

Light driven lipid peroxidation of coral membranes and a suggested role in calcification

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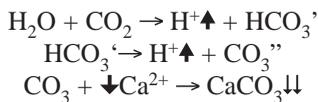
Abstract: Low concentrations of calcium ions (Ca^{2+}) are maintained in living cells by the Ca-ATPase calcium pump of the plasma membrane. The presence of a calcium pump in corals has been confirmed and its temperature related breakdown underlies coral bleaching. There is also mounting evidence from stable calcium isotope ratios in the skeleton for involvement of a calcium pump in coral calcification. Hydrogen peroxide (H_2O_2) is known to be generated by zooxanthellae during photosynthesis and passes easily through cell membranes. H_2O_2 is known to cause lipid peroxidation of the plasma membrane and make it leaky to Ca^{2+} . In this study lipid peroxidation was measured in the tissues of *Agaricia agaricites*. Lipid peroxides in the light doubled from 5.83 to 11.23 $\mu\text{Mol.cm}^{-2}$ ($n=17$) over the first 3-4 hours then slowly decreased over the next four hours. Glutathione, which in many organisms acts as a lipid peroxidation repair system, was also found at a mean level of 79 $\mu\text{g.cm}^{-2}$ ($n=14$) and probably accounts for the observed reduction of lipid peroxide levels. It is suggested that the lipid peroxidation makes the plasma membrane leaky to Ca^{2+} and thus provides a route by which Ca^{2+} enters cells of the calcicoblastic layer. A model of calcification, following Adkins *et al.* (2003), is presented in which calcification takes place from the extracellular calcifying fluid (ECF) between the calcicoblastic layer and the skeleton. The Ca-ATPase calcium pump drives calcification by actively transporting Ca^{2+} into the ECF and setting up pH and carbon dioxide (CO_2) gradients that enhance the passive diffusion of CO_2 and the formation of calcium carbonate. It is suggested that, in light, H_2O_2 produced by zooxanthellae makes the membranes leaky to Ca^{2+} . The calcium pump has to work harder to maintain low internal levels of calcium and thus more Ca^{2+} would be actively transported into the ECF to be deposited as calcium carbonate. Rev. Biol. Trop. 56 (Suppl. 1): 1-9. Epub 2008 May 30.

Key words: coral calcification, Ca-ATPase calcium pump, hydrogen peroxide, lipid peroxidation.

Calcium carbonate is generally saturated in seawater but the mechanism by which it is laid down in the skeleton of scleratinian corals is not completely understood. Calcification takes place from the extracellular calcifying fluid (ECF) in the space between the skeleton and the coral's calcicoblastic membrane (McConnaughey 1989, Adkins *et al.* 2003). Carbon dioxide, either metabolic in origin or from seawater, is able to diffuse from the calcicoblastic layer into the ECF to provide a source for carbonate. Charged calcium ions (Ca^{2+}), to which membranes are impermeable, would have to be actively transported into the

ECF using Ca-ATPase calcium pump suggested by Chalker (1976) and demonstrated by Ip *et al.* (1991), travel by pericellular pathways (Cohen and McConnaughey 2003) or by leaking from the surrounding seawater as in an open system (Adkins *et al.* 2003). The latter alternative seems unlikely as Furla *et al.* (2000) estimated that 70-75% of the carbon was metabolic in origin and corals produce six times the amount of CO_2 that is used for calcification. Tambutté *et al.* (1996) confirmed an intracellular pathway for Ca^{2+} transportation in corals and suggested that two types of cells were involved: Ca^{2+} transporting cells using L-type

voltage dependent Ca^{2+} channels with an energy consuming mechanism and cells with Ca^{2+} channels that permitted passive movement of Ca^{2+} . The involvement of voltage dependent Ca^{2+} channels is supported by molecular evidence (Zoccola *et al.* 1999). The operation of Ca-ATPase calcium pump/proton pump (Ca^{2+} out and H^+ in) (Al-Horani *et al.* 2003 and Zoccola *et al.* 2004) acts as an alkalinity pump and produces pCO_2 and pH gradients which enhance the passive diffusion of CO_2 into the ECF, driving the series of equations to the right, thus enhancing calcification.



(Note: CO_2 and H_2O enter the ECF by diffusion, Ca^{2+} is pumped in (\downarrow), H^+ out (\uparrow), and CaCO_3 is deposited ($\downarrow\downarrow$).

How Ca^{2+} enters the calcicoblastic layer is not clear. Goreau (1959) demonstrated a large, exchangeable pool in the coelenteron which may be exchanged with seawater from the outside mainly at night and a smaller pool in the tissue. Channels through which calcium can be selectively taken up were suggested by Clode and Marshall (2002). Ca^{2+} channels permitting the uptake of Ca^{2+} by the calcicoblastic and oral epidermal cells (Zoccola *et al.* 1999) and Ca^{2+} uptake involving special cells (Tambutté *et al.* 1996) have been suggested. Intracellular Ca^{2+} transport may be achieved by Ca^{2+} binding proteins or diffusion, but not by intracellular vesicles (Bohm *et al.* 2006). The organic matrix on which calcium carbonate is deposited has been shown by Tambutté *et al.* (2006) to consist of structural proteins which also play a catalytic role by eliminating the kinetic barrier to inter-conversion of inorganic carbon. Fractionation of the stable isotopes of calcium is to be expected if an ATPase calcium/proton pump is involved in actively transporting calcium ions into the ECF. However the results of stable isotope analysis of coral skeletons have proved somewhat ambiguous (Bohm *et al.*

2006). They found that scleratinian corals are enriched in $\delta^{44/40}\text{Ca}$ by 0.4-0.5 ‰ compared to slowly deposited sclerosponge and inorganic aragonite but are lower than might be expected from their high deposition rates. The $^{44}\text{Ca}/^{40}\text{Ca}$ ratios found by Gussone *et al.* (2005) and Bohm *et al.* (2006) were also positively correlated with temperature. Coral calcification rates are known to be higher in the light (Goreau 1959) and corals may, for example, calcify eleven times faster in light than in the dark (Erez 1978).

The Ca-ATPase pump of animal cell plasma membranes maintains low internal concentrations of Ca^{2+} . The effect of light on intracellular Ca^{2+} is not known and studies on the effects of temperature on intracellular Ca^{2+} levels in isolated coral cells have given conflicting results. Sawyer and Muscatine (2001) found no significant changes in with heat or cold shock while Fang *et al.* (1997) and Huang *et al.* (1998) found increases proportionate to the duration of the heat treatment. Disruption of the calcium pump in intact corals by high temperature has been shown (Sandeman 2006) to lead to high internal cellular concentrations of Ca^{2+} . This results in blebbing or ballooning out of the membrane and eventual fragmentation of the gastrodermal layer and loss of zooxanthellae (bleaching). The same end results are obtained at lower temperatures in light as the result of the accumulation of intracellular H_2O_2 . That study showed that both isolated zooxanthellae and whole corals release hydrogen peroxide (H_2O_2) during photosynthesis. At high temperatures H_2O_2 interferes with the calcium exclusion mechanisms, probably by combining with $-\text{SH}$ groups, and leads to bleaching. At lower temperatures, and on a localized basis, H_2O_2 probably plays a part in the regulation of algal populations in the coral. H_2O_2 is produced directly during photosynthesis of the coral, or indirectly by the dismutation of the superoxide radical ($\text{O}_2^{\bullet-}$). H_2O_2 is able to move easily through biological membranes and to cause lipid peroxidation (Halliwell and Gutteridge 1999). Neither H_2O_2 nor $\text{O}_2^{\bullet-}$ is sufficiently reactive on its own to initiate lipid

peroxidation (Halliwell and Gutteridge 1999). However, when they occur together in the presence of iron or other transition metal ions, hydroxyl radicals (OH^\bullet) can be produced by the Haber-Weiss reaction. Hydrogen peroxide and organic peroxides are also able to release iron ions from iron proteins and may also form hydroxyl radicals by Fenton chemistry. Hydroxyl radicals initiate lipid peroxidation in hydrocarbon side chains containing polyunsaturated fatty acids (PUFAs) by abstracting H and if oxygen is present peroxy radicals are formed which are capable of extracting H from other PUFAs to form more lipid peroxides and a chain reaction results. Thus a few hydroxyl radicals may lead to considerable lipid peroxidation in the absence of glutathione (GSH) to repair and minimize damage. In the repair process GSH is oxidized to GSSG. Glutathione reductase catalyses the reduction of GSSG back to GSH but requires NADPH. Lipid peroxidation makes cell membranes leaky to Ca^{2+} (Halliwell and Gutteridge 1999) and provides a possible route for Ca^{2+} to enter cells. With leakage taking place the calcium pump would have to work harder to maintain the low internal concentration of Ca^{2+} . For cells of the calicoblastic layer, during daylight hours, more of the leaked Ca^{2+} would be transported out into the EFC where it would be deposited as CaCO_3 . This mechanism would explain the higher coral calcification rates for daylight than those found at night (Goreau 1959) and may explain the higher calcification rates of hermatypic as compared to ahermatypic and deep-sea corals.

The aim of this study was to determine whether lipid peroxidation takes place in corals and to detect the expected presence of a repair system involving glutathione. These would give support to the role suggested above for H_2O_2 in coral calcification.

MATERIALS AND METHODS

Coral sampling and preparation:

All sea water used for experiments was passed through a column of 4-8 mesh activated charcoal (Sigma) and millipore-filtered (0.45 μ).

Small pieces of live coral, *Agaricia agaricites*, were collected in the back reef at Discovery Bay, Jamaica and were held in the sea water table at the Discovery Bay Marine Laboratory of the University of the West Indies. Small platelike *Agaricia* colonies with a thin outer edge proved to be a particularly useful source of material as the structure was simple, consisting of parallel septa, and pieces could be easily trimmed to a desired shape with small scissors. Small pieces from the edge of colonies of *Montastrea faveolata* were obtained and subdivided with a hammer and chisel. The area of pieces of coral used for experiments was estimated with aluminium foil (Marsh 1970). The sea water table and the laboratory in which the experiments were carried out both had low illumination ($< 5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

Lipid peroxidation: The production of lipid peroxide was estimated with the Fox2 technique (Jiang *et al.* 1991, Wolff 1994). Nine small pieces (approx 2 cm^2 each) from the same colony were used in each experiment. One piece was analysed at the start of the experiment (dark result), four of the remainder were placed in each of two small vessels of seawater, one held at 28° C, the other at 33° C in constant temperature baths. Irradiation (800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ measured with a model LI-250, LI-COR Quantum Radiometer) was provided by 60-watt halogen spotlights with heat filters and 80b optical filters. Pieces of coral were withdrawn at intervals of two hours for analysis. Each piece of coral was water-piked with millipore-filtered seawater which was then homogenised briefly to break up the mucus and centrifuged at low speed for four minutes to deposit the zooxanthellae. The supernatant fluid with suspended animal tissue, was drawn through a glass-fiber filter which retained the solid material. The filter was rinsed with distilled water to remove salt and extracted in a 15 ml centrifuge tube with 10 ml of Fox2 reagent (final concentrations: 100 μM xylene orange, 250 μM ammonium ferrous sulphate, 90 % methanol, 4 mM butylated hydroxytoluene and 25 mM H_2SO_4).

After centrifuging to remove suspended solids and standing for 30 minutes the absorbance of a two ml sub-sample was read at 560 nm in a spectrophotometer. Known concentrations of hydrogen peroxide were used to calibrate the analysis and results were corrected for the area of each piece of coral. Absorbances continued to increase slowly after 30 minutes at room temperature indicating that some chain oxidation, not inhibited by the butylated hydroxy toluene, might be taking place. A small portion (< 5 %) of the absorbance at 560 nm could be attributed to pigments in the few zooxanthellae that were not removed by centrifugation, this was ignored. Statistical procedures were carried out with NCSS 2000 statistical software (Dr Jerry L. Hintze, Kaysville, Utah 84037).

Glutathione: Glutathione levels in the tissues of *Agaricia*, before and during exposure to high light/temperature, was estimated using a technique following Cohn and Lyle (1966), McNeil and Beck (1968), and Hissin and Hilf (1976). This technique is based on the reaction of reduced glutathione (GSH) with *o*-phthalaldehyde (OPT) at pH 8 to give a fluorescent compound (emission at 425 nm). McNeil and Beck (1968) reported that with higher concentrations of extract a yellow quenching compound develops with the addition of OPT. This reaction was taking place with the coral extracts and, to minimise its effect, higher concentrations of OPT were used to lessen the time over which fluorescence reached a plateau before it started dropping again. For each estimation of GSH the initial coral extract was divided into two parts. To one part a known quantity of GSH was added and the two samples were processed together identically. From the results the concentration of GSH was calculated and corrected for the area.

Small samples (2-3 cm²) from three *Agaricia* plates maintained in seawater at 32 °C and exposed to illumination of 350 µmol.m⁻².sec⁻¹ were withdrawn for analysis at intervals of about three hours. Each was water-piked with approximately 10 ml seawater which was then homogenized with a high-speed

homogenizer (30 seconds) and centrifuged at low speed for three minutes to deposit the zooxanthellae. The supernatant was decanted, its volume recorded, and it was then divided into two parts, each of 4.5 ml. To one portion 0.1 ml of buffer containing 100 µg of GSH was added. To each part 0.25 ml of concentrated phosphoric acid was added and mixed well with a vortex mixer to precipitate any protein present. After centrifugation at 5000 g for eight minutes, sodium hydroxide (pellets and a strong solution) was added to bring the pH of each to 8.0 as measured with a pH meter. After further centrifugation at 5000 g for 4 minutes, 1 ml of supernatant of each was added to 4 ml of 0.1 M sodium phosphate buffer (pH8). To 0.7 ml of these solutions in sample vials, 0.1 ml of OPT solution in methyl alcohol (containing 300 µg OPT) was added. The fluorescence of the two vials was read with a fluorometer (Sandeman 2006) at one minute intervals until a plateau was reached after which the fluorescence decreased. The difference between the maximum values of fluorescence for the spiked and unspiked samples represents the fluorescence due to 100 µg of glutathione.

RESULTS

Lipid peroxidation: Typical results from Fox2 assays of lipid peroxide of coral pieces initially in the dark and held in seawater at 25-28 °C and 33-35 °C in light can be seen in Figure 1. All show lipid peroxides increasing to a maximum for the first 3-4 hours, this is followed by a period of reduction or recovery, either relatively quickly (Fig. 1A, C), or more slowly (Fig 1B). The experiments were originally carried out at two temperatures and under strong light to explore whether lipid peroxidation played a part in bleaching. Many of the pieces of coral held at the higher temperature exhibited signs of bleaching by the end of the experimental period but lipid peroxidation (Fig. 2A) was not significantly different for coral pieces held at the two temperatures. For the samples of *Agaricia* the means of lipid peroxidation at the start (dark) and after four hours

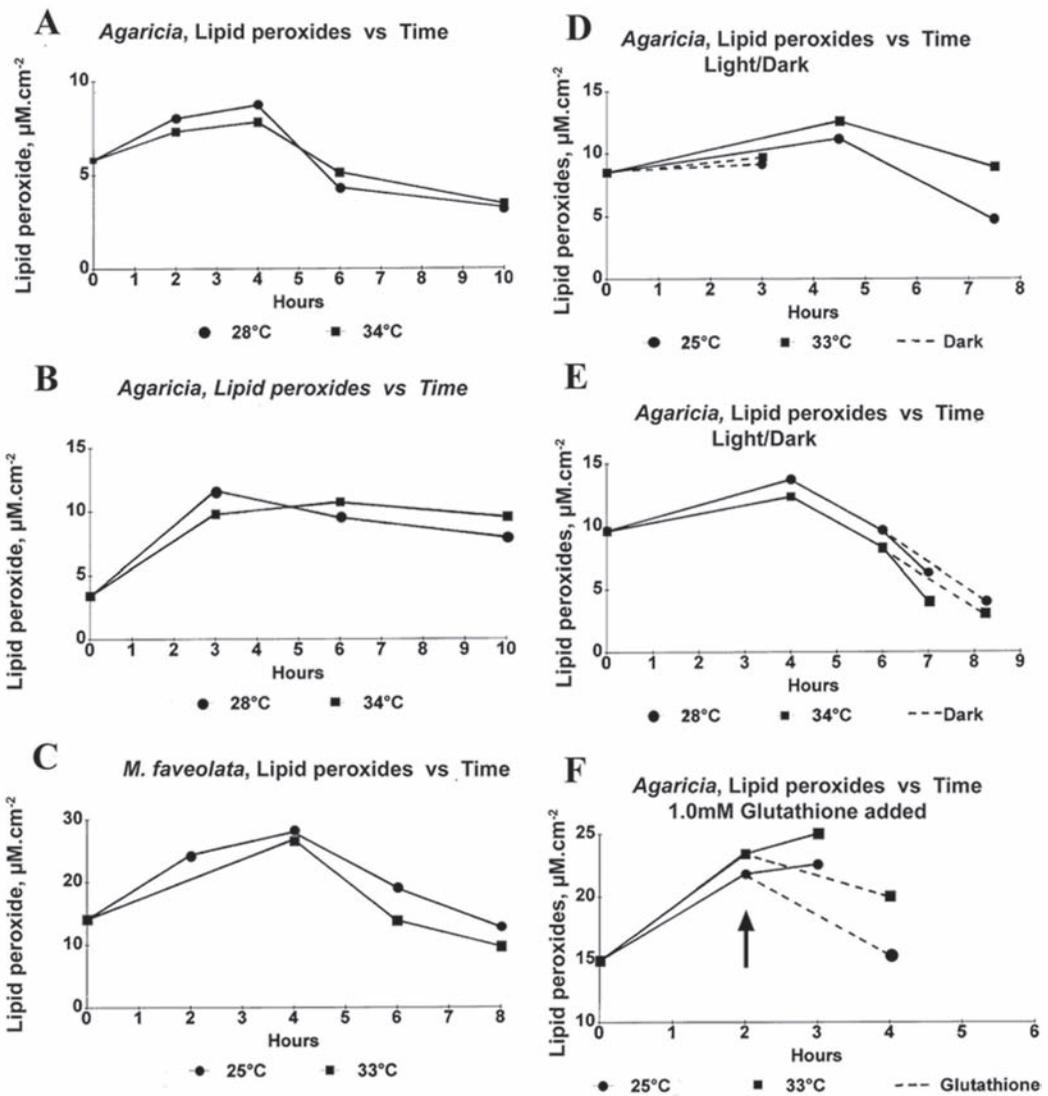


Fig. 1. Small (2 cm²) pieces of coral were incubated at 25-28° C and 33-34° C with 800 μMol.m⁻².s⁻¹ illumination. Pieces were removed at intervals and lipid peroxides were measured with the Fox2 technique. Lipid peroxides are plotted against time. A,B *Agaricia agaricites*. C. *Montastrea faveolata*. D. *Agaricia agaricites*, two pieces held in the dark. E. *Agaricia agaricites*, two pieces held in the light during repair phase. F. *Agaricia agaricites*, pieces with 1.0 mM glutathione added (arrow).

in light were significantly different ($p=0.0033$) for both temperatures. When the results for the two temperatures were combined together (Fig. 2B) the means of lipid peroxidation at the start (dark) and after four hours in the light were significantly different ($p=0.0033$). Pieces of coral ($n=4$) were simultaneously kept in the dark and

light (an example in Fig. 1D) there was little change in lipid peroxide from the initial level. When coral pieces ($n=2$) were held in the dark the rate of recovery was lower than that of the pieces held in the light (Fig. 1E). The addition ($n=2$) after two hours in the light, of 0.1 M glutathione (GSH) to the seawater containing

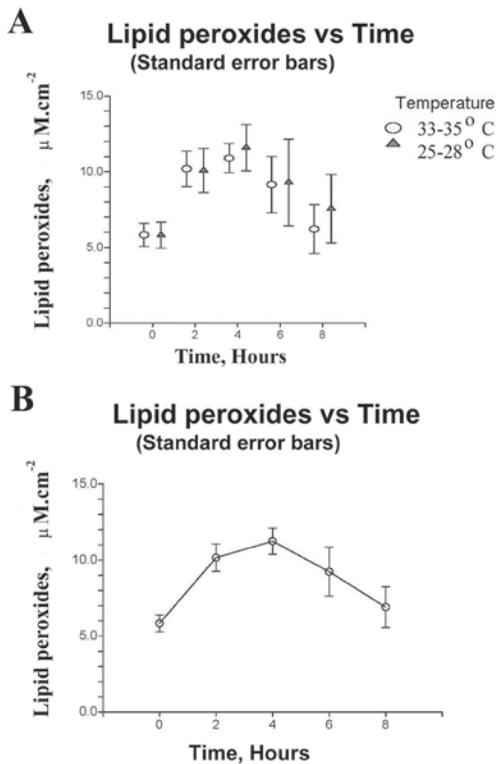


Fig. 2. Pieces of *Agaricites agaricites* incubated at 25-28°C and 33-34°C under illumination of 800 $\mu\text{Mol.m}^{-2}.\text{s}^{-1}$. Lipid peroxides are plotted against time. Standard error bars are shown. A. Results for the two temperatures compared. B. Means of all results (n=17) combined.

the corals (Fig. 1F), reversed the increase in lipid peroxide to a fairly rapid reduction. The combined results from all experiments (Fig. 2B) indicate that the mean lipid peroxides level (initially 5.83 $\mu\text{M.cm}^{-2}$, n=8) increased during the first 3-4 hours in light to a maximum (11.23 $\mu\text{M.cm}^{-2}$, n=17) then decreased to their original level. A single experiment with *Montastrea faveolata* (Fig. 1C) showed higher lipid peroxides levels but the pattern was similar to the results obtained for *Agaricia agaricites*.

Glutathione: Glutathione was present in all the samples analysed (mean 79.0 $\mu\text{g/cm}^5$, n=11). The results for the samples from the three colonies can be seen in Fig. 3. From the regression line and R⁵ value (0.00) for all the

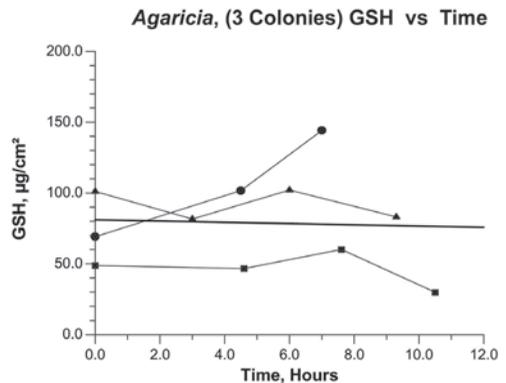


Fig. 3. Small (2-3 cm^2) samples from three *Agaricites agaricites* colonies were incubated at 32°C with 350 $\mu\text{Mol.M}^{-2}.\text{s}^{-1}$ illumination. Samples were removed at intervals for estimates of GSH. GSH is plotted against time. Regression line is shown.

points, it is clear that there is no difference in glutathione level over the ten hour period during which the samples were exposed to light.

DISCUSSION

These experiments indicate that lipid peroxides in the tissues of *Agaricia agaricites* increase in the light to a maximum in 3-4 hours then decrease over the next few hours (Figs. 1, 2). Confirmation that similar patterns are found in other corals was provided by the experiment using *M. faveolata*. A possible explanation for the reduction in lipid peroxide is that a repair mechanism is involved, such as the GSH-GSSG system that is commonly present in animals and is responsible for the repair of lipid peroxidation damage (Halliwell and Gutteridge 1999). The presence of GSH confirms that the same system is probably involved in corals. Further confirmation may come from the single experiment (Fig. 1F) in which lipid peroxidation was reversed and showed a high rate of repair when GSH was added to the seawater in which the coral was being held. Energy in the form of NADPH is required for the conversion of GSSG back to GSH (Halliwell and Gutteridge 1999). The higher recovery rate in

the light seen in Fig. 1E might be explained if recovery is indirectly light driven. Levels of lipid peroxidation and the rates of formation and repair showed considerable variation. This probably results from the interplay of many factors: light and temperature levels, the rate of H_2O_2 production and loss, the rate at which hydroxyl ions are generated, the quantity of GSH present and the presence of glutathione reductase and NADPH to regenerate glutathione from oxidised glutathione.

Lipid peroxidation is known to make the plasma membrane leaky to calcium ions (Halliwell and Gutteridge 1999) and in view of the presence of GSH and the light related formation of lipid peroxides, this is very likely to be the case for corals. There is no direct evidence that intracellular Ca^{2+} increases in the light and information on the effect of temperature on intracellular Ca^{2+} is conflicting. However, H_2O_2 , which can move easily through cell membranes and is the probable source of lipid

peroxidation, was shown to accumulate in coral tissues in light (Sandeman 2006).

Calcium ions are charged and are unable to pass easily through the plasma membrane. Suggestions for how Ca^{2+} enters the calicoblastic layers include “special” channels (Zoccola *et al.* 1999, Clode and Marshall 2002) or special cells (Tambutté *et al.* 1996). During the day, when photosynthetic production of H_2O_2 is high, leakage due to lipid peroxidation thus provides, without invoking “special” channels or cells, a route for Ca^{2+} to enter the calicoblastic layer before being actively transported out and into the ECF by the calcium/proton pump (Fig. 4). The availability of CO_2 in the calicoblastic layer is enhanced by the proton pump and CO_2 diffusion into the ECF is also enhanced (Adkins *et al.* 2003). The series of reactions leading to the formation of CO_3^{2-} is enhanced by the removal of protons, CO_3^{2-} combines with Ca^{2+} and is deposited as calcium carbonate (Fig. 4). These reactions probably take place on

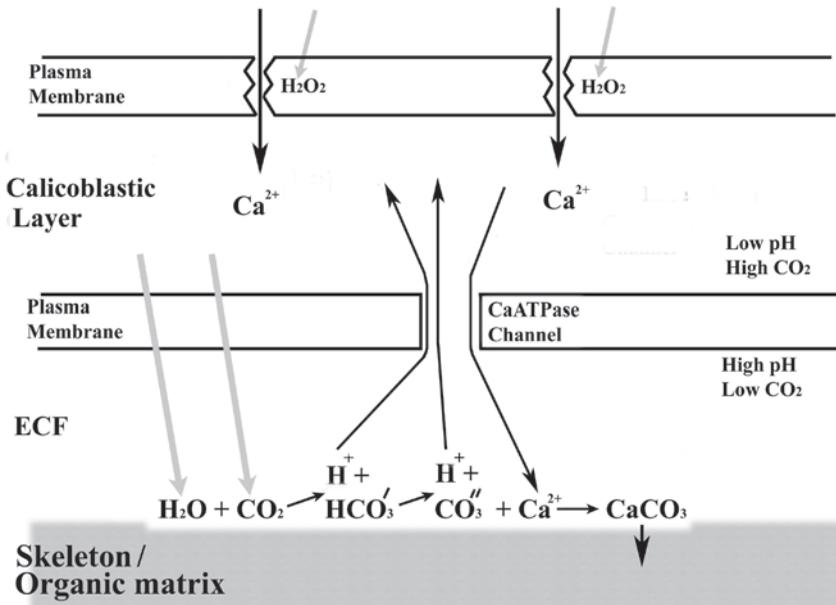


Fig. 4. A model of calcification (following Adkins *et al.* 2003). CO_2 and water diffuse into the calicoblastic layer and ECF. In daylight Ca^{2+} enters the calicoblastic layer by leakage caused by lipid peroxidation of the plasma membrane by H_2O_2 . The Ca-ATPase calcium pump actively transports Ca^{2+} into the ECF and protons out, setting up a pH gradient which enhances the diffusion of CO_2 . Removal of protons also enhances the conversion of CO_2 to CO_3^{2-} on the organic matrix. CO_3^{2-} combines with Ca^{2+} and is deposited as $CaCO_3$.

the surface of the protein organic matrix which has been shown to have the catalytic properties of carbonic anhydrase (Tambutté *et al.* 2007). At night and in azooxanthellate corals there is probably a basal rate at which Ca^{2+} leaks into the cells and is actively transported into the ECF by the calcium/proton pump. During the day in zooxanthellate corals H_2O_2 is produced, diffuses into the coelenteron and from there is distributed round the coral and can diffuse to the calciblastic layer (Sandeman 2006). It causes lipid peroxidation of the plasma membranes which is likely to make them leaky to Ca^{2+} . The calcium pump would have to work harder, resulting in more Ca^{2+} being actively transported into the ECF and resulting in higher calcification rates.

Hydrogen peroxide, generated directly by their symbionts or indirectly by the dismutation of superoxide, appears to play an important part in the life of corals. At low concentrations H_2O_2 may enhance calcification during daylight hours as described above. At intermediate levels it may provide a localized mechanism by which the numbers of zooxanthellae in the gastrodermis are regulated and at still higher levels it is involved in solar bleaching (Sandeman 2006).

RESUMEN

La bomba de calcio Ca-ATPase de la membrana de plasma mantiene bajas las concentraciones de iones de calcio (Ca^{2+}) en las células vivas. La presencia de una bomba de calcio en los corales ha sido confirmada, y su fallo en el funcionamiento debido al cambio de temperatura es lo que conlleva al blanqueamiento de coral. Existe también abundante evidencia obtenida de los proporciones de los isótopos estables del calcio en el esqueleto, de que hay una bomba de calcio involucrada en la calcificación del coral. Como se sabe, el peróxido de hidrógeno (H_2O_2) es generado por las zooxantelas durante la fotosíntesis y pasa fácilmente a través de las membranas celulares. Además, el H_2O_2 produce la peroxidación de lípidos de la membrana plasmática y lo hace permeable al Ca^{2+} . En el presente estudio, se midió la peroxidación lípida en los tejidos de *Agaricia agaricites*. Los peróxidos lípidos en la luz se duplicaban, de 5.83 a 11.23 $\mu\text{Mol.cm}^{-2}$ ($n=17$), durante las primeras 3 a 4 horas, y luego, disminuían lentamente durante las siguientes 4 horas. El glutatión, que en muchos organismos actúa como

parte del sistema de reparo de la peroxidación de los lípidos, fue encontrado en niveles significativos, 79 $\mu\text{g.cm}^{-2}$ ($n=14$), e indica probablemente la reducción observada en los niveles de peróxido lípido. Se sugiere que la peroxidación de lípidos hace que la membrana de plasma permita la fuga de Ca^{2+} , y por lo tanto, que el Ca^{2+} tenga una ruta para entrar en las células de la capa calcioblástica. El modelo de calcificación según Adkins *et al.* (2003), presenta cómo la calcificación se lleva a cabo entre el líquido calcificante extracelular (ECF: extracellular calcifying fluid), que está en la capa calcioblástica, y el esqueleto. La bomba de calcio Ca-ATPase dirige la calcificación en la medida en que transporta el Ca^{2+} al ECF y establece los gradientes de pH y dióxido de carbono (CO_2) que permiten la difusión pasiva del CO_2 y la formación de carbonato de calcio. Se sugiere que, en la luz, el H_2O_2 producido por las zooxantelas hace que las membranas permitan la fuga de Ca^{2+} . La bomba de calcio tiene que trabajar más para mantener bajos los niveles internos de calcio, y consecuentemente, más Ca^{2+} sería transportado activamente al ECF para ser depositado como carbonato de calcio.

Palabras clave: calcificación de coral, bomba de calcio Ca-ATPase, peróxido de hidrógeno, peroxidación de lípidos.

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