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Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach

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Abstract *Lucina pectinata* is a large tropical clam living deeply burrowed in the black, reducing mud of mangrove swamps. It is known to possess hemoglobin in the cytoplasmic areas of its bacteriocytes, which harbor sulfide-oxidizing bacteria. The bacteriocytes also possess lysosome-like microbodies containing either membrane whorls or electron-dense granules in which free heme compounds have been identified. The cytochemical detection of acid phosphatase and arylsulphatase through EDX (energy-dispersive X-ray) microanalysis strongly suggests that the bacteriocytes of *L. pectinata* contain, in fact, two different types of microbodies. Some of these (devoid of dense granules) possess a variable amount of lysosomal enzymes and occasionally a limited quantity of iron, which may result from a recycling process of hemoglobin. Their main function seems to be the digestion of a limited proportion of symbiotic bacteria. They represent genuine secondary lysosomes with a functionally acidic pH. The second type of microbodies is characterized by dense granules containing sulfur and iron hemes but no lysosomal enzymes. Their sulfide-oxidizing activity was substantiated by benzyl viologen assay, with Na_2S as a substrate. These microbodies appear to be similar to the sulfide-oxidizing bodies (SOBs) described in the bacteriocytes of other bivalve species with symbiotic thioautotrophic bacteria; however, their sulfide-oxidizing activity appears to be non-enzymatic. They are discrete organelles, characterized by a functionally basic pH and pseudoperoxidasic activity, and have been termed SOBs. Therefore, the bacteriocytes of *L. pectinata* possess at the same time functional lysosomes and functional SOBs.

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Introduction

Lucina pectinata is a large lucinid clam living deeply burrowed in the black, reducing mud of mangrove swamps, ranging from the Caribbean area (Warmke and Abbott 1962) to Brazil (Rios 1985). Its red gills were noted by Read (1962), who demonstrated that this color is due to a large supply of hemoglobin. As in other lucinid clams, *L. pectinata* harbors endosymbiotic sulfide-oxidizing bacteria, but, of the various species of Lucinidae studied, *L. pectinata* contains one of the highest concentrations of hemoglobin in gill tissue (Kraus 1995). However, this hemoglobin is not located in the yellow-brownish, dense granular inclusions as was concluded by Read (1962), but in patchy, dark cytoplasmic areas (Frenkiel et al. 1996).

Ultrastructural studies revealed that, in *L. pectinata*, bacteriocytes contain lysosome-like inclusions as large as the nucleus in their basal part. These inclusions appear to be crowded with membrane whorls and/or electron-dense granules in which heme compounds have been identified by using the diaminobenzidin (DAB) reaction; however, the inclusions do not contain hemoglobin but free hemes (Frenkiel et al. 1996). Non-protein-bound hemes present in the electron-dense granules of *L. pectinata* microbodies may, therefore, correspond to hematin, an active free heme compound (defined as an oxidized heme compound without associated protein) that appears to catalyse the oxidization of hydrogen sulfide in specific brown granules of *Urechis caupo* coelomocytes and *Calyptogena magnifica* gill cells (Powell and Arp 1989). Similar specific organelles, initially identified in the gill cells of the gutless clam *Solemya reidi*, in which a sulfide oxidase enzyme system can rapidly oxidize sulfide entering the cells into non-toxic forms of sulfur, were named sulfide-oxidizing bodies (SOBs) (Powell and Somero 1985). The initial metabolism of sulfide, in the symbiont-containing gills of this species, appears to occur not in the bacterial symbionts, but rather in the SOBs, which therefore represent a key structure of the bacteriocyte.

In addition to the internal sites of sulfide detoxification, a "peripheral defense" strategy against sulfide has been described in the superficial cell layers of symbiont-free tissues, such as the foot tissue of *S. reidi* (Powell and Somero 1985). However, this detoxification activity has not been attributed to a specific cell type.

On the other hand, cytoenzymologic studies have demonstrated that active lysosomal resorption of the symbionts occurs in chemoautotrophic symbioses, and may be a strategy for organic molecule transfer (Fiala-Médioni et al. 1989; Streams et al. 1997). Lysosomal digestion of symbionts could also be considered as a way to control the abundance of symbionts by elimination of senescent bacteria (Fiala-Médioni et al. 1989).

Previous identification of distinct lysosome-like microbodies, with or without electron-dense granules, in the bacteriocytes of *L. pectinata* (Frenkiel et al. 1996) has raised new questions. Thus, the present study was designed to characterize the large microbodies observed in the bacteriocytes of *L. pectinata* and to determine whether they are either secondary lysosomes, which may be involved in symbiont resorption or hemoglobin breakdown, or functional sulfide-oxidizing bodies, probably involved in sulfide detoxification.

Materials and methods

Adult specimens of *Lucina pectinata* (30–60 mm shell length) were collected by hand in the black mud of mangrove swamps in Guadeloupe (2 m average depth of water).

Histology and histochemistry

Cryosectioning of unfixed gill and foot tissues frozen at −30 °C in Tissue-Tek II O.C.T. compound in a cryostat was used for sulfide-oxidizing activity detection and related controls. Paraffin histology was used to compare information on mucosubstances with results obtained by cytoenzymological techniques. Fixation and staining methods were all described in a previous paper on *L. pectinata* gill structure (Frenkiel et al. 1996). Alcian blue staining, generally performed on paraffin sections, was adapted to cryosections in order to compare the exact location of mucocytes with the location of sulfide-oxidizing activities. Good results were obtained by fixation of the cryosections with 5% formalin in seawater and staining with Alcian blue at pH 3 and 0.5 for 2 h.

TEM preparation

Small pieces, dissected from various locations in the gills of freshly collected individuals, were fixed for TEM, for 2 h at 8 °C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer adjusted to pH 7.2 and to 1000 mOsM with NaCl and 2 mM $CaCl_2$; they were post-fixed for 1 h in 1% osmium tetroxide in the same buffer at the same temperature, and dehydrated through ascending ethanol and propylene oxide. After embedding in epoxy (Epon-Araldite) or in acrylic (LR White) resins, sections were cut on an Ultracut E ultramicrotome. Semi-thin sections (0.5 μm thick) were stained with 0.5% Toluidine blue in 1% borax; thin sections (60 nm thick) mounted on 100-mesh copper grids with pyroxylin film were contrasted with aqueous uranyl acetate and lead citrate before being observed in a Hitachi TEM H-8000 at an acceleration voltage of 100 kV.

For EDX (energy-dispersive X-ray) analysis, fixations were performed in a mixture of 1% paraformaldehyde and 2% glutaraldehyde in the same buffer, but pieces were dehydrated and embedded in Epon-Araldite without post-fixation in osmium tetroxide and without use of propylene oxide. The contrast of those unosmicated sections in TEM was improved by addition of picric acid (0.2%) to the fixative. The thickness of sections was tested, and 200 nm was selected to obtain the best signal. The sections were collected on 200-mesh copper grids, without supporting film, and observed, without additional treatment, within 2 days.

Purification of small vesicle fraction and microbody fraction

To obtain the small vesicle fraction, small pieces of delaminated gills, washed in sterile seawater, were crushed before centrifugation at 4000 *g* for 5 min. The supernatant was centrifuged at 8000 *g* for 5 min, and the new pellet was suspended and washed two times in sterile distilled water under the same conditions in order to remove salt from the seawater. The last pellet was suspended in 30 μl of sterile distilled water. Five microliters of this enriched fraction of refringent small vesicles, measuring approximately 1 μm, were loaded on copper grids coated with pyroxylin film and air dried before EDX microanalysis in the STEM mode, without delay.

To obtain the fraction containing microbodies, crushed gill tissue suspended in a 50 mM Tris-HCl buffer at pH 8.0 was centrifuged at 4000 *g* for 5 min to eliminate the cytoplasmic hemoglobin as well as the small vesicles in the supernatant. The pellet was resuspended in the same buffer and filtered on a 40 μm mesh nylon tissue to eliminate coarse debris. The filtrate was centrifuged at 300 *g* for 3 min. The last pellet, which contained 2–10 μm large, yellow or brown corpuscules, was resuspended in distilled water and loaded on copper grids as described for the small vesicles.

EDX microanalysis

Sections prepared from blocks especially fixed for microanalysis as well as isolated small vesicles and microbodies were analyzed in the STEM mode of the Hitachi H-8000, using a pentafet detector (Oxford Instrument), monitored by a Link Isis system, in the point mode. EDX results obtained from these sections were compared with those obtained from classical glutaraldehyde- and osmium-fixed tissues and from those submitted to cytoenzymological detection of hydrolases. The Temquant software of the Isis system was used for comparison between different structures in the same section, or between similar structures in different individuals. All these quantified comparisons are valid only if the thickness of sections and conditions of acquisition are similar. They were designed only to obtain relative abundance for the various fixation methods used and not to provide true quantification. As a positive control, sections of rat blood cells, obtained under the same conditions of inclusion and thickness, were used. A negative control was constituted by the various cells, devoid of any iron, present in the same sections as the bacteriocytes.

Cytochemical characterization of lysosomal activities

Two frequent hydrolase enzymes, acid phosphatase and arylsulphatase, which have already been detected in various mollusk tissues (Owen 1972; Pipe and Moore 1985) were chosen as lysosomal markers. Acid phosphatase activity was detected by the β-glycerophosphate and lead acetate method of Barka and Anderson (1962, modified by Pottu-Boumendil 1990) in Tris-maleate buffer adjusted to pH 5.2, modified for marine mollusks by addition of NaCl in the incubation mixtures. Arylsulphatase activity was detected by the method of Hopsu-Havu et al. (1967, cited in Lewis and Knight 1992), with P-nitrocatechol and barium salt in acetate buffer adjusted to pH 5, also adapted to the osmolarity of marine mollusks. For both procedures, incubation at 37 °C was main-

tained for 0.25–1.5 h, and a control test without substrate was performed for each incubation time. The most successful incubations were 0.75 and 1 h. A trial incubation at a lower temperature for a longer time was not successful. Thin sections (100 nm thick) were observed in TEM mode to take pictures and in STEM mode to analyze the cytoenzymological results obtained in the EDX mode. Thicker sections (200 nm thick) were also analyzed to compare the results with those obtained from unreacted and unosmicated sections. The good contrast obtained in TEM as well as in STEM mode allowed the identification of cytological structures without any lead citrate contrast, avoiding possible displacement of the metallic precipitate [cf. Lewis and Knight (1992)].

Cytochemical evaluation of sulfide-oxidizing activity

The artificial electron acceptor benzyl viologen was used to histochemically detect sulfide-oxidizing activities in various cells and specific organelles according to the method described by Powell and Somero (1985). Control sections were subjected to benzyl viologen without sulfide. The benzyl viologen assay is considered positive only if the color reaction develops within 5 min in the presence of Na_2S and does not develop in control sections. The thermal stability of sulfide-oxidizing activities was checked by heating the section at 70 °C in a humid chamber prior to the benzyl viologen assay.

Results

Sulfur and iron localization

A typical bacteriocyte (Fig. 1A, B) contains bacterial vacuoles scattered in a patchy clear and dark cytoplasm. Large microbodies as well as small, clear vesicles are located in the basal part of the bacteriocytes, in the same area as the nucleus.

EDX microanalysis revealed a high level of iron (2.14 ± 1.63%), as well as of sulfur (2.26 ± 0.75%), in the electron-dense granules of microbodies (Fig. 2A), with considerable variability in the various microbodies of the same section. The clear matrix of these microbodies does not contain detectable iron or sulfur. In microbodies devoid of dense granules (Figs. 1B, 2B), iron and sulfur levels were lower (0.74 ± 0.32% and 1.09 ± 0.11%, respectively) than in the well-defined granules of the other type of microbodies. Microanalysis of these microbodies from a partly purified fraction revealed a higher proportion of iron, possibly reaching up to 8%; this means that, even in the heme status, iron is not totally protected from extraction during the fixation and dehydration steps (Table 1).

TEM examination of gill sections revealed also electron-lucent, apparently empty vesicles (Fig. 1A, B). These small vesicles, approximately 0.5–1 μm in diameter, are consistently located in the basal part of bacteriocytes, in close proximity with the large microbodies, but their abundance varies among individuals. Sulfur was not detected by EDX microanalysis in these electron-lucent vesicles, and iron was found at a very low level (0.32 ± 0.04%). However, the partly purified fraction containing such small vesicles showed high levels of sulfur (2.05 ± 0.92%) and iron (1.62 ± 0.91%) (Fig. 2C), indicating that their content is dissolved during some steps of TEM preparation.

Iron can be detected by EDX microanalysis in the dark cytoplasmic areas of bacteriocytes, whereas clear cytoplasmic areas are always devoid of a significant iron signal. Nevertheless, iron content of dark cytoplasmic areas is lower than expected from heme detection by the DAB reaction. Iron is never detected in bacteria nor in intercalary and ciliated cells, which are DAB negative and constitute an internal negative test. A significant proportion of sulfur

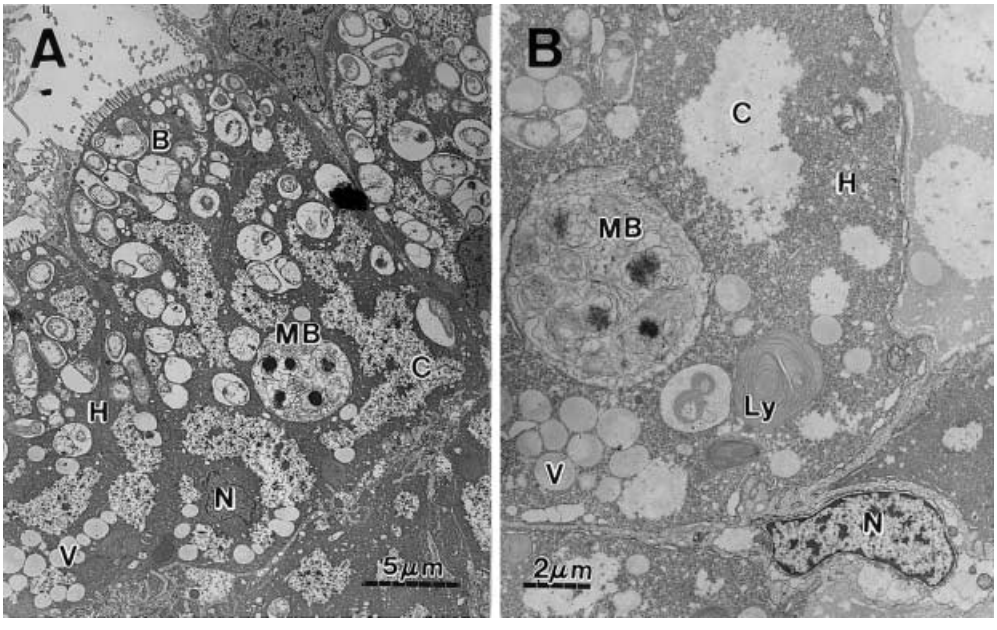

Fig. 1A, B *Lucina pectinata*. Typical structures of a bacteriocyte. **A** Bacteriocyte with patchy cytosol of dark hemoglobin-containing areas (*H*) and clear areas (*C*), bacterial vacuoles (*B*), small vesicles (*V*) and a large microbody (*MB*), characterized by electron-dense granules, near the nucleus (*N*). **B** Basal part of a bacteriocyte with clear and dark cytoplasmic areas, including small vesicles, a secondary lysosome (*Ly*), and a large microbody (*MB*) containing less mature electron-dense granules and more membrane whorls than the microbody present in A

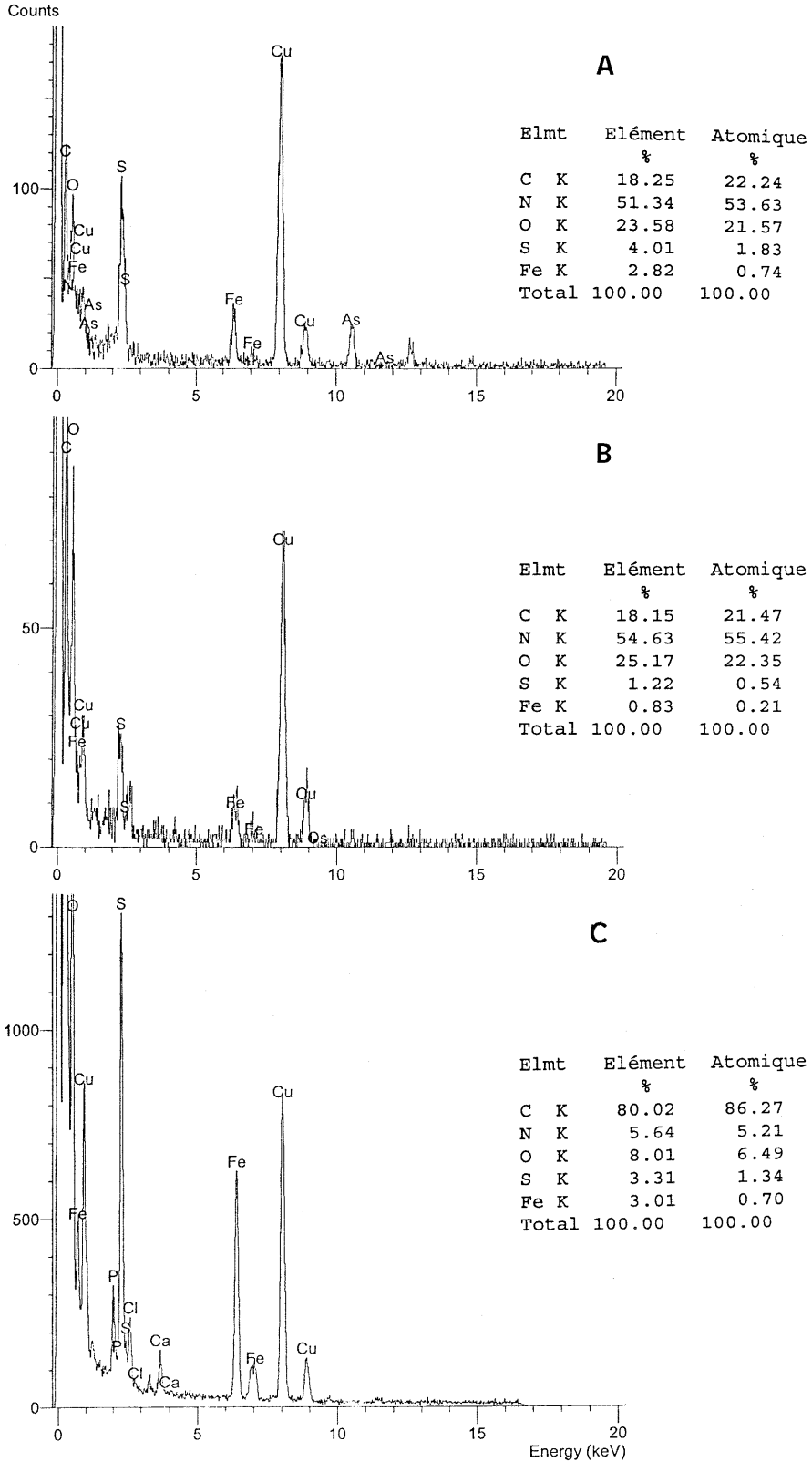

Fig. 2A–C *Lucina pectinata.* EDX microanalysis of the main inclusions of a bacteriocyte; typical spectra and corresponding quantitative analysis. Quantification is expressed in percent, which allows comparison of the ratios of iron (*Fe*) and sulfur (*S*) with the major elements, i.e. carbon (*C*), nitrogen (*N*) and oxygen (*O*). The copper (*Cu*) comes from the grid bars and is considered in the quantification for deconvolution only (*K* energy level of the X-ray spectrum). **A** Electron-dense granule from a microbody. **B** secondary lysosome. **C** small vesicle analyzed from a purified unfixed fraction

(1.45 ± 0.39%) is regularly detected in the dark cytoplasmic areas, and a lower proportion, in clear areas (0.15 ± 0.12%).

In all cases, there is a large range of individual variation in the sulfur and iron content of each type of organelle detected under the same experimental

Table 1 *Lucina pectinata*. Summary of highest values obtained for each structure under various experimental conditions. All values are expressed as a percentage of all elements, including carbon, nitrogen and oxygen. The arylsulphatase activity detected by barium (*Ba*) precipitation clearly discriminates the secondary lysosomes from the sulfide-oxidizing bodies (*SOBs*). The detection of acid phosphatase by lead precipitation, which gives similar results, is not shown (–, undetected; no analysis for the cytoplasm which is eliminated in the purified fractions)

		SOBs	Lysosomes	Small vesicles	Dark cytoplasm	Clear cytoplasm
Unosmicated sections	S	3.76	2.19	0.28	2.08	1.53
	Fe	5.78	1.36	0.37	0.39	0.27
Arylsulphatase detection	S	0.21	8.46	–	0.81	0.28
	Fe	0.14	0.48	–	0.45	0.16
	Ba	0.10	42.92	–	0.03	0
Purified, unfixed fractions	S	7.64	–	3.27		
	Fe	8.91	–	2.97		

conditions, which may be related with the cytophysiological status of the organelles.

Cytoenzymology

Lysosomal enzymatic activities

Cytoenzymologic reactions for acid phosphatase are positive around some single bacteria inside their endocytic vacuole (Fig. 3A). The two lysosomal enzyme-markers, acid phosphatase and arylsulphatase, characterized, respectively, by lead and barium salt deposit, are also detected surrounding aggregated bacteria at a more advanced stage of degradation (Fig. 3B), as well as in large lysosome-like inclusions containing partly destroyed bacteria (Fig. 3C). Cytochemical detection revealed that acid phosphatase and arylsulphatase activities are only present in microbodies devoid of electron-dense granules, therefore considered lysosomes, but not in all of them (Fig. 3D). Conversely, lysosomal markers were never detected in microbodies with dense granules containing iron and sulfur (Figs. 4A, B, 5A). The control reaction without substrate was negative in both cases.

EDX microanalysis gives a strong signal for barium when barium salt is the capturing agent in the detection of arylsulphatase (Fig. 5B). However, it appears the most variable element in different samples, according to the functional status of the lysosomes (2–43% of the total ions analyzed with carbon, nitrogen, oxygen, sulfur and iron included). There is no, or only a weak iron signal. The introduction of barium at a high ratio lowers the ratio of all the elements, expressed in percent of total content. However, these ratios are abnormally low even in the microbodies that do not contain barium, which demonstrates that the fixation of these elements is not optimal under the detection conditions for enzymatic activities. Therefore, the information obtained through EDX microanalysis of sections that have been subjected to cytoenzymologic reactions is only indicative of the presence of the elements, as, in similar positive locations, the detection of iron and sulfur is generally much stronger in tissues not subjected to hydrolase reactions (Table 1).

Sulfide-oxidizing activity

Cytochemical staining by benzyl viologen, in the presence of sulfide, of unfixed gill tissue resulted in the appearance of a reddish to purple color at the sites of sulfide oxidation. No color appeared when benzyl viologen was added without sulfide. In gill sections, microbodies with electron-dense granules stained positively for sulfide-oxidizing activity, and were, therefore, named sulfide-oxidizing bodies (SOBs), while those devoid of iron-containing granules (lysosomes) failed to show any sulfide-oxidizing activity.

Unexpectedly, mucocytes, located at the abfrontal end of each filament and in the intermediate zone between the ciliated heads of filaments and lateral zone containing the bacteriocytes, developed an intense positive reaction to the benzyl viologen assay. The sulfide-oxidizing activities of gill microbodies and mucocytes were heat resistant. The positive benzyl viologen reaction in gill mucocytes strictly coincides with Alcian blue staining at pH 0.5. Alcian blue staining is typical of proteoglycans; thus coincidence of both reactions proves that gill mucocytes are involved in sulfide oxidization. In contrast, the foot superficial mucous layer which contains mucocytes of various types, some positive and some negative to Alcian blue, was negative to the benzyl viologen assay. All the color reactions, which are not conspicuous on the black and white micrographs, are summarized in Table 2.

Discussion

Lysosomal activities

In the present study, secondary lysosomes have been identified through the cytoenzymologic characterization

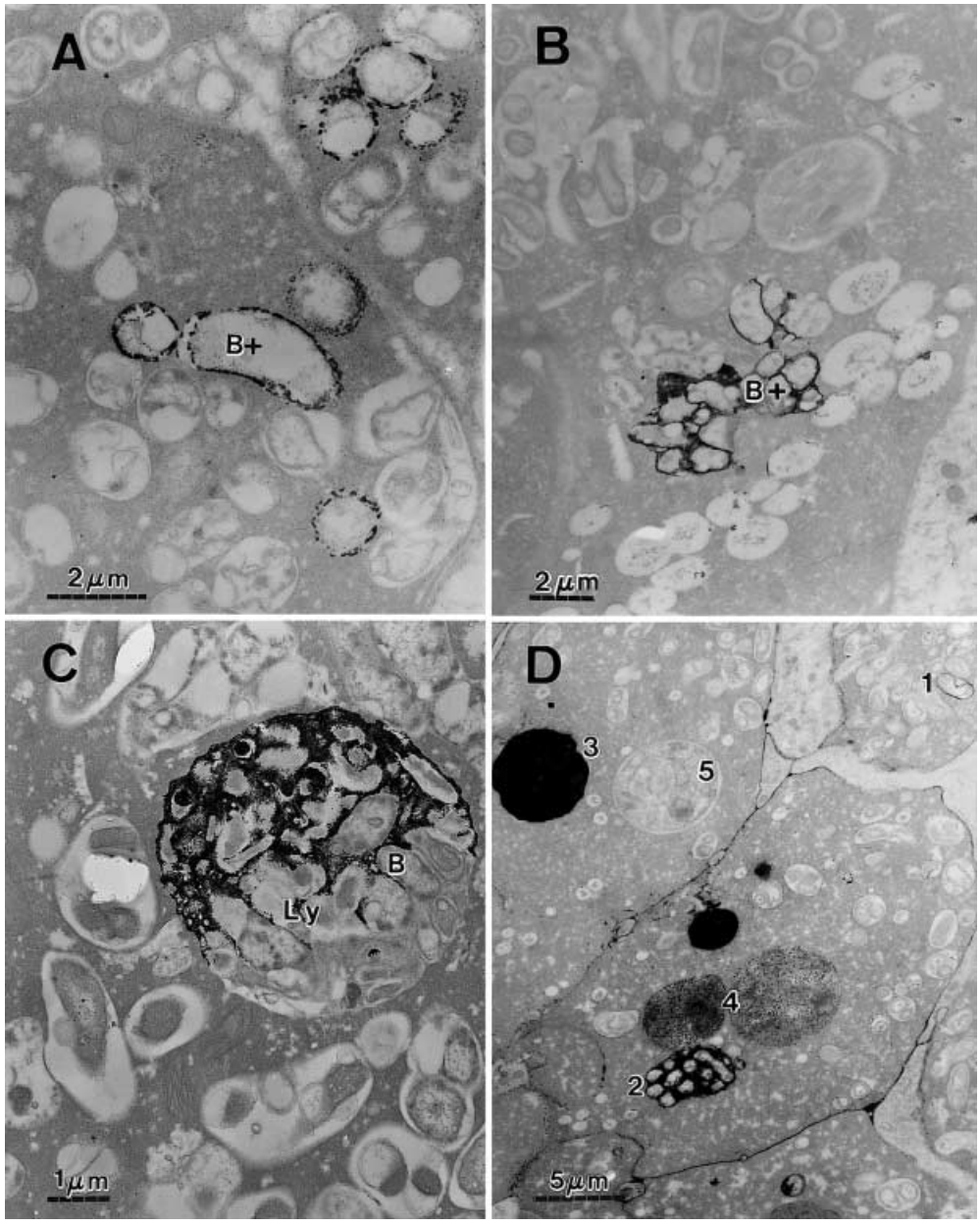

Fig. 3A–D *Lucina pectinata*. Detection of lysosomal enzymes in the bacteriocyte. **A** Cytoenzymologic detection of acid phosphatase in some bacterial vacuoles surrounding individual symbionts (B^+). **B** Aggregation of bacteria (B^+), digested by lysosomal enzymes, is an intermediary stage before contribution to a typical secondary lysosome. **C** Detection of arylsulphatase in a secondary lysosome (*Ly*) containing remains of bacteria (*B*). **D** Bacteriocytes at various stages of lysosomal evolution, including single envacuolated bacterium (*1*), aggregated bacteria (*2*), mature secondary lysosome (*3*), decreasing activity (*4*) and residual body devoid of any lysosomal activity (*5*). The enzymatic activity detected by barium precipitation is arylsulphatase

of two enzyme-markers, acid phosphatase and arylsulphatase. The demonstration of acid phosphatase activity around single bacteria inside their endocytic vacuole, as well as surrounding aggregated bacteria at a more advanced stage of degradation, indicates that lysosomal digestion of symbionts by the invertebrate host does occur, following the addition of hydrolases to the individual endocytic vacuoles. Subsequent progressive coalescence may then result in large secondary lysosomes. Similar lysosomal digestion of bacteria has been identified by Streams et al. (1997) in the bacteriocytes of seep mytilids associated with methanotrophic symbionts. However, these authors show that in the residual body condition, secondary lysosomes may no longer retain any hydrolase activity. Therefore, we have to consider as secondary lysosomes or as residual bodies a variety of microbodies characterized by a strong positive signal of acid phosphatase and arylsulphatase, as well as morphologically similar inclusions that may be negative, in the same bacteriocytes. Such residual bodies consistently contain myelin-like structures, but nothing is known about the evolution of the pH of their content.

Sulfide-oxidizing activities

An alternative interpretation has been put forward by Powell and Somero (1985) on the basis of the gill cells of *Solemya reidi*, which contain a similar type of large microbodies, also located in the basal part of bacteriocytes. According to these authors, yellow-brownish granules were characterized as sulfide-oxidizing bodies (SOBs) on unfixed cryosections by the benzyl viologen

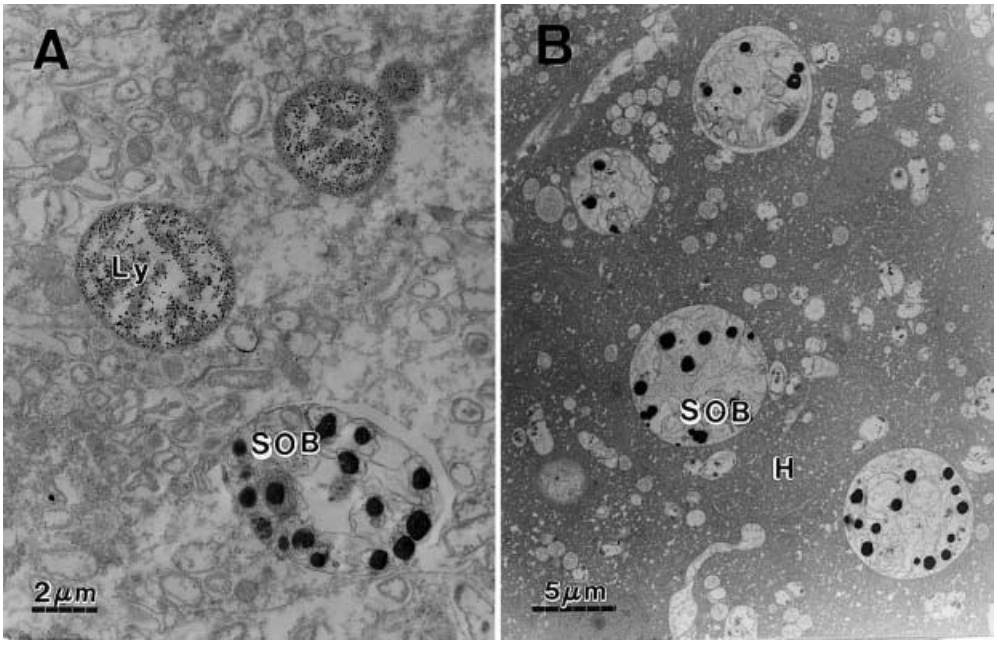

Fig. 4A, B *Lucina pectinata*. Secondary lysosomes and sulfide-oxidizing bodies. **A** Discrimination of two types of microbodies by acid phosphatase detection: secondary lysosome (*Ly*), positive to acid phosphatase reaction; sulfide-oxidizing body (*SOB*) with electron-dense granules, negative to lysosomal enzyme detection. **B** Numerous typical sulfide-oxidizing bodies, with electron-dense granules containing free hemes, located in the basal part of bacteriocytes which are occupied mostly by dark hemoglobin-rich cytoplasm (*H*)

Table 2 Cytochemical detection of proteoglycans through Alcian blue at pH 0.5, and sulfide-oxidizing activity through benzyl viologen purple coloration in various microbodies [sulfide-oxidizing bodies *SOBs*, or lysosomess] and mucocytes (+, positive reaction; −, negative reaction)

	Alcian blue	Benzyl viologen
Gill lysosomes	−	−
Gill SOBs	−	+
Gill mucocytes	+	+
Foot mucocytes	+	−

reaction. Brown granules, identified in *S. reidi* as well as in *Calyptogena magnifica* and *Lucinoma annulata*, have also been demonstrated to contain free heme, identified in connection with sulfide-oxidizing activity, and have, thus, been considered SOBs (Powell and Arp 1989).

In fresh gill-tissue squash of *Lucina pectinata*, conspicuous large brown corpuscules are numerous. They correspond to the peculiar microbodies which contain abundant free hemes concentrated in electron-dense granules and which develop a positive benzyl viologen reaction. On the other hand, iron and sulfur, detected by EDX microanalysis, are consistently more abundant in the electron-dense granules of these peculiar microbodies than in secondary lysosomes. Moreover, on sections treated for cytoenzymology, these microbodies containing electron-dense granules lack lysosomal markers, in contrast with nearby secondary lysosomes.

In conclusion, positive reactions to DAB and benzyl viologen, detection of iron and sulfur in the electron-dense granules of the microbodies, and lack of hydrolase reactions strongly suggest that the morphologically distinct inclusions identified in *L. pectinata* represent discrete organelles, independent from secondary lysosomes, that may be termed SOBs.

The sulfide-oxidizing activity within these SOBs is heat resistant; therefore, it is not an enzymatic sulfide oxidation. Such a non-enzymatic mechanism may be similar to the oxidizing process involving hematin described by Powell and Arp (1989) in the brown granules identified in the bacteriocytes of symbiotic bivalves and in coelomocytes of *Urechis caupo*, a large echiurian worm well adapted to sulfide-rich environments, apparently without symbiosis. However, a heat resistance control is necessary to ascertain whether or not the sulfide-oxidizing activity is enzymatic in the various species which possess SOBs.

Lysosomal SOB hypothesis

The involvement of microbodies with electron-dense granules, considered SOBs, in sulfide oxidation processes has been ascertained in *L. pectinata*. However, the origin of the free hemes contained in the electron-dense granules is unknown.

With regard to the origin of SOBs in *U. caupo*, an original hypothesis has been proposed by Menon and Arp (1993) and Arp et al. (1995). These authors consider that the numerous SOBs observed in the epidermis and respiratory hindgut of *U. caupo* may be secondary lysosomes containing phagocytosed sulfide-damaged mitochondria. Heme iron from these mitochondria would then be used to catalyse sulfide oxidization. The sulfide detoxification end products appear as electron-dense specks that may not be very different from the dense granules typical of SOBs in *L. pectinata*. Conversely to the *U. caupo* case, no mitochondria appear to be damaged or included in the lysosomes of *L. pectinata* or in the other lucinids studied (Frenkiel and Mouëza 1995; Frenkiel et al. 1996; Gros et al. 1996). A putative origin for iron-free hemes in *L. pectinata* SOBs could be the breakdown of cytoplasmic hemoglobin in the lysosomal compartment, both cytoplasmic hemoglobin and

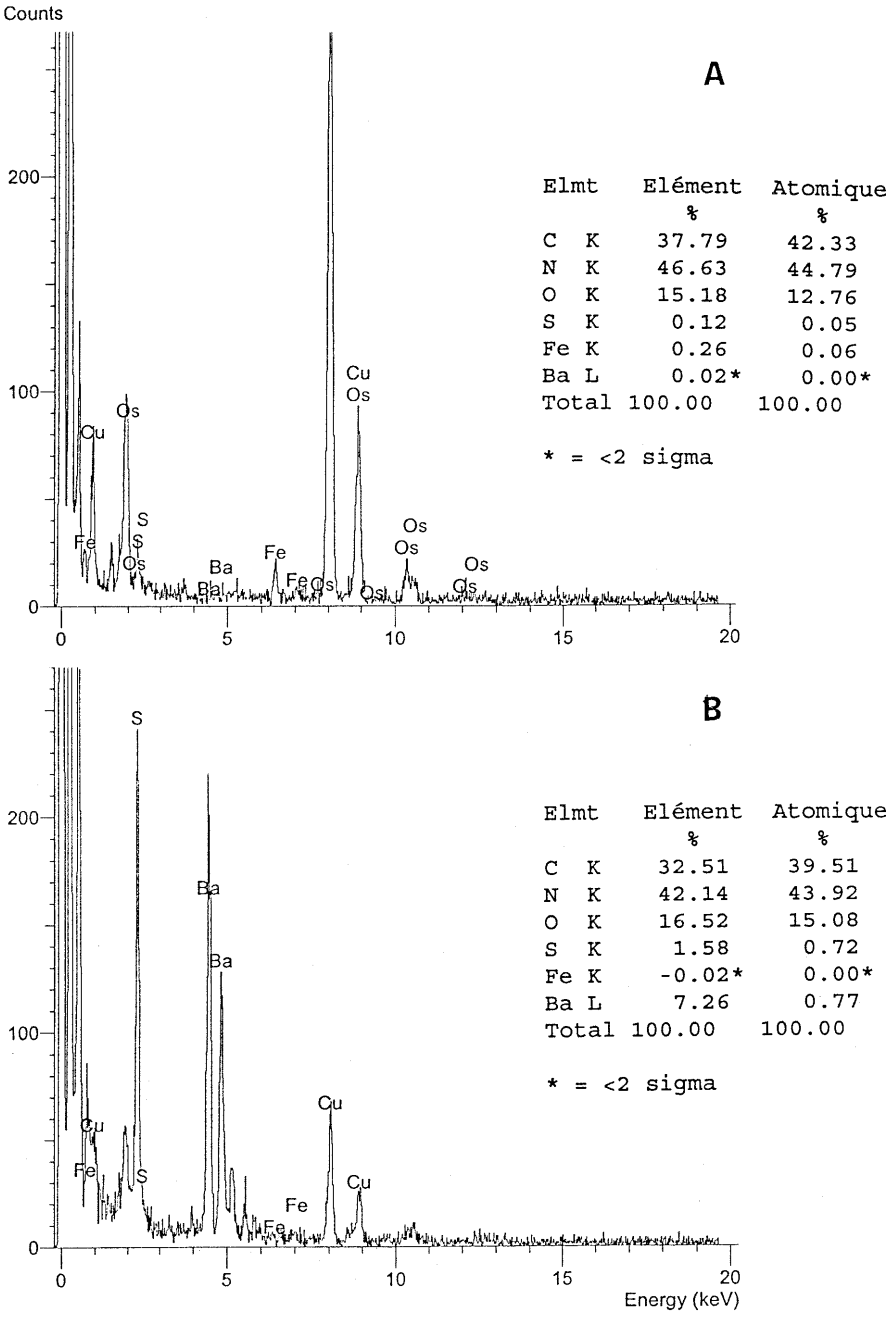

Fig. 5A, B *Lucina pectinata.* EDX microanalysis on cytochemical detection of arylsulphatase by barium precipitation. Quantification is expressed in percent, which allows comparison of the ratios of iron (*Fe*) and sulfur (*S*) with the major elements, i.e. carbon (*C*), nitrogen (*N*) and oxygen (*O*). The introduction of barium (*Ba*) at a high ratio lowers the relative ratios of all the elements. Copper (*Cu*), the signal of which comes from the grid bar, and osmium (*Os*), the signal of which comes from the fixative, are considered only for the deconvolution of the spectrum and do not appear in the quantification (*K* and *L* energy levels of the X-ray spectrum; asterisk ratio of the element non-significant) **A** Electron-dense granule of a sulfide-oxidizing body (Ba–); **B** secondary lysosome (Ba+)

free hemes in SOBs being particularly abundant in this species. Nevertheless, such a process would involve an obligatory step, i.e. a pH shift from acidic to basic, necessary for the shift from lysosomal function to SOB function.

Detection of hydrolase activities to determine the real nature and function of lysosomes appears to be a prerequisite before the questionable hypothesis of "lysosomal SOBs" can be accepted. Cytoenzymologic assays in *L. pectinata* have detected hydrolase activities in secondary lysosomes versus free hemes in SOBs. However, assays failed to identify autophagic vacuoles implicated in the breakdown of cytoplasmic hemoglobin which may generate those free hemes; although the presence of a small proportion of iron was detected in some secondary lysosomes.

In any case, *U. caupo* is very different from *L. pectinata* and other symbiotic bivalves, and may have developed very different detoxification mechanisms. In fact, even within the lucinid group, putative detoxification processes may be very variable. *Linga pensylvanica,* for example, has developed typical peroxisomes with unknown function (Gros et al. 1996). A possible congruence between such peroxisomes and SOBs has to be addressed, as the enzyme equipment of peroxysomes functions under basic pH conditions like the sulfide oxidase systems in SOBs. Moreover, the enzymological functions of peroxysomes are connected with oxidizing activities.

Cytoplasmic small vesicles

Another type of small vesicles has been identified in the dark cytoplasmic areas of the bacteriocytes close to SOBs. Similar vesicles have not been described in other lucinid species or in other symbiotic bivalves with sulfide-oxidizing chemoautotrophic bacteria. Obviously, the content of these small vesicles is washed away, probably by the inclusion procedures. Attempts to retain and analyze their real content have been successful by using isolated and partly purified vesicles in which large proportions of iron and sulfur have been detected. These vesicles might store sulfur and iron before possible incorporation in SOBs, as suggested by close associations between some of these vesicles and SOBs. The iron content of these vesicles, being washed away during classical TEM procedures and being negative to DAB identification of pseudoperoxidasic activity, may be in a transitory state not included in heme.

Sulfide-oxidizing activities in mucocytes

Another point of concern is the peripheral detoxification process and its relationship with the diversity of mucocytes and their function in detoxification processes. Powell and Somero (1985, 1986) have identified, by using the benzyl viologen assay, a peripheral oxidizing activity, diffusely distributed, in the foot of *S. reidi* as well as in the foot and mantle of *C. magnifica*. Such a reaction, in symbiont-free tissues, has been considered by these authors to be a peripheral protective mechanism against sulfide toxicity, produced by an unknown enzymatic system. Our observations on the foot of *L. pectinata* consistently lack such a benzyl viologen reaction. However, a similar area is occupied by numerous mucocytes of various types, most of them containing sulfomucins, as demonstrated by Alcian blue staining. In contrast, the mucocytes located at both ends of gill filaments are intensively reactive to benzyl viologen sulfide assay. This sulfide-oxidizing activity is non-enzymatic, as demonstrated by its resistance to heat. Thus, in *L. pectinata*, the gill mucocytes exhibit a sulfide-oxidizing activity, whereas the mucocytes of the foot fail to have such an activity. However, the numerous mucocytes of the foot, which contain various types of mucosubstances, may be physically protective, reducing the rate of sulfide diffusion (Arp et al. 1995). Moreover, the synthesis of sulfated mucopolysaccharides, which use sulfide present in the environment, may constitute a peripheral detoxification process. From such a perspective, the peculiar SOBs identified in *L. pectinata* but not in *Codakia orbicularis* and *Linga pensylvanica* (authors' personal observations) may represent an adaptive response to the reducing mud of mangrove swamps, which is a more selective environment with respect to sulfide resistance than are the sea grass beds where most other tropical lucinids are to be found.

A search for typical SOBs and other detoxification processes in other lucinids inhabiting various environments from low to high sulfide contents may shed new light on the protective functions of various types of mucocytes as well as on the development of SOBs in the evolution of the Lucinacea.

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