Measurement of internal pH in the coccolithophore *Emiliania huxleyi* using 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein acetoxy methyl ester and digital imaging microscopy

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Abstract. Internal pH (pHi) was determined in *Emiliania huxleyi* (Lohmann) using the probe 2',7'-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein acetoxy methyl ester (BCEF-AM) and digital imaging microscopy. The probe BCEF-AM was taken up and hydrolysed to the free acid by the cells. A linear relationship was established between pHi and the 490/450 fluorescence ratio of BCEF-AM over the pH range 6.0 to 8.0 using the ionophore nigericin. Two distinct pH domains were identified within the cell, the cytoplasmic domain (approx. pH 7.0) and the chloroplast domain (approx. pH 8.0). The average pHi was 7.29 (+0.11) for cells in the presence of 2 mM HCO₃⁻. In the absence of HCO₃⁻ the pH was decreased by 0.8 pH unit. The importance of these changes in pHi is considered in relation to inorganic-carbon uptake.

Key words: 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein – Digital imaging microscopy – *Emiliania* (coccolithophorid) – Intracellular pH

Introduction

Coccolithophores are marine unicellular algae that produce elaborate structures, the so-called coccoliths consisting of calcite. They have constituted a large proportion of the marine phytoplankton in recent and Paleozoic times (for a review, see Paasche 1968). Of the coccolithophorids, *Emiliania huxleyi* is the widest ranging and most ubiquitous species, often comprising 50% of the coccolithophorid population in tropical waters and nearly 100% in sub-arctic and sub-antarctic waters (McIntyre and Bé 1967). The coccolithophorids contribute a major proportion of total oceanic productivity (Holligan et al. 1983; Pentecost 1985), while the coccoliths are a major vehicle for the transport of carbon from the photic zone into marine sediments (Pentecost 1985).

Some marine microalgae can use bicarbonate, in addition to CO₂, as an exogenous carbon source for photosynthesis (Colman and Gehl 1983; Rees 1984; Patel and Merrett 1986; Dixon et al. 1987). In these algae, intracellular pH (pHi) will be important in determining the CO₂/bicarbonate equilibrium. Several methods have been used for the determination of intracellular pH, the most widely used being the distribution of the weak acid 5,5-dimethyl-2-[¹⁴C]oxazolidine-2,4-dione (DMO) between the cell and external medium (Beardall 1981; Beardall and Raven 1981; Beardall and Raven 1981; Trombulla 1983). This method has numerous disadvantages (see Roos and Boron 1981), including the necessity for destruction of the cells, the pH determined can only be an average value and only measurements of steady-state conditions or relatively slow pH transients can be made.

Recently, methods have been developed for the measurement of cytoplasmic calcium in plant and animal cells using fluorescent indicator dyes (Gryniewicz et al. 1985; Bush and Jones 1987; Brownlee and Pulford 1988; Clarkson et al. 1988) and similar methods have been used to measure intracellular pH in animal cells (Rink et al. 1982; Bright et al. 1987; Paradiso et al. 1987). In this paper we report on the determination of intracellu-
lar pH in *Emiliania huxleyi*, using the pH-sensitive fluorescent indicator dye 2',7-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein acetoxy-methyl-ester (BCECF-AM), in conjunction with dual-wavelength fluorescence microscopy and digital image analysis. The fluorescence properties of BCECF are such that the ratio of fluorescence (measured at 500 nm) following excitation at 490 and 450 nm is pH-dependent. The $pK_a$ of the dye is around 6.97 which makes it ideal for the measurement of intracellular pH (*Rink et al. 1982*). As far as we are aware this is the first measurement of intracellular pH in a plant cell by this method. The technique has the advantage that the spatial and temporal variation in pH of a single cell can be measured.

**Material and methods**

**Growth of cells.** Axenic cultures of *Emiliania huxleyi* (Lohmann) (Scottish Marine Biological Association; No. 279) were grown on ASP-2 medium (Provasoli et al. 1957) with the NaCl concentration decreased to 30 mM and buffered with glycyglycine $(1 \times 10^{-4} \text{ M})$ at pH 8. Cultures were grown at $20 \degree \text{C}$ $(\pm 1 \degree \text{C})$ on a 12/12 h light/dark cycle with a photon flux density of approx. 200 $\mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Carbon-starved cells were obtained by closing the chamber and allowing the $pH$ of a single cell to be corrected.

**Measurement of photosynthetic oxygen evolution.** Cells were harvested by centrifugation (500 g, 1 min), washed twice and resuspended in either full ASP-2 medium or 500 mM mannitol, 25 mM $4$-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and 30 mM NaCl at pH 8 to a final cell number of between $10^5$ to $10^7$ cells $\cdot \text{ml}^{-1}$.

Oxygen evolution was measured using a Clark-type oxygen electrode (Hansatech, King’s Lynn, Norfolk, UK). A 1-m1 volume of cell suspension was incubated at $20 \degree \text{C}$ with a photon flux density of approx. 200 $\mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Carbon-starved cells were obtained by closing the chamber and allowing the cells to deplete the endogenous carbon sources as determined by the cessation of $\text{O}_2$ evolution. Oxidation was measured using a Clark-type oxygen electrode (Hansatech, King’s Lynn, Norfolk, UK). A 1-m1 volume of cell suspension was incubated at $20 \degree \text{C}$ with a photon flux density of approx. 200 $\mu \text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Carbon-starved cells were obtained by closing the chamber and allowing the cells to deplete the endogenous carbon sources as determined by the cessation of $\text{O}_2$ evolution.

**Spectrofluorimetry.** Cell suspensions containing approx. $10^6$ to $10^7$ cells $\cdot \text{ml}^{-1}$ were transferred to 1-cm path-length quartz cuvettes. Fluorescence excitation was scanned from 400 to 550 nm with emission at 540 nm in a Perkin-Elmer (Beaconsfield, Buck., UK) 3000 fluorescence spectrometer. Spectra were obtained for cell suspensions before and after loading with 5 $\mu$M (final concentration) BCECF-AM (Molecular Probes Inc., Eugene, Oreg., USA).

This was obtained from a 1-m1 stock solution in pure dimethyl sulfoxide (DMSO). After 2-3 h with the dye, cells were centrifuged, washed three times in ASP-2 medium and resuspended in distilled water. The excitation spectrum of these washed cells was then obtained. Excitation spectra of the free acid and its acetoxy-methyl-ester (final concentration 5 $\mu$M) were also obtained in distilled water. Time-course measurements of BCECF-AM (final concentration 5 $\mu$ M) hydrolysis by intact, heat-treated $(80 \degree \text{C}, 30 \text{ min})$ or lysed cells $(0.1 \% \text{ w/v} \text{ Triton X-100})$ (octylphenoxypolyethoxyethanol) were determined using the fluorescence spectrometer. Fluorescence measurements (excitation 480 nm; emission 540 nm) were taken at frequent intervals over a period of 2 h.

**Fluorescence microscopy.** A small volume of cell suspension was settled in a glass cavity microscope slide and observed at $x$ 400 or 1000 magnification with a Leitz (Luton, Beds., UK) fluorescence microscope modified for dual-wavelength excitation. Excitation was at 490 and 450 nm obtained with a 200-W mercury-vapour lamp and line filters (bandwidth 8 nm, Schott, Stafford, UK). Filters were changed manually. Emitted light was passed through a 500-nm band filter (bandwidth 50 nm; Schott). Fluorescence of single cells was recorded with an image-intensified Newvicon CCTV camera (Panasonic, Osaka, Japan) operated in manual gain mode (Brownlee et al. 1987). Images were stored on videotape. Excitation time was kept to <5 s to minimize dye bleaching which was estimated to occur at approx. 3% s$^{-1}$.

**Analysis of fluorescent images.** Images from videotape were digitized using digital image analyser (Kontron, Munich, FRG) with IBAS (Kontron, Munich, FRG) software, giving a resolution of 512 x 512 pixels with 256 grey values. At least six frames per image were averaged to reduce noise and show out temporal variations in lamp output. Autofluorescence images were subtracted where necessary and the resulting 490-nm and 450-nm images were divided pixel by pixel to give 490/450-nm-ratio images (*Bright et al. 1987*). The resulting ratio image was multiplied by a constant factor such that a ratio of 1.0 gave a grey level of 30. Quantitative 490/450-nm ratios along any chosen profile were obtained. The software allows any slight misalignments of 490- or 450-nm images caused by movements of individual cells to be corrected.

**Calibration of ratio images.** In-vitro and in-situ standard curves were obtained in medium containing 100 mM KCl, 30 mM NaCl, 500 mM mannitol, 25 mM 2-(N-morpholino)ethanesulfonic acid (Mes) and 25 mM Heps. For the in-vitro standard curve 490/450-nm fluorescence ratios were obtained using the digital image analyser as described above for small volumes of the above buffers ranging from pH 6 to 8 containing 5 $\mu$M BCECF – free acid on a glass cavity microscope slide. A 5-mm-diameter pinhole was inserted in front of the image-intensified camera to produce a bright spot on the TV monitor during excitation.

The in-situ standard curve was prepared using the same buffers as for the in-vitro curve containing BCECF-loaded cells, and 10 $\mu$g ml$^{-1}$ nigericin (Sigma Chemical Co., Poole, Dorset, UK) (*Bright et al. 1987*). The nigericin was prepared as a stock solution (10 mg ml$^{-1}$) in 100% ethanol and stored at $-20 \degree \text{C}$. The nigericin was added 5 min before measurement of the fluorescence. Longer periods of incubation with the nigericin led to a loss of dye from the cells.

**Electron microscopy.** The cells were pelleted by centrifugation and fixed for 2 h at 4 $\degree \text{C}$ in 2% glutaraldehyde in 0.2 M 1,4-piperazinediethanesulfonic acid (Pipes) buffer, pH 7.7, containing 0.5 M sucrose. After three washes in buffered sucrose, the cells were post-fixed in 2% OsO$_4$ in Pipes buffer, pH 7.7, dehydrated in an alcohol series, and embedded in LR White acrylic resin (London Resin Co., Woking, Surrey, UK). Sections were stained with uranyl acetate and lead citrate, and examined in a JEOL (Tokyo, Japan) 1200 EX electron microscope at 80 kV.

**Results**

**Uptake and hydrolysis of BCECF-AM by *E. huxleyi*.** Suspenions of cells incubated with BCECF-