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A STUDY OF THE TEMPORARY ADHESION OF THE PODIA IN THE SEA STAR *ASTERIAS RUBENS* (ECHINODERMATA, ASTEROIDEA) THROUGH THEIR FOOTPRINTS

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Summary

Sea stars are able to make firm but temporary attachments to various substrata owing to secretions released by their podia. A duo-glandular model has been proposed in which an adhesive material is released by two types of non-ciliated secretory (NCS1 and NCS2) cells and a de-adhesive material is released by ciliated secretory (CS) cells. The chemical composition of these materials and the way in which they function have been investigated by studying the adhesive footprints left by the asteroids each time they adhere to a substratum. The footprints of *Asterias rubens* consist of a sponge-like material deposited as a thin layer on the substratum. Inorganic residues apart, this material is made up mainly of proteins and carbohydrates. The protein moiety contains significant amounts of both charged (especially acidic) and uncharged polar residues as well as half-cystine. The carbohydrate moiety is also acidic, comprising both uronic acids and sulphate groups. Polyclonal antibodies have been raised against footprint material and were used to locate the origin of footprint constituents in the podia. Extensive immunoreactivity was detected in the secretory granules of both NCS1 and NCS2 cells, suggesting that their secretions together make up the bulk of the adhesive material. No immunoreactivity was detected in the secretory granules of CS cells, and the only other structure strongly labelled was the outermost layer of the cuticle, the fuzzy coat. This pattern of immunoreactivity suggests that the secretions of CS cells are not incorporated into the footprints, but instead might function to jettison the fuzzy coat, thereby allowing the podium to detach.

Key words: temporary adhesion, podium, footprint, ultrastructure, biochemical composition, immunocytochemistry, Echinodermata, Asteroidea, *Asterias rubens*.

Introduction

Many marine benthic organisms are equipped with adhesive organs, the secretions of which allow them to attach to the substratum. Three types of adhesion may be distinguished: (1) permanent adhesion involving the secretion of a cement (e.g. the attachment of barnacles on rocks); (2) transitory adhesion permitting simultaneous adhesion and movement along the substratum (e.g. the foot secretions of gastropod molluscs); and (3) temporary adhesion allowing an organism to attach strongly but momentarily to the substratum (e.g. the adhesion of echinoderm podia) (Walker, 1987; Tyler, 1988; Flammang, 1996).

Although adhesion mechanisms are beginning to be deciphered in a few sessile organisms, including the byssal attachment of bivalve molluscs (Waite, 1983, 1992) and the cement attachment of cirripede crustaceans (Yule and Walker, 1987; Kamino *et al.* 1996), they essentially remain poorly understood. This is especially true for temporary adhesion, which generally relies on duo-gland organs requiring at least two antagonistic secretions, one adhesive and one de-adhesive (Tyler, 1988). Moreover, these duo-gland adhesive organs are found very frequently in microscopic invertebrates inhabiting the interstitial environment, e.g. turbellarians, gastrotrichs, nematodes and polychaetes (Tyler, 1988). The small size of the duo-gland organs in these organisms has precluded any biomechanical or biochemical studies on temporary adhesion. The study of macroinvertebrate adhesive systems is, therefore, a necessary and more promising way of understanding temporary adhesion.

Hermans (1983) has argued that the adhesive systems of echinoderms, which are usually associated with the podia, operate with the functions of adhesion and de-adhesion, and that two or more glands are involved. Accordingly, he applied the name 'duo-gland' to these systems. The duo-glandular nature of echinoderm podia has since been corroborated by

several morphological studies showing that the podia always enclose two types of secretory cells: non-ciliated secretory (NCS) cells, presumably releasing an adhesive material, and ciliated secretory (CS) cells, presumably releasing a de-adhesive material (for a review, see Flammang, 1996). However, the chemical compositions of these materials and the way in which the duo-gland adhesive systems operate are still largely unknown.

The aim of the present work was to investigate temporary adhesion of the podia in the sea star *Asterias rubens* by studying their footprints. Footprints are deposited each time podia adhere to a substratum (Chaet, 1965; Thomas and Hermans, 1985; Flammang *et al.* 1994). They appear to consist mainly of adhesive secretions, although they may also contain de-adhesive secretions and/or material from the cuticle (Flammang, 1996). In the present study, footprints were analysed in terms of their fine structure and gross chemical composition. Moreover, the cellular origin of footprint constituents in the podia was investigated by immunohistochemistry and immunocytochemistry using new antisera raised against footprint material.

Materials and methods

Collection and maintenance of sea stars

Individuals of *Asterias rubens* L. were collected intertidally in Audresselles (Pas-de-Calais, France). They were transported to the Marine Biology Laboratory of the University of Mons, where they were kept in a marine aquarium with closed circulation (13 °C, 33‰ salinity) and fed mussels (*Mytilus edulis* L.). Footprints were collected primarily during the first 2 or 3 days after the arrival of the sea stars. Indeed, although the asteroids survived for several weeks, their ability to produce abundant footprints appeared to decrease quickly when they were held in captivity.

Scanning electron microscopy

Footprints were prepared as follows. Podia were allowed to adhere firmly to clean aluminium stubs. After the podia had detached themselves, the footprints were briefly rinsed in distilled water, frozen in liquid nitrogen and freeze-dried. They were then coated with gold in a sputter-coater and observed with a JEOL JSM-6100 scanning electron microscope.

Collection of footprint material

Sea star footprints were obtained by allowing individuals to walk across and/or attach to the bottom of clean glass Petri dishes (diameter 15 cm) filled with artificial sea water (ASW). The asteroids and the ASW were renewed every hour for 8 h, after which the Petri dishes were thoroughly rinsed in distilled water and placed in a freeze-dryer. The lyophilized footprint material was then scraped off using a glass knife and stored at –20 °C. This method allows the collection of 50–300 µg of a red-brown powder per Petri dish. Attempts made to recover wet footprint material all failed. However, after scraping, this material appears as sticky, red-brown filaments.

Antiserum production and characterisation

Polyclonal antisera were raised in two female rabbits (R1 and R2) to the footprint material. The immunisation schedule was as follows. Approximately 1 mg of footprint material was suspended in 0.75 ml of phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant (Difco, Detroit, MI, USA). The rabbits were then injected subcutaneously at several sites in the back, with a total volume of 0.5 ml of the emulsion to R1 and 1 ml of the emulsion to R2. Three boosts were prepared identically, but with incomplete Freund's adjuvant (Difco, Detroit, MI, USA), and were administered, respectively, 20 days, 35 days and 50 days after the first injection. Antisera were harvested twice: 10 days after the second boost (AS1) and 20 days after the third boost (AS2). Pre-immune sera (PS) were obtained by bleeding the rabbits just before the first injection of footprint material.

The different antisera and pre-immune sera were tested for specific footprint-binding activity using a fluorescence immunoassay. Footprints were obtained by allowing sea stars to walk across clean glass microscope slides. The presence of footprints was checked by soaking a few slides for 1 min in a 0.05 % solution (in distilled water) of the cationic dye Crystal Violet (Flammang *et al.* 1994). The other slides were pre-incubated for 30 min in 10 % normal goat serum (BioCell, Cardiff, UK) in PBS to block non-specific antigenic sites. Antisera and pre-immune sera, diluted 1:100 in PBS containing 1 % Tween 20 and 3 % bovine serum albumin (BSA; PBS–Tween–BSA1), were then applied overnight at 4 °C. After several washes in PBS, the sections were incubated for 1 h in FITC-conjugated swine anti-rabbit immunoglobulins (DAKO A/S, Glostrup, Denmark) diluted 1:50 in PBS–Tween–BSA1. After a final wash in PBS, they were mounted in Vectashield (Vector, Burlingame, CA, USA) and observed with a Leica TCS 4D confocal laser scanning microscope.

An enzyme-linked immunosorbent assay (ELISA) was used for the comparison of specific antibody titres between the different antisera and pre-immune sera. The footprint material was digested with trypsin under the following conditions. Approximately 1 mg of footprint material was suspended in 2 ml of 0.1 mol l^{-1} sodium bicarbonate, pH 8.2, at a protease:protein ratio of approximately 1:100, under constant stirring at 22–24 °C for 180 min. The progress of the digestion was monitored at 30 min intervals by centrifuging the suspension at 3000 ***g*** for 5 min and measuring the absorbance of the supernatant at 280 nm. The digestion was terminated by centrifuging the suspension at 3000 ***g*** for 15 min and freezing the supernatant. The ELISA was carried out according to the following procedure at 37 °C. The soluble fraction of the trypsinised footprint material, diluted 25 times in 0.1 mol l^{-1} carbonate/bicarbonate buffer, pH 9.2, was loaded (200 µl per well) into 96-well microtitration plates (Nunc Maxisorb Immunoplates, Rochester, NY, USA) and incubated overnight. The plates were washed several times with 300 µl of PBS containing 0.05 % Tween 20 and 0.1 % BSA (PBS–Tween–BSA2) per well. Each antiserum (200 µl) and

each pre-immune serum (200 µl) (serial dilution in PBS) was then added and incubated for 1 h. After a second series of washes with PBS–Tween–BSA2, 200 µl of horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO A/S, Glostrup, Denmark) diluted 1:1000 in the same buffer was added and incubated for an additional hour. Finally, the plates were washed thoroughly with PBS–Tween–BSA2, and 200 µl of the enzyme substrate (a solution of 0.04 % *o*-phenylenediamine in phosphate/citrate buffer, pH 5.0, containing 0.012 % H_2O_2) was added. After 10 min at room temperature (22–24 °C), the reaction was stopped by the addition of 50 µl of 2.5 mol l^{-1} H_2SO_4, and the plates were read at 492 nm using a Titertek Multiskan plate reader.

Immunohistochemistry and immunocytochemistry

For light microscopy, podia were fixed in 4 % paraformaldehyde in PBS (pH 7.4) for 2 h at room temperature and then rinsed in the same buffer for 30 min. They were embedded using a routine method in paraffin wax, sectioned at 8 µm and mounted on clean glass slides. The sections were permeabilised in PBS with 0.25 % Triton X-100 for 1 h, and pre-incubated for 30 min in 10 % normal goat serum in PBS. Primary antisera, diluted 1:100 in PBS–Tween–BSA1, were then applied overnight at 4 °C. After several washes in PBS, the sections were incubated for 1 h in FITC-conjugated swine anti-rabbit immunoglobulin diluted 1:50 in PBS–Tween–BSA1. To make the tissue identification easier, propidium iodide (Calbiochem, La Jolla, CA, USA) was added to the secondary antibody solution at a final concentration of 5 µg ml^{-1} to stain the nuclei. Following five washes in PBS, the sections were mounted and observed with the confocal laser scanning microscope.

For transmission electron microscopy, podia were fixed in 3 % glutaraldehyde in cacodylate buffer (0.1 mol l^{-1}, pH 7.8) for 3 h at 4 °C, rinsed in cacodylate buffer, and post-fixed for 1 h in 1 % osmium tetroxide in the same buffer. Some podia were fixed according to the same protocol but with the addition of 0.05 % Ruthenium Red to all the solutions (Luft, 1971). This technique allows a better preservation of the cuticle as well as of the secreted material, when present (Flammang *et al.* 1994). After a final wash in buffer, the podia were dehydrated in graded ethanol and embedded in Spurr's resin. Ultrathin sections (40–70 nm) were cut, using a Leica UCT ultramicrotome equipped with a diamond knife, and collected on gold grids. The sections were blocked with 10 % normal goat serum in PBS for 30 min and incubated overnight at 4 °C with the primary antisera diluted 1:500 in PBS–Tween–BSA1. After rinsing in PBS, the sections were immunogold-stained for 1 h at room temperature in goat anti-rabbit immunoglobulin conjugated to 15 nm gold particles (BioCell, Cardiff, UK) diluted 1:100 in PBS–Tween–BSA1. Following several washes in PBS, and then distilled water, they were further stained with aqueous uranyl acetate and lead citrate and observed using a Zeiss EM 10 transmission electron microscope.

For both light and transmission electron microscopy, two types of control were carried out: (1) substitution of the primary antiserum with PBS–Tween–BSA1 and (2) substitution of the primary antiserum with the pre-immune sera diluted 1:100 in PBS–Tween–BSA1.

Analytical methods

All analyses were performed on duplicate samples (0.5–1 mg each) of freeze-dried footprint material.

For amino acid and hexosamine analysis, samples were suspended in 6 mol l^{-1} HCl and hydrolysed under vacuum in sealed tubes for 24 h at 110 °C. The hydrolysates were evaporated to dryness, and the residual solids were dissolved in citrate buffer (0.2 mol l^{-1}, pH 2.2). Amino acid, glucosamine and galactosamine concentrations were measured on a Beckman 120C amino acid analyser. Total protein was estimated by adding the masses of the individual amino acids calculated from the amino acid analysis and expressing the total mass of the amino acids as a percentage of the dry mass of the sample. Total hexosamine was estimated similarly, correcting the masses from standard curves constructed using glucosamine and galactosamine subjected to exactly the same hydrolytic conditions as the footprint samples.

Neutral sugars were determined by the anthrone method, as described by Jermyn (1975). D-Glucose was used as the standard. Uronic acids were determined according to the *m*-hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (1973) using glucuronic acid as the standard. Total sulphate was assayed by the benzidine method, as modified by Antonopoulos (1962). The lipid determination was based on the method of Holland and Gabbott (1971) using tripalmitin as the standard.

For inorganic residue analysis, samples were ashed in a muffle furnace for 3 h at 550 °C. The mass of the residual ash expressed as a percentage of the sample mass was taken as the total inorganic residue.

Results

Ultrastructure of the footprints

Asterias rubens footprints are thin, disc-shaped films of material deposited on a substratum each time a podium has adhered to it. These films, however, are not solid, homogeneous layers, but instead are sponge-like with numerous holes in the matrix (Fig. 1A). The thickness of the film may vary between different footprints or even within the same footprint, presumably depending on the amount of material secreted (see also Flammang *et al.* 1994). In the thinnest areas, the footprint material has the shape of a network with a mesh ranging from 3 to 10 µm and with a wall thickness of approximately 0.4 µm (Fig. 1A). When more secretory material is added, the footprint takes on a felt-like appearance.

Antiserum characterisation

Preliminary solubility tests carried out on the footprint material of *A. rubens* showed that it was insoluble in sea water, distilled water and salt solutions (e.g. buffers) (results not

shown). These solubility characteristics being incompatible with quantitative assays for specific interactions, a qualitative fluorescence assay was used to evaluate the presence of footprint-binding antibodies in the antisera. Both immunised rabbits (R1 and R2) generated antibodies to footprint material. Non-fixed footprints freshly deposited on glass microscope slides were strongly labelled by the four antisera (e.g. Fig. 1B), but not by the pre-immune sera.

To estimate the antibody titre in each serum, footprint material was digested with trypsin to allow the collection of a soluble fraction which could be used in an ELISA. Trypsin was used since it has previously been shown to remove footprints from a substratum (Thomas and Hermans, 1985; Flammang, 1996). Moreover, owing to its specificity for cleavage after lysine and arginine residues, it was expected that the peptides generated would be large enough to be recognised by the specific antibodies. This was found to be the case, and serum dilution curves were constructed (Fig. 2). For both R1 and R2, there were no differences in the specific antibody levels between the two successively collected antisera (AS1 and AS2). The antisera from R2, however, appeared to have slightly higher titres than those from R1. This is not surprising since R2 was immunised with twice the amount of footprint material as was used for R1.

Immunohistochemistry

The presence and location of footprint constituents in the podia of *A. rubens* was investigated by immunofluorescent labelling using light microscopy. There was a strong and reproducible immunolabelling of podia with the antisera, whereas there was no labelling with the pre-immune sera.

The podia consist of an extensible cylindrical stem ending distally at a somewhat wider, flat and specialised disc (Fig. 3A). The stem allows the podium to lengthen, flex and retract, and the disc makes contact with the substratum (Flammang *et al.* 1994). The podia are hollow organs consisting of a central, fluid-filled ambulacral lumen surrounded by quite a thin wall. The podium wall is made up of four tissue layers: an inner mesothelium, a connective tissue layer, a nerve plexus and an outer epidermis covered by a cuticle (Fig. 3B). These layers are, however, organised differently according to whether they belong to the stem or to the disc, those within the disc being specialised for adhesion (Flammang *et al.* 1994). Immunoreactivity (green labelling) was restricted to the epidermal layer; no immunoreactivity was detected in the nerve plexus, in the connective tissue layer or in the mesothelium (Fig. 4). Immunofluorescent labelling was strongest at the level of the disc, being present extensively in epidermal cell secretory granules (Fig. 4A–C). Labelled granules were particularly abundant in the apical and basal parts of the disc epidermis, occurring as conspicuous clusters, whereas the central part of the epidermis was occupied by a thick layer of nuclei. In the stem, some secretory cells also enclosed immunoreactive secretory granules (Fig. 4D). However, these granules differed from those of the disc in the shape, size and intensity of the immunofluorescent labelling,

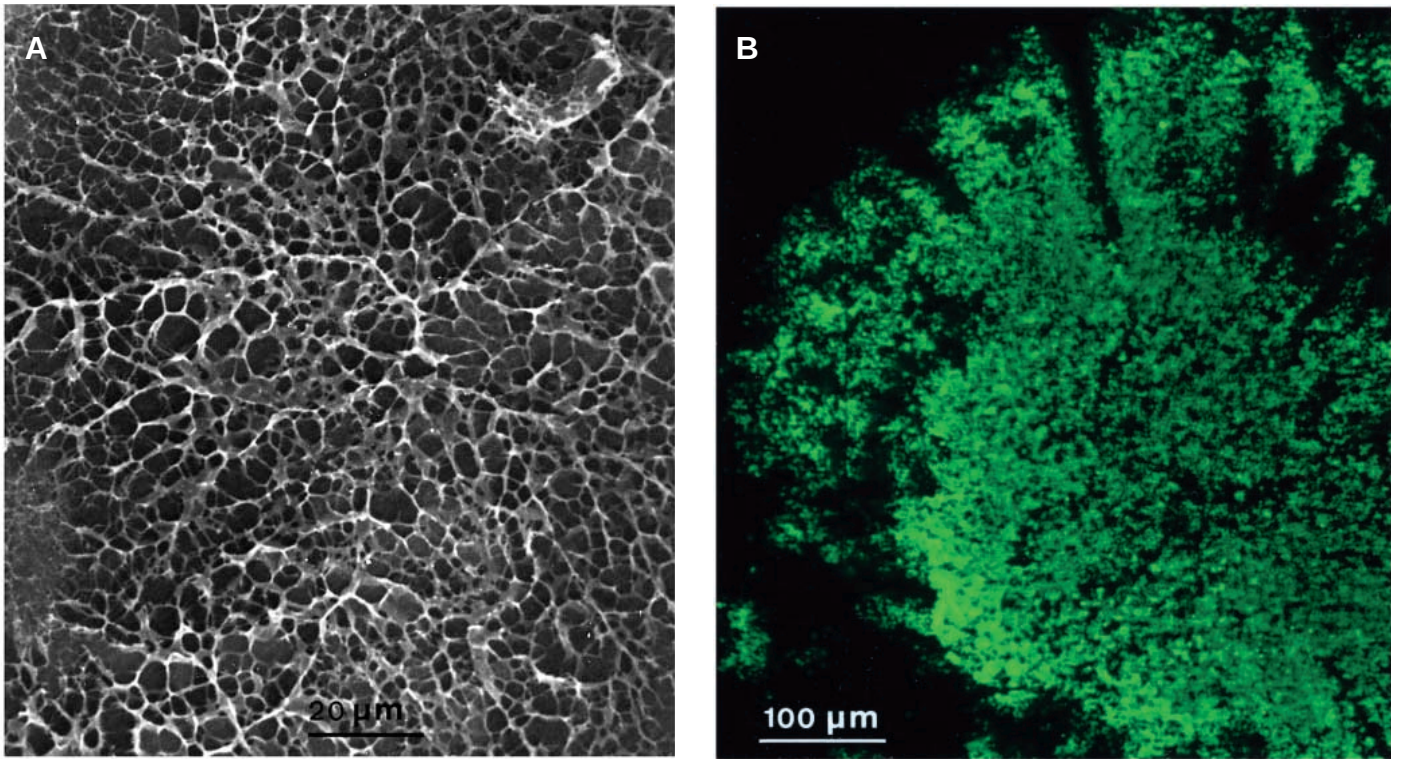

Fig. 1. Footprints of *Asterias rubens*. (A) Detail of a footprint from a scanning electron micrograph. (B) Freshly deposited, non-fixed footprint immunolabelled with R2AS1.

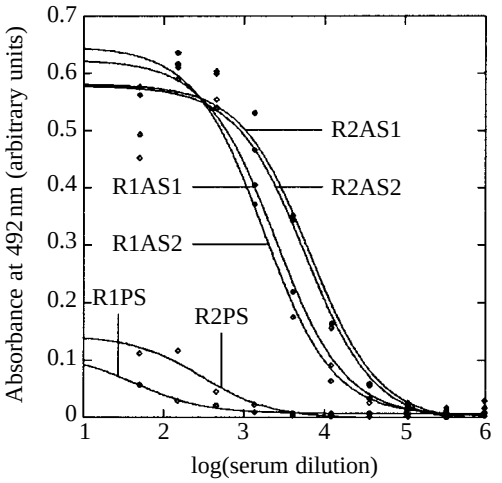

Fig. 2. Binding in enzyme-linked immunosorbent assay (ELISA) of the antisera raised against footprint material of *Asterias rubens* with peptides generated by trypsin digestion of this material (see text). R1PS, R1AS1, R1AS2, R2PS, R2AS1, R2AS2, pre-immune serum, first antiserum and second antiserum collected in rabbits R1 and R2, respectively.

which was much weaker in the former than in the latter (see Fig. 4A). In both the disc and the stem, the only other immunoreactive structure was the cuticle (Fig. 4A,D). The asteroid cuticle is generally poorly preserved with aldehyde fixatives (Holland and Nealson, 1978; McKenzie, 1988*a*), and this may explain why it was no longer present on some micrographs (see Fig. 4B, for example). In the areas where the cuticle was preserved, e.g. in the wrinkles of the stem epidermis, at least two layers can be distinguished, the outermost being the most strongly labelled (Fig. 4D).

Immunocytochemistry

To locate more precisely the footprint constituents in the epidermis of *A. rubens* podia, immunogold labelling and transmission electron microscopy were used. As in light microscopy, the antisera were strongly immunoreactive, all labelling the same structures, and there was no labelling with the pre-immune sera.

The disc epidermis is made up of five cell types: non-ciliated secretory (NCS) cells of two different types (NCS1 and NCS2); ciliated secretory (CS) cells; non-secretory ciliated (NSC) cells; and support cells (Fig. 5A,B) (for a more detailed description of the disc epidermis of the podia of *A. rubens*, see Flammang *et al.* 1994).

Non-ciliated secretory (NCS1 and NCS2) cells are flask-shaped, their enlarged cell bodies being located basally. Each cell body sends out a long apical process that reaches the apex of the podium (Fig. 5A,B). The cytoplasm of both the cell body and the apical process is filled with densely packed membrane-bound secretory granules. At the end of the apical processes, the granules are extruded through a duct delimited by a ring of microvilli and opening onto the disc surface as a pore. The secretory granules of NCS1 cells are ellipsoids approximately 1 μm long and 0.6 μm in diameter. They have a complex ultrastructure. Most of their volume is occupied by a bundle of parallel rods. Each bundle of rods is surrounded by a ring of the same density as the rods and separated from the granule membrane by a clear cortex (Fig. 6A). The secretory granules of NCS2 cells are spherical (approximately 550 nm in diameter). They contain electron-lucent, finely granular material surrounded by a clear cortex (Fig. 6B). The secretory granules of both NCS1 and NCS2 cells were strongly immunoreactive (Fig. 6A,B), and gold particles were observed all over the granules, i.e. both over the core and over the cortex. However, the immunolabelling appeared to be fixation-dependent, the amount decreasing with improved fixation. This fixation artefact affected especially the core of NCS1 cell granules, sometimes giving the impression that the cortical regions of the granules were labelled preferentially (Fig. 6A).

Ciliated secretory (CS) cells have the same shape and size as NCS cells. They possess an enlarged nucleus-containing cell body and a long and narrow apical process that ends with a bulge just beneath the cuticle (Fig. 5). This bulge is devoid of microvilli but bears a subcuticular cilium (Fig. 6B). The entire cytoplasm of the cell is filled with membrane-bound secretory granules. These granules are spherical and 300–450 nm in diameter. They enclose an electron-dense homogeneous material surrounded by a thin clear cortex (Fig. 5). No

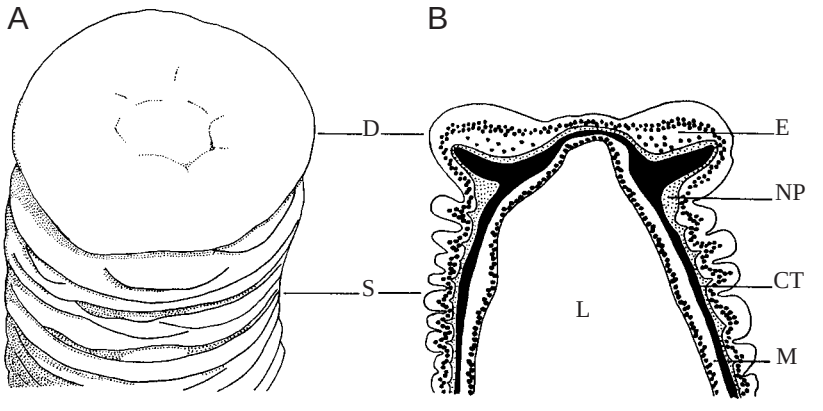

Fig. 3. Schematic representation of a podium of *Asterias rubens*. (A) External aspect of the distal portion; (B) longitudinal section through this portion. CT, connective tissue layer; D, disc; E, epidermis; L, ambulacral lumen; M, mesothelium; NP, nerve plexus; S, stem.

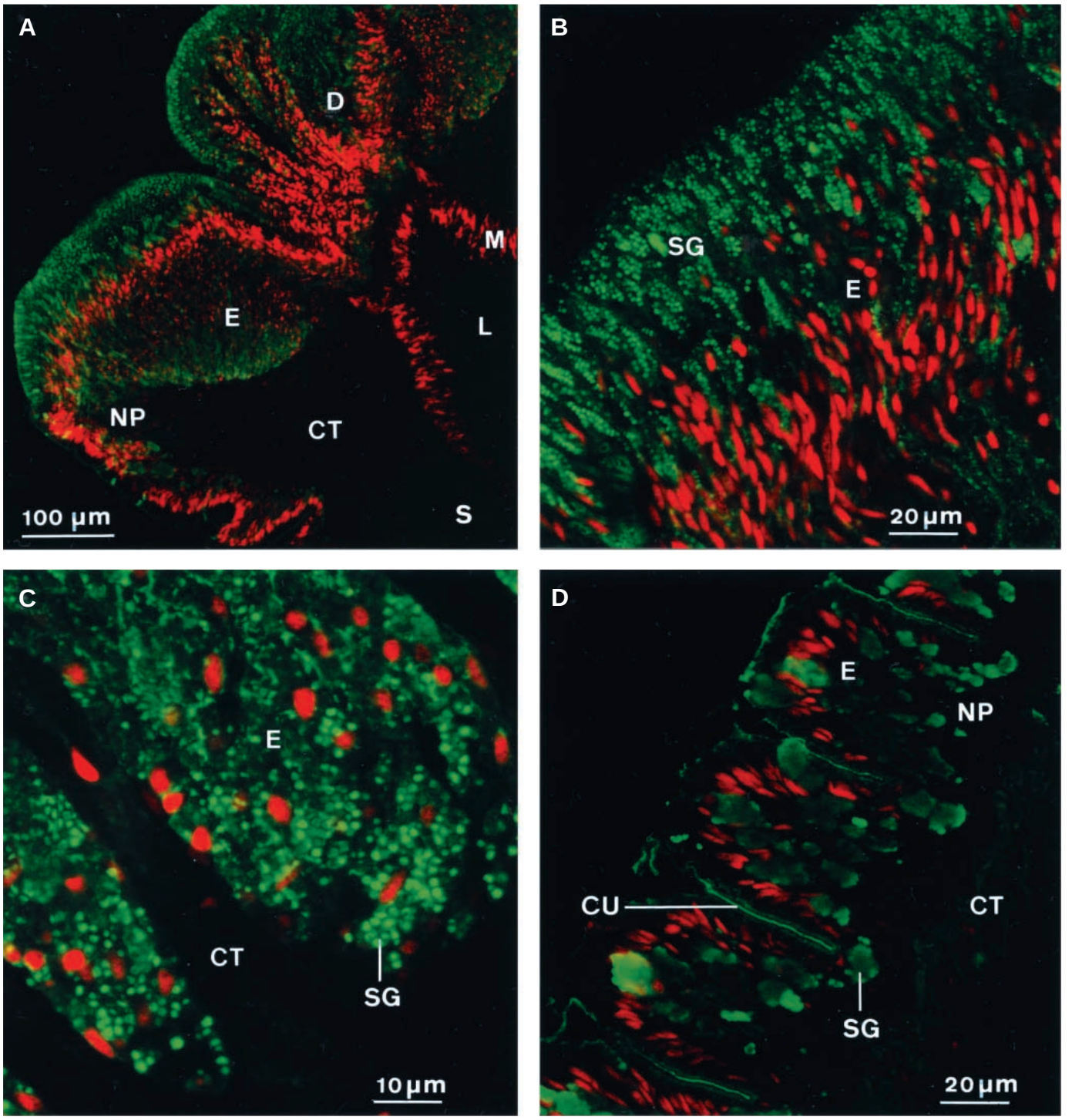

Fig. 4. Immunohistochemical location of footprint constituents in the podia of *Asterias rubens* (immunoreactive structures are labelled in green while nuclei appear in red). (A) Longitudinal section through the distal part of a podium (labelled with R1AS1). (B) Longitudinal section through the apical part of the disc epidermis labelled with R1AS2. (C) Longitudinal section through the basal part of the disc epidermis labelled with R1AS1. (D) Longitudinal section through the stem epidermis (labelled with R1AS1). CT, connective tissue layer; CU, cuticle; D, disc; E, epidermis; L, ambulacral lumen; M, mesothelium; NP, nerve plexus; S, stem; SG, secretory granules.

immunogold labelling was observed on the secretory granules of CS cells (Fig. 6B).

Non-secretory ciliated (NSC) cells are narrow and have a centrally located nucleus. Their characteristic feature is a single short cilium whose apex protrudes into the outer medium (Fig. 5B). NSC cells did not contain any immunoreactive structure.

Support cells have a centrally located nucleus. Their apical

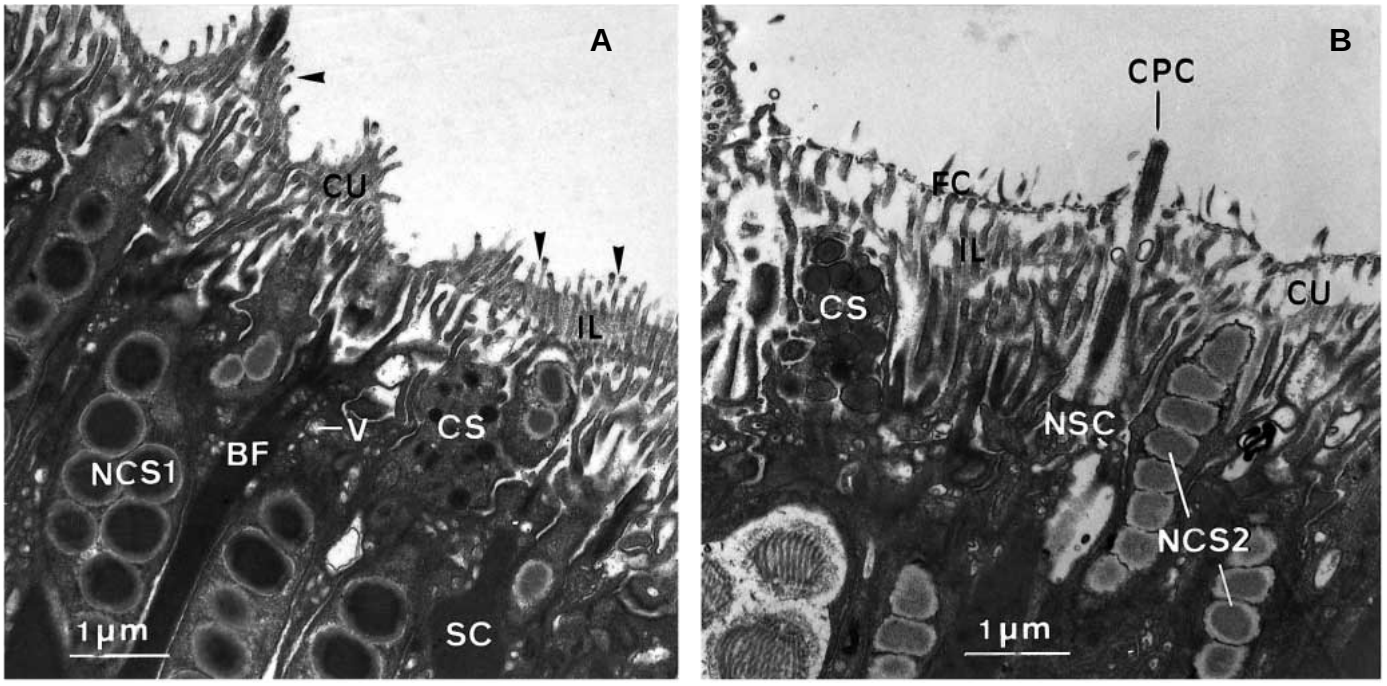

Fig. 5. Ultrastructure of the disc epidermis in the podia of *Asterias rubens* fixed (A) without and (B) with Ruthenium Red. Arrowheads in A indicate where the cuticle fuzzy coat should lie. BF, bundle of filaments; CPC, cuticle-protruding cilium; CS, ciliated secretory cell; CU, cuticle; FC, cuticle fuzzy coat; IL, cuticle inner layers; NCS1, type 1 non-ciliated secretory cell; NCS2, type 2 non-ciliated secretory cell; NSC, non-secretory ciliated cell; SC, support cell; V, vesicle.

surface bears numerous microvilli (Figs 5, 6A,B). Their cytoplasm encloses one longitudinal bundle of intermediate filaments that traverses the cell and joins its apical and basal membranes. It also contains several clear vesicles (ranging from 100 to 200 nm in diameter), some of which were immunolabelled (Fig. 6B).

A three-layered cuticle covers the epidermal cells of the disc (Fig. 5). Its two innermost layers (approximately 200 nm thick for the proximal one and 350 nm thick for the distal one) lie between the microvilli of the epidermal cell, while the outermost layer (approximately 200 nm thick) covers and embeds the tips of the microvilli (Figs 5B, 6C). The latter, the so-called fuzzy coat, is generally poorly preserved using the classical transmission electron microscopy fixation (Fig. 5A), and special fixatives must be used to visualise it (compare Fig. 5A,B) (Holland and Nealson, 1978; McKenzie, 1988*a*). When preserved, however, the cuticle was strongly immunoreactive. The immunogold labelling was almost exclusively restricted to the fuzzy coat, the inner layers being only lightly labelled (Fig. 6C).

Some podia, which were fixed while they were attached to a substratum, still had adhesive material bound to their distal surface (Fig. 6C). This material, which appeared as an electron-dense, compact fibrillar matrix, was strongly immunoreactive although, once again, the immunolabelling seemed to be fixation-dependent.

At the level of the stem epidermis, only one cell type was found to be immunoreactive. These cells are mucocyte-like secretory cells which were also occasionally observed in the disc epidermis. Their cytoplasm is packed with ovoid, heterogeneous secretory granules measuring approximately 1.3 µm×0.75 µm (Fig. 6D). Only the contents of these granules, a finely granular material of medium electron-density enclosing electron-dense patches, were immunolabelled (Fig. 6D). These mucocytes are morphologically identical to the type B mucocytes described by Souza Santos and Silva Sasso (1968) in the podial epidermis of the asteroid *Asterina stellifera*. The stem cuticle was identical to the disc cuticle with respect to both its organisation and its immunolabelling pattern (Fig. 6E).

Biochemical composition of the footprints

The results of the chemical analyses of footprint material are presented in Table 1 together with the results from analyses carried out on the adhesive secretions of other marine invertebrates for comparative purposes. Total protein calculated from the amino acid analysis was 20.6 % of dry mass, representing the main organic component of the footprint material. The amino acid composition is given in Table 2. It shows significant amounts of both charged (especially acidic) and uncharged polar residues (amounting to 52 % together) as well as of half-cystine (3.2 %). The carbohydrate content was 8 % of dry mass (10.5 % if sulphates are taken into account), and the lipid content was 5.6 % of dry mass. The inorganic residue (ash) accounted for a large part of the footprint material (40 % of dry mass). When combined, these values give a recovery of approximately 75 %. Because of the insolubility of the footprint material, all the analyses had to be performed under hydrolytic conditions. The relatively low recovery could

Table 1. *Biochemical composition of the footprint material of* Asterias rubens

	Asterias rubens[1] (Echinodermata, Asteroidea) Footprints	*Patella vulgata*[2] (Mollusca, Gastropoda) Pedal mucus	*Mytilus edulis*[3] (Mollusca, Bivalvia) Byssal attachment discs	*Balanus crenatus*[4] (Crustacea, Cirripedia) Basal cement
Total protein	20.6	32.8	99.4	84.43
Total lipid	5.6	ND	8	0.69
Total carbohydrate	ND	12	0	1.05
Neutral sugars	3	2.11	ND	ND
Amino sugars	1.5	1.13	ND	ND
Uronic acids	3.5	0.15	ND	ND
Sulphates	2.5	16.8	ND	ND
Inorganic residue	40	30–40	ND	4.18

For comparative purposes values are included for the adhesive secretions of other marine invertebrates.
Results are expressed as percentages of dry mass (ND, not determined).
[1]Present work; [2]Grenon and Walker (1980); [3]Cook (1970); [4]Walker (1972).

therefore be explained by losses or incomplete hydrolysis. The insolubility of the footprint material also precluded any filtration or dialysis treatment prior to chemical analyses so that the results will include values for any contaminant associated with the footprint material.

Discussion

Adhesion is the joining together of two dissimilar materials, the adherends, with a sticky material, the adhesive. The surface properties of the adherends and the chemical and physical properties of the adhesive determine the strength of adhesion (Waite, 1983). In adhesion of sea star podia, the adherends are the fuzzy coat (the outermost layer of the epidermal cuticle) on one side and a solid substratum on the other. The adhesive is present as a thin film between the cuticle and the substratum and appears to be secreted by two types of non-ciliated secretory cells (NCS1 and NCS2 cells) (Flammang *et al.* 1994). Since this adhesion is temporary, the podia are able to detach voluntarily from the substratum. The breaking of the adhesive bond always occurs between the surface of the podium and the adhesive material. It has been suggested that this breakage could be brought about by the release of a so-called ‘de-adhesive’ secretion by ciliated secretory (CS) cells (Flammang *et al.* 1994). After detachment, the adhesive material remains strongly attached to the substratum as a footprint (Chaet, 1965; Thomas and Hermans, 1985; Flammang *et al.* 1994; Flammang, 1996). The study of these footprints could therefore provide new insights into the temporary adhesion of sea star podia.

Table 2. *Amino acid composition of the footprint material of* Asterias rubens *expressed as residues of amino acid per 1000 residues*

Amino acid	
Asx	118
Thr	78
Ser	76
Glx	102
Pro	61
Gly	97
Ala	62
Cys/2	32
Val	67
Met	17
Ileu	45
Leu	61
Tyr	27
Phe	38
Lys	56
His	21
Arg	41

Ultrastructure of the footprints

Footprints of *A. rubens* that have been quickly frozen and lyophilised appear in scanning electron micrographs as thin, disc-shaped films made up of a material having a sponge-like appearance, with numerous holes in the matrix. This appearance is identical to that observed in light micrographs of unfixed, stained footprints (Thomas and Hermans, 1985) and in scanning electron micrographs of footprints that have been chemically fixed, dehydrated and critical-point-dried (Flammang *et al.* 1994), suggesting that the trabecular pattern is not an artefact of preservation, but represents the actual structure of the footprint material. Interestingly, the adhesive secretions of some marine invertebrates using permanent adhesion also have a spongy organisation, as in the case of the byssal plaque matrix of mussels (Benedict and Waite, 1986; Waite, 1986) and the basal cement of barnacles (Naldrett, 1993).

Origin of footprint constituents in the podia

Although the footprints appear to consist mainly of adhesive secretions (Flammang *et al.* 1994), it is possible that they also

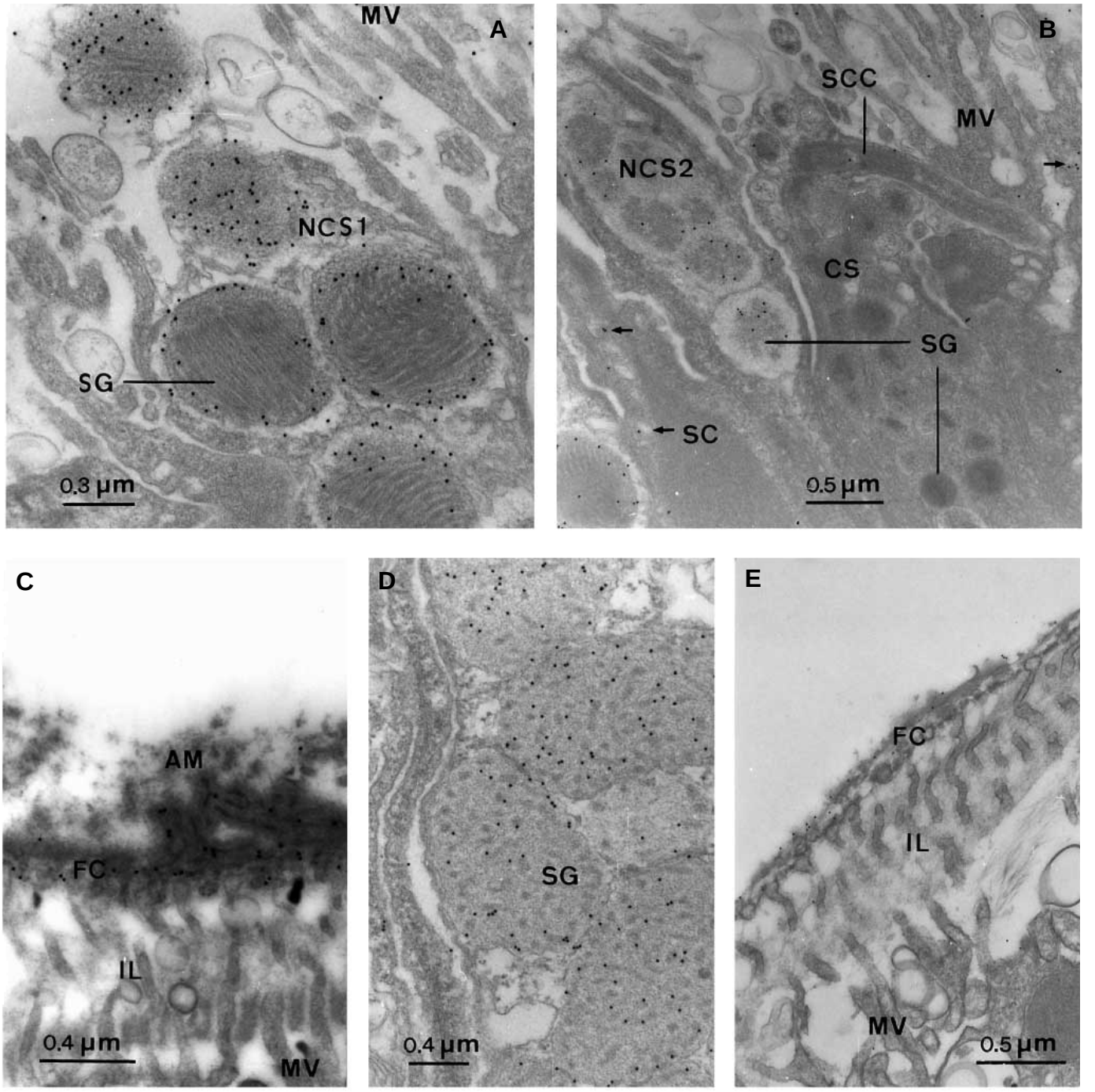

Fig. 6. Immunocytochemical location of footprint constituents in the epidermis of the podia of *Asterias rubens*. (A,B) Apex of the disc epidermis labelled with R1AS1 (arrows indicate immunoreactive vesicles in support cells). (C) Disc cuticle labelled with R1AS1 (fixative, buffer and post-fixative all include Ruthenium Red). (D) Cytoplasm of a stem mucocyte-like cell labelled with R2AS1. (E) Stem cuticle labelled with R2AS1 (fixative, buffer and post-fixative all include Ruthenium Red). AM, adhesive material; CS, ciliated secretory cell; FC, cuticle fuzzy coat; IL, cuticle inner layers; MV, microvilli; NCS1, type 1 non-ciliated secretory cell; NCS2, type 2 non-ciliated secretory cell; SC, support cell; SCC, subcuticular cilium; SG, secretory granules.

enclose de-adhesive secretions, material from the cuticle or even general epidermal secretions. To address this issue, we have raised four antisera to the footprint material of *A. rubens* and used them to locate the origin of footprint constituents in the podia by taking advantage of the polyclonal character of the antibodies generated. Indeed, being raised against footprints as a whole, these antibodies may react with a wide variety of the substances originally present in the footprint material.

In sections of the podia, the immunoreactivity was restricted

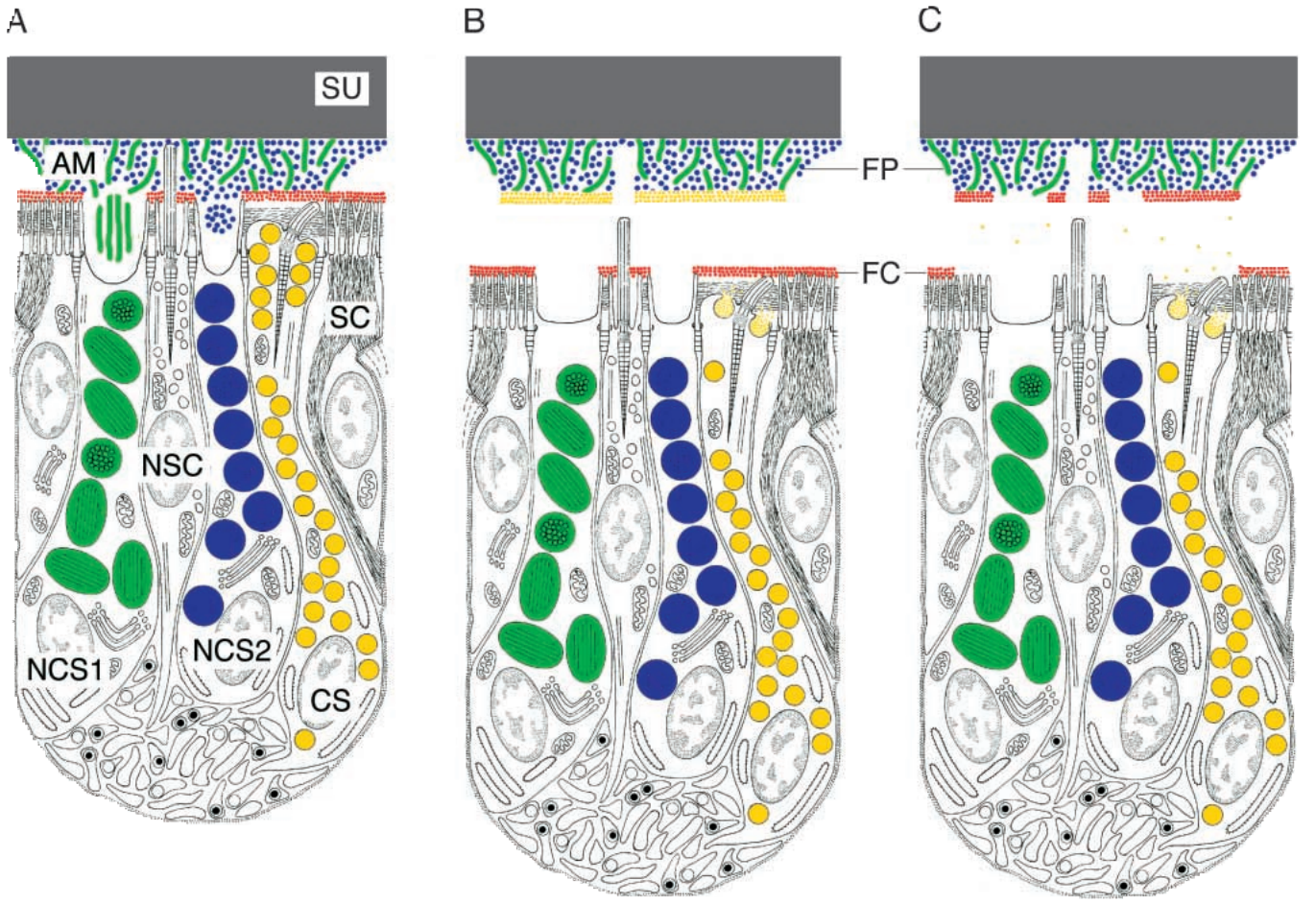

Fig. 7. Diagrammatic representations of the different models proposed for the attachment (A) and detachment (B, 'competition model'; C, 'enzyme model') of a podium of *Asterias rubens* using reconstructions of longitudinal sections through the disc epidermis (not to scale; see text for detailed explanations). AM, adhesive material; CS, ciliated secretory cell; FC, cuticle fuzzy coat; FP, footprint; NCS1, type 1 non-ciliated secretory cell; NCS2, type 2 non-ciliated secretory cell; NSC, non-secretory ciliated cell; SC, support cell; SU, substratum.

to the epidermis and, although both the stem and the disc contained immunolabelled cells, the labelling was several times stronger at the level of the disc. Most of it was localised in the secretory granules of the two types of non-ciliated secretory cells (NCS1 and NCS2 cells), demonstrating that their secretions make up the bulk of the footprint material. Conversely, no immunolabelling was found in the secretory granules of ciliated secretory (CS) cells. This could suggest that CS cells are not involved in adhesion or de-adhesion, or that their secretions are not immunogenic or, more probably, that they are not incorporated into the footprints.

The only other strongly immunoreactive structure was the fuzzy coat, i.e. the outermost layer of the cuticle, indicating that it is probably a relatively common constituent of the footprint material. The presence of cuticular material in the footprints could also explain the slight immunoreactivity observed in support cells and type B mucocytes, two types of cells apparently not involved in the adhesion mechanism. Indeed, several studies have suggested that support cells could participate in the synthesis of the cuticle (Bargmann *et al.* 1962; Engster and Brown, 1972; Holland, 1984). The immunolabelled vesicles in the cytoplasm of these cells would then enclose fuzzy coat precursors. Similarly, Souza Santos and Silva Sasso (1968) described type B mucocytes as the only secretory cells in sea star podia found in the epidermis of both the stem and the disc; they concluded from their histochemical study that cuticular material originated mainly from the secretory granules of these cells. Alternatively, it is also possible that type B mucocytes secrete a general-purpose mucus that could occasionally be incorporated into the footprints.

Biochemical composition of the footprints

Inorganic residues apart, the footprints are made up mainly of proteins, although carbohydrates and lipids are also present in significant amounts. The protein moiety contains significant amounts of both charged (especially acidic) and uncharged polar residues as well as half-cystine. The carbohydrate moiety is likely to be acidic, comprising both uronic acids and sulphate groups. This gross composition is in accordance with the

results of dye-binding tests that have shown sea star footprints to include both proteins and acid mucopolysaccharides (the presence of lipids has never been investigated) (Chaet, 1965; Flammang *et al.* 1994). Since adhesive secretions appear to make up the bulk of the footprint material, the biochemical composition given in Table 1 probably reflects the composition of these secretions. Interpretation of the biochemical composition, however, must take into account the fact that, in addition to adhesive material, footprints also seem to contain cuticular material. Perpeet and Jangoux (1973) have published a detailed histochemical study of the podia of *A. rubens*. Their results indicate that both the secretory granules of NCS cells and the fuzzy coat consist of carboxylated and sulphated acid mucopolysaccharides as well as proteins. Lipids have not been detected either in NCS cell granules or in the cuticle. These histochemical characteristics seem to be constant in all asteroid species studied so far (Chaet and Philpott, 1964; Souza Santos and Silva Sasso, 1968, 1970; Engster and Brown; 1972; Flammang *et al.* 1994). Unfortunately, no distinction can be made between the adhesive secretion and the material from the fuzzy coat; both seem to involve protein and acidic glycans as glycoproteins, proteoglycans or polysaccharide–protein complexes. As for the lipid fraction detected in the footprints, its origin is questionable. This fraction might not be detectable by classical histochemical stains or might come from the membranes of the NCS cell secretory granules (these membranes appear to be incorporated in the adhesive matrix; P. Flammang, unpublished observations). It could also be a contaminant in the footprint material.

As there are no other studies published on the biochemical composition of secretions used in temporary adhesion either in echinoderms or in other phyla, the composition of the footprints of *A. rubens* has been compared with data obtained for marine invertebrates using other types of adhesion: those cemented to the substratum with a solid adhesive (bivalve molluscs and cirripede crustaceans) and those crawling along the substratum on a viscous film (gastropod molluscs) (Table 1). The permanent adhesives of mussels and barnacles consist almost exclusively of proteins, while the transitory adhesive of limpets is made up of an association of proteins and glycans whose chemical composition resembles that of the footprint material of *A. rubens*. In the limpet *Patella vulgata*, the pedal mucus is a protein–carbohydrate complex with the two moieties linked by electrovalent bonds, the carbohydrate moiety being in the form of sulphated acid mucopolysaccharides (Grenon and Walker, 1980). It possesses viscoelastic properties, and Grenon and Walker (1980) have proposed that it behaves like an elastic solid during adhesion and like a viscous fluid during locomotion (see also Denny, 1983). However, Smith (1991, 1992) showed that limpets may use different attachment mechanisms depending on whether they are moving or are stationary for a long time. The glue-like attachment used when stationary gives far greater adhesive strength to the limpet (Smith, 1991), but it also implies that, at the end of the stationary period, the limpet must detach before it resumes crawling. This is close to temporary adhesion and could explain the similarity between sea star and limpet adhesive secretions.

Implications for the duo-gland model of adhesion/de-adhesion of sea star podia

When integrated with the morphological information already available, the data obtained from the study of footprints give some new clues on how the duo-gland adhesive system of asteroid podia might function.

Non-ciliated secretory cells have always been considered to be the adhesive cells of sea star podia (Chaet and Philpott, 1964; Chaet, 1965; Harrison and Philpott, 1966; Souza Santos and Silva Sasso, 1968; Engster and Brown, 1972; McKenzie, 1988*b*; Flammang *et al.* 1994). Indeed, these cells are the only secretory cells shown to release some of their secretory granules in attached podia, i.e. podia that were amputated and fixed while firmly attached to a substratum (Flammang *et al.* 1994). The secretions of these cells appear to form the adhesive layer joining the podium to the substratum (Flammang *et al.* 1994) and, indeed, the immunocytochemical study shows that they make up the bulk of the footprint material remaining on the substratum after the podium has become detached. The fact that both NCS1 and NCS2 cells are strongly immunolabelled demonstrates that the secretions from both types are incorporated into the adhesive material. Equal numbers of NCS1 and NCS2 cells are present in the disc epidermis, and they are homogeneously interspersed (Flammang *et al.* 1994). This suggests that, when the contents of their secretory granules are released, they would coalesce and mix physically (it is not possible to tell whether they actually react chemically) to form a sponge-like matrix: the adhesive layer (Fig. 7A). Adhesive interactions with the substratum and the fuzzy coat could be through ionic bonds, presumably involving the acidic residues of both the carbohydrate and protein moieties, while cohesive strength could be achieved by intermolecular disulphide bonds. In mussels, a cystine-rich protein is believed to be responsible for the sponge-like matrix formation in byssal plaques (Rzepecki *et al.* 1992). Using an adhesive in the form of a meshwork may present several advantages: it is economic (more coverage with less material and with better gap-filling properties on rough substrata) and it could prevent the propagation of cracks in the adhesive (cohesive failure) (Waite, 1986; Denny, 1988).

It has been suggested that de-adhesion is brought about by the release of the contents of CS cell granules. These cells have not been shown to release any granules in attached podia but have released their most apical secretory granules in detached podia, i.e. podia that were amputated and fixed just after they voluntarily became detached from a substratum (Flammang *et al.* 1994; Flammang, 1996). Although the composition of this secretion and the way it operates are not known, different hypotheses have been put forward. It has been proposed that the de-adhesive secretion might either compete with the fuzzy coat for ionic bonds on the adhesive layer (Hermans, 1983; Thomas and Hermans, 1985) or act as an enzyme releasing the fuzzy coat from the podium distal surface (Flammang, 1996).

In the first hypothesis, one would expect to find the de-adhesive secretion retained on the adhesive secretion within the footprint, but there is no reason for cuticular material to be present (competition model; Fig. 7B). Conversely, in the second hypothesis, the fuzzy coat would remain attached to the adhesive secretion, while the de-adhesive secretion (perhaps a soluble enzyme) would fail to be incorporated into the footprint (enzyme model; Fig. 7C). The immunocytochemical data presented in this report support the second hypothesis, which is further supported by the ultrastructural evidence showing that the CS cells release their secretion just under the cuticle and that the fuzzy coat can no longer be distinguished in transmission electron micrographs of detached podia (Flammang *et al.* 1994; Flammang, 1996).

Future studies should allow the isolation and purification of adhesive molecules from either the footprints or the podia by taking advantage of the new antisera raised against footprint material. The elucidation of their structures and physico-chemical characteristics, together with the morphological data, should provide the necessary basis for understanding how sea star adhesive systems are used in temporary adhesion.

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References

ANTONOPOULOS, C. A. (1962). A modification for the determination of sulphate in mucopolysaccharides by the benzidine method. *Acta chem. scand.* **16**, 1521–1522.

BARGMANN, W., HARNACK, M. AND JACOB, K. (1962). Uber den Feinbau des Nervensystem des Seesternes (*Asterias rubens* L.). Ein Beitrag zur Vergleichenden Morphologie der Glia. *Z. Zellforsch. mikrosk. Anat.* **56**, 573–594.

BENEDICT, C. V. AND WAITE, J. H. (1986). Composition and ultrastructure of the byssus of *Mytilus edulis*. *J. Morph.* **189**, 261–270.

BLUMENKRANTZ, N. AND ASBOE-HANSEN, G. (1973). New method for quantitative determination of uronic acids. *Analyt. Biochem.* **55**, 484–489.

CHAET, A. B. (1965). Invertebrates adhering surfaces: Secretions of the starfish, *Asterias forbesi* and the coelenterate, *Hydra pirardi*. *Ann. N.Y. Acad. Sci.* **118**, 921–929.

CHAET, A. B. AND PHILPOTT, D. E. (1964). A new subcellular particle secreted by the starfish. *J. Ultrastruct. Res.* **11**, 354–362.

COOK, M. (1970). Composition of mussel and barnacle deposits at the attachment interface. In *Adhesion in Biological Systems* (ed. R. S. Manly), pp. 139–150. New York: Academic Press.

DENNY, M. (1983). Molecular biomechanics of molluscan mucous secretions. In *The Mollusca*, vol. 1, *Metabolic Biochemistry and Molecular Biomechanics* (ed. P. W. Hochachka), pp. 431–465. New York: Academic Press.

DENNY, M. (1988). *Biology and the Mechanics of the Wave Swept Environment*. Princeton, NJ: Princeton University Press.

ENGSTER, M. S. AND BROWN, S. C. (1972). Histology and ultrastructure of the tube foot epithelium in the phanerozonian starfish, *Astropecten*. *Tissue & Cell* **4**, 503–518.

FLAMMANG, P. (1996). Adhesion in echinoderms. In *Echinoderm Studies*, vol. 5 (ed. M. Jangoux and J. M. Lawrence), pp. 1–60. Rotterdam: Balkema.

FLAMMANG, P., DEMEULENAERE, S. AND JANGOUX, M. (1994).) The role of podial secretions in adhesion in two species of sea stars (Echinodermata). *Biol. Bull. mar. biol. Lab., Woods Hole* **187**, 35–47.

GRENON, J.-F. AND WALKER, G. (1980). Biochemical and rheological properties of the pedal mucus of the limpet, *Patella vulgata* L. *Comp. Biochem. Physiol.* **66**B, 451–458.

HARRISON, G. AND PHILPOTT, D. (1966). Subcellular particles in echinoderm tube feet. I. Class Asteroidea. *J. Ultrastruct. Res.* **16**, 537–547.

HERMANS, C. O. (1983). The duo-gland adhesive system. *Oceanogr. mar. Biol. A. Rev.* **21**, 281–339.

HOLLAND, D. L. AND GABBOTT, P. A. (1971). A micro-analytical scheme for the determination of protein, carbohydrate, lipid and RNA levels in marine invertebrate larvae. *J. mar. biol. Ass. U.K.* **51**, 659–668.

HOLLAND, N. D. (1984). Echinodermata: epidermal cells. In *Biology of the Integument*, vol. 1, *Invertebrates* (ed. J. Bereiter-Hahn, A. G. Matoltsy and K. S. Richards), pp. 756–774. Berlin: Springer Verlag.

HOLLAND, N. D. AND NEALSON, K. H. (1978). The fine structure of the echinoderm cuticle and the subcuticular bacteria of echinoderms. *Acta zool.* **59**, 169–185.

JERMYN, M. A. (1975). Increasing the sensitivity of the anthrone method for carbohydrate. *Analyt. Biochem.* **68**, 332–335.

KAMINO, K., ODO, S. AND MARUYAMA, T. (1996). Cement proteins of the acorn barnacle, *Megabalanus rosa*. *Biol. Bull. mar. biol. Lab., Woods Hole* **190**, 403–409.

LUFT, J. H. (1971). Ruthenium red and violet. II. Fine structural localisation in animal tissues. *Anat. Rec.* **171**, 369–416.

MCKENZIE, J. D. (1988*a*). Echinoderm surface coats: Their ultrastructure, function and significance. In *Echinoderm Biology* (ed. R. D. Burke, P. V. Mladenov, P. Lambert and R. L. Parsley), pp. 697–706. Rotterdam: Balkema.

MCKENZIE, J. D. (1988*b*). The ultrastructure of tube foot epidermal cells and secretions: their relationship to the duo-glandular hypothesis and the phylogeny of the echinoderm classes. In *Echinoderm Phylogeny and Evolutionary Biology* (ed. C. R. C. Paul and A. B. Smith), pp. 287–298. Oxford: Clarendon Press.

NALDRETT, M. J. (1993). The importance of sulphur cross-links and hydrophobic interactions in the polymerization of barnacle cement. *J. mar. biol. Ass. U.K.* **73**, 689–702.

PERPEET, C. AND JANGOUX, M. (1973). Contribution à l'étude des pieds et des ampoules ambulacraires d'*Asterias rubens* (Echinodermata, Asteroides). *Forma et Functio* **6**, 191–209.

RZEPECKI, L. M., HANSEN, K. M. AND WAITE, J. H. (1992). Characterization of a cystine-rich polyphenolic protein family from the blue mussel *Mytilus edulis* L. *Biol. Bull. mar. biol. Lab., Woods Hole* **183**, 123–137.

SMITH, A. M. (1991). The role of suction in the adhesion of limpets. *J. exp. Biol.* **161**, 151–169.

SMITH, A. M. (1992). Alternation between attachment mechanisms by limpets in the field. *J. exp. mar. Biol. Ecol.* **160**, 205–220.

SOUZA SANTOS, H. AND SILVA SASSO, W. (1968). Morphological and histochemical studies on the secretory glands of starfish tube feet. *Acta anat.* **69**, 41–51.

SOUZA SANTOS, H. AND SILVA SASSO, W. (1970). Ultrastructural and histochemical studies on the epithelium revestment layer in the tube feet of the starfish *Asterina stellifera*. *J. Morph.* **130**, 287–296.

THOMAS, L. A. AND HERMANS, C. O. (1985). Adhesive interactions between the tube feet of a starfish, *Leptasterias hexactis* and substrata. *Biol. Bull. mar. biol. Lab., Woods Hole* **169**, 675–688.

TYLER, S. (1988). The role of function in determination of homology and convergence – examples from invertebrates adhesive organs. *Fortschr. Zool.* **36**, 331–347.

WAITE, J. H. (1983). Adhesion in byssally attached bivalves. *Biol. Rev.* **58**, 209–231.

WAITE, J. H. (1986). Mussel glue from *Mytilus californianus* Conrad: a comparative study. *J. comp. Physiol.* B **156**, 491–496.

WAITE, J. H. (1992). The formation of mussel byssus: anatomy of a natural manufacturing process. In *Structure, Cellular Synthesis and Assembly of Biopolymers* (ed. S. T. Case), pp. 55–74. Berlin: Springer-Verlag.

WALKER, G. (1972). The biochemical composition of the cement of two barnacle species, *Balanus hameri* and *Balanus crenatus*. *J. mar. biol. Ass. U.K.* **52**, 429–435.

WALKER, G. (1987). Marine organisms and their adhesion. In *Synthetic Adhesives and Sealants* (ed. W. C. Wake), pp. 112–135. Chichester: John Wiley & Sons.

YULE, A. B. AND WALKER, G. (1987). Adhesion in barnacles. In *Crustacean Issues*, vol. 5, *Biology of Barnacles* (ed. A. J. Southward), pp. 389–402. Rotterdam: Balkema.