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Polyphosphates in the red macroalga *Chondrus crispus* (Rhodophyceae)

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SUMMARY

Plants of *Chondrus crispus* Stackhouse, collected from mid-littoral tidepools, were treated as follows. Some plants were kept for a few weeks under controlled starvation conditions in order to decrease their initial content of total tissue phosphorus, then incubated for up to 48 h in phosphorus (15 μM) and nitrogen (25 μM)enriched sea water. Other plants were directly incubated in enriched sea water. Chemical analyses showed that the total phosphorus content of fresh and starved plants remained stable, reflecting the nutritional status of the plants. The predominant acid-soluble phosphate fraction was larger in fresh than in starved plants. The content of acid-soluble polyphosphates, similar in both types of plants at the beginning of the experiment, doubled in starved plants, and increased by a factor of 2·7 in fresh plants, over 48 h. The content of acid-insoluble polyphosphates was lower than that of acid-soluble polyphosphates.

Transmission electron microscopy and energy dispersive X-ray microanalysis confirmed the presence, mostly in medullary cells, of acid-insoluble polyphosphates in the form of cytoplasmic granules and precipitates along the plasmalemma, particularly near pit plugs. This is the first report of such phosphorus storage structures in a red macroalgal species.

Key words: *Chondrus crispus*, phosphorus, polyphosphates, Rhodophyceae, X-ray microanalysis.

INTRODUCTION

Phosphorus and nitrogen can be limiting factors for the growth of *Chondrus crispus* Stackhouse (Chopin, Gallant & Davison, 1995). When N is limited, fronds become pale, and bleach to a pinkish white colour (Neish & Fox, 1971). Under P limitation, fronds become weakened, and increased fragmentation often occurs (Neish *et al.*, 1977). An inverse relationship between P and N enrichment of sea water and the content of the economically important carrageenans was demonstrated in *C. crispus* and other species (Neish *et al.*, 1977; Chopin *et al.*, 1995). Chopin *et al.* (1990) demonstrated that the main storage pools in *C. crispus* are not the inorganic P fraction, but the organic and acid-insoluble P fractions, the latter fraction possibly containing polyphosphates (Bieleski & Ferguson, 1983).

Polyphosphates isolated from biological material are linear polymers in which orthophosphate residues are linked through energy-rich phosphoanhydride bonds similar to those connecting terminal phosphate residues in the molecule of ATP and other nucleoside oligophosphates (Kulaev, 1975). They may be separated into acid-soluble and acid-insoluble polyphosphate fractions. The latter are longer and more condensed than the former.

Polyphosphates, and the energy that can be released during their hydrolysis, are involved in dynamic P storage, growth control, synthesis of proteins, nucleic acids and phospholipids, and cell division (Harold, 1966). They are able to sequester and release cations (e.g. calcium, magnesium and sodium), whose controlled release regulates the activity of various enzymes. They are also involved in cellular detoxification by complexing metals and cations (Hashemi, Leppard & Kushner, 1994). A reversible transformation between the insoluble and soluble fractions is a fundamental reaction during the mobilization of polyphosphates (Kuhl, 1960). Polyphosphates are cleaved to orthophosphates by various intracellular phosphatases (Harold, 1966). Environmental factors such as pH of the medium, S starvation, N deficiency, irradiance, photoperiod and temperature affect polyphosphate content (Healey, 1973). The phosphate content of the medium, however, has the most striking effect (Aitchison & Butt, 1972).

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Polyphosphate granules (also termed 'volutin' or 'metachromatic' granules/bodies) are a form of P storage and have been thoroughly investigated in bacteria, fungi and unicellular algae (Kulaev & Vagabov, 1983), but very little in macroalgae. Polyphosphates were reported in the green algae *Enteromorpha*, *Cladophora* and *Ulva* (Langen, 1958; Lin, 1977; Lundberg *et al.*, 1989), *Acetabularia* (Niemeyer, 1977), *Codium* (Rutter & Cobb, 1983), and *Prasiola* (Bock *et al.*, 1996), and in a single red seaweed, *Ceramium* (Langen, 1958; Lundberg *et al.*, 1989). However, no mention of a granular structure was made.

To contribute to the understanding of P metabolism in *C. crispus*, and especially of P storage, this study first investigated the distribution of P in different phosphorylated fractions. These chemical analyses were then combined with transmission electron microscopy and energy dispersive X-ray microanalysis to demonstrate the presence of acid-insoluble polyphosphates in this species as cytoplasmic granules and precipitates along the plasmalemma.

MATERIALS AND METHODS

Plant culture

Plants of *Chondrus crispus* were collected in June and October 1994 from mid-littoral tidepools at Maces Bay, in the Bay of Fundy, New Brunswick, Canada. Fresh material was transported to the laboratory and was either used immediately or kept for a few weeks under controlled starvation conditions in a large holding tank (5343 L) until its total P content was between 2·5 and 3 mg P g^{-1} d. wt (Chopin *et al.*, 1995). A photoperiod of 12/12 h L/D per day was provided with a photon irradiance of 100 μmol m^{-2} s^{-1} at the centre of the tank; the sea water was maintained at 13–14 °C.

For the experiment, eight small tanks (50 L), in a temperature- and light-controlled room providing the same conditions as above, were filled with enriched sea water. Phosphorus was supplied (as NaH_2PO_4, H_2O) in one pulse at 15 μmol per tank; N was supplied (as $NaNO_3$) at 25 μmol per tank. In seven of these tanks, 100 g wet weight (w. wt) plants were placed for 3, 6, 9, 12, 15, 24 and 48 h, respectively. In the last tank, two plants for each incubation period were placed in the enriched sea water in reverse order so that all could be collected at the same time to be processed for electron microscopy. These plants were coded with wire of different colours so that those receiving different incubation periods could easily be identified.

Chemical analyses

Triplicate samples of apical parts of plants were taken immediately after collection every second day during the starvation period and at the beginning and end of the experimental incubation in order to determine total tissue P content by the method of Murphy & Riley (1962) after acidic mineralization (H_2SO_4 and HNO_3) in a Büchi 430 digester.

At the end of each treatment period, triplicate samples were frozen in liquid nitrogen. The different phosphorylated fractions were separated by extrac-

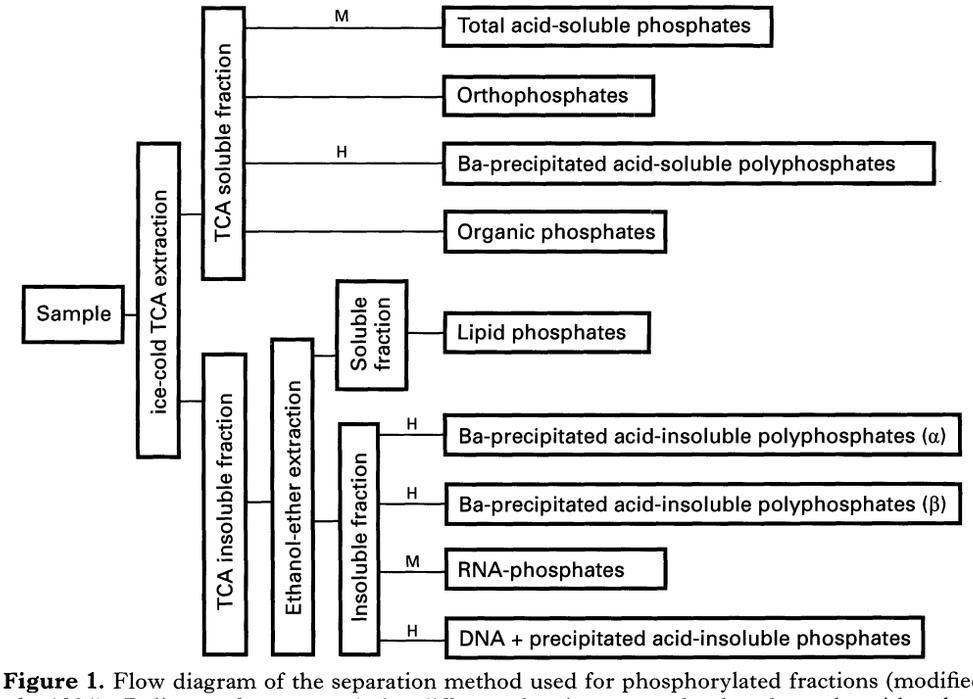

Figure 1. Flow diagram of the separation method used for phosphorylated fractions (modified from Rolin *et al.*, 1984). Rolin *et al.* converted the different fractions to orthophosphates by either hydrolysis (H) or mineralization (M); in this study only hydrolyses were performed.

Table 1. *Contents of different phosphorylated fractions in starved and fresh plants of* Chondrus crispus

Phosphate-containing fractions	Type of plants	Phosphorus content (μg P g^{-1} w. wt) Beginning of experiment		End of experiment
Acid-soluble phosphates				
Total acid-soluble phosphates	Starved		226·94 ± 30·21	
	Fresh		297·36 ± 41·19	
Ba-precipitated acid-soluble polyphosphates	Starved	67·63 ± 9·04		138·36 ± 32·26
	Fresh	84·57 ± 26·68		224·68 ± 22·74
Orthophosphates	Starved	76·34 ± 6·32		116·55 ± 11·24
	Fresh		120·05 ± 18·91	
Acid-insoluble phosphates				
DNA- and precipitated acid-insoluble phosphates	Starved		53·35 ± 10·03	
	Fresh		80·66 ± 14·51	
Ba-precipitated acid-insoluble polyphosphates, method α	Starved		14·63 ± 3·42	
	Fresh		17·04 ± 4·17	
Ba-precipitated acid-insoluble polyphosphates, method β	Starved		19·31 ± 4·65	
	Fresh		24·15 ± 5·81	
Lipid phosphates	Starved	2·89 ± 0·69		0·00 ± 0·00
	Fresh	2·34 ± 1·55		0·02 ± 0·02
RNA-phosphates	Starved		0·12 ± 0·12	
	Fresh		0·07 ± 0·07	

When significant differences ($P \leqslant 0{\cdot}05$) were recorded, means ($n = 6$) ± SD are indicated for the beginning and the end of the experiment. When no significant differences were observed, means ($n = 48$) ± SD, for the eight sampling times combined, are reported in a central column.

tion and precipitation using the modified techniques of Aitchison & Butt (1972) and Rolin, Tacon & Larher (1984). The seaweed material was first ground to frozen powder in liquid nitrogen with a Retsch Vibratory Mixer Mill Type MM-2 (Retsch GmbH, Haan, Germany). The fractions were all converted to orthophosphates by hydrolysis alone instead of by a mixture of mineralization and hydrolysis (Fig. 1). Contents were measured by the method of Murphy & Riley (1962).

The concentrations of dissolved inorganic P (DIP) and dissolved inorganic N (DIN; as the sum of $NH_4^+ + NO_3^- + NO_2^{2-}$) in sea water were measured by the methods of Murphy & Riley (1962) and Grasshoff, Ehrhard & Kranling (1983), respectively, using a Technicon Autoanalyzer II segmented flow analyser (Tarrytown, New York, USA).

Transmission electron microscopy

Apical tissues of *C. crispus* were fixed for 1 h in 5 % (v/v) glutaraldehyde with 0·1 M cacodylate buffer and 0·2 M sucrose, post-fixed for 3 h in 0·1 M cacodylate buffer with 0·7 M sucrose, dehydrated in an ethanol series, and embedded in Spurr's resin (hard type). After 2 d of constant agitation on a rotator, tissues were transferred to specimen blocks; the resin was polymerized at 70 °C for 12 h. Thin sections (80–100 nm) were mounted on 300 mesh copper grids, stained (lead citrate/uranyl acetate) according to the method of Daddow (1983), and observed with a Philips EM 400T transmission electron microscope (TEM).

Energy dispersive X-ray microanalysis

Sections were deposited on 100 mesh copper grids coated with a Formvar resin film, and were either left unstained, or stained only with uranyl acetate. Energy dispersive X-ray analyses were made using a Link eXL microanalyser with a LZ-5 Pentafet windowless detector (Link Systems Ltd., High Wycombe, Bucks, UK) mounted on the Philips EM 400T TEM operated at 60 kV. A spot size of *c.* 200 nm was used, and the beam current was adjusted to give count rates of *c.* 1000 cps.

Statistical analyses for the chemical data

Differences between contents at the different time intervals were tested using Student's *t*-test (Zar, 1984). When examination of the complete data set indicated that no difference occurred between the different times, only the mean content (± SD) for all the times combined is indicated in Table 1. When significant differences ($P \leqslant 0{\cdot}05$) were noted, they were observed only between the beginning (0 h) and the end (48 h) of the experiment and are reported in Table 1. As similar results were obtained with plants from spring and autumn, data were pooled ($n = 6$ for each point).

RESULTS

Tissue total P content

The total P content of both fresh (unstarved) and starved plants remained stable during the 48 h of the

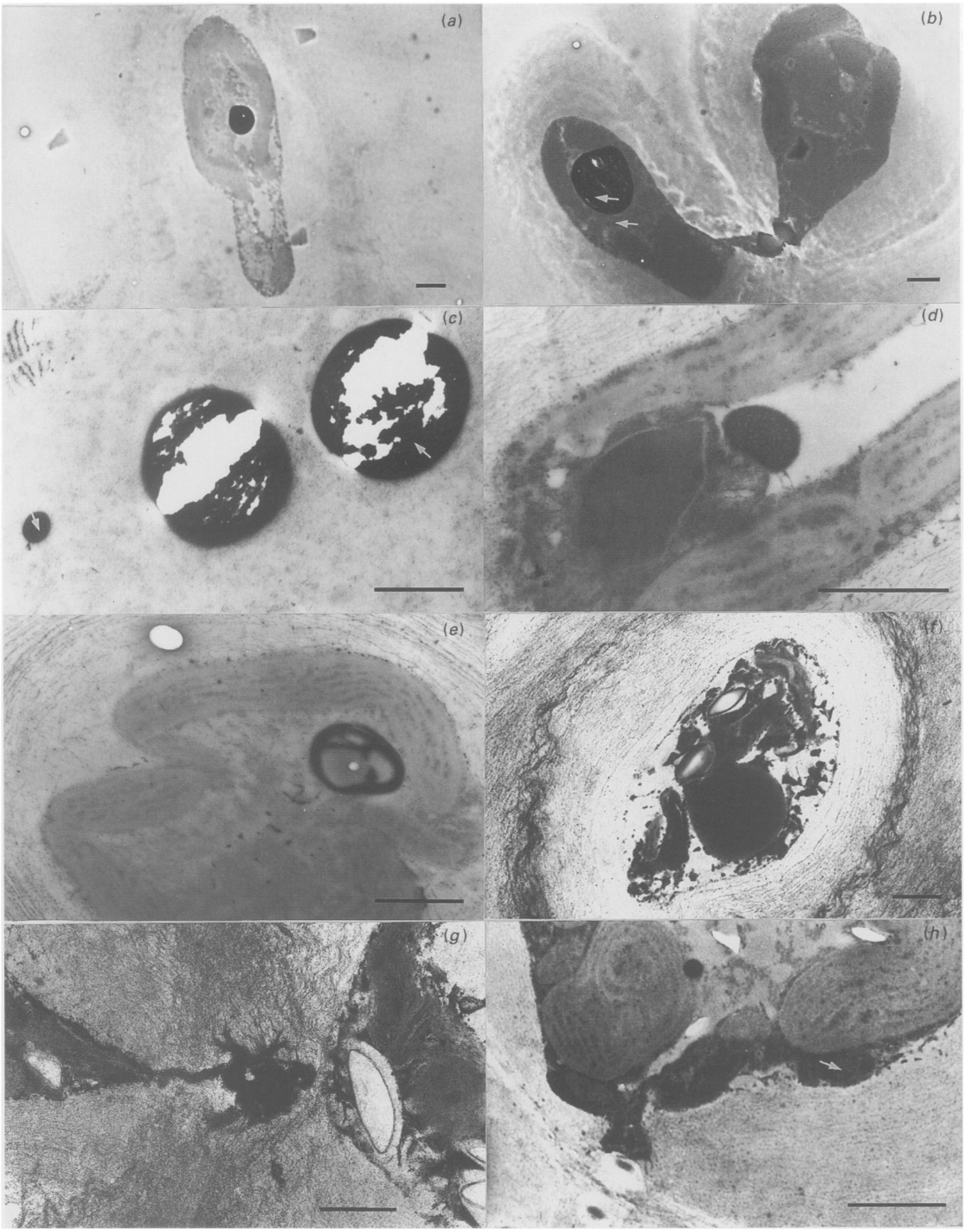

Figure 2. Transmission electron micrographs of cells of *Chondrus crispus* containing polyphosphate granules and precipitates of P. Arrows indicate where energy dispersive X-ray microanalysis was performed. Bar, 1 μm. (*a*) A single polyphosphate granule in a medullary cell of fresh material incubated in enriched sea water for 9 h; single staining (uranyl acetate). (*b*) Two medullary cells, connected by a pit plug, of fresh material incubated for 9 h; no staining. The large polyphosphate granule contained large amounts of P (Fig. 3*b*). (*c*) Medullary cell of fresh material incubated for 6 h with two large damaged and one small dense polyphosphate granules; single staining. (*d*) Medullary cell of fresh material incubated for 9 h with one reticulated polyphosphate granule; single staining. (*e*) Inner cortical cell of starved material incubated for 9 h with one reticulated polyphosphate granule; single staining. (*f*) Medullary cell of starved material incubated for 9 h with one large and uniformly electron-opaque polyphosphate granule; triple staining (lead, uranyl acetate, lead). (*g*) Two

experiment at $3{\cdot}73 \pm 0{\cdot}46$ and $2{\cdot}75 \pm 0{\cdot}20$ mg P g^{-1} d. wt, respectively. The difference between these values reflected the nutrient prehistory of the plants (fresh vs. starved).

To ensure that neither P nor N were limiting factors during the experiment with either fresh or starved plants, DIP and DIN concentrations in sea water were monitored (data not shown). At the end of the experiment, these concentrations were still high ($6{\cdot}8 \pm 0{\cdot}1$ μM P and $19{\cdot}7 \pm 0{\cdot}6$ μM N, respectively).

Phosphorylated fractions

Both fresh and starved plants contained more acid-soluble than acid-insoluble phosphates (Table 1). Acid-soluble phosphates were more abundant in fresh than in starved plants. The contents of acid-soluble polyphosphates in starved and fresh plants were similar at the beginning of the experiment. However, by the end of the experiment, the content had doubled in starved plants and had increased by a factor of 2·7 in fresh plants. The orthophosphate content increased in starved plants to reach a value of $116{\cdot}55 \pm 11{\cdot}24$ μg P g^{-1} w. wt, similar to that in fresh plants, in which it remained stable at $120{\cdot}05 \pm 18{\cdot}91$ μg P g^{-1} w. wt during the entire experiment.

The DNA- and precipitated acid-insoluble phosphates were the largest fraction of the acid-insoluble phosphates and were present in larger quantities in fresh plants. The content of acid-insoluble polyphosphates was lower than that of acid-soluble polyphosphates. In agreement with Rolin *et al.* (1984), less acid-insoluble polyphosphates were found with method α than with method β (see Fig. 1). In both fresh and starved plants, only low contents of lipid and RNA- phosphates were measured. After 48 h of experimental treatment, lipid phosphates had virtually disappeared.

Transmission electron microscopy

Conspicuous large and electron-dense polyphosphate granules were found in sections of fresh and starved plants (Fig. 2*a–f*). Some granules were larger than 2 μm in diameter; they were generally smaller in starved plants. Most of the granules were located in medullary cells, with only a few observed in cortical cells. Their distribution appeared to be random, some cells being devoid of granules, some containing as many as four. The appearance of these granules varied from large and circular (often damaged during sectioning or by the electron beam: Fig. 2*c*), to reticulated (Fig. 2*d*, *e*), and to uniformly electron-opaque (Fig. 2*c*, *f*).

Other P-containing structures (as evidenced below by X-ray microanalysis), found both in fresh and starved plants, were precipitates along the plasmalemma, particularly near the pit plugs (Fig. 2*g*, *h*).

Energy dispersive X-ray microanalysis

Regions of cytoplasm near polyphosphate granules (arrow in Fig. 2*b*) did not show significant peaks for P when subjected to energy dispersive X-ray microanalysis (Fig. 3*a*). Small amounts of copper were often detected because of the proximity of a grid bar. No peak above background was recorded in spectra from the resin (data not shown). When the probe was placed on granules, strong P, Cl, and Ca peaks were detected, confirming the polyphosphate nature of these granules (Fig. 3*b*). In the same cell (Fig. 2*c*), a higher level of P, and associated Ca, was observed in a small dense granule (Fig. 3*c*) than in a dense area of one of the large granules (Fig. 3*d*), inviting caution regarding the use of energy dispersive X-ray microanalysis in a quantitative manner. The uranium peaks were due to the uranyl acetate single staining.

Energy dispersive X-ray spectra from precipitates along the plasmalemma, particularly near the pit plugs (Fig. 3*e*), displayed peaks of P, Si, S, Cl, and K. Peaks of Al, Ca, and Mg were also present in spectra from precipitates of starved plants (data not shown).

DISCUSSION

For the chemical analyses, starved and fresh plants were studied, and the contents of total tissue P and the different phosphorylated fractions were measured over a period of 48 h in order to investigate whether the occurrence of polyphosphates in *C. crispus* could be influenced by the nutritional status of the plants and by the time of incubation in enriched medium. The phenomenon of overcompensation (transient increase of P content immediately after transfer to a P-enriched medium) was not recorded in this pluricellular alga, in contrast with findings with unicellular algae like *Chlorella vulgaris* (Aitchison & Butt, 1972), *Plectonema boryanum* (Sicko-Goad & Jensen, 1976), and *Microcystis aeruginosa* (Jacobson & Halmann, 1982), and the simple multicellular cyanobacterium *Anabaena flos-aquae* (Thompson, Oh & Rhee, 1994). The overcompensation phenomenon is dependent on the orthophosphate concentration of the medium and the duration of P starvation. Previous studies used cells starved for a few days and transferred to media enriched to up to 300 μM P. In our study, plants

medullary cells of starved material incubated for 9 h with P precipitates along the plasmalemma and near the pit plug; triple staining. (*h*) Inner cortical cell of fresh material incubated for 9 h with precipitates along the plasmalemma and near the pit plug, and one polyphosphate granule; single staining.

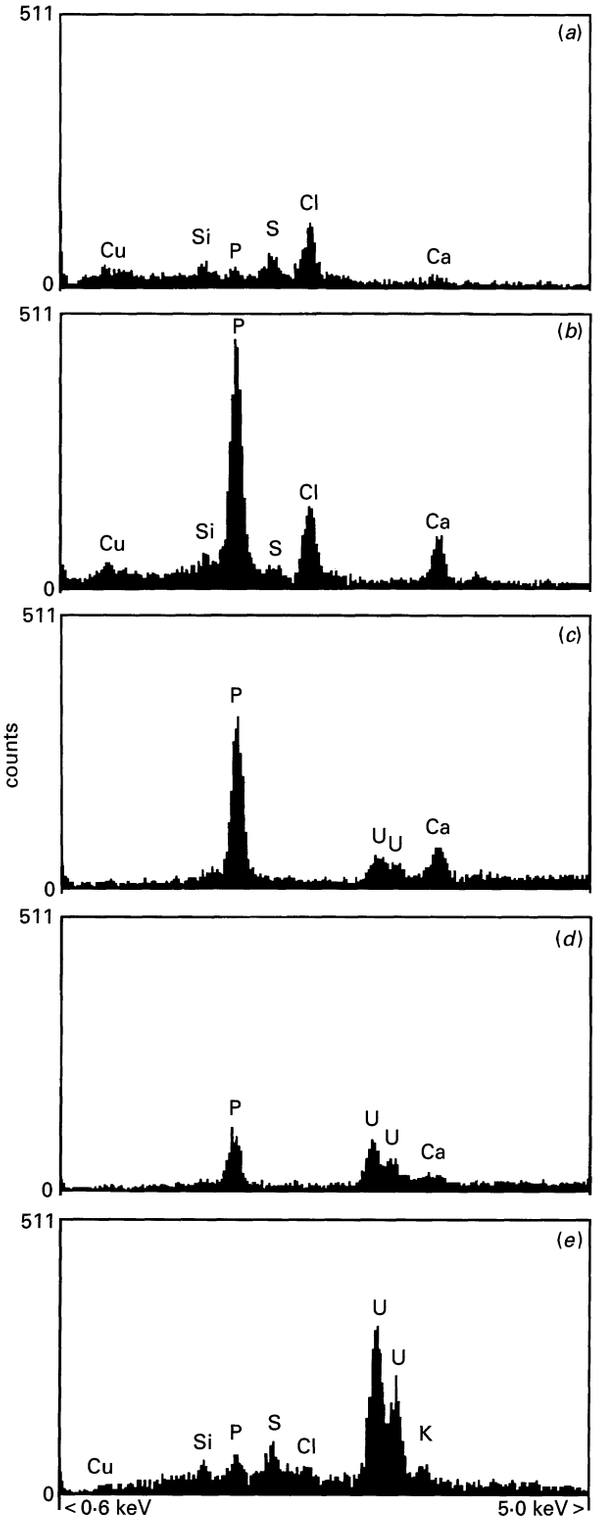

Figure 3. Energy dispersive X-ray spectra from areas indicated on Figure 2. (*a*) Cytoplasm near the polyphosphate granule of Figure 2*b*. (*b*) Polyphosphate granule of Figure 2*b*. (*c*) Small polyphosphate granule of Figure 2*c*. (*d*) Large polyphosphate granule of Figure 2*c*. (*e*) Precipitates along the plasmalemma, near a pit plug, of Figure 2*h*.

were starved for several weeks, because of their large capacity for nutrient storage, and were transferred to sea water enriched by 15 μM P. It is therefore possible that overcompensation could have been observed at higher enrichment. However, in a previous study (Chopin *et al.*, 1995), we demonstrated that, in similar culture conditions, an enrichment of 6 μM P was enough to saturate the total P content of tissues of *C. crispus*.

The nutritional status of the plants appeared to have an impact at several levels. Fresh plants were richer in acid-soluble phosphates than starved plants, mostly because of a much larger acid-soluble polyphosphate fraction, especially at the end of the experiment. Polyphosphate synthesis is an energy-requiring process which could be dependent on the physiological state of the plants. The uptake of orthophosphates in starved plants was stimulated by transfer to P-enriched medium. After 48 h, the orthophosphate pool size of the starved plants had reached that of the fresh plants, which remained constant throughout the experiment, indicating saturation of this pool in plants freshly collected at times of the year when nutrients have not reached limiting levels (Chopin *et al.*, 1995). Concerning acid-insoluble phosphates, the only difference between starved and fresh plants was that the latter were richer in DNA- and precipitated acid-insoluble phosphates.

Chemical analyses showed that acid-soluble and, to a lesser extent, acid-insoluble polyphosphates exist in *C. crispus*. Confirmation of the presence of acid-insoluble polyphosphates, in the form of cytoplasmic granules and precipitates along the plasmalemma, was obtained by combining transmission electron microscopy and energy dispersive X-ray microanalysis with chemical analyses. To our knowledge, this is the first time that such structures have been reported in a red macroalga.

Conspicuous electron-opaque granules were easy to observe. However, their polyphosphate nature, based only on their structure, was first questionable because of their cytoplasmic localization. In bacteria, fungi and unicellular algae, in which polyphosphates have been most studied, these granules are generally seen in vacuoles (Kulaev & Vagabov, 1983). Numerous sections of tissues of *C. crispus* revealed that its cytoplasm is compact and that vacuoles are rare. Confirmation of the polyphosphate nature of these cytoplasmic granules and precipitates along the plasmalemma was provided by energy dispersive X-ray microanalysis. Some ^{31}P-NMR studies (Tijssen & van Steveninck, 1984; Sianoudis *et al.*, 1986; Kjeldstad *et al.*, 1991) presented evidence that some polyphosphates can also occur on the outside of the cytoplasmic membrane. It was not, however, possible to detect these in this study, perhaps because of the techniques used. Histochemical staining methods are not generally able to detect such small amounts of aggregated phosphates. Lead staining results in better visualization of polyphosphates (Tijssen & van Steveninck, 1984), but its use would have

complicated the interpretation of the X-ray spectra by generating a peak close to that of P, possibly masking it. During preparation for conventional electron microscopy, phosphates may also be solubilized and washed out (Tijssen, van Steveninck & De Bruijn, 1985). Moreover, the diameter of the X-ray probe (200 nm) is also a limiting factor for very fine and precise localization.

Quantification of polyphosphate content by electron microscopy and X-ray microanalysis was not possible because of the random distribution of the number and size of the granules and precipitates close to pit plugs. Because sequential sectioning of whole cells was not carried out, a cell apparently without granules could in fact contain some. Moreover, 'small' granules could be tangential sectionings of larger granules. Electron microscopy also revealed non-uniform packing of the granules, which was confirmed by X-ray microanalysis showing a stronger peak of P in a 'small' granule than in a 'large' one.

Peaks of Si, S, Cl, Ca, K, Mg and Al were consistently present in X-ray spectra from both granules and precipitates. These peaks are frequently seen in association with polyphosphates, and the role of the latter as cation- and metal-traps has been cited frequently (Strullu *et al.*, 1982; Lapeyrie, Chilvers & Douglas, 1984). The presence and intensity of these peaks can, however, be influenced by the choice of fixation technique. Baxter & Jensen (1980) reported that glutaraldehyde fixation of cells of *Plectonema boryanum* resulted in loss and underestimation of K and enhancement of Ca. Mg was lost during embedding in epoxy resin; there is, however, to our knowledge, no reference available concerning the impact of Spurr's resin on Mg.

The combination of chemical analyses and electron microscopy, both showing the presence of acid-insoluble polyphosphates, allowed us to confirm that cytoplasmic polyphosphate granules and precipitates near the plasmalemma in *C. crispus* are not an artefact of specimen preparation at early stages of ethanol dehydration, as reported by Orlovich & Ashford (1993) in the more acidic vacuoles of the ectomycorrhizal fungus *Pisolithus tinctorius*. These authors discussed the possibility of P transport, as soluble polyphosphates, along hyphae through the vacuole system, which in *P. tinctorius* is mobile and interconnected via tubular elements (Shepherd, Orlovich & Ashford, 1993). In *C. crispus*, precipitates of P along the plasmalemma were particularly abundant near pit plugs, reinforcing the concept that these wall features, characteristic of the Florideophycidae and sporophytes of the Bangiophycidae, might be involved in intercellular transport (Wetherbee, 1979).

In conclusion, this study showed clearly the existence in *C. crispus* of a significant amount of acid-soluble polyphosphates and acid-insoluble polyphosphates, the latter as cytoplasmic granules and precipitates along the plasmalemma. In 1958, Langen commented that the scale at which polyphosphates are common in macroalgae was not known. Polyphosphates were sought but not found in the green alga *Chara*, and the brown algae *Fucus*, *Ectocarpus*, and *Pilayiella* (Langen, 1958; Lundberg *et al.*, 1989). However, a systematic analysis of polyphosphates in macroalgae is still warranted if we are to understand the uptake, turnover, storage and translocation of P in these organisms, and the ecophysiological influence of biotic and abiotic factors on these mechanisms. The presence of polyphosphate granules in a rhodophycean macrophyte is also of interest from an evolutionary viewpoint of P metabolism because it supports the assertion that high-molecular-weight polyphosphates in primitive organisms were able to fulfil the functions that, in higher plants and animals, are mainly carried out by ATP (Kulaev & Vagabov, 1983).

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