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Low temperature X-ray microanalysis of calcium in a scleractinian coral: evidence of active transport mechanisms

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Summary

Element concentrations were measured by X-ray microanalysis in seawater (SW) compartments and mucocytes in bulk, frozen-hydrated preparations of the scleractinian coral *Galaxea fascicularis*. Quantitative X-ray microanalysis of polyps sampled in the daytime revealed that concentrations of the elements Na, S, K and Ca were all significantly higher in a thin (10–20 μm) external SW layer adjacent to the oral ectoderm ($P<0.05$, <0.05, <0.0001 and <0.01, respectively) than in standard SW. In polyps sampled during night-time, concentrations of Ca and S in this external SW layer were significantly reduced ($P<0.05$). Ca concentration in the coelenteron and extrathecal coelenteron was significantly higher ($P<0.001$) than in the external SW layer, regardless of time of sampling, suggesting that Ca^{2+} transport across the oral epithelium occurs *via* an active, transcellular route. X-ray microanalyses of mucocytes revealed that the concentration of S was high and did not vary between epithelial layers, while that of Ca increased in an inward gradient toward the skeleton. We suggest that throughout the day, secreted mucus behaves as a Donnan matrix at the oral ectoderm–SW interface, facilitating intracellular Ca^{2+} uptake. The accumulation within internal SW compartments of high concentrations of Ca relative to standard SW levels, however, appears to be independent of mucus secretion and is likely to be a consequence of active transport processes.

Key words: Low temperature, X-ray microanalysis, calcium, mucus, coral, *Galaxea fascicularis*, active transport Donnan equilibria, frozen-hydrated.

Introduction

The study of calcification dynamics in scleractinian corals is made difficult by the presence of a massive $CaCO_3$ skeleton, to which the thin layers of soft tissue are tightly apposed. This significant mineralised component precludes the use of many conventional preparatory and investigative techniques. Consequently, the processes involved in, and responsible for, the control of $CaCO_3$ precipitation, crystallisation and deposition within the skeleton, and the mechanisms of Ca accumulation within cells, compartments and organelles are not well understood (reviewed by Marshall, 2002).

In scleractinian corals, the ability to concentrate Ca at or near the site of mineralisation necessitates migration of Ca^{2+} ions from the SW across the epithelial layers. This may be achieved either directly from the surrounding SW or *via* the coelenteron, which maintains direct contact with the external SW environment *via* the mouth of the polyp. To date, much of the research upon calcification in scleractinian corals has involved the use of metabolic and enzymatic inhibitors and their effects upon rates of ^{45}Ca deposition into the skeleton (Marshall, 1996; Tambutté et al., 1995, 1996). These studies have indicated the involvement of an active transport process in the movement of Ca^{2+} across the coral epithelia. Whilst it is probable that active Ca^{2+} transport occurs in the aboral epithelia, immediately adjacent to the skeleton (McConnaughey, 1995; Tambutté et al., 1996), the mechanism of Ca^{2+} transport across the outer, or oral, epithelium is less certain.

The mode of Ca^{2+} movement across the oral epithelium of scleractinian corals has been variously reported to be both active (Wright and Marshall, 1991) and passive (Bénazet-Tambutté et al., 1996). The former studies were made upon sheets of isolated epithelia from two scleractinian corals (Wright and Marshall, 1991) and the latter upon isolated tentacles from a heliofungid scleractinian coral and an anemone (Bénazet-Tambutté et al., 1996). Since the evidence from these two investigations is contradictory, we have attempted to measure *in situ* transepithelial Ca concentrations in an effort to further elucidate the nature of Ca^{2+} transport across the oral epithelium. This investigation was prompted by previous studies on coral mucus (Marshall and Wright, 1991, 1995) and the realisation that mucus could play a major role at the diffusive boundary layer of coral epithelia. Such boundary layers have been suggested to have a marked influence on ion transport in several ion-transporting epithelia (reviewed

by Shephard, 1989; Verdugo, 1990; Lichtenberger, 1995; Werther, 2000).

Using a technique for preparing bulk, frozen-hydrated coral samples similar to that described by Clode and Marshall (2002), we have been able to quantitatively determine element concentrations within distinct morphological regions in the scleractinian reef coral *Galaxea fascicularis*. This has allowed us to identify sites of calcium accumulation and to suggest possible modes of Ca^{2+} transport. These bulk, frozen-hydrated preparations are highly suitable for quantitative elemental analyses of specialised and unspecialised cell types, distinctive cellular features and SW compartments (coelenteric cavities) using low temperature X-ray microanalysis (Marshall, 1987, 1998).

Materials and methods

Coral collection and maintenance

Colonies of *Galaxea fascicularis* L. were collected from the reef flat at Heron Reef, Great Barrier Reef, Australia and transported in buckets of SW to Heron Island Research Station. Colonies were maintained in semi-shaded outdoor flow-through aquaria [Photosynthetic Photon Flux Density (PPFD) 500–1500 μmol photons s^{-1} m^{-2}; 23–25°C] and allowed to recover for 2 days. Polyps were easily separated using forceps and placed in trays of running SW (PPFD 50–150 μmol photons s^{-1} m^{-2}; 23–25°C) to recover for a further 2 days. Small separated polyps were incubated for 2 h in jars containing 200 ml filtered SW (0.25 μm) that were partially submerged in shallow, flow-through aquaria in full sunlight (PPFD 800–1900 μmol photons s^{-1} m^{-2}; 23–25°C). Polyps were then frozen at midday in liquid propane (–190°C) that had been cooled by liquid nitrogen (LN_2), as previously described (Marshall and Wright, 1991). Polyps were also sampled at midnight and rapid-frozen in liquid propane. All samples were transferred to La Trobe University, Melbourne, in a dry shipper (CryoPak) at –180°C and stored in LN_2 until required.

X-ray microanalysis

The basic method of X-ray microanalysis of frozen-hydrated bulk samples has been described previously (Marshall, 1980a, 1987, 1998; Marshall and Xu, 1998) and is a well established technique (Echlin, 1992). Polyps were fractured under LN_2 and small, suitable pieces mounted in a modified sub-stage, so that the plane of the fractured surface ran parallel to the stage. Strips of indium foil were wrapped around the sample to improve conductivity and to provide flexible compression when clamped in the vice-like sub-stage. Fractured preparations were etched at –92°C before being coated with 200 Å aluminium in a Cryo-Preparation System (CT 1500; Oxford Instruments). Samples of standard SW in hollow rivets were frozen by plunging into liquid propane (–190°C). The rivets were mounted in an appropriate substage and the samples were fractured and coated in the cryo-preparation chamber. Both etched and unetched samples were analysed.

Selected area analyses were conducted in a JEOL JSM 840A scanning electron microscope (SEM) fitted with a Link exL X-ray analyzer and LZ5 detector. Analyses were carried out with a Be window in place, for a period of 100 s livetime, at 15 kV and a beam current (measured by a Faraday cup) of 2×10^{-10} A, on a custom built LN_2-cooled stage maintained at –174°C. Areas of analysis were no less than 25 μm^2. Selection of regions for analysis, where local surface tilt angle was close to 0°, was aided by using backscattered electron images, as described by Marshall (1981).

Spectra from selected area analyses were processed to yield peak integrals by linear least-squares fitting to library peaks and quantitative data were calculated using the PhiRhoZed model for matrix corrections (Marshall, 1982; Marshall and Condron, 1987). The standards used were polished microprobe standards (Biorad) of pure elements or minerals of well-defined composition. For mucocyte analyses, C and H concentrations were fixed at 10% and N at 3.3%, based on the elemental composition of a 20% aqueous solution of generalised protein. Protein composition was according to Engström (1966). Oxygen was calculated by difference. For SW analyses (standard SW and SW compartments of polyps), H was fixed at 10%, O was calculated by difference and C and N were omitted from the calculations.

Because the highly mineralised coral polyps had to be fractured under LN_2 external to the cryo-preparation chamber associated with the microscope, superficial frost had to be sublimed in order to visualise any morphological features. This resulted in surface etching, which compromises the accuracy of X-ray microanalytical data (Marshall, 1981). Therefore, the results of SW analyses were standardised by reference to Cl concentration of SW at 33‰ salinity (Rankin and Davenport, 1981), since preliminary experiments indicated that Cl concentrations in the different SW compartments analysed were not significantly different from those of standard SW samples. Similarly, no significant differences (P>0.05) were detected for any elements between etched samples of standard SW, after analyses were standardised, and unetched samples of standard SW in which analyses were not standardised. The results from X-ray microanalyses of mucocytes were ratioed to S and expressed as %S, because S was the predominant and invariable (P=0.99) element, and because the other elements present are primarily cations, presumed to be involved in charge neutralisation of sulphate groups.

Strontium analysis was based on the use of the Sr Lα X-ray spectral lines. These correspond in X-ray energy almost exactly to the *M* lines of tungsten X-rays. It was necessary, therefore, to correct for the extremely small amount of tungsten deposited on the sample during evaporation of Al from the tungsten filament. This was particularly important for the analysis of Sr in SW compartments. Since the quantity of tungsten deposited was constant under the same conditions of evaporation, the correction consisted of the subtraction of a measurement of Sr concentration from a frozen sample (NaCl solution), that did not contain Sr, from the Sr concentrations obtained from the coral samples.

The analytical depth resolution at 15 kV is estimated to be approximately 2 μm and the lateral resolution similar (Marshall and Condron, 1985). Thus the volume from which X-rays are detected is roughly approximated by a sphere of radius 1 μm. Results were expressed as mean ± standard error of the mean (S.E.M.) and all statistical tests (both one-way analysis of variance, ANOVA, with *post-hoc* analysis using Student's *t*-test and Wilcoxon 2-group non-parametric test) were performed using the computer software package JMP (SAS Institute Inc.). *N* is the number of analyses, derived from 12 midday polyps and 4 midnight polyps.

Corallite samples

Soft tissue was removed from *G. fascicularis* polyps by submersion in 5 mol l^{-1} NaOH at 60°C for 20 min. The resultant corallites were rinsed in running water for 25 min and twice in distilled water for 10 min, before being infiltrated with 1000 mosmol kg^{-1} NaCl in 15% polyvinyl-pyrrolidone (PVP) for 40 min. Preparations were then frozen in liquid propane and stored as above. For analysis, PVP-infiltrated corallites were fractured, etched and coated as described for frozen-hydrated polyps. Digital linescans across the skeletal/NaCl-PVP interface were collected at 15 kV and a beam current of 2×10^{-10} A with a Be window in place. The linescans recorded counts from an X-ray energy window covering the Ca Kα peak (3.663–3.743 keV) and a region of background (3.423–3.503 keV) at the low energy side of the peak. Subsequent to acquisition, the linescans were arithmetically processed to yield linescans of peak-background/background ratios.

Freeze-substituted sections

Frozen *G. fascicularis* polyps were freeze-substituted in 10% acrolein in ether and embedded in araldite as described by Marshall (1980b). Soft tissue was dissected from skeleton, re-embedded and sectioned according to Marshall and Wright (1991). Sections were stained with an aqueous $BaCl_2$ solution (10 mmol l^{-1}) and imaged on a JEOL 1200EX scanning transmission electron microscope at 80 kV.

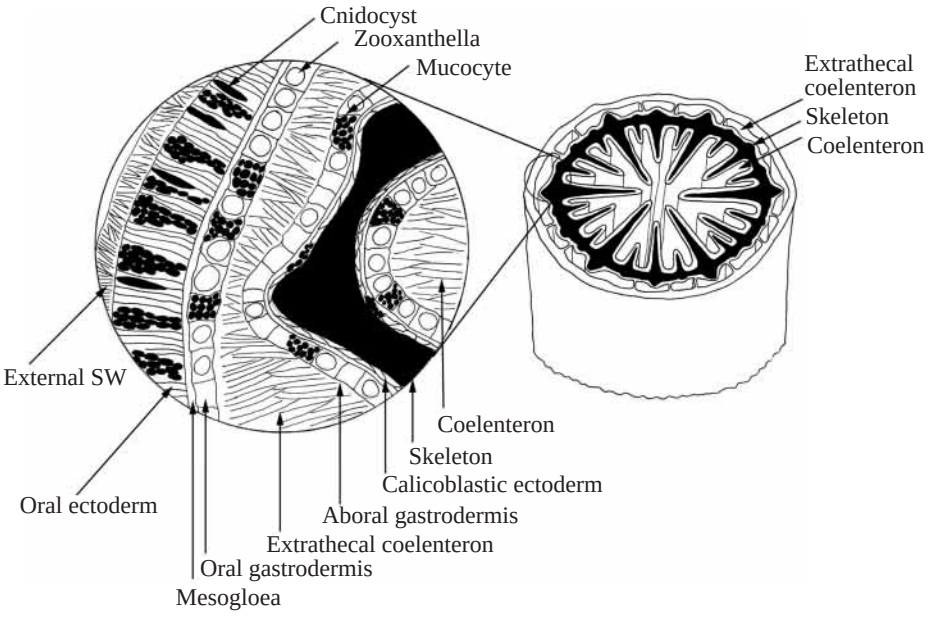

Fig. 1. Schematic diagram of a transverse section through a *Galaxea fascicularis* polyp, detailing the regions analysed by X-ray microanalysis, using selected area analyses. See text for details.

Results

Nature of samples

The samples analysed were fractured polyps and fractured exsert septa. Exsert septa are extensions of the skeletal septa at the crown of the polyp. As such, the samples of exsert septa did not contain an internal coelenteron. In all other respects the two types of samples were similar. The structure of the polyp, as revealed in a fractured surface, is shown semi-diagrammatically in Fig. 1. Micrographs from etched, frozen-hydrated samples show the oral epithelium (Fig. 2) and aboral epithelium (Fig. 3) and the associated SW compartments and skeleton. In frozen-hydrated samples, the SW layer external to the oral ectoderm contains a mucus layer secreted by the epithelium. This cannot be distinguished in fractured, frozen-hydrated samples, but is readily visible in frozen samples that have been freeze-substituted and sectioned (Fig. 4).

X-ray microanalysis of seawater compartments

Quantitative X-ray microanalysis of the external SW layer and extrathecal and internal coelenteric SW compartments of freeze-fractured, frozen-hydrated and etched *G. fascicularis* polyps revealed significant differences in element concentrations between these compartments and standard SW (Fig. 5). The major elements present in standard SW were Na, Mg, S, Cl, K and Ca (Fig. 5). In polyps sampled during daytime, sulphur was present in significantly higher concentrations ($P<0.05$) in the external SW layer (60±9 mmol kg^{-1} wet mass; $N=33$) than in standard SW (29±0.8 mmol kg^{-1}; $N=17$). Potassium concentrations were significantly higher in both the external SW layer (34±6 mmol kg^{-1}; $N=33$) and the extrathecal coelenteron (19±2 mmol kg^{-1}; $N=38$) than in standard SW (11±0.6 mmol kg^{-1}; $N=17$) ($P<0.0001$ and $P<0.01$, respectively). Sodium concentrations in the external SW layer (546±12 mmol kg^{-1}; $N=33$) and extrathecal coelenteron (549±10 mmol kg^{-1}; $N=38$) were also significantly higher than in standard SW (501±12 mmol kg^{-1}; $N=17$) ($P<0.05$ and $P<0.01$, respectively).

Significantly higher calcium concentrations were observed in all SW compartments of daytime-sampled

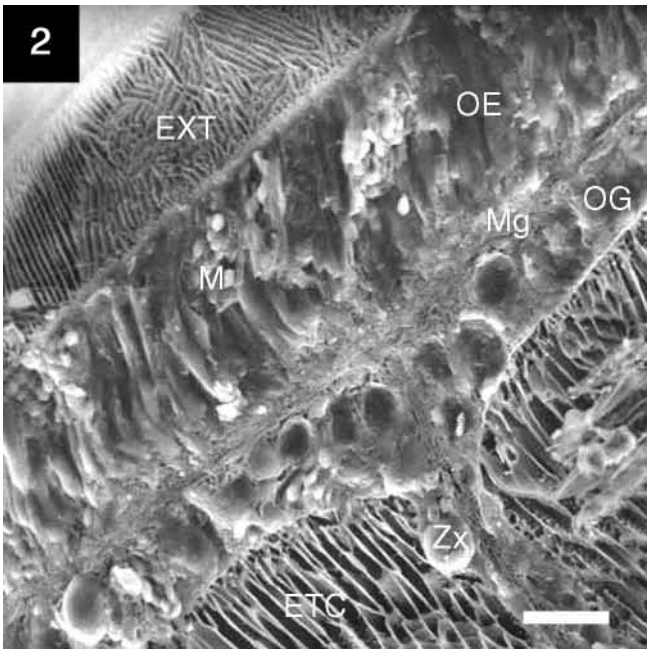

Fig. 2. Scanning electron micrograph of a typical fracture surface of the oral epithelium of a frozen-hydrated and superficially etched *Galaxea fascicularis* polyp prepared for selected area analyses. The external seawater layer (EXT), oral ectoderm (OE), mesogloea (Mg), oral gastrodermis (OG), zooxanthellae (Zx), mucus granules (M) and extrathecal coelenteron (ETC) are clearly distinguishable. Scale bar, 10 μm.

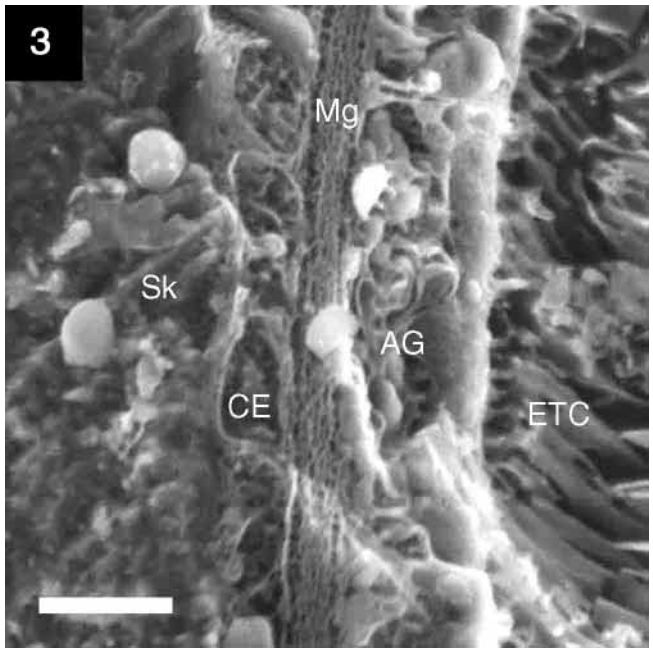

Fig. 3. Scanning electron micrograph of a typical fracture surface of the aboral epithelium of a frozen-hydrated and superficially etched *Galaxea fascicularis* polyp prepared for selected area analyses. The skeleton (Sk), calicoblastic ectoderm (CE), mesogloea (Mg), aboral gastrodermis (AG) and extrathecal coelenteron (ETC) are visible. Scale bar, 10 μm.

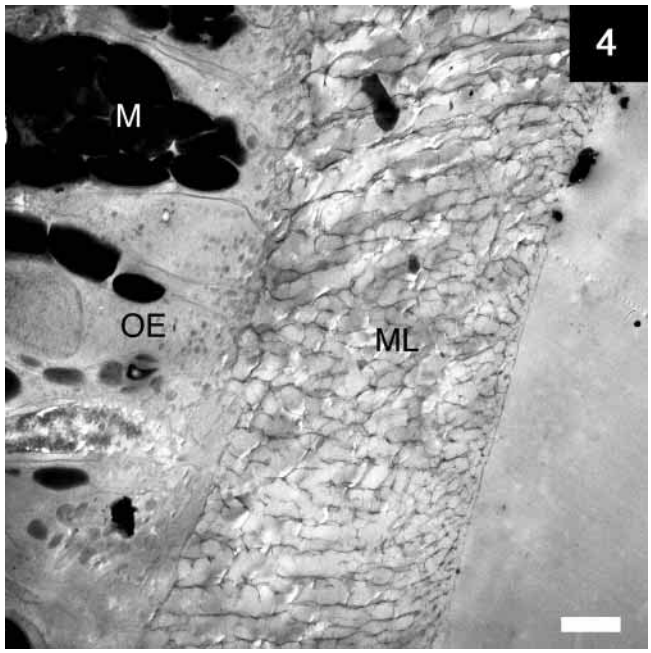

Fig. 4. Transmission electron micrograph showing the mucus layer (ML) located within the external seawater layer at the outer surface of oral ectodermal cells (OE), in a freeze-substituted, sectioned *Galaxea fascicularis* polyp. Abundant mucocytes (M) within the oral ectoderm are also visible. Scale bar, 2 μm.

polyps in comparison to standard SW. The calcium concentrations of the external SW layer (16±0.7 mmol kg^{-1}; N=33), extrathecal coelenteron (22±1 mmol kg^{-1}; N=38) and internal coelenteron (23±2 mmol kg^{-1}; N=18) were all significantly higher (P<0.01 for external SW layer and P<0.0001 for both internal compartments) than the calcium concentrations of standard SW (12±1 mmol kg^{-1}; N=17) (Fig. 5). In addition, SW within the coelenteric compartments contained significantly higher (P<0.001) calcium concentrations than the external SW layer. An example of this gradient in calcium concentration is shown as raw X-ray counts in Fig. 6, for a single polyp. The gradient is apparent without conversion of X-ray counts to concentration values and standardisation. Notably, the major elemental components of mucus (S, K and Sr) all decreased in concentration within SW compartments in an inward gradient across the polyp (Fig. 5).

A comparison of element concentrations between polyps frozen in the daytime and night-time revealed that the concentrations of both S and Ca were significantly higher (P<0.05) in the external SW layer during the day (Fig. 7A). While not significant, Sr also showed a trend of lower concentration in the external SW layer in polyps sampled at night-time. No significant differences (P>0.05) in Na, Mg or K were observed within the external SW layer between midnight and daytime samples (Fig. 7A). In the extrathecal coelenteron SW, Mg concentration was significantly higher (P<0.05) in polyps sampled at night-time. No significant differences (P>0.05) in Na, S, K, Ca or Sr concentrations were observed within the extrathecal coelenteron SW between

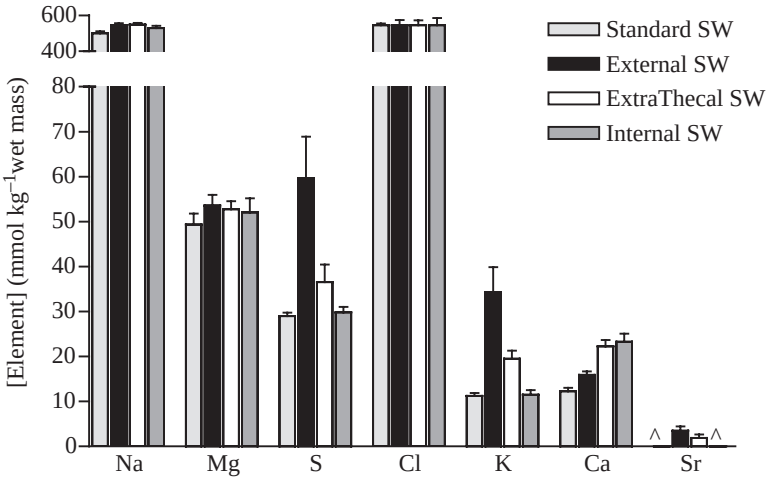

Fig. 5. Concentrations of elements present in standard seawater (SW) (*N*=17), and the external SW layer (*N*=33), extrathecal coelenteron SW (*N*=38) and internal coelenteron SW (*N*=18) of bulk, frozen-hydrated *Galaxea fascicularis* polyps sampled during daytime. Values are means ± S.E.M. All results were standardised to 546 mmol kg^{-1} Cl. See Results for significant differences. ^Not detectable.

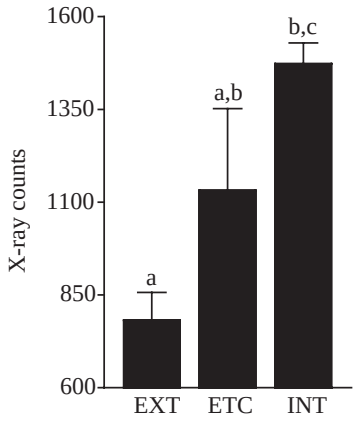

Fig. 6. Raw Ca X-ray counts (mean ± S.E.M.) for the external (EXT; *N*=6), extrathecal (ETC; *N*=5) and internal (INT; *N*=3) seawater compartments, obtained from a single *Galaxea fascicularis* polyp. Different letters denote statistical significance (*P*<0.05; ANOVA).

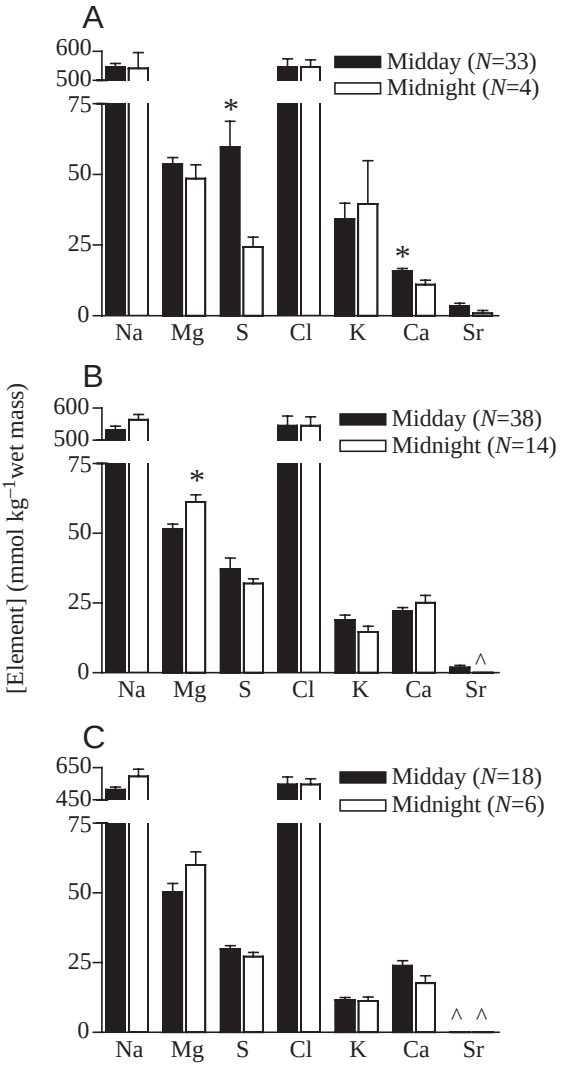

Fig. 7. Comparison of element concentrations (mean ± S.E.M.) within seawater (SW) compartments obtained from bulk, frozen-hydrated *Galaxea fascicularis* polyps sampled during daytime and night time. (A) External SW; (B) extrathecal coelenteron SW; (C) internal coelenteron SW. All results were standardised to 546 mmol kg^{-1} Cl. **P*<0.05 (Wilcoxon). ^Not detectable.

midnight and daytime samples (Fig. 7B). Within the internal coelenteron SW, no significant differences (*P*>0.05) in concentration were detected between daytime and night-time collected samples for any element (Fig. 7C).

X-ray microanalysis of mucocytes

Since there were no significant differences between analyses of mucocytes in polyps frozen in the daytime or night-time, the data were pooled. The primary elements detected in mucocytes of *G. fascicularis* were Na, S, Cl, K, Ca and Sr (Fig. 8), with S being the predominant and invariable element. Sodium, Cl and Ca all increased in concentration (relative to S) in an inward gradient, with the lowest concentrations detected in mucocytes of the oral ectoderm and the highest in mucocytes of the calicoblastic ectoderm (Fig. 8). Concentrations differed significantly (*P*<0.0001) between the oral and aboral epithelium for all three elements. In addition, the amount of Ca detected in mucocytes of the calicoblastic ectoderm was significantly higher (*P*<0.0001) than the amount of Ca present in mucocytes from any other cell layer. The two other primary elements, K and Sr, were concentrated in particular epithelial layers. Potassium was significantly higher (*P*<0.0001) in concentration in ectodermal mucocytes compared to gastrodermal mucocytes, while the reverse was true for Sr (*P*<0.0001) (Fig. 8). No other significant differences (*P*>0.05) were observed.

The ratios of K and Ca relative to S were calculated from the analytical data for mucocytes from the oral ectoderm, oral gastrodermis and aboral gastrodermis. Actual differences in

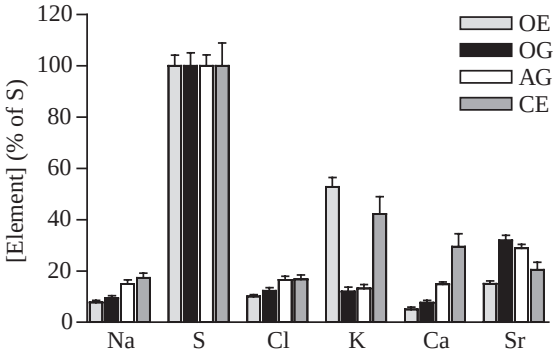

Fig. 8. Ratios (% of S, mean ± S.E.M.) of the primary elements present in mucocytes of the oral ectoderm (OE; *N*=45), oral gastrodermis (OG; *N*=43), aboral gastrodermis (AG; *N*=36) and calicoblastic ectoderm (CE; *N*=11), of bulk, frozen-hydrated *Galaxea fascicularis* polyps. See Results for significant differences.

Table 1. *Actual and expected concentrations of K and Ca relative to S (mmol kg^{-1}), based upon ratios calculated from X-ray microanalytical data, for standard seawater (SW) containing mucus from oral ectodermal mucocytes (external SW layer), or standard SW containing mucus from oral and aboral gastrodermal mucocytes (extrathecal coelenteron SW), in bulk, frozen-hydrated preparations of the scleractinian coral* Galaxea fascicularis

Region	Result	S	K	Ca
External SW layer	Actual	59.6	34.3	15.9
	Expected	59.6	27.5	13.8
Extrathecal coelenteron SW	Actual	37.5	19.5	22.3
	Expected	37.5	12.3	13.2
Standard SW	Actual	29.0	11.3	12.3

element concentrations evident in SW compartments were then compared with expected concentrations, calculated on the basis that SW compartments contained standard SW and mucus secretions from mucocytes located within the surrounding epithelial layer(s). This calculation is based on the simplistic assumption that the mucus and associated cations dissolve in the SW. The concentrations of K and Ca (relative to S) observed in the external SW layer were slightly higher than the predicted ratios of standard SW containing secreted mucus from oral ectodermal mucocytes (Table 1). However, K and Ca concentrations in the extrathecal coelenteron were considerably higher than the expected ratios for standard SW containing mucus secretions from the oral and aboral gastrodermal mucocytes (Table 1), assuming that the mucocytes from each layer contributed equally to mucus within the SW.

Line scans – resolution test

X-ray counts for Ca across the skeleton/NaCl-PVP interface of appropriately prepared *G. fascicularis* corallites, revealed that when analysing features close to the $CaCO_3$ skeleton by

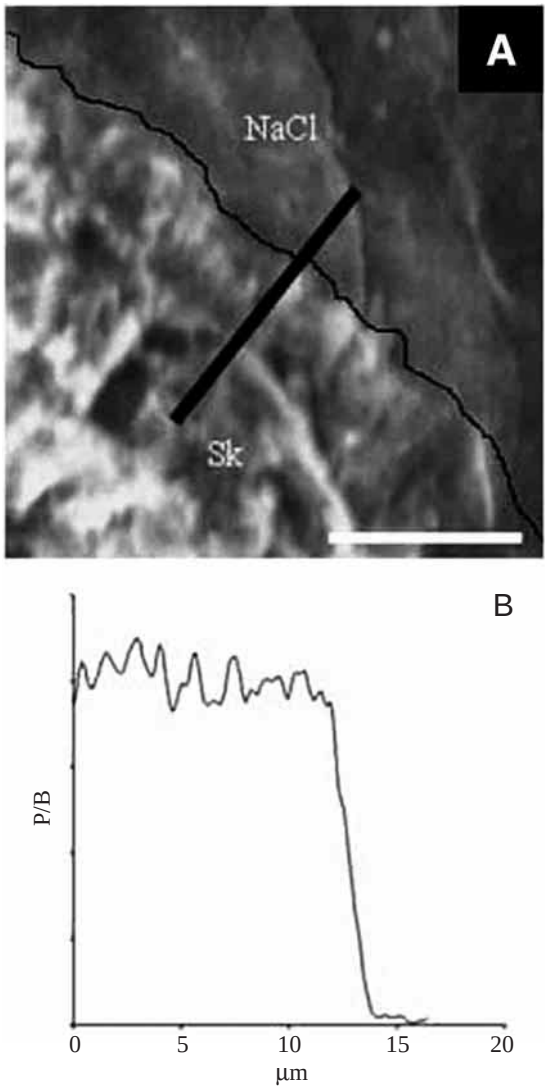

Fig. 9. (A) Fracture surface of a bulk, frozen-hydrated *Galaxea fascicularis* skeleton infiltrated with a NaCl-PVP solution. The interface between the skeleton (Sk) and the NaCl-PVP solution (NaCl) is depicted by a thin line. The position of the line scan across this interface is depicted by the broad line. Scale bar, 10 μm. (B) Peak to background X-ray counts (P/B; 0–2500 full scale) for Ca along the line scan shown in A.

X-ray microanalysis, Ca X-rays generated from the bulk of the skeleton are minimal and contribute little to the Ca signal. From the skeletal surface, the Ca X-ray signal was, as expected, high; but as the beam moved away from the skeleton and into the NaCl-PVP solution, this signal rapidly decreased (Fig. 9). Almost no Ca X-ray signal was detected when the beam was focussed more than 1 μm from the skeletal surface.

Discussion

Methodology

The accuracy of X-ray microanalysis is a function of X-ray counting, the accuracy with which empirical

mathematical models account for differences in X-ray generation and emission between the sample and standards, and the number of analyses. The physics of X-ray emission and detection of Na Kα X-rays inevitably means that in an analysis of a frozen-hydrated bulk sample, Na will be measured with the least accuracy of any element (see Marshall, 1982). For a standard SW sample, the value given for the concentration of Na varies from one literature source to another. We have taken the value as approximately 468 mmol kg^{-1} (Rankin and Davenport, 1981). Thus, our measurement of Na concentration (501 mmol kg^{-1}) is indicative of an overestimate with an approximate error of 7%. These deficiencies in Na determination will only affect absolute values, not relative values. Consequently Na:Cl ratios in SW compartments will be overestimates, but they will be correct relative to each other.

In the mucus layer, which is located within the external SW layer, there did not appear to be a detectable reduction in Cl concentration relative to SW. This was also determined in our preliminary experiments and has additionally been observed in freeze-substituted sections (A. T. Marshall and O. P. Wright, unpublished data). The reason for this is not obvious, but the markedly lower S concentration in the mucus layer, compared with mucus granules in mucocytes, indicates a considerable degree of swelling and a concomitant reduction in negative charge density. Any reduction in Cl concentration, compared to SW, as a result of Donnan equilibria may, therefore, be very small and possibly masked by analytical variation. We estimate that any errors in determining Ca concentration resulting from standardising measurements to a fixed Cl concentration, would be less that 10%. Such an error would not significantly affect the subsequent calculations or change our conclusions. The possibility that the standardisation procedure could generate an artefactual Ca concentration gradient was eliminated by showing that the gradient persists in the unprocessed Ca X-ray counts across a single polyp.

For the quantitative analysis of mucocytes, matrix correction factors were calculated on the basis of an assumed composition of 10% C and 10% H. The content of C and H will vary depending upon the ratio of protein to oligosaccharide side chains and the water content. Both of these ratios are unknown. However, at the extreme limit, if the former is assumed to be 1.5 and the latter 20%, then the composition will be approximately 18% C and 8% H. Substitution of these values in the calculation for matrix correction factors results in essentially no change in the measured concentrations of the other elements present, with the exception of O.

The volume of the sample from which X-rays are emitted under the analytical conditions used approximates a sphere 2 μm in diameter (Marshall, 1982; Marshall and Condron, 1985). In the analysis of frozen-hydrated corals we measured Ca concentration in the mucocytes within the calicoblastic ectoderm, which varies from 1–5 μm in thickness (Clode and Marshall, 2002) and is immediately adjacent to the $CaCO_3$ skeleton. It was therefore necessary to demonstrate that the analyses could be accomplished without detecting extraneous Ca X-rays from the skeleton. Line scan data clearly indicate that any Ca signal generated by scattering of electrons to the bulk of the skeleton was minimal, and that concentrations measured in mucocytes close to the skeleton were not artefactual.

Calcium entry

A coral polyp is essentially a sac-like organism with the interior of the sac communicating with the external SW *via* the mouth. However, water exchange *via* the mouth is probably extremely small. Consequently, the coelenteron can essentially be regarded as a sealed compartment with an internal medium that is isolated from the surrounding SW (Wright and Marshall, 1991; Bénazet-Tambutté et al., 1996; Furla et al., 1998). Coupled with the low water permeability of the oral tissue (Bénazet-Tambutté and Allemand, 1997), the ionic environment of the coelenteric cavities may become highly modified by active transport of particular ions, thereby generating electrochemical gradients across both the oral and aboral epithelial layers.

Several authors have suggested that Ca^{2+} transport across the oral epithelium is an active, transcellular process (Chalker, 1976, 1981; Wright and Marshall, 1991; Marshall and Wright, 1998). This is supported by our results, which describe significantly higher Ca concentrations in the extrathecal and internal coelenteron in comparison to an external SW layer, adjacent to the outer surface of the polyp, and to standard SW. These findings are consistent with those of Wright and Marshall (1991), but inconsistent with those of Weber (1973) and Bénazet-Tambutté et al. (1996), who suggested that Ca^{2+} transport across the oral epithelium occurred *via* simple, paracellular diffusion.

Wright and Marshall (1991) measured Ca^{2+} transport across isolated oral epithelia from the theca of the scleractinian coral *Lobophyllia hemprichii* and the vesicles of *Plerogyra sinuosa*, whereas Bénazet-Tambutté et al. (1996) used the oral epithelia from the tentacles of an anemone *Anemonia viridis* and an anemone-like scleractinian coral, *Heliofungia actiniformis*. On the one hand, Bénazet-Tambutté et al. (1996) found that Ca^{2+} moved passively across the tentacle epithelium and that the epithelium was permeable to Na^+ and Cl^-. On the other hand, Wright and Marshall (1991) showed that Ca^{2+} was transported actively across the oral epithelium of the body wall. Oral epithelial preparations from *L. hemprichii* were impermeable to Na^+ (using the Ussing chamber protocol described by Wright and Marshall, 1991; O. P. Wright and A. T. Marshall, unpublished data). The flux of Na^+ was measured in one paired experiment – ectoderm to gastrodermis and gastrodermis to ectoderm – and four unpaired experiments, each of ectoderm to gastrodermis and gastrodermis to ectoderm. The radioactivity in the cold half of the Ussing chamber remained, in each experiment, at little more than background levels after 320 min incubation, and in one case after 10 h incubation. Typically, the hot side contained 180×10^3 d.p.m. ml^{-1} whereas the cold side contained $<0.5\times10^3$ d.p.m. ml^{-1}. It seems that the oral epithelia from the body wall and the tentacles may have different permeability properties.

Mucus composition

Meikle et al. (1987) reported that mucus in scleractinian corals was composed of a glycoprotein chain with numerous side chains of sulphated oligosaccharides. This observation is supported by X-ray microanalytical data from mucocytes in freeze-substituted coral preparations (Marshall and Wright, 1991, 1995; Marshall and Clode, 1998) and by our analyses of frozen-hydrated preparations. As mucins are large, negatively charged molecules that are not covalently linked, condensation of mucins into mucus granules requires neutralisation of the mutually repulsing polyanionic (sulphated) charges (see Verdugo, 1990; Bansil et al., 1995). In *G. fascicularis*, high concentrations of the cations K^+ and Sr^{2+} in mucus granules suggests that these cations are likely to play a primary role in neutralising the polyanions of mucin molecules. While Ca^{2+} concentrations may be high (Warner and Coleman, 1975; Takano and Akai, 1988; Verdugo, 1990) and play a major role in neutralising polyanionic charges of mucins in some secretory epithelia (see Verdugo, 1990), it appears that this role is reduced in coral mucocytes. The detection of high Na and Cl concentrations in *G. fascicularis* mucocytes is difficult to explain. High Na and Cl concentrations have previously been reported in mucus granules (Sasaki et al., 1983), including those of *G. fascicularis* (Marshall and Wright, 1991, 1995), yet the function of their association with mucin polymers remains unexplained.

Mucus secretion and the distribution of Ca

In *G. fascicularis*, the layer of mucus covering the oral ectoderm is of the order of 10–20 μm thick (Marshall and Wright, 1995), and we suggest that this layer may play an important role in ion transport. Mucus layers are known to influence the distribution of ions at epithelial surfaces (reviewed by Shephard, 1989; Verdugo, 1990; Lichtenberger, 1995; Werther, 2000), this being a consequence of the effect of the mucus on diffusion rates of ions and also of the participation of the constituent negatively charged polyanions in Donnan equilibria.

Extracellular mucus may be regarded as forming a Donnan matrix, the interactions of which are complex and not readily predicted (Comper and Laurent, 1978). As pointed out by Gupta (1989), the extracellular mucus matrix is not a quasi-permanent matrix supporting an absolute Donnan equilibrium, but is continually being secreted and demonstrates a complex steady state Donnan situation. In general, it would be expected that the concentration of counterions (to polyanions) would be increased, relative to the surrounding medium, and the concentration of coanions would be reduced. Furthermore, most extracellular polyanions show a strong preference for K^+ over other cations, including Ca^{2+} and Na^+, although Na^+ may be in much higher concentration in extracellular fluid (Scott, 1989).

The distribution of Ca and K in the external SW layer and the extrathecal coelenteron SW may be the result of a combination of Donnan equilibria and transport across the oral epithelium. However, the ratios of Ca and K to S in the external SW layer were very close to those measured in mucus granules within mucocytes. This suggests that, unlike many secreted mucins (Gupta, 1989), there is not an exchange of counterions (eg. Ca^{2+} from mucin with Na^+ from external medium) during the secretion process. Ion activities in mucoidal solutions may be lower than in SW because the dissociation constants of counterions with polyanions may differ from their constants in free solution (Gupta, 1989). However, measurements of Ca^{2+} concentration with a mini ion-selective electrode on the surface of the oral epithelium (A. T. Marshall and P. L. Clode, unpublished data) are consistent with the concentration measurements obtained by X-ray microanalysis. [The Ca^{2+} concentration measured by ion-selective electrode in the SW layer at the surface of the epithelium in the light (14.5 ± 0.7 mmol l^{-1}; $N=3$) was always higher than in standard SW (11.3 ± 0.3 mmol l^{-1}; $N=3$)]. Since the Ca^{2+} concentration measured by this type of electrode is directly proportional to activity, it follows that there can be no significant reduction in activity due to binding to the polyanion, when compared with SW.

The establishment of a mucus layer at the SW–oral ectoderm interface may facilitate the uptake of Ca^{2+} into the oral ectodermal cells, with Ca^{2+} able to exist in higher concentrations in a Donnan state within this mucus matrix, in comparison to the normal SW environment. At night-time when calcification rates fall (Marshall, 1996), the demand for Ca^{2+} is reduced and the presence and influence of this mucus layer at the apical surface of the oral ectodermal cells, also declines. This is indicated by lower S (indicative of mucus) and Ca concentrations detected within the external SW layer in polyps sampled during night-time. A reduction in the secretion of mucus and mucus-lipids at night time has also been observed in the corals *Acropora acuminata* and *Acropora variabilis* (Crossland et al., 1980; Crossland, 1987). The K concentration in the external SW layer could also be expected to be reduced at night-time. Failure to observe such a reduction can be attributed to the presence of one, possibly anomalous, measurement of a very high K concentration out of a total of four. It seems probable that a greater number of analyses would have shown a reduction in K concentration concomitant with the reduced S and Ca concentration.

Conclusions

The effects of an external mucus layer upon the microenvironment of the oral ectoderm–SW interface and its effect upon the rates of ionic uptake and exchange have not been previously recognised. Consideration has only been given to the boundary layer in relation to O_2 diffusion (Kuhl et al., 1995; Gardella and Edmunds, 1999; De Beer et al., 2000). The presence of a mucus layer within an external SW compartment on the surface of the oral ectoderm may facilitate the maintenance of a Ca^{2+} concentration next to the ectodermal cells that is higher than that in standard SW. This in turn may favour a high rate of entry of Ca^{2+} into the ectodermal cells during daytime. At night-time, calcification rate is markedly reduced, but calcification still occurs

(Marshall, 1996; Marshall and Wright, 1998). Thus, it is not surprising that a high Ca concentration persists in the internal SW coelenteric compartments. A continual presence of elevated Ca concentration, detected within internal coelenteric compartments in corals sampled during both daytime and night-time, suggests that maintenance of high Ca concentrations within internal SW compartments is independent of the formation of the mucus layer and Donnan state in the external SW layer. The latter, however, may affect the rate of transport across the epithelium. Certainly, Ca concentration appears to be limiting for calcification rate (Marshall and Clode, 2002). In addition, it appears that mucus secretion into the coelenteric cavities is minimal and that a substantial mucus secretion does not normally persist in these internal compartments. This is indicated by the concentrations of S detected within coelenteric cavities being only slightly higher than those of standard SW. From this, it appears that mucus is not responsible for establishing the higher Ca concentrations detected within internal SW compartments. It is likely, therefore, that active transport mechanisms are involved in the movement of Ca^{2+} across the oral epithelium, resulting in the accumulation of significant amounts of Ca within internal SW compartments.

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References

Bansil, R., Stanley, E. and LaMont, J. T. (1995). Mucin biophysics. *Ann. Rev. Physiol.* **57**, 635-657.

Bénazet-Tambutté, S., Allemand, D. and Jaubert, J. (1996). Permeability of the oral epithelial layers in cnidarians. *Mar. Biol.* **126**, 43-53.

Bénazet-Tambutté, S. and Allemand, D. (1997). Water permeability of the oral epithelial layers of the sea anemone, *Anemonia viridis*. *J. Exp. Zool.* **279**, 1-8.

Chalker, B. E. (1976). Calcium transport during skeletogenesis in hermatypic corals. *Comp. Biochem. Physiol.* **54A**, 455-459.

Chalker, B. E. (1981). Skeletogenesis in scleractinian corals: The transport and deposition of strontium and calcium. In *Handbook of Stable Strontium* (ed. S. C. Skoryna) pp. 47-63. New York: Plenum Press.

Clode, P. L. and Marshall, A. T. (2002). Low temperature FESEM of the calcifying interface of a scleractinian coral. *Tiss. Cell* **34**, 187-198.

Comper, W. D. and Laurent, T. C. (1978). Physiological function of connective tissue and polysaccharides. *Physiol. Rev.* **58**, 255-315.

Crossland, C. J. (1987). In situ release of mucus and DOC-lipid from the corals *Acropora variabilis* and *Stylophora pistillata* in different light regimes. *Coral Reefs* **6**, 35-42.

Crossland, C. J., Barnes, D. J. and Borowitzka, M. A. (1980). Diurnal lipid and mucus production in the staghorn coral *Acropora acuminata*. *Mar. Biol.* **60**, 81-90.

De Beer, D., Kuhl, M., Stambler, K. and Vaki, L. (2000). A microsensor study of light enhanced Ca^{2+} uptake and photosynthesis in the reef building coral *Favia* sp. *Mar. Ecol. Prog. Ser.* **194**, 75-85.

Echlin, P. (1992). *Low-Temperature Microscopy and Analysis*. New York: Plenum Press.

Engström, A. (1966). X-ray microscopy and X-ray absorption analysis. In *Physical Techniques in Biological Research* (ed. A. W. Pollister), pp 87-171. New York: Academic Press.

Furla, P., Bénazet-Tambutté, S., Jaubert, J. and Allemand, D. (1998). Diffusional permeability of dissolved inorganic carbon through the isolated oral epithelial layers of the sea anemone, *Anemonia viridis*. *J. Exp. Mar. Biol. Ecol.* **221**, 71-88.

Gardella, D. J. and Edmunds, P. J. (1999). The oxygen microenvironment adjacent to the tissue of the scleractinian *Dichocoenia stokesii* and its effects on symbiont metabolism. *Mar. Biol.* **135**, 289-295.

Gupta, B. L. (1989). The relationship of mucoid substances and ion and water transport, with new data on intestinal goblet cells and a model for gastric secretion. In *Mucus and Related Topics* (ed. E. Chantler and N. A. Ratcliffe), pp. 81-110. Cambridge: Company of Biologists Ltd.

Kuhl, M., Cohen, Y., Dasgaard, T., Jorgensen, B. B. and Revsbech, N. P. (1995). Microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O_2, pH and light. *Mar. Ecol. Prog. Ser.* **117**, 159-172.

Lichtenberger, L. M. (1995). The hydrophobic barrier properties of gastrointestinal mucus. *Ann. Rev. Physiol.* **57**, 565-583.

Marshall, A. T. (1980a). Quantitative x-ray microanalysis of frozen-hydrated bulk biological specimens. *Scan. Electron Microsc.* **1980 II**, 335-348.

Marshall, A. T. (1980b). Freeze-substitution as a preparation technique for biological x-ray microanalysis. *Scan. Electron Microsc.* **1980 II**, 395-408.

Marshall, A. T. (1981). Simultaneous Use of EDS, Windowless EDS, BE and SE Detectors and Digital Real-Time Line Scanning for the X-Ray Microanalysis of Frozen-Hydrated Biological Specimens. *Scan. Electron Microsc.* **1981**, 327-343.

Marshall, A. T. (1982). Application of $\phi(\rho z)$ curves and a windowless detector to the quantitative x-ray microanalysis of frozen-hydrated bulk specimens. *Scan. Electron Microsc.* **1982**, 243-260.

Marshall, A. T. (1987). Scanning electron microscopy and x-ray microanalysis of frozen-hydrated bulk samples. In *Cryotechniques in Biological Electron Microscopy* (ed. R. A. Steinbrecht and K. Zierold), pp. 240-257. Berlin: Springer Verlag.

Marshall, A. T. (1996). Calcification in hermatypic and ahermatypic corals. *Science* **271**, 637-639.

Marshall, A. T. (1998). X-ray microanalysis of frozen-hydrated biological bulk samples. *Mikrochim. Acta* **15**, 273-282.

Marshall, A. T. (2002). The occurrence, distribution and localisation of metals in cnidarians. *Microsc. Res. Tech.* **56**, 341-357.

Marshall, A. T. and Clode, P. (1998). Mapping of elemental distributions in coral tissues by x-ray microanalysis. In *Symp. J. 14th Int. Conf. Electron Microsc.* pp. 337-338. Bristol: Institute of Physics Publishing.

Marshall, A. T. and Clode, P. (2002). Effect of increased calcium concentration in seawater on calcification and photosynthesis in the scleractinian coral *Galaxea fascicularis*. *J. Exp. Biol.* **205**, 2107-2113.

Marshall, A. T. and Condron, R. J. (1985). X-ray microanalytical resolution in frozen-hydrated biological bulk samples. *J. Microsc.* **140**, 109-118.

Marshall, A. T. and Condron, R. J. (1987). A simple method of using $\phi(\rho z)$ curves for the x-ray microanalysis of frozen-hydrated biological bulk samples. *Micron Microsc. Acta* **18**, 23-26.

Marshall, A. T. and Wright, A. (1998). Coral calcification: autoradiography of a scleractinian coral *Galaxea fascicularis* after incubation in ^{45}Ca and ^{14}C. *Coral Reefs* **17**, 37-47.

Marshall, A. T. and Wright, O. P. (1991). Freeze-substitution of scleractinian coral for confocal scanning laser microscopy and x-ray microanalysis. *J. Microsc.* **162**, 341-354.

Marshall, A. T. and Wright, O. P. (1995). X-ray microanalysis of coral mucus. *Microbeam Anal.* **4**, 305-315.

Marshall, A. T. and Xu, W. (1998). Quantitative elemental x-ray imaging of frozen-hydrated biological samples. *J. Microsc.* **190**, 305-316.

McConnaughey, T. (1995). Ion transport and the generation of biomineral supersaturation. *Bull. Ins. Oceanogr. Monaco* **14**, 1-18.

Meikle, P., Richards, G. N. and Yellowlees, D. (1987). Structural determination of the oligosaccharide side chains from a glycoprotein isolated from the mucus of the coral *Acropora formosa*. *J. Biol. Chem.* **262**, 16941-16947.

Rankin, J. C. and Davenport, J. A. (1981). *Animal Osmoregulation*. London: Blackie.

Sasaki, S., Nakagaki, I., Mori, H. and Imai, Y. (1983). Intracellular calcium store and transport of elements in acinar cells of the salivary gland determined by electron probe x-ray microanalysis. *Jap. J. Physiol.* **33**, 69-83.

Scott, J. E. (1989). Ion-binding: Patterns of 'affinity' depending on types of acid groups. In *Mucus and Related Topics* (ed. E. Chantler and N. A. Ratcliffe), pp. 111-115. Cambridge: Company of Biologists Ltd.

Shephard, K. L. (1989). The effects of mucus and mucilaginous materials on ion-distributions at epithelial surfaces. In *Mucus and Related Topics* (ed. E. Chantler and N. A. Ratcliffe), pp. 123-130. Cambridge: Company of Biologists Ltd.

Takano, Y. and Akai, M. (1988). Histochemical, ultrastructural and x-ray microprobe analytical studies of localization of calcium in the mucous lining of the rat duodenum. *Histochemistry* **89**, 429-436.

Tambutté, E., Allemand, D., Bourge, I., Gattuso, J.-P. and Jaubert, J. (1995). An improved ^{45}Ca protocol for investigating physiological mechanisms in coral calcification. *Mar. Biol.* **122**, 453-459.

Tambutté, E., Allemand, D., Mueller, E. and Jaubert, J. (1996). A compartmental approach to the mechanisms of calcification in hermatypic corals. *J. Exp. Biol.* **199**, 1029-1041.

Verdugo, P. (1990). Goblet cells secretion and mucogenesis. *Ann. Rev. Physiol.* **52**, 157-176.

Warner, R. R. and Coleman, J. R. (1975). Electron probe analysis of calcium transport by small intestine. *J. Cell Biol.* **64**, 54-74.

Weber, R. (1973). Incorporation of strontium into reef coral skeletal carbonate. *Geochim. Cosmochim. Acta* **37**, 2173-2190.

Werther, J. L. (2000). The gastric mucosal barrier. *Mount Sinai J. Med.* **67**, 41-53.

Wright, O. P. and Marshall, A. T. (1991). Calcium transport across the isolated oral epithelium of scleractinian corals. *Coral Reefs* **10**, 37-40.