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RETENTION OF P-NITROPHENOL AND 4-METHYLUMBELLIFERONE BY MARINE MACROALGAE AND IMPLICATIONS FOR MEASUREMENT OF ALKALINE PHOSPHATASE ACTIVITY[1]

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ABSTRACT

During standardization of the methodology for estimating "cell-bound" alkaline phosphatase activity (APA: phosphomonoesterase) in Fucus spiralis *L. (Phaeophyta), some of the nonphosphate moiety of the original phosphomonoester was found to be released to the medium subsequent to completion of the routine assays. This occurred with the two substrates generally employed in APA measurements: p-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (MUP). Other marine macrophytes tested showed the same phenomenon. The conditions influencing retention were investigated to establish the simplest procedure for measuring APA. When using pNPP, the release of the product (p-nitrophenol) after the assays was maximum when assays were run for longer than 20 min and at slightly acid pH or at high pNPP concentrations. When using MUP, the leakage of the product (4-methylumbelliferone) after the assays was maximum when APA measurements were run for longer than 40 min and at neutral pH or at high MUP concentrations. The significance of the leakage of the nonphosphate moiety after APA assays is discussed.*

Key index words: 4-methylumbelliferone; alkaline phosphatase; Fucus spiralis; *Phaeophyta; phosphomonesterase; p-nitrophenol; polyphenols*

Phosphorus is probably often a limiting nutrient in shallow marine water ecosystems (Littler et al. 1991), and evidence that this can apply to macroalgae includes measurement of C:N:P tissue ratios (Atkinson and Smith 1983, Hernández et al. 1993). Thus, interest in factors influencing phosphorus availability is increasing. Most of the available phosphate in the sea can sometimes exist as dissolved organic P (Thingstad and Rassoulzadegan 1995), of which phosphomonoesters, the natural substrates of alkaline phosphatase, may be an important component. High phosphatase activity may contribute to the success of macroalgae in some oligotrophic waters (Delgado et al. 1994). The measurement of alkaline phosphatase activity (APA) in marine macrophytes is therefore receiving increased attention (Lapointe et al. 1992, Delgado and Lapointe 1994).

For alkaline phosphatase to be of biological importance to an organism, it must bring about the hydrolysis of organic phosphates in the environment and the incorporation of at least some of the released phosphate into the cell. Such APA has been described as "cell-bound" activity (Whitton et al. 1991), and use of electron microscopy has shown that the enzyme is located in the periplasm of cyanobacteria (Doonan and Jensen 1977). In microalgae the enzyme has been located in the cell wall (Flynn et al. 1986), whereas in macroalgae the situation is less clear-cut. So far, the evidence for the location of the enzyme mostly depends on staining (Gibson and Whitton 1987, Hernández et al. 1994), and the external location is suggested because the phosphomonoesters in the medium can be hydrolyzed. However, in some macroalgae (e.g. the red algae *Gelidium sesquipedale* (Clem.) Born. et Thur. or *Porphyra umbilicalis* (L.) Kütz.), histochemical studies have localized the enzymatic activity at the outer surface of the thallus, in either the cell wall or the periplasmic space (Hernández et al. 1994).

The two most widely used substrates for assays are p-nitrophenyl phosphate (pNPP) and 4-methylumbelliferyl phosphate (MUP), leading to the release of p-nitrophenol (pNP) and 4-methylumbelliferone (MU), respectively. Assays are typically run for periods of 30–120 min using pNPP. pNP is measured spectrophotometrically (McComb et al. 1979). Usually, shorter assay periods are run using MUP, with MU measured fluorometrically (Pettersson 1980). In both cases it is assumed that all of the nonphosphate moiety of the original phosphomonoester is present in the medium when absorbance or fluorescence is measured. Because there is no evidence in the literature of macroalgae to support this and our preliminary studies showed that the assumption was not justified, a more detailed study on possible retention of pNP and MU was conducted using the brown alga *Fucus spiralis,* followed by comparative studies on other macroalgae. The aims were to assess

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the possible errors in previous studies and to recommend simple procedures to minimize errors during routine assays.

MATERIALS AND METHODS

Fucus spiralis thalli were collected from the upper intertidal at Tyne Sands, East Lothian, Scotland (56°0′N, 2°35′W), where the estuary of the (Scottish) River Tyne opens to the North Sea. Accounts of the site, including phosphorus (P) chemistry of the pools into which the terminal parts of the thalli extend, have been given by Khoja et al. (1984) and Yelloly and Whitton (1996). Other algae and a marine angiosperm (Table 1) were collected from the intertidal of St. Mary's Island, N-E. England (55°5′N, 1°27′W).

After collection, plants where transferred to the laboratory in an ice chest. The plants were kept for a maximum of 5 days in aerated cultures at 18° C and under continuous light (20 μmol photons·m^{-2}·s^{-1}) in filtered (Whatman GF/F) seawater offshore from the sampling sites (mean phosphate concentration <1.5 μM). The water was renewed daily.

Before the experiments on *F. spiralis,* 1.5-cm tips (approximately 0.03 g dry weight) were cut and kept for 12 h in aerated, filtered seawater in order to minimize the presence of dissolved polyphenolic compounds (phlorotannins, Yates and Peckol 1993) released when the thalli were cut. Receptacles were not observed during the study period.

Assay procedure. APA was measured using pNPP or MUP as substrate with three replicates in each case. Except for adding another substrate (MUP), the method followed that of Hernández et al. (1994). The tips were incubated in 10 mL assay medium containing 500 μM pNPP or 50 μM MUP as substrate and 50 μM HEPPS-NaOH buffer (Sigma) at pH 8.3. P-free artificial seawater (Woelkerling et al. 1992) of 33‰ salinity was used as a solvent. The tips were incubated in a water bath at 20° C with constant shaking. Standard assays using pNPP were made for 1 h, with absorbance read at 410 nm. Any small effect due to polyphenolics derived from *F. spiralis* (which absorb at 410 nm) was compensated by using controls (plants in medium without pNPP). Another control (assay medium without plant) was run to account for any pNPP hydrolysis not due to the alga. Standard assays using MUP were run for 40 min. Fluorescence was measured using a BA Fluoripoint model instrument with wavelength settings of 356 nm for excitation and 444 nm for emission. Tests with cut thalli showed that there was no leakage of fluorescent materials (such as polyphenolics) at this wavelength. APA was expressed as product released·g^{-1} dry weight (dry wt)·h^{-1}.

Immediately after reading the absorbance, plants were rinsed vigorously in tap water (10 s) and seawater (10 s) and placed in 5 mL assay medium (without substrate), subsequently termed postassay medium. Absorbance or fluorescence was read after 45 min to quantify possible further release of pNP or MU. The rinse in tap water apparently did not cause an osmotic effect, as the amount of product released after the assays was similar when only seawater was used to rinse the thalli. Controls that had not been exposed to substrate were again included to compensate for possible release of polyphenolics.

The influence of substrate incubation time on subsequent leakage was tested using periods of 1–10, 20, 30, 40, 50, and 60 min. After a vigorous rinse (as earlier), the thalli were incubated in the postassay medium to follow the time course of the product release (pNP or MU). The influence of substrate concentration was tested over the range 5–700 μM.

The influence of pH was tested in two ways, during the assay period and during the postassay period. For the former, APA assays were run at pH values over the range 6.0–9.3 using the following buffers: dimethylglutaric acid–NaOH (pH 6.0–7.3), EPPS (*N*-[2-hydroxyethyl] piperazine-*N′*-[3-propanesulfonic acid])–NaOH (pH 7.5–8.5), and glycine–NaOH (pH 8.8–9.3).

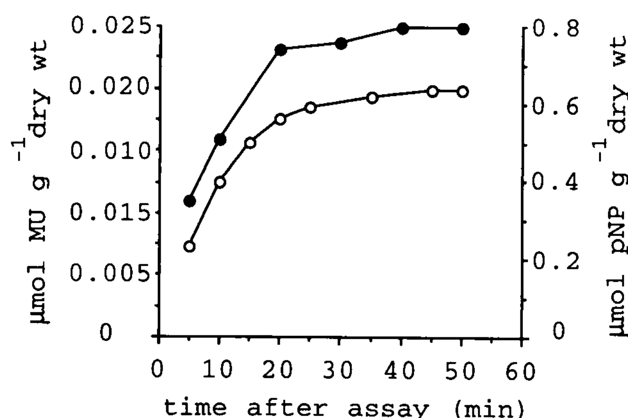

FIG. 1. Time course of release of product (nonphosphate moiety) by *Fucus spiralis* tips after completion of a standard phosphatase assay. pNP (●), MU (○).

Tips were then placed in a postassay medium at the same pH as used for the assay. The second approach involved measurement of APA at a fixed pH (8.3) and then testing the effects of postassay media at pH values from 6.0 to 9.3.

To test whether retarded release of pNP or MU involves its binding to the cell wall, *F. spiralis* tips were incubated for 1 h in artificial seawater containing 50 μM EPPS–NaOH, pH 8.3, and 20 μM pNP or 2 μM MU, and the time course of absorbance (410 nm) or fluorescence was followed. Immediately after a vigorous rinse (as earlier), the tips were placed in postassay medium, and any increase in absorbance or fluorescence again was followed.

Other plants. The procedures for preparation of the other marine macrophytes tested were similar, apart from slight differences in the length of the terminal portion of alga selected; in the case of *Zostera marina,* the terminal 3 cm (approximately 0.036 g dry wt) of young leaves was used.

Statistical analysis. Differences between more than two means were analyzed by a single-factor analysis of variance. Groups of means were compared by a sum of squares, simultaneous test procedure (SS-STP, Fry 1993). Comparisons between two linear regression equations were performed by a test of comparison of slopes and elevations (Zar 1984).

RESULTS

Similar patterns of release of the nonphosphate moiety of pNPP and MUP were observed after completion of the standard assay and transfer to postassay medium (Fig. 1). The leak was detectable for periods up to 30–40 min. Expressed as a percentage of nonphosphate moiety released at the end of the standard assay, pNP released after the assay was 12%, compared to 15% for MU.

The pNP released after the APA assay apparently arose from pNPP bound inside or on the cells or from pNPP temporarily retained inside or on the cells following hydrolysis, because negligible pNP was released if plants had previously been incubated in 20 μM pNP solution (data not shown). Furthermore, the pNP spectrum showed no qualitative or quantitative change after 1 h incubation of the algae in the pNP solution. Similarly, negligible MU was released if plants had previously been incubated in 2 μM MU solution (data not shown).

Leakage of the nonphosphate moiety depended

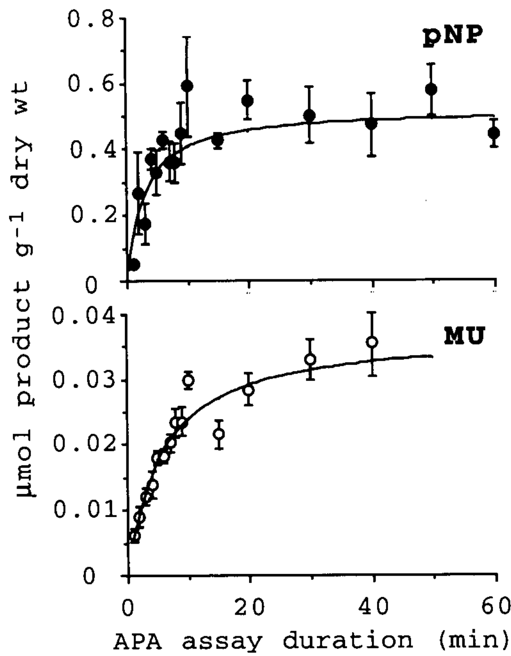

FIG. 2. Amount of product (nonphosphate moiety) released by *Fucus spiralis* tips during a 45-min period following phosphatase assays carried out for different times. In both figures, data were fitted by a saturation curve. Values represent means (n = 3) ± SD.

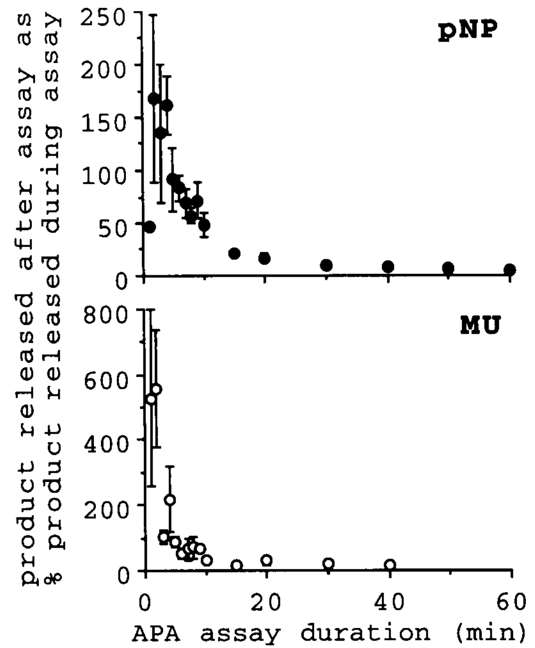

FIG. 3. Changes in product (nonphosphate moiety) released from *Fucus spiralis* tips after the standard phosphatase assay expressed as percentage of product released during the postassay period. Values represent means (n = 3) ± SD.

markedly on the duration of the previous APA assay (Fig. 2). The amount of pNP released after assays was saturated following assay of greater than 20 min duration. In the case of MU, saturation was reached when previous assays were run for approximately 40 min.

The significance of this further release of product can be evaluated if the product released in the post-assay medium is compared with the product released during the phosphatase assays (Fig. 3). When APA assays were carried out for short periods (<5 min), the total product released after the assays was greater than the reaction product released during the assay (up to 3-fold greater for MU). APA was markedly underestimated using short assay periods (<20 min), but the effect of postassay release became less important the longer the period of incubation (Fig. 3). When APA was assayed for 40 min, pNP and MU released after the assay were only 7.5 and 15%, respectively, of the values released during the assay. By 2 h, the values had become negligible (<3%, data not shown).

The pNP leaking from *Fucus* was constant (mean 0.1 μmol pNP$\cdot$g^{-1} dry wt) using low previous pNPP concentrations but gradually became more important at higher pNPP concentrations (Fig. 4). In contrast, the leakage of MU after the assays was directly related to the MUP concentration in the assay. The percentage of pNP released after the assay to pNP released during the assay was quite low at all substrate concentrations using the standard assay period (1 h, Fig. 5). The maximum (20%) was observed at the lowest pNPP concentration assayed (5 μM). The minimum percentage (<5%) occurred between 50 and 125 μM, with a gradual increase at higher

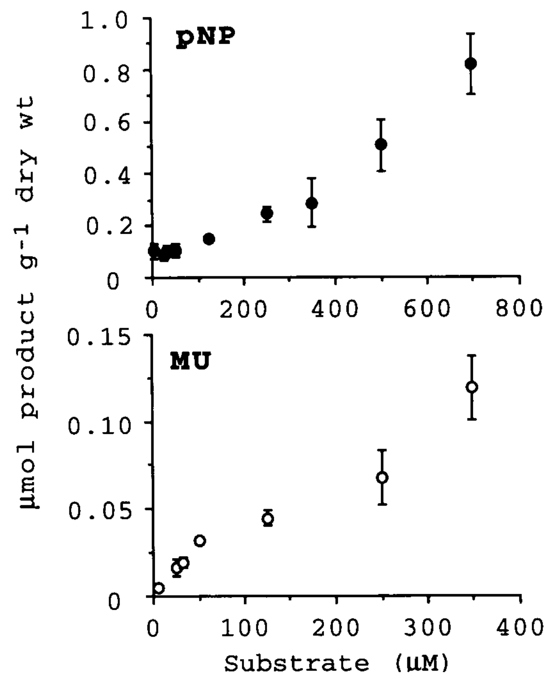

FIG. 4. Amount of product (nonphosphate moiety) released by *Fucus spiralis* tips during a 45-min period following standard phosphatase assays (60-min incubation period when using pNPP and 40 min when using MUP) at different substrate concentrations. Values represent means (n = 3) ± SD.

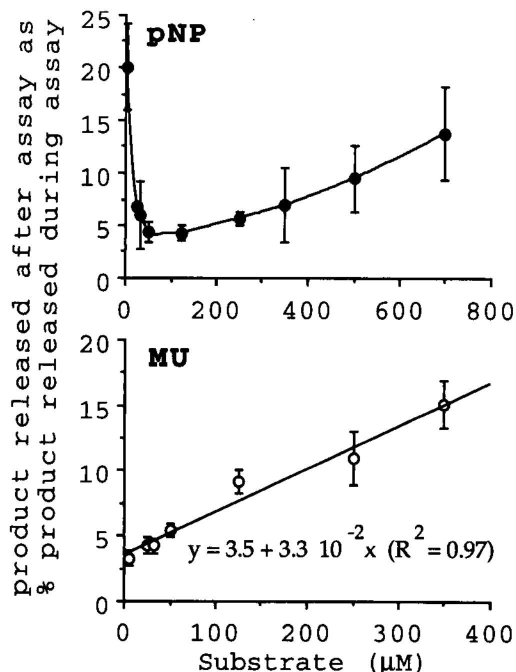

FIG. 5. Changes in product (nonphosphate moiety) released by *Fucus spiralis* tips after phosphatase assays testing different substrate concentrations expressed as percentage of product released during the assay.

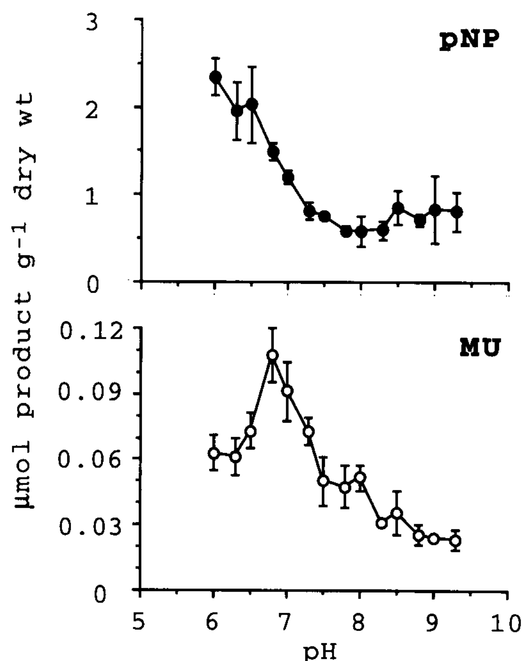

FIG. 6. Amount of product (nonphosphate moiety) released by *Fucus spiralis* tips during a 45-min period following a standard phosphatase assay (60-min incubation period when using pNPP and 40 min when using MUP) when both the assay medium and the postassay medium were prepared at the same pH. Values represent means (n = 3) ± SD.

pNPP levels. In contrast to pNP, the percentage of MU released after the assay to MU released during the assay showed a direct relationship to MUP concentration during the assays (Fig. 5).

The release of the nonphosphate moiety after the APA assay was also influenced markedly by pH. The highest leakage of pNP after the phosphatase assays was at pH 6–6.5 (Fig. 6). pNP release per unit dry weight decreased with rise in pH, reaching a constant value above pH 7.3. MU release per unit dry wt was maximum at neutral pH (Fig. 6). However, the product leakage after the APA assays caused a small change in the pH profile of APA. The inclusion of product released after the assay caused relatively minor changes in the pH spectrum, the effect being most obvious at the lower pH values tested (data not shown).

The ratio of product released after the assay to product released during the assay increased at low pH, causing a clear underestimate of APA at low pH (Fig. 7). At pH 6–6.5, more pNP was released after the assay than during the assay. Both pNP and MUP showed a continuous decrease in the percentage value with rise in pH, being <15% at pH > 8.3.

The influence of pH during the postassay period was tested on algae that were incubated under standard conditions (pH 8.3, etc.). The values for pNP and MU release at any particular pH were significantly correlated ($P < 0.05$), although the absolute values for the former were about 10 times those for

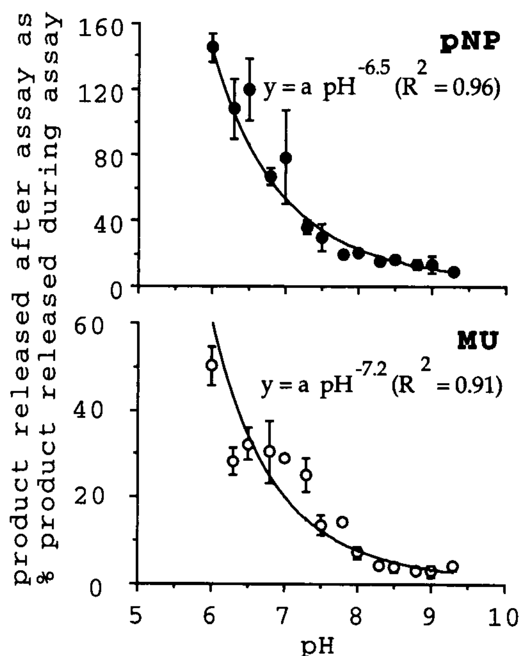

FIG. 7. Changes in product (nonphosphate moiety) released by *Fucus spiralis* tips after the standard phosphatase assay expressed as percentage of product released during the assay when both media were prepared at the same pH. Data were fitted to an inverse exponential curve (percentage = $a \cdot pH^{-b}$; *a*, *b*, constants). Values represent means (n = 3) ± SD.

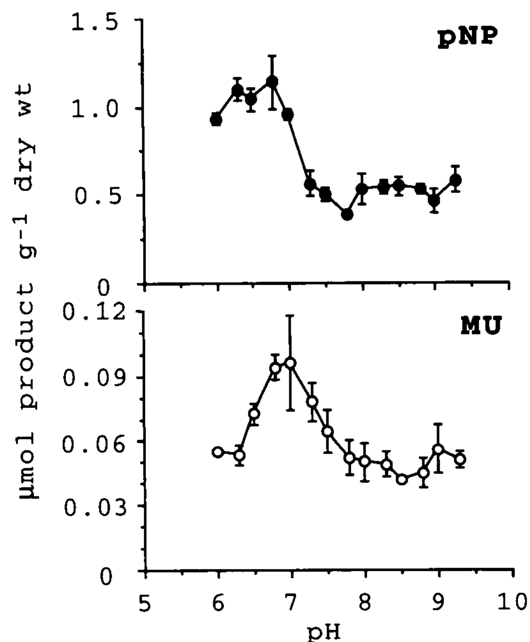

FIG. 8. Influence of pH on amount of product (nonphosphate moiety) released by *Fucus spiralis* tips during a 45-min period following a standard phosphatase assay (60-min incubation period when using pNPP and 40 min when using MUP) at pH 8.3. Values represent means (n = 3) ± SD.

the latter (Fig. 8). The results in this figure suggest that more product is retained at alkaline than neutral or slightly acid pH. If it is assumed that all the product still retained in the plants was released when maximum leakage was observed, about 0.5 μmol pNP$\cdot$g^{-1} dry wt or 30 nmol MU$\cdot$g^{-1} dry wt are retained and not accounted for when assays are performed at the typical seawater pH. These values are 1 order of magnitude lower than the product released during assay for 1 h. Figure 8 also raises the possibility that complete release of the nonphosphate moiety may not be observed under any pH.

Leakage of pNP or MU during the postassay period was also observed in nine other algae and the leaves of a marine angiosperm (Table 1). The significance of the postincubation release of product varied between species. The value was >10% with one or both substrates for five species (including *F. spiralis*) and >20% for three species. There was no obvious relationship to taxonomic group. For instance, retention of the product was negligible in *Cladophora rupestris* (L) Kütz. but high in another green alga, *Monostroma grevillei* (Thur.) Wittrock.

DISCUSSION

These results show that APA of *Fucus spiralis* and some other algae may be underestimated markedly due to the release of the nonphosphate moiety of the hydrolysis product after the standard assay has been completed. This occurs with both pNPP and MUP. The data show that factors influencing the release of pNP and MU are similar. This applies to the responses to time, high substrate concentration, and pH, but the response to a marked reduction in substrate concentration appears to differ between the two (Fig. 5).

The least suitable conditions include short assays (<15 min), high substrate concentration, and, in the case of pNPP, also a low concentration (i.e. low for this substrate: <25 μM). Such conditions have been used in some previous APA studies, such as substrate concentrations greater than 2 mM (Lubián et al. 1992), short incubation times (Hino 1988, Uchida 1992), or low pNPP concentration (Jones 1972). The present work suggests that the problem of product release subsequent to the APA assay should be considered if the studies deal with eukaryotes. If critical comparisons are to be made of a range of species, as done by Lapointe et al. (1992) and Hernández et al. (1994), it is especially important to consider this source of possible error.

In the case of *F. spiralis*, we suggest that the fol-

TABLE 1. *Amount of product (nonphosphate moiety) released during and after a standard phosphatase assay by different marine macrophytes. Assays were run for 60 min when using pNPP and 40 min when using MUP. The product released after the standard phosphatase assay is also given as percentage of product released during the assay. Values are means of three replicates ± SD. P, Phaeophyta; C, Chlorophyta; R, Rhodophyta; SG, seagrass.*

	MU			pNP		
	µmol·g⁻¹ dry wt			µmol·g⁻¹ dry wt		
Species	During assay	After assay	%	During assay	After assay	%
Fucus spiralis (P)	0.13 ± 0.02	0.02	14.9 ± 1.7	8.3 ± 0.04	0.80 ± 0.06	9.6 ± 0.52
Halidrys siliquosa (P)	0.75 ± 0.14	0.07 ± 0.003	9.7 ± 1.3	12.2 ± 2.7	0.20 ± 0.01	1.8 ± 0.33
Pelvetia canaliculata (P)	1.2 ± 0.12	0.02	1.9 ± 0.16	51 ± 3.2	3.6 ± 0.13	7.2 ± 0.96
Cladophora rupestris (C)	0.51 ± 0.01	0.001	0.16 ± 0.03	19.8 ± 1.2	0.15 ± 0.06	0.76 ± 0.34
Monostroma grevillei (C)	0.34 ± 0.03	0.08 ± 0.01	25.2 ± 3.1	2.3 ± 0.55	0.65 ± 0.31	27.0 ± 7.1
Corallina elongata (R)	0.22 ± 0.01	0.004 ± 0.001	1.8 ± 0.07	1.0 ± 0.10	0.03 ± 0.01	3.1 ± 0.68
Porphyra umbilicalis (R)	1.4 ± 0.08	0.02	1.1 ± 0.12	42.2 ± 3.2	0.14 ± 0.03	0.33 ± 0.01
Mastocarpus stellatus (R)	0.40 ± 0.01	0.03	7.2 ± 0.61	20.5 ± 1.3	1.26 ± 0.05	6.0 ± 0.52
Dumontia consorta (R)	0.47 ± 0.07	0.08 ± 0.01	16.3 ± 0.25	1.7 ± 0.51	0.51 ± 0.14	30.7 ± 1.6
Laurencia pinnatifida (R)	1.0 ± 0.18	0.04 ± 0.002	4.2 ± 0.56	1.5 ± 0.04	0.33 ± 0.08	22.2 ± 6.6
Zostera marina (SG)	0.55 ± 0.13	0.03	5.4 ± 1.2	11.8 ± 1.8	0.36 ± 0.09	3.0 ± 0.48

lowing are suitable conditions for standard assays: 20° C (or field temperature), 60 min (pNPP) or 30 min (MUP), 250 μM (pNPP) or 50 μM (MUP), pH 8.3 (or field pH). These substrate concentrations correspond to about 75% maximum velocity. With 5 μM MUP, less than 10% substrate was hydrolyzed during the assay and the percentage of postassay product to product released during the standard assay was very low (Fig. 5: 4%). This will minimize the effects of product inhibition or substrate depletion (McComb et al. 1979). However, in the case of pNPP, more than 10% was hydrolyzed when the substrate concentration was less than 125 μM, and with low (<25 μM) pNPP concentration a much higher percentage (20%) of postassay product was released. Therefore, where substrate concentrations nearer to those occurring in most natural environments are needed, we recommend that MUP be used. Assays with the two substrates, however, do not always give the same results (Yelloly and Whitton 1996), suggesting that more than one enzyme may be involved in hydrolysis.

There are several possible explanations for release of some of the nonphosphate moiety of an organic phosphate molecule after the assay. If hydrolysis takes place inside the cell (whether or not in addition to hydrolysis in the cell wall), the product released inside the cell might move to the exterior only slowly, perhaps even requiring active transport. However, staining in macroalgae does not suggest an intracellular location or internal hydrolysis when plants are exposed to phosphomonoesters (Hernández et al. 1994). It seems unlikely that simple adsorption of the product to the cell wall can account for postassay release, because there was no evidence for binding of pNP or MU when they were added to medium. We cannot rule out the possibility that some substrate hydrolysis takes place after the algae were washed, this substrate either being inside the cell or firmly bound to the wall. However, incubations at low substrate concentration (similar to the ones produced by the amount of product released after the assays) showed slow linear increases in absorbance or fluorescence for at least 2 h (data not shown), whereas the release of product after a standard phosphatase assay occurred rapidly and in a short time (Fig. 1). Finally, *Fucus*, like other macroalgae, has a mucilaginous cuticle covering the epidermal layer of cells (Lee 1992), which may contribute to the short-term retention of the product after hydrolysis has taken place. This cuticle comprises polysaccharide moieties that possess abundant carboxyl and hydroxyl groups, which act as binding ligands for dissolved compounds or retain their diffusion (Decho 1990). More work is needed, as the experiments presented here do not permit a satisfactory explanation of the results observed. In any case, and whatever the possible explanation, the effect of the postassay product release becomes negligible when the preceding recommendations are followed.

Studies using phytoplankton and radiolabeled glucose-6-phosphate (Taft et al. 1977, Heath and Edinger 1990) have shown that glucose (the nonphosphate moiety) was accumulated much more slowly than the phosphate, suggesting that in this case hydrolysis took place outside the cell, followed by subsequent uptake of phosphate. Parallel studies using radiolabeled substrates are needed to explain fully the phenomenon described here; for instance, is pNP accumulated by the cells or is it trapped in the cell wall?

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PHYSIOLOGICAL RESPONSES TO PHOSPHORUS LIMITATION IN BATCH AND STEADY-STATE CULTURES OF *DUNALIELLA TERTIOLECTA* (CHLOROPHYTA): A UNIQUE STRESS PROTEIN AS AN INDICATOR OF PHOSPHATE DEFICIENCY[1]

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ABSTRACT

A protein unique to phosphorus stress observed in Dunaliella tertiolecta *Butcher was studied in the context of phosphate-limited cell physiology and is a potential diagnostic indicator of phosphate deficiency in this alga. Cells were grown over a range of limited, steady-state growth rates and at maximum (replete) and zero (phosphate-starved) growth rates. The stress protein, absent in nutrient-replete cells, was produced under all steady-state phosphate-limited conditions and increased in abundance with increasing limitation (decreasing growth rate). Cellular carbon : phosphorus ratios and the maximum uptake rate of phosphate (V_m) increased with increasing limitation, whereas the ratio of chlorophyll* a *: carbon decreased. Alkaline phosphatase activity did not respond to limitation but was measurable in starved, stationary-phase cells. F_v/F_m, a measure of photochemical efficiency, was a nonlinear, saturating function of μ, as commonly observed under N limitation. The maximum F_v/F_m of 0.64 was measured in nutrient-replete cells growing at μ_{max}, and a value of zero was measured in stationary-phase starved cells. When*

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