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Investigations on the cuticle of the polychaete elytra using energy dispersive X-ray analysis*

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ABSTRACT: Energy dispersive X-ray analysis of elements with $Z > 11$ in a SEM (scanning electron microscope) was used to investigate the elytra of *Lagisca extenuata*, *Lepidonotus clava*, and *Harmothoe areolata* from Naples (Italy) and Banyuls (France). High concentrations of halogens and a few other elements were found in certain papillae in samples from both locations. Additional TEM-examinations and X-ray analysis of thin sections revealed that the halogen concentration is inversely related to the collagen content of the matrix. The halogens are presumably bound to tyrosines, which occur in these structures. In addition, accumulation of Mn^{2+} and possibly Fe^{3+} in the papillae might depend on environmental conditions. The results show that valuable information about the chemical composition of biological structures can be obtained by energy dispersive X-ray analysis. Moreover, the results indicate that this method may be useful for environmental investigations.

INTRODUCTION

In several studies on the structure and chemical composition of the cuticle of polychaetes chemical and histochemical staining methods were used to investigate its composition or local variations in composition, respectively (Mill, 1978). These variations are suggested by the locally varying collagenfiber and matrix distributions in the cuticle.

Normally, the proximal zone of the cuticle consists of layers of collagen fibers arranged at right angles to one another. In the case of the elytron cuticle we found that the fibers of two subsequent layers always lie at an angle of 120° to one another. These fibers are housed in a homogeneous matrix, which also forms the distal zone of the elytron cuticle. The relative amount of fibers in the cuticle of certain papillae is found to be much less than in the cuticle between the papillae.

The polychaete cuticle is reported to be composed of two classes of organic material, a carbohydrate component and a protein component in the form of collagen. The collagen fibers are unbanded, which specifically refers to the binding of a carbohydrate component to the tropocollagen dimers (Maser & Rice, 1963). The matrix is composed of neutral and acidous mucopolysaccharides of which the acidous position is carboxylated. This may confer an increased viscosity to the matrix (Manavalaramanujam & Sundara

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Rajulu, 1974). Presumably there is some non-collageneous protein in the distal cuticle zone. In Nereis the susceptibility of the fuchsinophilia together with the presence of aromatic, tyrosine containing protein and the demonstration of disulphide bridges indicate a hardening of the protein moiety (Manavalaramanujam & Sundara Rajulu, 1974).

This study is the first to use X-ray microanalysis techniques for obtaining further information on local changes in the chemistry of the elytron cuticle. This technique is especially helpful in relating differences in the morphology of the cuticle, with and without fiber-structures, to their chemical composition. The present investigations were carried out by small area energy-dispersive X-ray analysis in a scanning electron microscope, combined with transmission-electron microscopy of thin sections.

MATERIALS AND METHODS

The species studied *(Lepidonotus clava, Harmothoe areolata, Lagisca extenuata)* were collected from the Mediterranean Sea near Naples, Italy, and near Banyuls-sur-mer, France. The total elytra-objects were fixed and dehydrated with methanol in steps from 70 to 100 %. The elytra from which cross-sections were to be obtained, were fixed for 2 hours by 2 % glutaraldehyde in cacodylate buffer (0.2 M; pH 7.4) with dissolved sucrose to 900 mOsm at 5 °C. After rinsing in the same buffer, the specimens were postfixed in 1 % osmium tetroxide dissolved in cacodylate buffer (0.2 M; pH 7.4), and with dissolved NaCl to 850 mOsm for 2 hours at 5 °C. Then the specimens were dehydrated in ethanol, embedded in Spurr, and stained with uranyl acetate in 50 % methanol and lead citrate.

By means of the energy dispersive X-ray analysis in a scanning electron microscope (SEM) the elements with atomic number $Z > 11$ can be determined qualitatively and, under certain conditions, quantitatively. An advantage of this technique is the unambiguous identification of the analysed area by the secondary electron image and comparison with X-ray mapping images, taken with selected characteristic X-ray lines of the elements analysed. All the tables in this work list "quantitative estimates" of the compositions neglecting the amounts of biological matrix, despite the fact that biological specimens mainly consist of elements with $Z < 11$, and the detection efficiency for the heavier elements depend on their distribution in the biological matrix. The concentration values for the analysed elements were calculated by quantitative analysis programs for bulk specimens or thin sections, respectively, as provided by the manufacturer of the detector system (EDAX). In these programs, the relative exitation and detection efficiencies for the elements, dependent on the exitation energy, the energy of the analysed X-ray lines, the geometrical orientation, etc., are taken into account. However, because of the influences mentioned above, the results can only be considered as rough estimates, as the contribution of the low Z biological matrix was unknown. For the comparison of different areas on the same sample only the ratios of the count rates for each element were analyzed in order to demonstrate relative concentrations.

Two representative spectra of the coneshaped papilla cuticle and of the elytron surface cuticle of *Lagisca extenuata* from Banyuls are shown in Figure 1.

For both spectra, areas of about 0.25 μm^2 on a bulk specimen were analysed. The comparison already demonstrates a spectacular concentration of mainly the halogens Br,

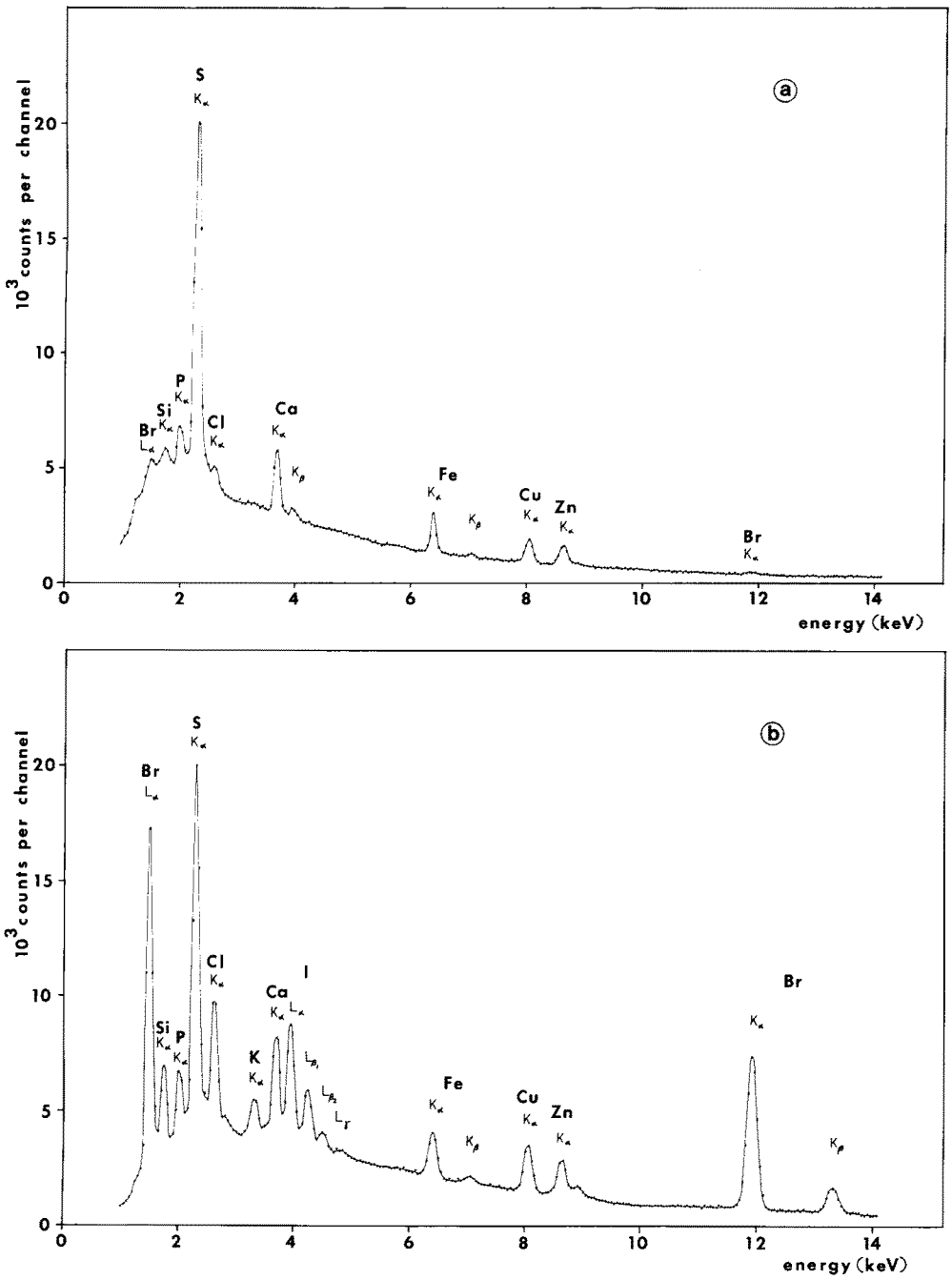

Fig. 1. *Lagisca extenuata* (Banyuls): (a) X-ray spectrum of the elytron surface cuticle between the papillae. The primary beam energy was 25 keV; the analysed area was about 0.25 μm^2. (b) X-ray spectrum of the cone-shaped papilla cuticle; same conditions as in (a)

I, and Cl in the papilla. Careful investigations by means of a blank specimen holder and other specimens revealed, that only part of the elements present were contained in the specimen itself. These were the halogens, as mentioned, K, Ca, P, S, Fe, and Mn. Sometimes, indications of Na and Mg were also found, but the intensities were not to be determined with reasonable accuracy due to the strongly varying background in this low-energy portion of the spectrum. Some other elements, mainly Cu and Zn in the case of bulk samples, were due to scattering from the holder, namely a brass plate on which the sample was mounted. In the case of thin sections, which were originally prepared for TEM-investigations, Cu-radiation originated from the grid, Al and Si from the holder. In addition, especially during the analysis of thin sections, a small amount of Fe was found and determined as due to scattering by some steel parts in the microscope chamber. This was only detectable because of the long accumulation times needed for the analysis of thin sections to obtain a reasonable signal-to-noise ratio, and a low background level. In addition, for thin sections, which were treated with Os-solution, the P-K-radiation interfered with the Os-M-line, so that the P-intensities could not be determined accurately. Nevertheless, even with thin sections, the strong concentration of the halogens in the cuticle of the papilla was detectable, as will be shown on the following pages.

RESULTS

Although numerous individuals of polychaetes were analysed, the representative results obtained from only three species will be presented here. Two samples of every species from each collecting site (Banyuls and Naples) are compared.

Lagisca extenuata

A massive, total elytron was investigated. The cuticle of the coneshaped papillae and of the elytron surface between the papillae were analysed. A SEM-image of the

Table 1. X-ray analyses of two types of cuticle of the elytra of *Lagisca extenuata* (Banyuls)

element	Coneshaped papilla		Surface of elytron		Ratio of count rates papilla/elytron
	count rate*	mass (%)	count rate	mass (%)	
Br	48.5	54.6	0.9	4.3	53.9 : 1
Cl	32.5	4.2	7.9	4.0	4.1 : 1
I	15.3	5.4	3.4	4.8	4.5 : 1
K	16.7	2.4	7.5	4.3	2.2 : 1
Ca	43.6	6.6	14.7	9.4	3.0 : 1
P	64.1	8.0	44.0	24.2	1.5 : 1
S	74.3	9.2	92.6	47.2	0.8 : 1
Mn	13.7	3.3	1.3	1.3	10.5 : 1
Fe	35.4	6.3	3.3	3.5	10.7 : 1

* The count rates are given in arbitrary units, but spectra which were compared with one another were accumulated under the same conditions (primary beam energy, beam current, etc.)

elytron is shown in Figure 2. In Table 1, the results of the X-ray analysis of two representative areas of these types of cuticle are shown. For the elements, the actual count rates and their ratio, e.g. comparing the surface of the papilla to the area in between, are listed.

Even when one takes into account, that the efficiencies in both analyses depend somewhat on the surface topography, the results show a conspicuous concentration of halogens, mainly Br, and in addition, Fe and Mn in the cuticle of the papilla. By elemental mapping, using the Br-K_α-line, as shown in Figure 2, the considerable Br-accumulation in the papilla is clearly demonstrated by comparison with the corresponding SEM-image. TEM-images of thin sections of these elytra show, that both types of cuticle exhibit different structures, mainly through the different content of collagen fibers and matrix (Fig. 2 c, d).

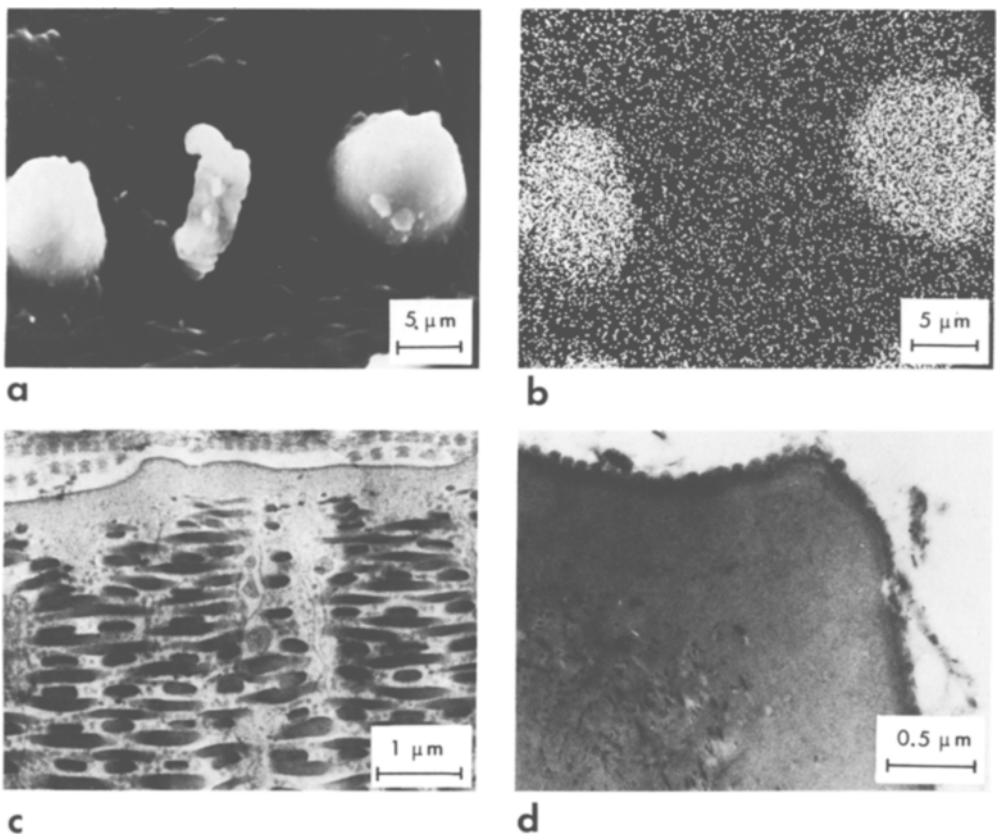

Fig. 2. *Lagisca extenuata* (Banyuls): (a) SEM-image of the elytron showing two types of papillae, two with cone-shaped, one with thin cylindrical habit (center). The cuticle structure of the latter is the same as in the elytron surface. (b) Corresponding elemental mapping image taken with the Br-K_α-line. The primary beam energy was 25 keV, the spot size 0.125 μm. The cone-shaped papillae with different cuticle structure from the elytron surface show a considerable Br-concentration. (c) TEM-image of a thin cross-section through the elytron surface cuticle between the papillae, mainly consisting of collagen fibers. (d) TEM-image of a thin cross-section through the cuticle of the cone-shaped papillae mainly consisting of matrix substance

Lepidonotus clava

Both a massive, total elytron and a thin cross-section were analyzed. For the total object, the cuticle composition of the papilla with small prickles and of the elytron surface between the papillae were compared. A SEM-image of the surface structure of this elytron is shown in Figure 3.

As for *Lagisca extenuata*, the halogens are strongly concentrated in the papilla cuticle. The distribution of Br all over the papilla surface is clearly visible from the X-ray mapping image, taken with the Br-K_α-line and from the comparison with the SEM-image (Fig. 3 c, b). Allowing for experimental error, and taking the restrictions mentioned earlier into the account, no pronounced concentration of the other elements was found. They seem to be more or less uniformly distributed in the cuticle of papilla and surface. TEM-investigations of both types of cuticle again revealed that the papilla cuticle contains much less fiber material than the elytra cuticle, as shown in Figure 3 c and d. A careful X-ray analysis of the cross section of the cuticle between the papillae revealed that in the proximal zone which is rich in fibers no Br and I were found. Some Cl was detected, but this can be attributed to the fixing agent during the preparation.

Table 2. X-ray analysis of two types of cuticle of the elytra of *Lepidonotus clava* (Naples)

	Papilla with prickles		Surface of the elytron		Ratio of the count rates
element	count rate	mass (%)	count rate	mass (%)	papilla/elytron
Br	65.0	66.8	0.74	3.0	87.8 : 1
Cl	45.5	5.2	9.4	4.3	4.8 : 1
I	29.2	9.5	5.0	6.8	5.8 : 1
K	14.9	1.9	7.0	3.7	2.1 : 1
Ca	25.7	3.6	21.7	12.0	1.2 : 1
P	30.1	3.3	22.3	9.9	1.4 : 1
S	74.1	8.6	92.5	46.0	0.8 : 1
Mn	–	–	–	–	–
Fe	16.8	4.1	13.8	14.3	1.2 : 1

Harmothoe areolata

The elytron surface of *Harmothoe areolata* is dotted with large, spine-shaped papillae with small prickles. The area between the papillae, the cuticle of the elytron surface, ist covered with a network of narrow joints which are only about 3 μm in width. A SEM-image of these structures is shown in Figure 4. X-ray investigations of different areas on the papillae and joints revealed, that even with a very small spotsize of the primary electron beam (16 nm) the lateral resolution was not sufficient to separate the expected low intensity of the narrow joint from that of the surrounding papillae completely. This effect was due to X-ray scattering in the bulk material. Therefore, the analysis of the joints was omitted; in Table 3, only the results for the papilla cuticle are listed.

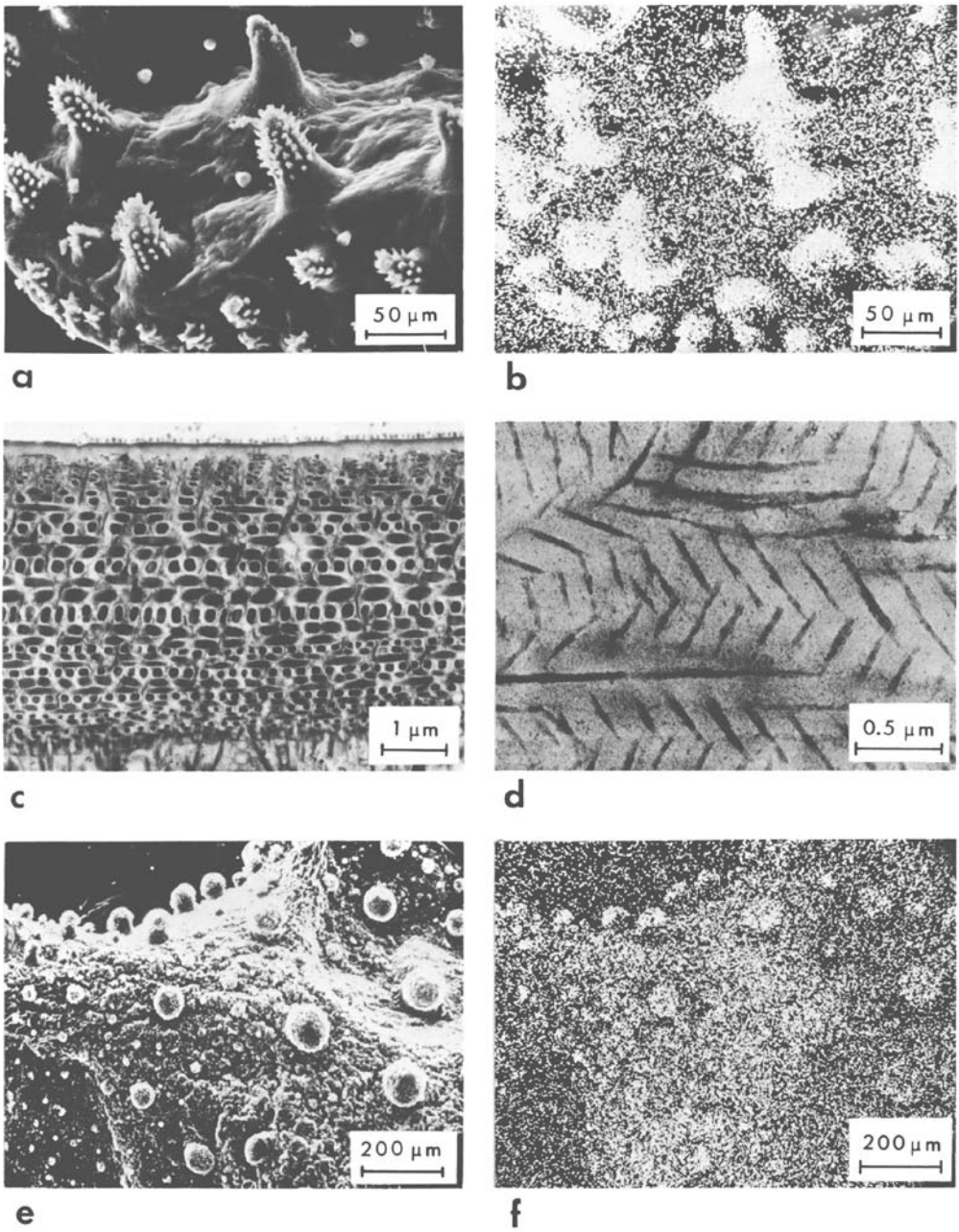

Fig. 3. *Lepidonotus clava* (Naples): (a) SEM-image of the elytron with large papillae with prickles and a few very small papillae, which are composed of both cuticle kinds, whereas the cuticle of the large papillae with prickles contains mainly matrix substance. (b) Corresponding Br-K_α-line elemental mapping of the same area as in (a). Both types of papillae show high Br-concentrations. (c) TEM-image of a cross-section of the elytron surface cuticle, mainly consisting of collagen fibers. (d) TEM-image of a horizontal section through the proximal cuticle zone of the papillae with far fewer fibers and more matrix substance. *L. clava* (Banyuls): (e) SEM-image of papillae with prickles. The X-ray analysis shows, besides the Br-accumulation, the occurrence of Mn in these papillae, which was typical for specimens from Banyuls. (f) X-ray elemental mapping with the Mn-K-line of the same area as in (e)

The values given in Table 3 are very similar to the corresponding analysis of the papillae of *Lagisca extenuata* and *Lepidonotus clava*, especially regarding the high amount of Br.

As the narrow joints between the papillae were expected to exhibit a different chemical composition, detailed X-ray analyses of thin cross sections of these zones were made. The scattering of X-rays was shown to be far less within thin foils than within bulk material and the lateral resolution accordingly increased. Moreover, the reduction of the total X-ray intensity is more than compensated by a strongly increased signal-to-noise

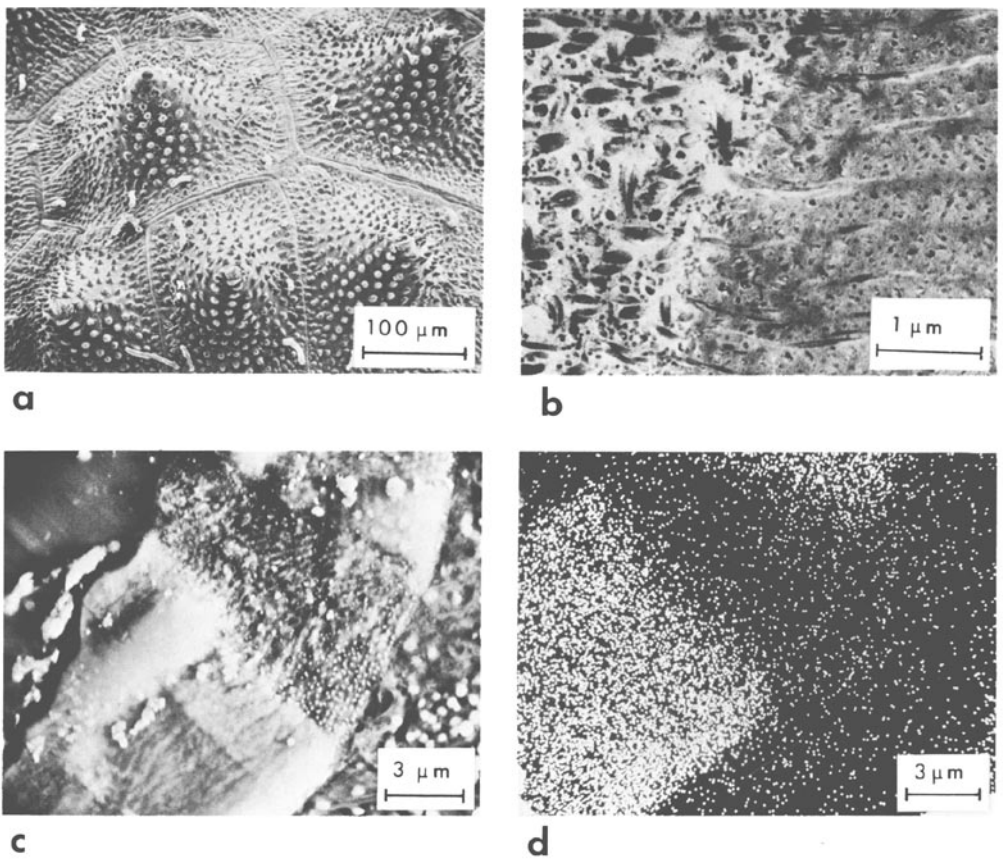

Fig. 4. *Harmothoe areolata* (Naples): (a) SEM-image of an elytron with large, spine-shaped papillae with small prickles and thin cylindrical papillae. The elytron surface is covered with a network of narrow joints between the papillae. (b) TEM-image of a cross-section at the border of a papilla (right) and beginning of a joint. The surface cuticle (left) is mainly composed of collagen fibers, whereas the papilla cuticle contains more matrix substance than collagen fibers. This appears darker presumably because of the contrasting Br-content, as was frequently found in cross-sections of papillae and elytra cuticles. (c) SEM-image of a cross-section of a joint between two papillae. The joint contains mainly fibers, as is typical for the elytron surface cuticle. The adjacent papilla cuticle contains mainly matrix. (d) Corresponding X-ray elemental mapping image of the cross-section shown in c), using the Br-K_{α}-line

Table 3. X-ray analysis of the cuticle of the spineshaped papilla of *Harmothoe areolata* (Naples)

Element	Count rate	Mass (%)
Br	74.2	84.4
Cl	46.4	6.0
I	4.0	1.4
K	1.8	0.3
Ca	0.6	0.1
P	8.8	1.1
S	48.6	6.1
Mn	–	–
Fe	1.0	0.6

Table 4. X-ray analysis of a thin cross section of two types of proximal cuticle of the elytra of *Harmothoe areolata* (Naples)

	Spineshaped papilla		Surface of the elytron		Ratio of the count rates
element	count rate	mass (%)	count rate	mass (%)	papilla/elytron
Br	0.338	39.9	0.027	1.0	12.5 : 1
Cl	(0.104)*	(6.4)	(1.515)	(30.1)	(0.069 : 1)
I	–	–	–	–	–
K	–	–	–	–	–
Ca	(0.003)	(0.2)	(1.07)	(11.1)	(0.003 : 1)
P/Os	(0.298)	(21.1)	(1.035)	(24.3)	(0.25 : 1)
S	0.074	4.8	0.45	9.6	0.16 : 1
Mn	–	–	–	–	–
Fe	(0.464)	(27.6)	(0.527)	(0.2)	(0.88 : 1)

* Values in brackets are possibly due to artefacts as discussed earlier. All mass (%) values may therefore be erroneous

ratio due to the low background. In Table 4, the results for the joints and the papilla cuticle are listed.

Despite the possibility that the Br-content may be influenced by the preparation, the concentration in the spineshaped papilla is obvious. The measured ratio of the count rates papilla/elytron of 12.5 : 1 may be even higher, because the Br-count rate on the elytron surface may only be due to scattering from the adjacent papilla with its high Br-content. The results for Cl, Ca, P/Os, and Fe are given in parentheses, because they are also constituents of the preparation agents and they are present in greater concentration in the surface cuticle. The TEM-image show that this region consists of more fibers than the papilla cuticle (Fig. 4b). As before, the X-ray elemental mapping image (Fig. 4d) shows clearly the Br-concentration in the papilla cuticle.

DISCUSSION

As revealed by TEM-images, the cuticle structures of the papillae and of the elytron surface between the papillae are different. The cuticle of the papillae investigated here consists almost entirely of a matrix substance, and only very few collagen fibers are present. The surface cuticle, however, is made up mainly of those fibers, and the matrix is present only between the fibers and forms a thin distal layer. As demonstrated by X-ray analysis these two sorts of cuticle can be distinguished by their chemical composition. In Figure 5, the compositions of the massive total elytra of the three polychaete species investigated here are shown graphically. The individual count rates of the analyzed elements are represented by hatched columns for the papilla cuticle and by white columns for the surface cuticle.

Comparing the data for *Lagisca extenuata* (Banyuls) and *Lepidonotus clava* (Naples), the ratios of the count rates for each element, except Mn and Fe, between the papilla and the surface cuticle are of the same order of magnitude, respectively. While for *Lagisca extenuata* (Banyuls) the ratios of Mn and Fe are about 10 : 1 each, for *Lepidonotus clava* (Naples) Mn was not detectable at all and Fe only with much lower intensity. Moreover, the lateral distribution seemed to be nearly uniform. By investigating several additional species, these results were found to be typical for the sample places Banyuls and Naples and related to a possible environmental influence. In Figure 3f, the highly non-uniform Mn-distribution is demonstrated by an X-ray elemental mapping image.

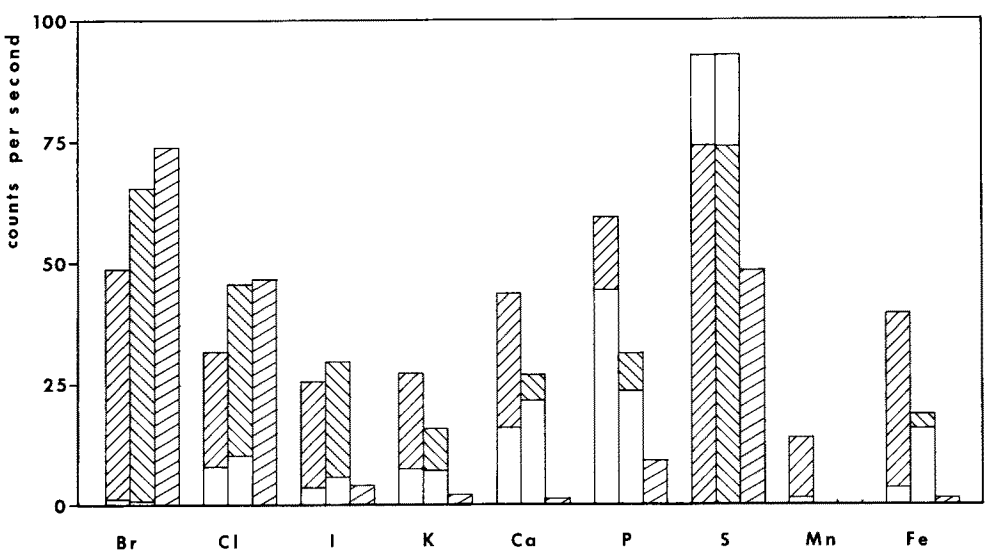

Fig. 5. Comparison of the compositions of the massive, total elytra of three polychaeta species. For each element, the first column represents *Lagisca extenuata* (Banyuls), the second *Lepidonotus clava* (Naples), and the third *Harmothoe areolata* (Naples), respectively. The hatched colums stand for the papilla cuticle, and the white, overlapping colums for the elytra surface cuticle. Remarkable is the high content of halogens in the papillae of all specimens, and the occurrence of Mn in the specimen from Banyuls

The most interesting result is, that in the case of *Lagisca extenuata* and *Lepidonotus clava* at least the halogens Br, Cl, and I are locally concentrated in the papilla cuticle, and only very small amounts are found in the surface cuticle between the papillae (see Fig. 5). Especially where Br is concerned, the ratios of concentration in the papilla and the surface are in the range of 50 to 90, so that high contrast elemental mapping images were obtained.

Comparing the samples from Banyuls and Naples, no significant difference was found for the halogen distributions on the elytra. The elements K, Ca, and P appeared to be concentrated in the papillae, too, but by more than one order of magnitude lower than Br. Only S seemed to be more or less uniformly distributed.

In *Harmothoe areolata* (Naples) the quantities of Br and Cl show values similar to those in *Lagisca extenuata* (Banyuls) and *Lepidonotus clava* (Naples), respectively, whereas the other elements occur with much lower intensities.

For the thin cross sections, as already pointed out, the results of the X-ray analysis are possibly defective due to the fixing and contrasting agents. It must be mentioned that in these cases the analyses were taken from areas in the proximal cuticle zone excluding the distal zone. Although the concentration of Br may be influenced by some chemical reactions, it is higher in the structure without fibers, as expected. In the surface cuticle between the papillae of *Lepidonotus clava* (Naples) no Br was found. For *H. areolata* (Naples) the Br intensity in the narrow joints of the surface cuticle is minimal and probably originates from scattering by the closely adjacent papilla cuticle. These results admit the possibility, that Br is not present in the proximal zone of the cuticle. On the other hand, the analyses of the total, massive objects for the surface cuticle of *Lepidonotus clava* (Naples) and *Lagisca extenuata* (Banyuls) always show Br, even though in relatively small amounts. In these cases, the Br intensity can not be explained by scattering from adjacent areas. Therefore it follows, that Br is present in the whole papilla cuticle as well as in the most distal zone of the surface cuticle. Both areas consist mainly of carbohydrate components (see also Fig. 6).

The absence of I in the proximal cuticle zone of the cross section specimens is probably also caused by some distal concentration. Those elements, of which the distribution and quantity is influenced by the mentioned preparation methods, namely Cl, Ca, P, and Fe, are without exception present in higher concentrations in the surface cuticle with its high fiber portion than in the papilla cuticle. This seems to be due to the

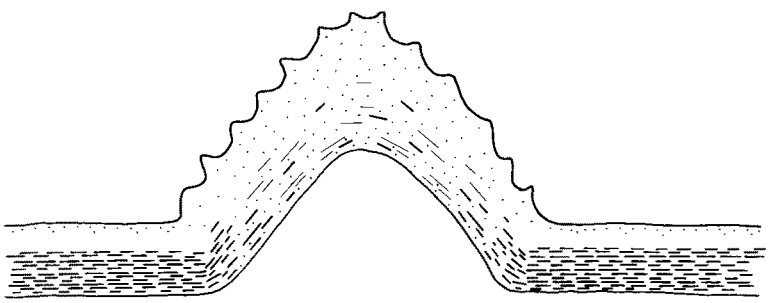

Fig. 6. Scheme of the cross-section of an elytron-surface with different fiber and Br-distributions (dotted) in the papilla and in the elytron-surface cuticle. Br is found in the whole cuticle of the papilla, also between the few collagen fibers, whereas in the elytron-surface cuticle, Br is present only in the distal cuticle zone

structure which possibly enables the agents to penetrate and adhere better between the fibers than in the matrix substance.

Swan (1950) and Gorbman et al. (1954) have shown in the case of several polychaetes that iodine 131 added to seawater is concentrated in the setae or cells of the bases of setae and the pharyngeal teeth, both composed of scleroproteins, in the form of monoiodotyrosine and diiodtyrosine. Siuda (1973) gives a survey of known organic structures containing halogen atoms, which are deemed " naturally occurring". The 3-chlorotyrosine was found by hydrolysis of the scleroproteine of *Buccinum undatum* and also of the cuticle of *Limulus polyphemus*. The latter species also exhibited 3.5-dichlorotyrosine, 3-chloro-5-bromo-tyrosine, and other dibromotyrosines. Monoiodtyrosine or diiodtyrosine are also well known constituents. In *Nereis*, in the distal cuticle zone, aromatic tyrosine containing protein was found (Manvalaramanujam & Sundara Rajulu, 1974). It can be presumed from these facts that the halogen found in the distal surface cuticle and in the papillae cuticle is also bound to tyrosine.

CONCLUSION

In this work the energy dispersive X-ray analysis with high local resolution was used to analyse the cuticle compositions of polychaetes. The present investigation has shown that the halogens Br, Cl, and I are mainly concentrated in the carbohydrate component of the cuticle, in this case in the papilla cuticle and in the distal zone of the surface cuticle of the elytra. The local accumulation of halogens was attributed to the presence of aromatic, tyrosine containing proteins in the distal cuticle zone, as determined by other authors with the aid of specific chemical methods. The additional knowledge about the occurrence of a few Br-, Bl-, and I-containing tyrosines within some scleroproteins indicates that the halogen accumulation is a natural effect. This assumption is confirmed by the fact that no difference was found in the samples from different places. In contrast to that, the concentration of Mn and probably of Fe in the papilla cuticle might be dependent on environmental conditions.

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