colorful colonial ascidian, *Botrylloides simodensis*, and discuss the phylogenetic homology or analogy of pigment cells and pigment substances in ascidians and vertebrates.

MATERIALS AND METHODS

Animals

Colonies of *Botrylloides simodensis* Saito et Watanabe, 1981 were collected from the vicinity of Shimoda (Izu Peninsula, Japan). They were attached to glass plates and reared in cages floating in Nabeta Bay near the Shimoda Marine Research Center, University of Tsukuba. Colonies were removed from the glass plates before use.

Microscopy of live specimens

Pigment cells in a colony were directly observed under a binocular stereomicroscope through transparent integument without dissection. For observation at higher magnifications, thin specimens were prepared by cutting out the tissue around the branchial siphon or atrial languet of zooids with a razor blade and micro-scissors. The thin specimens were observed under a light microscope (Optiphot, Nikon, Tokyo) equipped with epifluorescence. Autofluorescence of the pigment cells was examined under blue excitation (450–490 nm).

Isolation and culture of pigment cells

The ascidian tissue was sterilized with 70% ethanol for a few seconds and with 1% sodium hypochlorite for a few seconds. After being rinsed for several times with sterile artificial seawater (ASW; Aquamarin, Yashio, Osaka), fine notches were made in the upper surface of the colonies using a sharp blade. The free cells were isolated by rinsing the tissue with ASW without digestion by enzymes, followed by filtration through 100 μm nylon mesh. The collected cells were rinsed again and cultured in Leibovitz 15 based medium (× 1.1 concentration) containing 10% fetal calf serum, 100 μg/ml kanamycin and 100 IU/ml penicillin in a 96-well plate for 16 days at 20°C. Cell numbers were counted every fourth day after plating.

Microscopy of fixed specimens

Polychromatic tissue containing the epithelium and peribranchial wall was dissected from the upper side of the colony and fixed in 2.5% glutaraldehyde–0.1M sodium cacodylate–0.45 M sucrose (pH 7.4) for 2 hr on ice. Then these specimens were washed in 0.1 M sodium cacodylate–0.45 M sucrose (pH 7.4) and postfixed in 1% osmium tetroxide–0.1 M sodium cacodylate (pH 7.4) for 1 hr on ice. The specimens were dehydrated through an ethanol series, cleared with *n*-butyl glycidyl ether, and embedded in low viscosity epoxy resins. For light microscopy, sections that were about 3 μm thick were observed without staining, and some sections that were 0.5 to 1 μm thick were observed after being stained with 1% toluidine blue. For transmission electron microscopy, thin sections were mounted on grids with or without collodion membrane. They were stained with uranyl acetate and lead citrate and examined in a Hitachi HS-9 transmission electron microscope at 75 kV.

Solubilization test for pigment substances

Polychromatic parts of a colony were fixed in 10% formalin–seawater for 30 min or 1 hr. The fixed specimens were soaked in a test solution or in a combination of solutions (80% ethanol, absolute ethanol, acetone, benzene, petroleum ether, toluene, chloroform, 1% NH_4OH, or 1% HCl–methanol) for 1 or 2 hr at room temperature. The solubilization assay was carried out under a binocular stereomicroscope and the process of color change in a colony was recorded by photographs before and after the assay.

Separation and identification of pigments

Colony pieces of *B. simodensis* were homogenized in 50-fold volume (v/w) of 80% ethanol, extracted at 60°C for 10 min, and centrifuged at 3000 rpm for 10 min (Fukushima, 1980). The extraction was repeated three times. Using this treatment, the yellow-orange pigments were completely extracted from the tissue. The combined extracts were concentrated to dryness in vacuo below 50°C. Equal volumes of ether and water were added to the residue. Using this treatment, carotenoid pigments are transferred to the ether fraction, and purines, pteridines, and the other water-soluble color components remain in the aqueous layer.

The aqueous fraction was concentrated in vacuo below 50°C. The color components were separated by successive column chromatography with Ecteola-Sephadex (pH 7.0), CM-Sephadex (H^+), and Sephadex G-25 (fine; Pharmacia, Uppsala) with monitoring of their fluorescence and UV absorption (modification of Fukushima, 1980: P-Sephadex was changed to CM-Sephadex). The water-soluble color components were identified by their behavior on column chromatography by comparison of UV spectra and the mobilities on thin-layer chromatography (TLC) of standards Cellulose plates (Polygram cel 300, Macherey Nagel, Doren) and solvent systems A (*n*-butanol : acetic acid : water, 4:1:2), B (*n*-propanol : 1% NH_4OH, 2:1), and C (6% acetic acid) were used for TLC analysis.

The ether fraction was evaporated and used directly for analysis of carotenoid pigments by TLC. The cellulose plate was dipped in 8% olive oil in petroleum ether and dried at room temperature. With this treated cellulose plate, solvent D (methanol : acetone : water, 74:20:6, saturated with olive oil) was used. Furthermore, silica gel TLC (Kiesel ghur, Sigma, St. Louis, MO) was also used for TLC analysis with solvent system E (*n*-hexane : ether : acetone, 6:3:2).

The black and violet pigments remained in the residue of the 80% ethanol extraction. We used a separation method for the black pigment(s) that has been used in *Bufo* (Obika and Negishi, 1972). The residue of the 80% ethanol extraction was hydrolyzed with a threefold volume of concentrated HCl at room temperature for 48 hr. After addition of water to the reaction mixture, it was centrifuged at 4000 rpm for 10 min. The precipitate was washed with water, ethanol, and ether. The precipitate was dissolved in 0.1N NaOH, and we examined its absorption spectrum at 450–500 nm. In addition, eumelanin and phaeomelanin in the residue of the 80% ethanol extraction were quantified according to Ito and colleagues (1985, 1993); the samples were oxidized with permanganate to give pyrrole-2,3,5,-tricarboxylic acid (PTCA) from eumelanin or hydrolyzed with hydriodic acid to give aminohydroxyphenylalanine (AHP) from phaeomelanin. The amounts of PTCA and AHP were determined by HPLC with ultraviolet detection and electrochemical detection, respectively. For PTCA, a C_{18} 7-μm column was used and the mobile phase was 0.1 M potassium phosphate buffer (pH 2.1): methanol, 94:6 (v/v). For AHP, a C_{18} 10-μm column was used and the mobile phase was 0.1 M sodium citrate buffer (pH 4.0) containing 1 mM octanesulfonate and 0.1 mM EDTA 2Na: methanol, 97: 3 (v/v). The flow rate was 0.7 ml/min.

RESULTS

Morphological characteristics of pigment cells

B. simodensis forms a sheet-like colony. Several zooids surround a common cloacal aperture (Fig. 1). The entire colony is covered by a transparent tunic, an integumentary extracellular matrix, that is overlaid on the epidermis. The colonies in this study were usually colored yellowish brown (Fig. 2); this

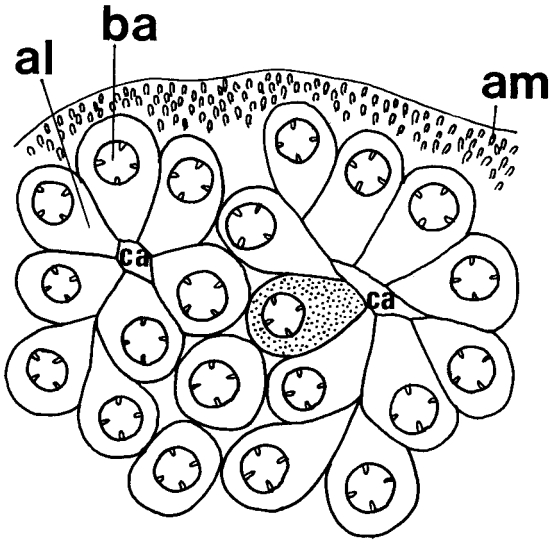


Fig. 1. Schematic drawing of the edge of a botryllid ascidian colony. Dotted area indicates one zooid. al, atrial languet; am, ampulla (terminus of interzooidal vascular vessel); ba, branchial aperture; ca, cloacal aperture.

color was probably due to the orange-colored stomach walls of zooids and to the white and yellow cells sparsely distributed in the upper sides of zooids. Furthermore, a brilliant polychromatic part appears particularly in the marginal area of the colony (Fig. 3). This polychromatism results from yellow, orange, red, violet, black, and white cells deposited around the branchial apertures and the atrial languets of zooids. These colored cells are classified as a kind of blood cell called nephrocytes (white) or pigment cells (other colors) in several ascidians (Wright, 1981). Strictly speaking, pigment cells are generally defined as cells that produce and retain pigment representing body color, thus these ascidian pigment cells are perhaps more appropriately called "colored cells" at present. However, because they are conventionally called "pigment cells" in ascidians, we refer to these colored cells, including the white ones, as pigment cells.

Although there were various color patterns in the polychromatic area of *B. simodensis*, the microscopical studies below were carried out on the orange area and the violet area. In the orange area (Fig. 3, large arrow), yellow, orange, red, or black cells were dominant, whereas white or violet cells were minor counterparts (Fig. 4A). Under reflected light, yellow, orange, and red cells were reflective, whereas black cells and pale yellow cells were not (Fig. 4B). Under blue excitation light (450–490 nm), yellow cells showed intense yellow autofluorescence, and orange or red pigment cells and pale yellow cells, respectively, showed yellowish brown or light green autofluorescence (Fig. 4C). The other pigment cells showed no autofluorescence. In the violet area (Fig. 3, small arrow), violet or white cells were dominant and both were reflective but did not show any autofluorescence under blue excitation light (data not shown).

Although the osmium fixation appeared to affect the colorations to some extent, they did not change significantly and remained even in thick sections (Figs. 5 and 6), except for the white cells. We observed the pigment cells in histological sections. Pigment cells were distributed in the mesenchymal space between the epidermis and the peribranchial wall and were usually bound to the epidermis (Figs. 5 and 6, large arrows). Each cell contained many colored granules and the colors of the cells seemed to be caused by these granules. In some cells, colored granules formed a mass (Fig. 5, arrowhead). In these histological sections, the pigment cells exhibited similar morphology although they had different colors. There were also other colorless blood cells commonly circulating in the mesenchymal space or vascular lumen. Although some of these blood cells were well stained with osmium fixative, these cells were morphologically distinct from the pigment cells.

Occurrence of pigment cells in the blood cell culture

Free cells were isolated from *B. simodensis* tissue without any digestive treatment and cultured for 16 days. Most of these cells seemed to be blood cells circulating in the mesenchymal space in this ascidian. Most of the isolated cells were almost colorless when the culture was started. During 16-day culture, the cells proliferated gradually (Table 1), reaching a final cell number that was 10 times of the initial cell number. From the fourth day, pigmented cells appeared and some of them were similar in color to the pigment cells in the ascidian colony (Fig. 7). These pigment cells in culture emitted yellow autofluorescence under blue light excitation, as did the pigment cells in the colony (data not shown). This indicates that the coloration of the cultured cells is not due to cell death but rather to the production of a color substance that is autofluorescent, such as pteridine.

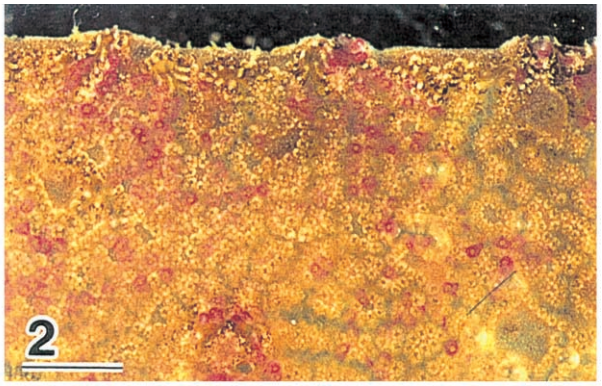
Electron microscopic observation

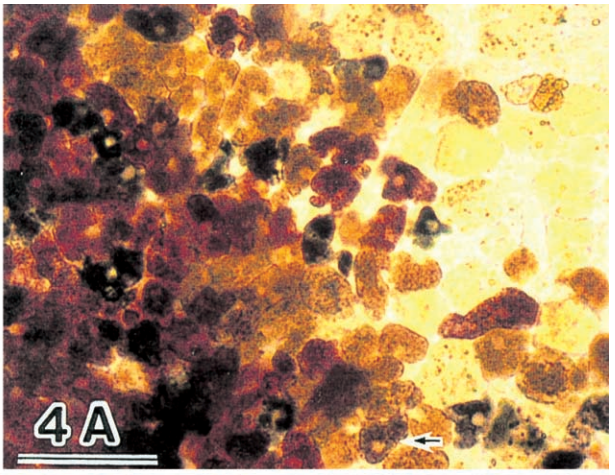
The pigment cells were globular cells without filopodia, and many of them were bound to the basal lamina of the epidermis. Fibrous materials were also found around the cells (Figs. 8 and 9). All types of pigment cells commonly contained a large cytoplasmic vacuole, and the nucleus and the bulk of the cytoplasm were situated at the periphery. There were many granules in the vacuole, and they did not have an individual limiting membrane. In some pigment cells, the granules formed a mass with filamentous materials (Figs. 10 and 11C). The spaces or holes seen in the electron micrographs were similar to those due to the sublimation of purines under electron beam in iridophores of vertebrates. The granules varied in size and shape (Fig. 11), and each pigment cell appeared to contain only one or two types of granules (see also Figs. 8–

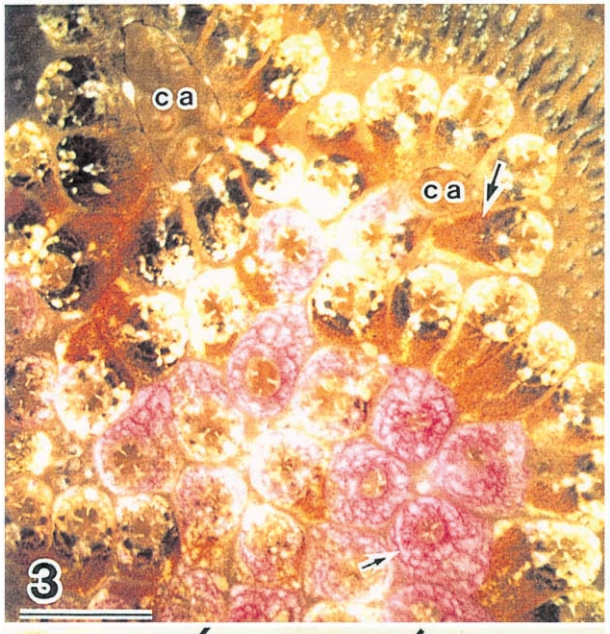
Table 1. Proliferation of cultured free cells in *Botrylloides simodensis*

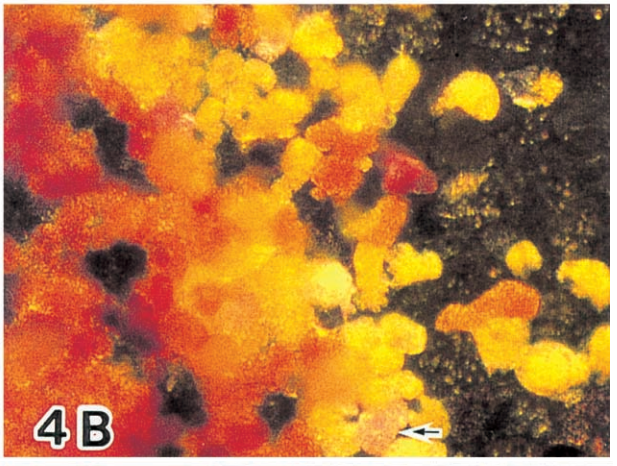
	Culture period (day)				
	0	4	8	12	16
Cell no./well*	1,384	4,754	10,380	12,143	15,026
S.D.	± 96.2	± 344.3	± 566.4	± 983.6	± 860.2

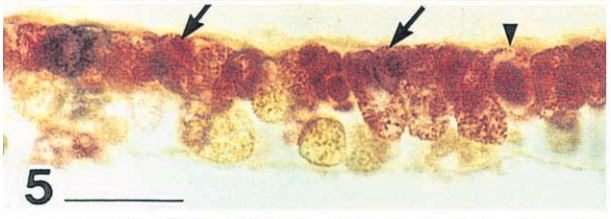
* Average of three replicates.

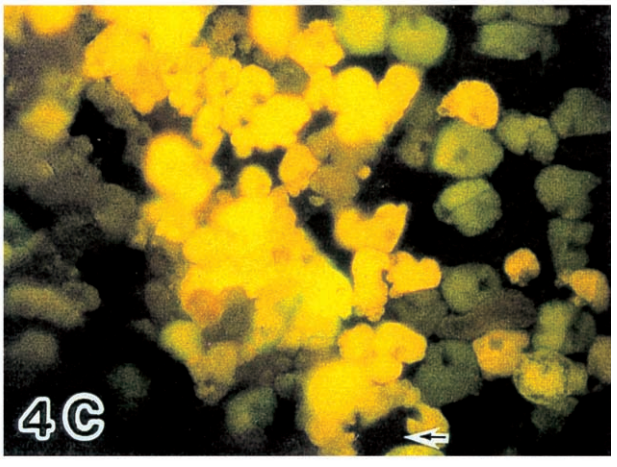


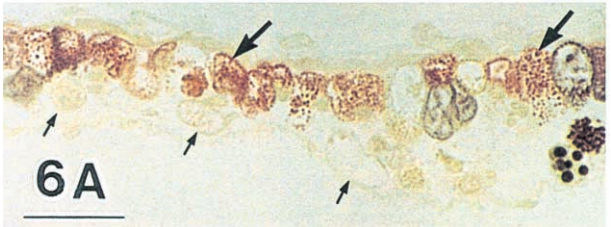


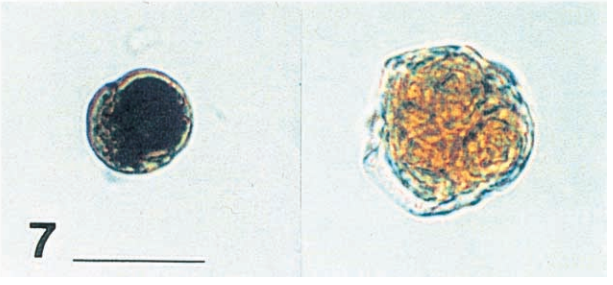












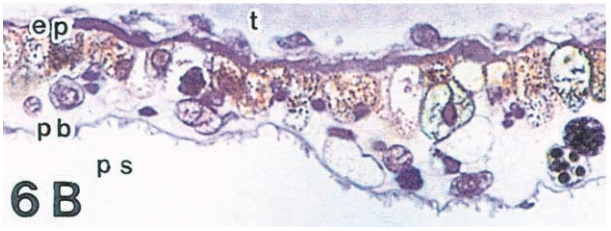


Table 2. Solubility of colors in pigment cells*

Pigment	organic solvents#	10% formalin	1% NH_4OH	1% HCl-methanol
White	–	+	+	+
Yellow	–	–	+	+
Orange	–	–	+	+
Red	–	–	+	+
Violet	–	–	–	+
Black	–	–	–	–

* Plus sign means the indicated pigments was extracted or decolorized. Minus sign means the indicated pigment remained in the pigment cells.
# 80% ethanol, absolute ethanol, acetone, benzene, petroleum ether, toluene or chloroform. In these media, the yellowish color was extracted from the colony, but colors in the pigment cells were not.

Table 3. Rf. values of the isolated and standard color components of ether fraction on thin-layer chromatography (TLC)

Color	*B. simodensis*	Sample shrimp*	Lutein
a. Silica gel TLC			
Orange	0.52	0.51	
	0.33	0.33	
Yellow	0.26		0.27
b. Cellulose TLC			
Orange	0.52	0.50	
	0.06	0.06	
Yellow	0.62		0.62

* The shrimp contains astacine and/or astaxanthins (Kuhn *et al*., 1939; Howell and Matthews, 1991).

10). Many types of granules appeared in the orange areas and in the violet areas.

Solubility of pigment substances

Solubility of the colors in the pigment cells is summarized in Table 2. In 10% formalin–seawater, most of the colors remained, whereas the color of white cells gradually faded in prolonged fixation. The white color completely disappeared in 3 to 10 hr. When the fixed colony pieces were immersed in 80% ethanol, absolute ethanol, acetone, benzene, petroleum ether, toluene, or chloroform at room temperature, the yellowish color was extracted, but colors in the pigment cells remained in these solvents. Therefore, the yellowish color was mainly extracted from stomach wall in which orange color completely disappeared. Under these conditions, carotenoids would be extracted, but pteridines, purines, and melanin are expected to be poorly soluble or insoluble.

Yellow, orange, and red colors of pigment cells were bleached in both 1% NH_4OH and 1% HCl–methanol (Table 2). Violet was soluble in 1% HCl–methanol but not in 1% NH_4OH. Black was not soluble in any of the media tested here.

Chemical characterization

In 80% ethanol at 60°C, yellow, orange, red, and white pigments were extracted from the pigment cells as well as from other tissues. Three yellow-orange pigments were detected in the ether fraction, in which carotenoids were expected to be transferred from the residue (Fig. 12, Table 3). Two orange pigments (arrows in Fig. 12C) were determined to be astacine and/or astaxanthins by comparison of their mobilities on TLC to the mobilities of components in the shrimp extract reported to contain these carotenoids (Kuhn *et al*., 1939; Howell and Matthews, 1991). The yellow pigment (arrowhead in Fig. 12C) was identified as lutein by comparison of silica gel and cellulose TLC with a lutein standard. This yellow spot migrated with a slight delay caused by the effect of upper spots when compared to the mobility of the lutein standard (arrowhead in Fig. 12B).

In addition, two pteridines were isolated from the ether insoluble–80% ethanol soluble fraction. They were identified as riboflavin and isoxanthopterin by analysis of their behavior on successive column chromatography and TLC with cellulose (Table 4, a and b). The UV absorption spectrum of the isolated isoxanthopterin was measured (λ_{max} = 287 and 339 nm in H_2O). In the same manner, uric acid (λ_{max} = 236 and 290 nm in H_2O) was also detected as a purine component (Table 4, c). The content of the purine base, however, was relatively small (160 μg/g tissue) by comparison with the value in other

Fig. 2. Part of a colony of *Botrylloides simodensis*. The polychromatic areas are distributed mainly along the colony periphery (see upper edge in the figure). Scale bar = 1 cm.
Fig. 3. Polychromatic part of a colony. Color patches consist of many pigment cells lodged in the zooids. The large arrow and the small arrow indicate an orange area and a violet area, respectively. ca, cloacal aperture. Scale bar = 2 mm.
Fig. 4. Reflective and fluorescent properties of pigment cells. (**A**) Under transmitted light. (**B**) Under reflected light (same field as in **A**). (**C**) Autofluorescence under blue excitation light (same field as in **A**). Arrows indicate the only violet pigment cell found here. Scale bar = 50 μm.
Fig. 5. Pigment cells in an unstained fixed section (about 3 μm thick). Arrowhead indicates a cell containing a large mass of color material. Scale bar = 20 μm.
Fig. 6. Paired images of a histological section (about 1 μm thick), unstained (**A**) and stained with toluidine blue (**B**). Large arrows indicate red or orange pigment cells binding to the epidermis, and small arrows indicate pigment cells of pale colors in the section. ep, epidermis; pb, peribranchial wall; ps, peribranchial space; t, tunic. Scale bar = 20 μm.
Fig. 7. Pigment cells that appeared in a 1-week culture of isolated, colorless, free cells. Scale bar = 20 μm.

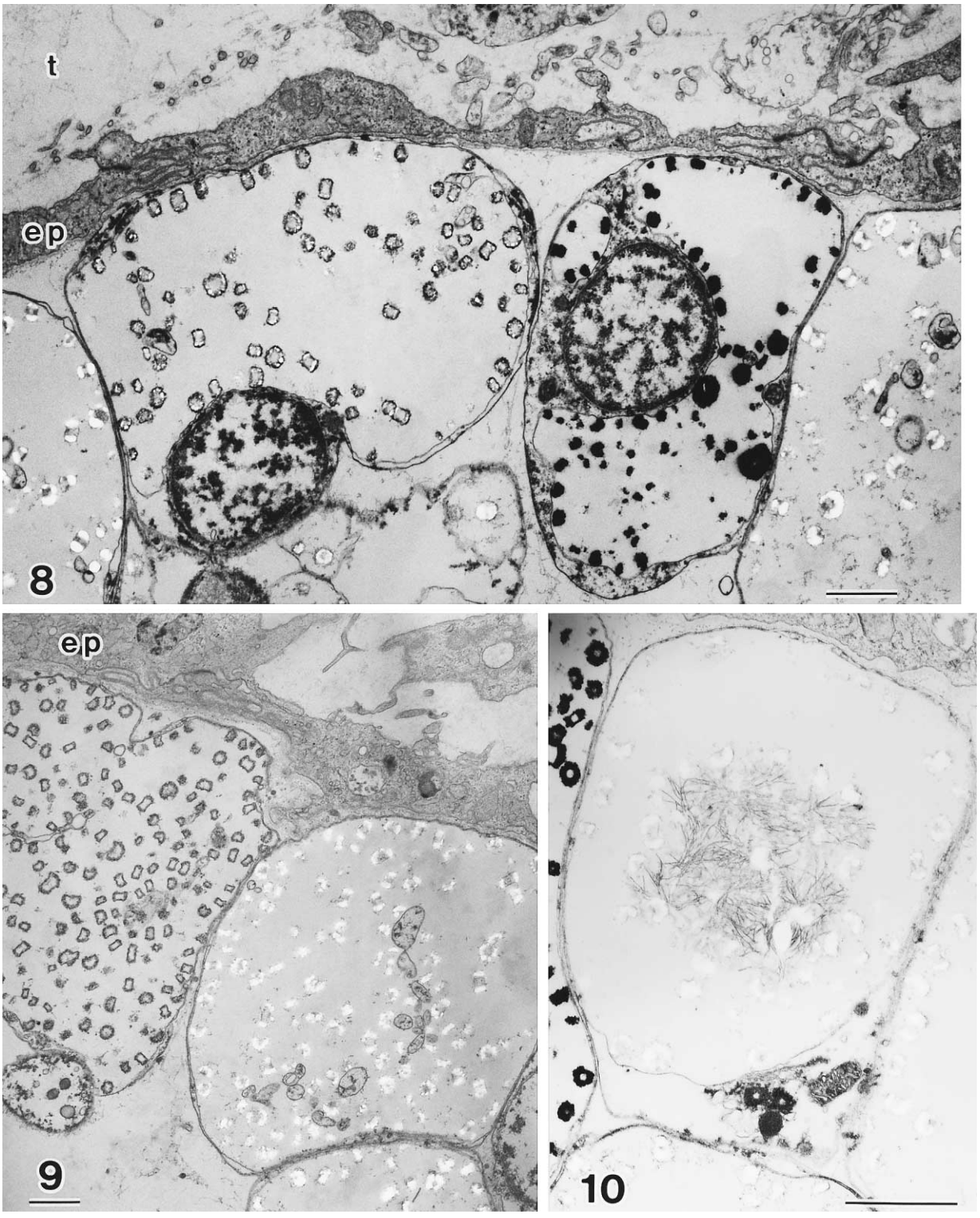


Fig. 8. Electron micrograph of pigment cells in an orange area. Pigment cells are generally bound to the epidermis, and some fibrous materials are found around the cells. ep, epidermis; t, tunic. Scale bar= 1 μm.
Fig. 9. Electron micrograph of pigment cells in a violet area. ep, epidermis. Scale bar= 1 μm.
Fig. 10. Electron micrograph of a pigment cell found in an orange area, containing a mass of granules; there are filamentous materials in its vacuole. Scale bar= 1 μm.

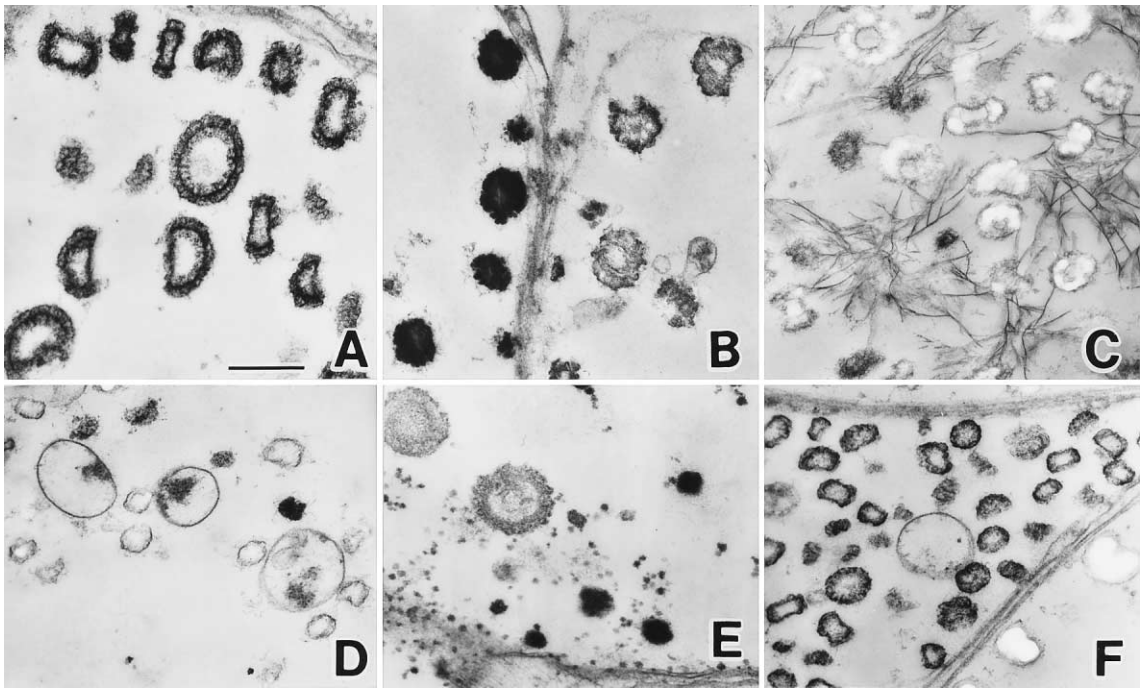


Fig. 11. Various types of granules in the vacuoles of pigment cells, as observed by electron microscopy. There are filamentous materials in **C**. The round, vesicular structures in **D** and **F** may be transverse sections of the cytoplasmic extensions crossing the vacuoles. Scale bar= 0.5 μm.

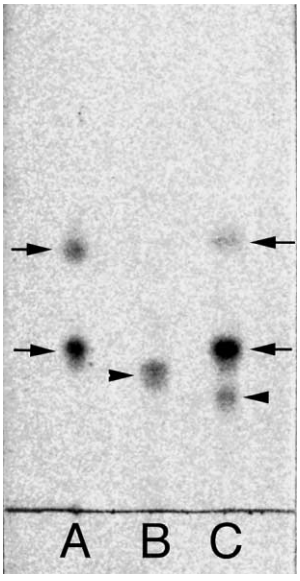


Fig. 12. Thin-layer chromatography (silica gel) of the ether fraction from the extraction of *Botrylloides simodensis* pigments. Lane A, shrimp; lane B, lutein (standard); lane C, *B. simodensis*. Arrows, orange spots; arrowheads, yellow spots.

Table 4. Rf. values of the isolated and standard color components of the ether insolbule fraction on cellulose thin-layer chromatography

Pigment	Solvent system* A	B	C
a. Riboflavin			
Isolated	0.44	0.26	0.32
Standard	0.44	0.26	0.32
b. Isoxanthopterin			
Isolated	0.29	0.16	
Standard	0.28	0.16	
c. Uric acid			
Isolated	0.31	0.33	0.54
Standard	0.29	0.33	0.54

* Solvent systems are explained in Materials and Methods.

invertebrates.

The black and violet pigments could not be extracted with 80% ethanol. Using the methods of melanin analysis in *Bufo* (Obika and Negishi, 1972), the black pigment(s) was dissolved and seemed to be melanin or melanin-like pigment. However, quantitative analysis (Ito and Fujita, 1985; Ito and Wakamatsu, 1993) showed that 1 mg of the residue of the 80% ethanol extraction contained only 45 ng of PTCA (degradation product from eumelanin) and 490 ng of AHP (degradation product from phaeomelanin). This result indicates that the melanin content of this material is less than 1%. Therefore, the main component of the black pigment(s) is still undetermined.

DISCUSSION

B. simodensis shows colorful polychromatism, particularly at the periphery of the colony, and its polychromatic color is due to white, yellow-orange, red, violet, and black pigment cells located in the mesenchymal space. These pigment cells are classified as a type of blood cell, and they appeared in cultures of colorless blood cells. Therefore, we believe that the pigment cells are derived from a type(s) of the colorless

blood cells and that the pigment cells produce the pigments. These ascidian pigment cells seem to fulfill the general definition of pigment cells, that is, the cells that produce pigments and retain them to represent body (or colony) color.

Many of the pigment cells do not circulate in the blood but bind loosely to the epidermis and adjacent pigment cells. These lodging pigment cells seem to bind to the basal lamina of the epidermis or to fibrous materials around the cells (Figs. 7–9). It is possible that the polychromatic color pattern is due to the affinity of each color type for the basal lamina. Mukai (1974) reported light-induced accumulation of brown pigment cells in *Botryllus primigenus*, and he assumed that this accumulation was caused by the light-induced intensification of affinity of tissues (epidermis) for pigment cells. It is assumed that the pigment cells move passively in the blood, because they lack dendrites and there are few microfilaments and microtubules in their cytoplasm.

B. simodensis belongs to the family Botryllidae, and in this family, colony color and pigment cells have been well studied in *Botryllus schlosseri* from the viewpoints of genetics (Sabbadin, 1982), ultrastructure (Burighel *et al*., 1983), and chemistry (Lederer, 1934; Sabbadin and Tontodonati, 1967). As for pigment cell morphology, the pigment cells in *B. schlosseri* and *B. simodensis* have the same basic structures, characterized by large vacuoles that contain granules. Under light microscopic observation, these pigment cells are very similar in morphology except for the colors of the granules. Under transmission electron microscope observation, the granules in the vacuoles show several variations in ultrastructure, and each pigment cell appears to contain only one or two types of granule. It is possible that these granule types correspond to separate colors. Burighel *et al*. (1983) showed that the differences in granule color are paralleled by differences in granule ultrastructure by studying several color strains of colonies in *B. schlosseri*.

Some pigment substances were extracted from the colonies and were partially characterized as carotenoids (lutein, astacine and/or astaxanthine), pteridines (riboflavin and isoxanthopterin), and purines (uric acid). As for carotenoids, Lederer (1934) separated similar substances from *B. schlosseri*. In our study, the results of the solubility test suggest that the carotenoids were mainly derived from the stomach wall and the pteridines were from yellow, orange, and red pigment cells. The black pigment may be a melanin-like substance, but its major components are neither eumelanin nor phaeomelanin. The autofluorescence of yellow, orange, and red pigment cells was probably due to the presence of pteridines in these cells. In *B. schlosseri*, Sabbadin and Tontodonati (1967) identified uric acid as a white, reflective pigment substance. Because uric acid was detected in *B. simodensis*, the white pigment cells in this ascidian possibly contain this substance. It is possible that white pigment granules contain another pigment substance, because the amount of uric acid obtained here was relatively small. Although the violet color extracted in 1% HCl–methanol was probably from violet pigment cells, it was not identified.

In ascidians, white pigment cells are sometimes discriminated from the other pigment cells as nephrocytes, because they are thought to have the functions of storage and excretion of nitrogenous wastes (see Wright, 1981). However, some other pigment cells also have nitrogenous compounds, such as pteridines, as pigment substances, and these cells also may have an excretory function. At present, we think that nephrocytes (i.e., white pigment cells) do not need to be distinguished from the other pigment cells because of their morphological similarity.

A recent study of molecular phylogeny supports the view that ascidians are primitive chordates (Wada and Satoh, 1994). The pigment cells described here are probably the most likely counterparts of vertebrate pigment cells, with the following shared characteristics: (1) They are mesenchymal cells. (2) Color granules are present in membrane-limited spaces in the cytoplasm. (3) The ascidian pigment cells contain a melanin-like substance, pteridines, purines, or unknown pigment substances, and some of them are common color molecules in lower vertebrates. For instance, riboflavin and isoxanthopterin identified here are found in melanophores (Obika and Negishi, 1972) and erythrophores of vertebrates, respectively, and uric acid is also reported in leucophores as a purine component (Bagnara and Hadley, 1973). There is, however, an apparent difference in cell structure: the color granules in vertebrates are individually limited by a membrane, whereas those described here are not individually limited but are contained in a large vacuole. In addition, the ultrastructure of color granules are different between vertebrates and ascidians.

In vertebrates, a chromatoblast is thought to differentiate into various types of pigment cells, such as melanophores, xanthophores, erythrophores, and iridophores (Bagnara *et al*., 1979; cf. Fujii, 1993), and the chromatoblasts originate from the neural crest. In *B. simodensis*, as well as in *B. schlosseri*, although there are several different color types of pigment cells, these cells have similar morphology. This structural similarity suggests that perhaps all of these pigment cells originate from a common precursor cell, as do the vertebrate pigment cells.

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