A PHYSIOLOGICAL EVALUATION OF CARBON SOURCES FOR CALCIFICATION IN THE OCTOCORAL *LEPTOGORGIA VIRGULATA* (LAMARCK)

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Summary

The union of calcium cations with carbonate anions to form calcium carbonate ($CaCO_3$) is a fundamentally important physiological process of many marine invertebrates, in particular the corals. In an effort to understand the sources and processes of carbon uptake and subsequent deposition as calcium carbonate, a series of studies of the incorporation of ^{14}C-labeled compounds into spicules was undertaken using the soft coral *Leptogorgia virgulata*. It has been surmised for some time that dissolved inorganic carbon in sea water is used in the biomineralization process. Furthermore, it was suspected that metabolically generated CO_2 is also available for calcification. As a means of testing these possible sources of carbon in spicule calcification, key enzymes or transport systems in each pathway were inhibited. First, the enzyme carbonic anhydrase was specifically inhibited using acetazolamide. Second, the active transport of bicarbonate was inhibited using DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid). Third, CO_2 generation resulting from glycolysis and the citric acid cycle was arrested using iodoacetic acid, which interferes specifically with the enzyme glyceraldehyde-3-phosphate dehydrogenase. The results indicate that dissolved CO_2 is the largest source of carbon used in the formation of calcitic sclerites, followed by HCO_3^- from dissolved inorganic carbon. In *L. virgulata*, the dissolved inorganic carbon is responsible for approximately 67 % of the carbon in the sclerites. The other 33 % comes from CO_2 generated by glycolysis. Two important conclusions can be drawn from this work. First, carbon for spiculogenesis comes not only from dissolved inorganic carbon in the environment but also from metabolically produced carbon dioxide. While the latter has been theorized, it has never before been demonstrated in octocorals. Second, regardless of the carbon source, the enzyme carbonic anhydrase plays a pivotal role in the physiology of spicule formation in *Leptogorgia virgulata*.

Key words: cnidaria, Anthozoa, octocoral, *Leptogorgia virgulata*, carbonic anhydrase, acetazolamide, DIDS, iodoacetic acid, spicule, calcification.

Introduction

The regulation of the transport and accumulation of calcium is fundamental to all living systems and it is, arguably, nowhere more important than in the process of calcification. Calcified hard tissues and skeletons provide structural support and protection for species in all classes of animals. Calcification has been well studied in a number of invertebrate phyla, including Crustacea (Roer, 1980; Cameron and Wood, 1985; Henry and Kormanik, 1985), Echinodermata (Sikes *et al.* 1981; Decker and Lennarz, 1988) and Mollusca (Wilbur and Saleuddin, 1983; Kawaguchi and Watabe, 1993), in which calcium carbonates are the principal minerals produced. Paramount among species undergoing calcification, on the basis of mass alone, are the members of the phylum Cnidaria. This phylum includes reef-building corals, which produce calcium carbonate skeletons so massive that they are globally important in carbon cycling and ecosystem interactions. While coral species in a number of orders have been used to investigate the physiology of calcification, the mechanisms involved in this process are not well understood. To date, most of the research involving coral calcification has focused either upon the structure and composition of matrices of skeletons and sclerites (also referred to as spicules) (Young *et al.* 1971; Dunkelberger and Watabe, 1974; Allemand *et al.* 1994) or on the uptake, regulation and mechanisms of deposition of calcium ions (Goreau and Bowen, 1955; Allemand and Grillo, 1992; Tambutté *et al.* 1995, 1996; Allemand and Tambutté, 1996).

As important as calcium is for invertebrate mineralization, the availability of carbonate ions plays an equally important role. It has been known for some time that the carbon used for calcium carbonate ($CaCO_3$) production comes, at least in part, from dissolved inorganic carbon (DIC) in the surrounding sea water, predominantly dissolved CO_2 and bicarbonate (HCO_3^-). Whether the DIC enters the calcifying areas of coral as dissolved CO_2 or as HCO_3^- has not been determined; in all likelihood, both routes are utilized. Another potential source

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for the carbon in $CaCO_3$ is from metabolically generated CO_2, derived in large part from the catalysis of glucose. However, except for research by Pearse (1970), very few attempts have been made to address the means by which CO_2 generated by metabolic activities may be utilized.

A useful organism for the study of invertebrate calcification is the anthozoan octocoral *Leptogorgia virgulata* (Lamarck), commonly known as sea whips, which are ahermatypic azooxanthelic soft corals. *L. virgulata* have been described as irregularly laterally branched colonies often reaching lengths of up to 1 m. Colonies consist of a horny central axis embedded with amorphous nonspicular calcareous matter. The axis is surrounded by an axial sheath of tissue that thickens into the coenchyme. Polyps extend outwards from the coenchyme and have eight pinnately compound tentacles. Scleroblasts, the sclerite- or spicule-producing cells of octocorals, are found within the mesoglea of the coenchyme (Fig. 1A). Spicules are made of the calcite form of the mineral salt calcium carbonate and are formed within a spicule vacuole inside the scleroblasts; upon maturity, they are extruded into the outer layer of coenchyme. The sclerites of *L. virgulata* have been described as having a disk-spindle shape (Fig. 1B); that is, a 'straight monaxial sclerite pointed at both ends with tubercles of four or more whorls fused more or less completely into disks' (Bayer *et al.* 1983). The route through which it is proposed that calcium is transferred to the scleroblast has been reported by Watabe and Kingsley (1992). It involves import of ions through the polyps to the axis, and recruitment of calcium from the axis for utilization by scleroblasts in the formation of spicules. The route followed by sources of carbon has not been examined.

Fundamentally, there are three pathways for carbon to be incorporated into sclerites formed by *Leptogorgia virgulata*, as indicated in the simplified model presented in Fig. 2. The first two are involved with the transport of DIC. The simplest of these is the direct diffusion of dissolved CO_2 across the plasma membranes of the several cell types of the polyp, epidermal and mesenchymal cells of the colony, including the scleroblasts. Once inside the scleroblast, CO_2 can be acted upon by carbonic anhydrase in the presence of H_2O to form carbonic acid (H_2CO_3), which dissociates rapidly into HCO_3^- and H^+. This reaction also occurs in the absence of carbonic anhydrase, but at a much slower rate. Inhibition of carbonic anhydrase with acetazolamide over a wide range of concentrations (Maren, 1977) has been used to elucidate the role of this pathway in carbonate production, biomineralization and acidification. Acidification of the local cellular or tissue environments through the action of carbonic anhydrase occurs as the dissociation of carbonic acid releases H^+. The buffering capacity of the bicarbonate is largely responsible for maintaining the pH in the cytoplasmic and interstitial fluids of animals. This process of regulating acid–base conditions is known to influence gas exchange in blood delivery systems (Maren, 1977) and to help in the establishment of the hydrogen ion gradients important in driving membrane transport (Dodgson, 1991; Simkiss and Wilbur, 1989), but how this specifically affects calcification in coral is not known.

The second pathway involving DIC relies on the extracellular, nonenzymatic conversion of CO_2 to HCO_3^- and its subsequent transport into a cell (Dodgson, 1991). HCO_3^- has been shown to be actively transported into the cell through a number of anion transporters and antiporters. These membrane channels can be inhibited using DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid) (Madshus, 1988), which has been demonstrated to contribute to the blockage of this transport pathway.

The third pathway of carbon utilization may be viewed as depending upon the availability of metabolically generated CO_2 (Pearse, 1970). To evaluate this pathway, [14C]glucose has been employed in an effort to follow the incorporation of
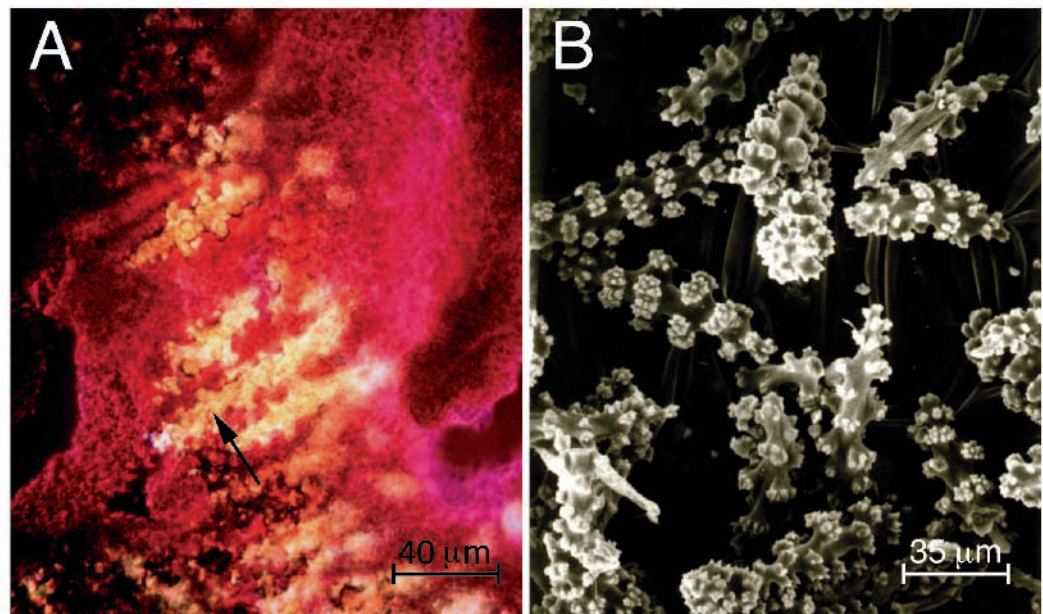

Fig. 1. Light and scanning electron micrographs showing examples of spicules in *Leptogorgia virgulata*. (A) Polarized light micrograph demonstrating the position and orientation of a spicule (arrow) embedded in the mesoglea (pink coloration) of the colony. (B) Scanning electron micrograph depicting the disk-spindle morphology of isolated spicules. Scale bars, 40 μm in A and 35 μm in B.
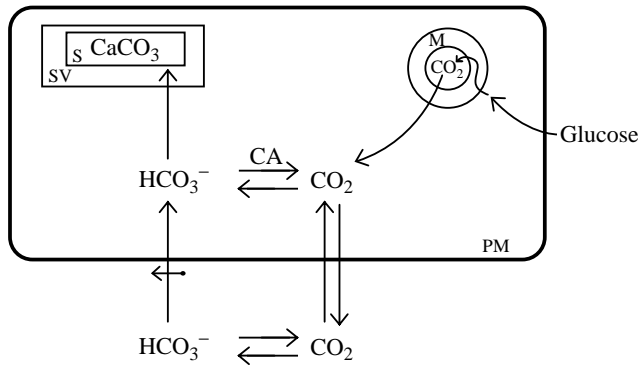
Fig. 2. A diagrammatic model of a scleroblast, depicting the potential pathways of carbon utilization for spiculogenisis. Dissolved inorganic carbon can be actively transported ($\leftarrow\bullet$) across the plasma membrane (PM) as HCO_3^-, or CO_2 can diffuse across the PM. In the cell, CO_2 can be acted upon by carbonic anhydrase (CA) to convert it into carbonic acid (H_2CO_3), which dissociates to release HCO_3^- and H^+. Both sources of HCO_3^- can then be joined with Ca^{2+} in the spicule vacuole (SV) to form the spicule (S). Another source of carbon (as CO_2) is from glycolysis and the citric acid cycle. Glucose is transported through the PM and undergoes glycolysis. Pyruvate then enters the mitochondria (M) where it is used in the citric acid cycle, thus generating several molecules of CO_2. This CO_2 can then be acted upon by CA and follow the pathway described above until it ends up as part of the $CaCO_3$ in a spicule.

$^{14}CO_2$ into carbonates and subsequently into mineralized hard tissue. When glucose is taken up by *L. virgulata*, the glucose can be used in glycolysis and CO_2 will be generated as pyruvate is used by the citric acid cycle. The CO_2 is converted to H_2CO_3 by carbonic anhydrase and ultimately to HCO_3^- as a consequence of the dissociation of the acid. This pathway has been shown to be inhibited early on in glycolysis by iodoacetic acid (Webb, 1966). Using these three classes of inhibitors, it is possible to distinguish the influence of each of the pathways on octocoral calcification.

Leptogorgia virgulata is an excellent organism for the study of calcification. First, there is a substantial body of biochemical and morphological information available in the literature (Kingsley and Watabe, 1984, 1985, 1987; Lucas and Knapp, 1996) upon which to interpret the results of physiological studies on calcification. Second, unlike many other corals, *L. virgulata* does not contain zooxanthellae, symbiotic algae. The lack of zooxanthellae means that any study of calcification in *L.virgulata* deals solely with the physiology of the coral. In corals containing zooxanthellae, the contribution of the algae to the regulation of calcification must be taken into account. The process of photosynthesis undergone by intracellularly resident algae complicates enormously the analysis of the physiology of coral calcification. In fact, the debate continues as to whether actively photosynthesizing zooxanthellae inhibit or stimulate the mineralization of their coral symbionts (Marshall, 1996*a,b*; Carlon, 1996; Goreau *et al.* 1996).

Much of what is known about calcification in *Leptogorgia virgulata* deals with the uptake and transport of calcium ions

into the scleroblast, but very little is known with respect to the details of the sources and pathways of carbon for its eventual deposition as calcium carbonate. The purpose of this study is to evaluate mechanisms of carbon uptake and utilization for calcification by *L. virgulata* using labeled isotopes of carbon in carbonate (as DIC) and in glucose (as a metabolic source). This was facilitated by the use of enzyme- or transport-specific inhibitors whose effects on the incorporation of these isotopic compounds could be quantitatively measured by the degree to which they inhibit calcification.

Materials and methods
Biological materials

Colonies of *Leptogorgia virgulata* (Lamarck) were collected at low tide from the estuary of the University of South Carolina at the Belle W. Baruch Institute for Marine Biology and Coastal Research in Georgetown, South Carolina. Living colonies were transported in aerated sea water and subsequently maintained in heavily aerated aquaria in the laboratory. The water was periodically exchanged with sea water transported from the estuary. The colonies were fed once a week with a commercially available coral food (zooplankton obtained from the Sweetwater Company, Luxembourg), but were not fed within 3 days of planned use in the experiments. Even though *L. virgulata* can be maintained in a laboratory environment for up to 8 months, all specimens tested in this experiment were used within 2 weeks of their collection.

Media and chemicals

Filtered sterilized sea water (FSW) served as the medium for all incubations. FSW was prepared using sea water siphoned from the aquaria which was then passed through $0.2\,\mu m$ bottle filters (Costar Scientific Corporation, Cambridge, MA, USA). Acetazolamide and DIDS (4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid) were prepared as $10\times$ concentrated solutions in FSW and required gentle heating and vigorous agitation to go into solution. This solution was diluted into FSW for use. Iodoacetic acid was dissolved in $5\,mmol\,l^{-1}$ Tris (pH 7.8) prepared in FSW. Tris was required as a buffer because of the highly acidic nature of iodoacetic acid. Furthermore, a pH of 7.8 was chosen for the tissue incubations because it corresponded to the pH of the sea water in the aquaria and in the estuary at the time of colony collection. All of the inhibitory solutions were prepared freshly and subsequently filter-sterilized using $0.2\,\mu m$ filters. All of the inhibitors as well as β-phenylethylamine were obtained from the Sigma Chemical Company (St Louis, MO, USA).

$^{45}CaCl_2$, $NaH^{14}CO_3$, D-[U-^{14}C]glucose and EcoLume were obtained from ICN Biomedicals, Inc. (Irvine, CA, USA).

Microsopy

For light microscopy, 5 mm pieces of *Leptogorgia virgulata* arms were fixed in modified Carnoy's solution (Knapp *et al.* 1991), dehydrated in alcohol and toluene, and embedded in paraffin. $10\,\mu m$ paraffin histological sections were cut using an

AO microtome and mounted on acid-cleaned glass slides. Tissue sections were deparaffinized, rehydrated and mounted unstained under glass coverslips. Sections were viewed using polarizing light microscopy on a Zeiss Universal microscope.

For scanning electron microscopy, dissected arms of *Leptogorgia virgulata* colonies were treated for 1 h in bleach (5 % sodium hypochlorite) to dissolve organic materials and to release spicules and axes. Intact spicules and axes were pelleted at 500 g and the bleach was removed. Spicules were separated from axial material, rinsed five times in distilled water and oven-dried at 60 °C for 1 h. Dried spicules were gently mounted on double-sided tape affixed to aluminum stubs, sputter-coated with gold and observed using a Hitachi S-2500 scanning electron microscope.

Measurement of ^{45}Ca and ^{14}C incorporation into sclerites

Pieces (1 cm) of the growing tips of the yellow color morph of *Leptogorgia virgulata* were washed with FSW and preincubated, with moderate shaking, in 1 ml of unlabeled FSW with or without 5 mmol l^{-1} Tris (pH 7.8) or in one of the following inhibitors or combinations of inhibitors dissolved in FSW: 1 mmol l^{-1} acetazolamide, 1.0 mmol l^{-1} DIDS and 1 mmol l^{-1} iodoacetic acid for 30 min. Each experimental set consisted of six tips incubated separately in 2 ml microfuge tubes placed in an 81-space cardboard storage box so that the tubes were parallel to a shaker table platform.

24 h incubations began with the addition of 1 µCi (3.7×10^3 Bq) of $^{45}CaCl_2$ and either 1 µCi of NaH$^{14}CO_3$ or 1 µCi of [^{14}C]glucose (3.3×10^{-6} mmol l^{-1}). After 24 h of incubation, the tips were then incubated for 30 min in FSW. Each tip was then rapidly washed four times in FSW and placed in 1 ml of bleach for a minimum of 30 min or until all of the tissue was dissolved, leaving only the sclerites and the axis intact. Materials in solution and the axis were removed and discarded. The sclerites were then washed three times in FSW and absorbed onto preweighed Whatman 934-AH glass microfiber filter circles (24 mm diameter), using a vacuum filter apparatus, and washed briefly with double-distilled water. The filters and sclerites were dried overnight in an oven at 37 °C. Once dried, the filters were weighed so that the dry mass of the sclerites could be determined. Each filter was then placed in a scintillation vial and processed according to the method of Barnes and Crossland (1977).

Briefly, ^{14}C was evolved from the sclerites as CO$_2$ by the addition of 1 ml of 1 mol l^{-1} HCl. The CO$_2$ was trapped using 200 µl of β-phenylethylamine absorbed onto two discs of Whatman no. 1 filter paper (22 mm diameter) placed in a second scintillation vial and connected to the first by a rubber sleeve stopper with a hole punched through the center of the stopper. 5 ml of EcoLume was added to both the HCl fraction (^{45}Ca fraction) and the ^{14}C fraction, and radioactivity was measured as counts min^{-1} in a Beckman LS-230 scintillation counter.

Initially, there was no significant effect on ^{45}Ca incorporation when acetazolamide and DIDS were added simultaneous and incubated for 24 h. In order to overcome this

anomalous result, the incubation procedure was modified in all cases involving the combination of these two inhibitors. Specifically, when used in combination, acetazolamide was added first and allowed to incubate for 12 h, at which point DIDS was added to the incubation mix and the coral pieces were allowed to continue their incubation for another 12 h. Likewise, when all three inhibitors were used, acetazolamide was added simultaneously with iodoacetic acid and allowed to incubate for 12 h before DIDS was added, and the incubation was then continued for another 12 h.

Statistical analyses

Radioactive incorporation readings were normalized to cts min^{-1} g^{-1} dry sclerites. Means were determined for each set of experiments, and an analysis of variance (ANOVA) was used to distinguish the differences between the experimental groups and controls, with $P<0.05$ considered to be significant.

Results

In an effort to determine the potential sources of carbon for spiculogenisis, double-labeling experiments were performed using ^{45}Ca and ^{14}C, in the presence of a number of enzymatic and metabolic inhibitors. Double-labeling with ^{45}Ca was necessary since calcium incorporation is considered to be a more reliable indicator of overall calcification rate than is carbon incorporation (Barnes and Crossland, 1977). Tables 1 and 2 present the numerical data, variance and statistical significance of incorporation of ^{45}Ca and ^{14}C under the experimental conditions described below.

Incorporation of ^{45}Ca and ^{14}C as carbonate

Fig. 3A shows ^{45}Ca incorporation into sclerites in the presence of a number of pharmacological agents and NaH$^{14}CO_3$. Over a 24 h period, the amount of ^{45}Ca incorporated into sclerites in the presence of acetazolamide was 45.5±10.9 % of the FSW control, indicating the importance of carbonic anhydrase in the calcification process. While not as impressive, the amount of ^{45}Ca incorporated into sclerites in the presence of DIDS was 78.8±3.3 % of the FSW control. However, the combined effects of acetazolamide plus DIDS showed an incorporation value that was a substantial 34.7±11.0 % of the control value. When compared with the Tris–FSW control, coral tips incubated in iodoacetic acid showed an incorporation value that was 67.2±11.0 % of the control. *Leptogorgia virgulata* incubated in acetazolamide plus iodoacetic acid, DIDS plus iodoacetic acid and a combination of acetazolamide, DIDS and iodoacetic acid incorporated 52.0±4.5 %, 71.7±6.6 % and 28.8±10.4 % of the control value, respectively.

The double-label companion to Fig. 3A is Fig. 3B, which shows the amount of ^{14}C incorporated into spicules when NaH$^{14}CO_3$ was the labeled carbon source. In this case, in the presence of acetazolamide, ^{14}C incorporation was reduced to 65.7±4.2 % of the control value. In the presence of DIDS, ^{14}C incorporation was a considerable 52.8±5.6 % of the control

Table 1. *Mean, standard deviation and significance for incorporation of ^{45}Ca and ^{14}C into spicules in the presence or absence of inhibitors*

	Treatment	Mean	S.D.	P
^{45}Ca incorporation	Control	2.03×10^6	5.65×10^5	
into sclerites	Ac	9.23×10^5	2.22×10^5	0.0006
when NaH^{14}CO$_3$	D	1.60×10^6	6.79×10^4	0.0005
is the labeled	AD	7.04×10^5	2.24×10^5	0.004
carbon source	Tcontrol	1.98×10^6	4.11×10^5	
	I	1.33×10^6	2.17×10^5	0.003
	AI	1.03×10^6	8.89×10^4	0.009
	DI	1.42×10^6	1.31×10^5	0.001
	ADI	5.70×10^5	2.06×10^5	0.0001
^{14}C incorporation	Control	1.21×10^5	4.57×10^3	
into sclerites	Ac	7.95×10^4	5.08×10^3	0.022
when NaH^{14}CO$_3$	D	6.39×10^4	6.76×10^3	0.0004
is the labeled	AD	2.87×10^4	2.53×10^4	0.0041
carbon source	Tcontrol	1.10×10^5	2.65×10^4	
	I	7.47×10^4	1.32×10^4	0.006
	AI	6.47×10^4	1.86×10^4	0.017
	DI	7.08×10^4	1.41×10^4	0.009
	ADI	3.42×10^4	7.40×10^3	0.003
^{45}Ca incorporation	Control	2.00×10^6	3.69×10^5	
into sclerites	Ac	1.06×10^6	1.24×10^5	0.002
when [^{14}C]glucose	D	1.68×10^6	1.16×10^5	0.015
is the labeled	AD	5.88×10^5	1.51×10^5	0.0014
carbon source	Tcontrol	2.01×10^6	9.90×10^4	
	I	1.43×10^6	1.54×10^5	0.006
	AI	1.08×10^6	8.30×10^4	0.0005
	DI	1.36×10^6	1.41×10^5	0.0053
	ADI	6.47×10^5	2.09×10^5	0.0098
^{14}C incorporation	Control	5.31×10^4	2.46×10^3	
into sclerites	Ac	3.19×10^4	4.79×10^3	0.029
when [^{14}C]glucose	D	4.73×10^4	5.06×10^3	0.171
is the labeled	AD	3.57×10^4	2.65×10^3	0.009
carbon source	Tcontrol	5.27×10^4	1.96×10^3	
	I	3.39×10^4	2.02×10^3	0.007
	AI	2.10×10^4	2.64×10^3	0.0008
	DI	3.77×10^4	4.96×10^3	0.0063
	ADI	2.12×10^4	3.16×10^3	0.0034

Filtered sterilized sea water (FSW) (Control); acetazolamide (Ac); DIDS (D); acetazolamide and DIDS (AD); Tris in FSW (Tcontrol); iodoacetic acid (I); acetazolamide and iodoacetic acid (AI); DIDS and iodoacetic acid (DI); acetazolamide, DIDS and iodoacetic acid (ADI).

Values are mean cts min^{-1} g^{-1} spicule; N=6.

Table 2. *Mean, standard deviation and significance value for incorporation of ^{45}Ca and ^{14}C into spicules in response to concurrent or sequential addition of inhibitors*

	Treatment	Mean	S.D.	P
Incorporation of ^{45}Ca	Control	2.03×10^6	5.65×10^5	
in the presence	AD	1.89×10^6	5.00×10^4	0.431
of NaH^{14}CO$_3$	A+D	7.04×10^5	2.24×10^5	0.004
Incorporation of ^{45}Ca	GControl	2.00×10^6	3.69×10^5	
in the presence	GAD	1.90×10^6	2.22×10^4	0.335
of [^{14}C]glucose	GA+D	5.88×10^5	1.51×10^5	0.0041
Incorporation of ^{14}C	Control	1.21×10^5	4.57×10^3	
in the presence	AD	7.99×10^4	7.52×10^3	0.011
of NaH^{14}CO$_3$	A+D	2.87×10^4	2.53×10^3	0.0014
Incorporation of ^{14}C	GControl	5.31×10^4	2.46×10^3	
in the presence	GAD	3.6×10^4	5.50×10^3	0.027
of [^{14}C]glucose	GA+D	3.57×10^4	2.65×10^3	0.009

Filtered sterilized sea water (FSW) (Control); acetazolamide and DIDS added concurrently (AD); acetazolamide added 12 h before DIDS (A+D), in the presence of H^{14}CO$_3$; FSW (GControl); acetazolamide and DIDS added concurrently (GAD); acetazolamide added 12 h before DIDS (GA+D), in the presence of [^{14}C]glucose.

Values are mean cts min^{-1} g^{-1} spicule; N=6.

Incorporation of ^{45}Ca and ^{14}C as glucose

A second group of double labeling experiments was performed in order to determine whether metabolic sources of carbon end up as calcite in *Leptogorgia virgulata* sclerites. In this case, tips were incubated with ^{45}CaCl$_2$ and [^{14}C]glucose. Fig. 4A shows the amount of ^{45}Ca incorporated into sclerites. Under these conditions, the values for those incubated in acetazolamide alone, DIDS alone and acetazolamide plus DIDS were 53.0±6.2 %, 84.0±5.8 % and 29.4±7.6 % of the control value, respectively. When incubated with iodoacetic acid, the value was 71.1±7.7 % of the Tris–FSW control value. Iodoacetic acid combined with acetazolamide resulted in a level of incorporation that was 53.7±4.1 % of the control value. Iodoacetic acid combined with DIDS gave a value that was 67.7±7.0 % of the control and, when combined with both acetazolamide and DIDS, the result was 32.2±10.4 % of the control value.

With [^{14}C]glucose as the only available labeled carbon source (Fig. 4B), the values for ^{14}C incorporation in the presence of either acetazolamide or DIDS were 60.1±9.0 % and 89.1±9.5 % of the control value, respectively. The value for incorporation in the presence of DIDS alone was not significantly different from the control value. When acetazolamide was combined with DIDS, the amount of ^{14}C incorporated into sclerites was 67.2±5.0 % of the control amount. Tips incorporating ^{14}C from glucose in the presence of iodoacetic acid, acetazolamide plus iodoacetic acid, DIDS plus iodoacetic acid, and the triple combination of acetazolamide, DIDS and iodoacetic acid had values that were
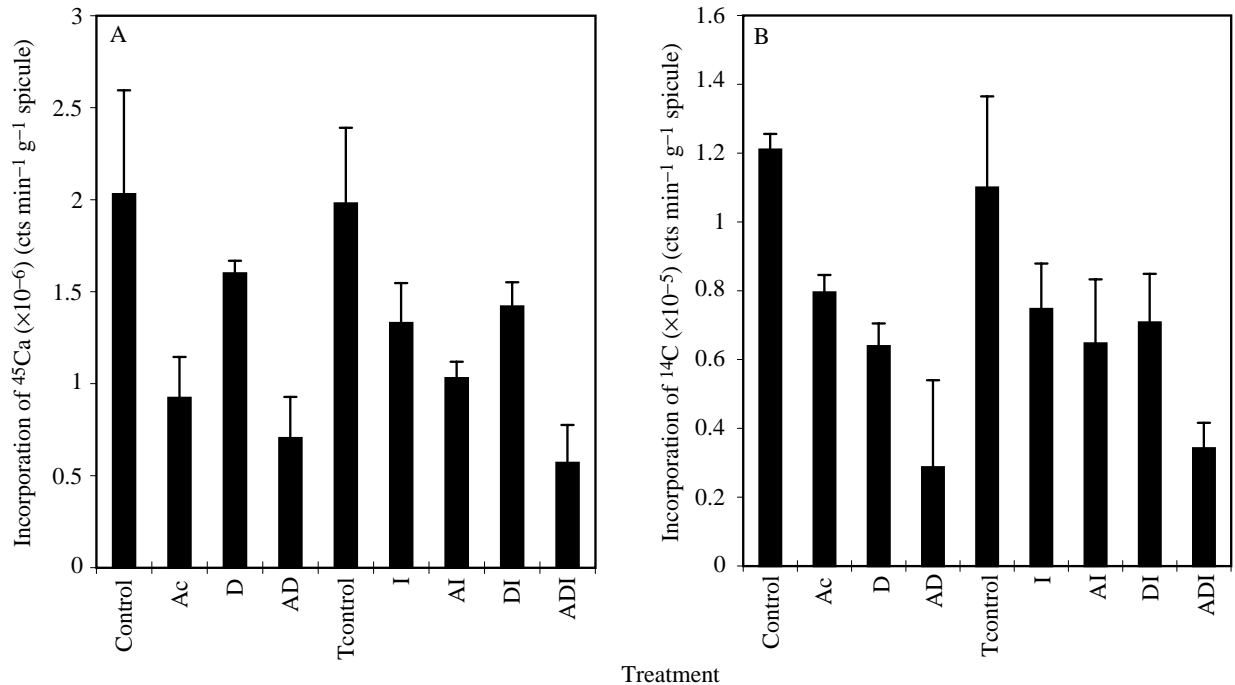
value. The combined effects of acetazolamide plus DIDS generated a value that was 23.7±20.9 % of the control value. In comparison, tips incubated with iodoacetic acid had a value that was 67.9±12.0 % of the Tris–FSW control. The combination of acetazolamide and iodoacetic acid had a value that was 58.8±16.9 % of the control. When combined, DIDS and iodoacetic acid had a value that was 64.4±12.8 % of the control. The triple combination of acetazolamide, DIDS and iodoacetic acid had a value that was 31.1±6.1 % of the control value.

Fig. 3. (A) Incorporation of ^{45}Ca expressed as counts per minute per gram of spicules when *Leptogorgia virgulata* tips are incubated with ^{45}CaCl$_2$ and NaH^{14}CO$_3$ for 24 h in the presence of the following: FSW (Control); 1 mmol l^{-1} acetazolamide (Ac); 1 mmol l^{-1} DIDS (D); 1 mmol l^{-1} acetazolamide plus 1 mmol l^{-1} DIDS (AD); 5 mmol l^{-1} Tris in FSW (Tcontrol); 1 mmol l^{-1} iodoacetic acid (I); 1 mmol l^{-1} acetazolamide plus 1 mmol l^{-1} iodoacetic acid (AI); 1 mmol l^{-1} DIDS plus 1 mmol l^{-1} iodoacetic acid (DI); and a combination of 1 mmol l^{-1} acetazolamide, 1 mmol l^{-1} DIDS and 1 mmol l^{-1} iodoacetic acid (ADI). Values are given as mean + s.d., N=6. All treatments are significantly different from the control values (P<0.05). (B) Incorporation of ^{14}C expressed as counts per minute per gram of spicules when *Leptogorgia virgulata* tips are incubated with ^{45}CaCl$_2$ and NaH^{14}CO$_3$ for 24 h in the presence of the same conditions as in A. Values are given as mean + s.d., N=6. All treatments are significantly different from the control values (P<0.05).

64.3±3.8 %, 39.8±5.0 %, 71.5±9.4 % and 40.2±6.0 % of the control values, respectively.

Sequential addition of acetazolamide and DIDS

During the development of this procedure, a few minor problems were encountered. Most problematic and possibly most interesting was the likelihood of interactions between the inhibitors acetazolamide and DIDS. Under the conditions described in the Materials and methods section, when acetazolamide and DIDS were added concurrently, no effect on ^{45}Ca incorporation was observed. However, when acetazolamide was added first, followed 12 h later by the addition of DIDS, an additive inhibitory effect was seen. Fig. 5A shows these results. With NaH^{14}CO$_3$ as the labeled carbon source, the value for concurrent addition was 93.1±2.5 % of the control value, indicating no inhibition. When added sequentially, this value was 34.7±11.0 % of the control. Also shown in Fig. 5A is the amount of ^{45}Ca incorporated into spicules when [^{14}C]glucose was the labeled carbon source. In this case, when acetazolamide and DIDS were added concurrently or sequentially, the values for ^{45}Ca incorporation were 95.0±1.1 % and 29.4±7.6 % of the control value, respectively, indicating that neither acetazolamide nor DIDS was effective when presented to tips simultaneously.

Similarly impressive differences were seen when ^{14}C incorporation was examined (Fig. 5B). With NaH^{14}CO$_3$, the concurrent and sequential values were 66.0±6.2 % and 23.7±2.1 % of the control value, respectively. With [^{14}C]glucose as the labeled carbon source, the value for the concurrent addition of acetazolamide and DIDS was 67.8±10.4 % of the control, while for the sequential addition of these inhibitors the value was 67.2±5.0 % of the control value.

Similar differences were recorded in both the incorporation of ^{45}Ca and ^{14}C, regardless of carbon source, when acetazolamide, DIDS and iodoacetic acid were added simultaneously or when acetazolamide and iodoacetic acid were added first, followed 12 h later by the addition of DIDS (data not shown).

Discussion

For a number of years, the potential sources of carbon for calcium carbonate formation in corals has been debated (Goreau, 1959; Pearse, 1970). The current study was undertaken in an effort to shed some light on this issue. Key to the success of these experiments are the use of specifically labeled sources of carbon and the specificity of the physiological inhibitors in which the tips of *Leptogorgia*
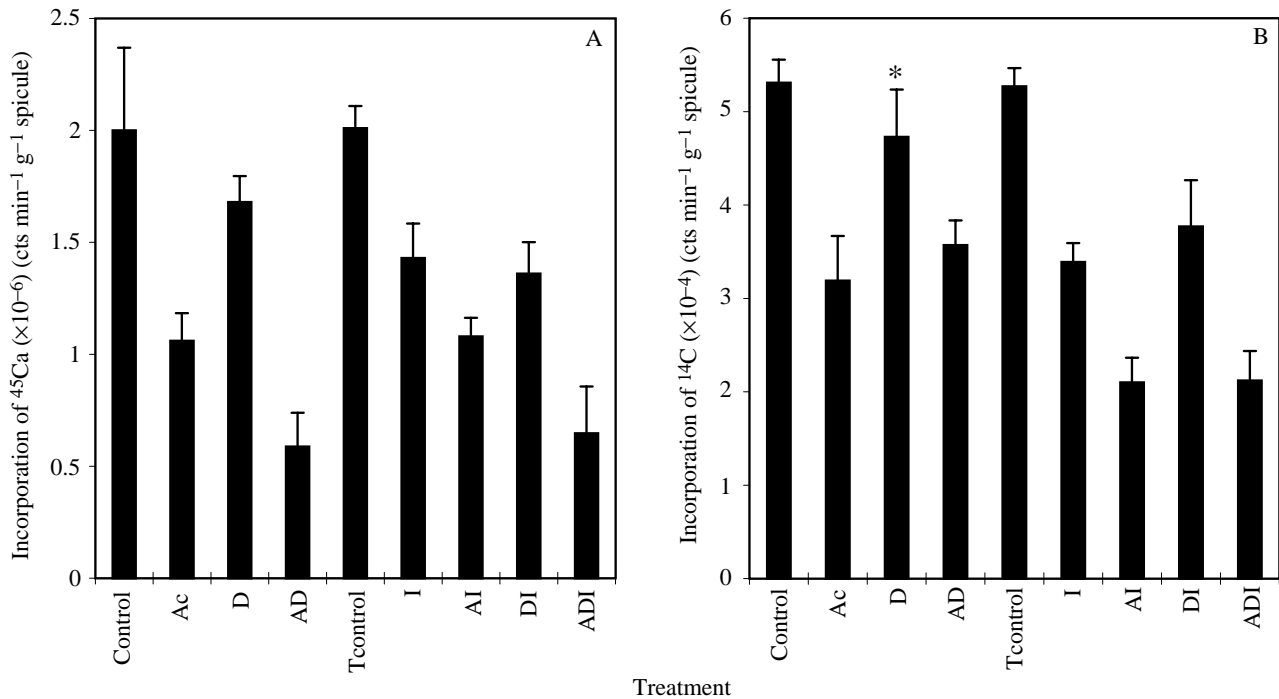
Fig. 4. (A) Incorporation of ^{45}Ca expressed as counts per minute per gram of spicules when *Leptogorgia virgulata* tips are incubated with ^{45}CaCl$_2$ and [^{14}C]glucose for 24 h in the presence of the same conditions as in Fig. 3A. Values are given as mean + S.D., N=6. All treatments are significantly different from the control values (P<0.05). (B) Incorporation of ^{14}C expressed as counts per minute per gram of spicules when *Leptogorgia virgulata* tips are incubated with ^{45}CaCl$_2$ and [^{14}C]glucose for 24 h in the presence of the same conditions as in Fig. 3A. An asterisk indicates a value that is not significantly different from the control value. Values are given as mean + S.D., N=6.

virgulata were incubated. Since the work of Goreau (1959), it has been known that the enzyme carbonic anhydrase is central to the calcification process in corals. Through the use of the specific carbonic anhydrase inhibitor acetazolamide, at concentrations up to 1 mmol l^{-1}, calcification in *L. virgulata* is effectively reduced to approximately 50 % of control values. This reduction in calcium incorporation does not correspond to the results reported by Kingsley and Watabe (1987), but is in agreement with the effects of acetazolamide on scleractinian corals (Goreau, 1957) and other gorgonians (Allemand and Grillo, 1992). We were concerned that the uptake of acetazolamide into coral cells was limiting so, to overcome this possible limitation, acetazolamide was used at the highest concentration utilized by Goreau (1959), 1 mmol l^{-1}, which was sufficient to inhibit calcification maximally, yet had no adverse effects on the survival of the organisms. This is significantly more concentrated than the 10^{-6} mol l^{-1} acetazolamide that has been shown to inhibit purified *L. virgulata* carbonic anhydrase completely *in vitro* (Lucas and Knapp, 1996), but *in vivo* treatment took into consideration the possible restriction on transport of this inhibitor into the scleroblast cells of the colony. It is interesting to note that, in the presence of DIDS, 1 mmol l^{-1} acetazolamide added concurrently has no effect on calcification (Fig. 5A,B). The reverse was also true: DIDS was not effective in the presence of acetazolamide. In addition, the long-term viability of octocorals, as measured by Trypan Blue dye exclusion, is not

affected by exposure to 1 mmol l^{-1} acetazolamide for the 2–3 days over which such studies were carried out (data not presented).

In terms of carbon sources, acetazolamide is able to reduce the amount of dissolved inorganic carbon incorporated into spicules by 34.3 %. Furthermore, acetazolamide inhibits the incorporation of metabolically derived carbon by 39.9 %. Therefore, regardless of the carbon source, carbonic anhydrase plays a pivotal role in the pathways involved in calcification.

Carbonic anhydrase has been localized by the cobalt phosphate method and immunolocalized using antiserum raised against chicken carbonic anhydrase (Kingsley and Watabe, 1987) and, more recently, using antiserum raised against purified *Leptogorgia virgulata* carbonic anhydrase (Lucas *et al.* 1996). All of these techniques have localized carbonic anhydrase to the spicule vacuole membrane and to the inside of electron-dense bodies, which are Golgi-derived vesicles. The vesicles eventually fuse to the spicule vacuole and provide the materials needed to continue the growth of both the spicule and the vacuole. This places carbonic anhydrase in close proximity to the site of calcification. Carbonic anhydrase is known to be important in the production of hydrogen ions and bicarbonate ions and, thus, in regulating local pH. However, acidification tends to dissolve calcium carbonates (Simkiss and Wilbur, 1989), and an environment in which hydrogen ions are produced and retained would not be conducive to calcification. Carbonate ions not utilized in the
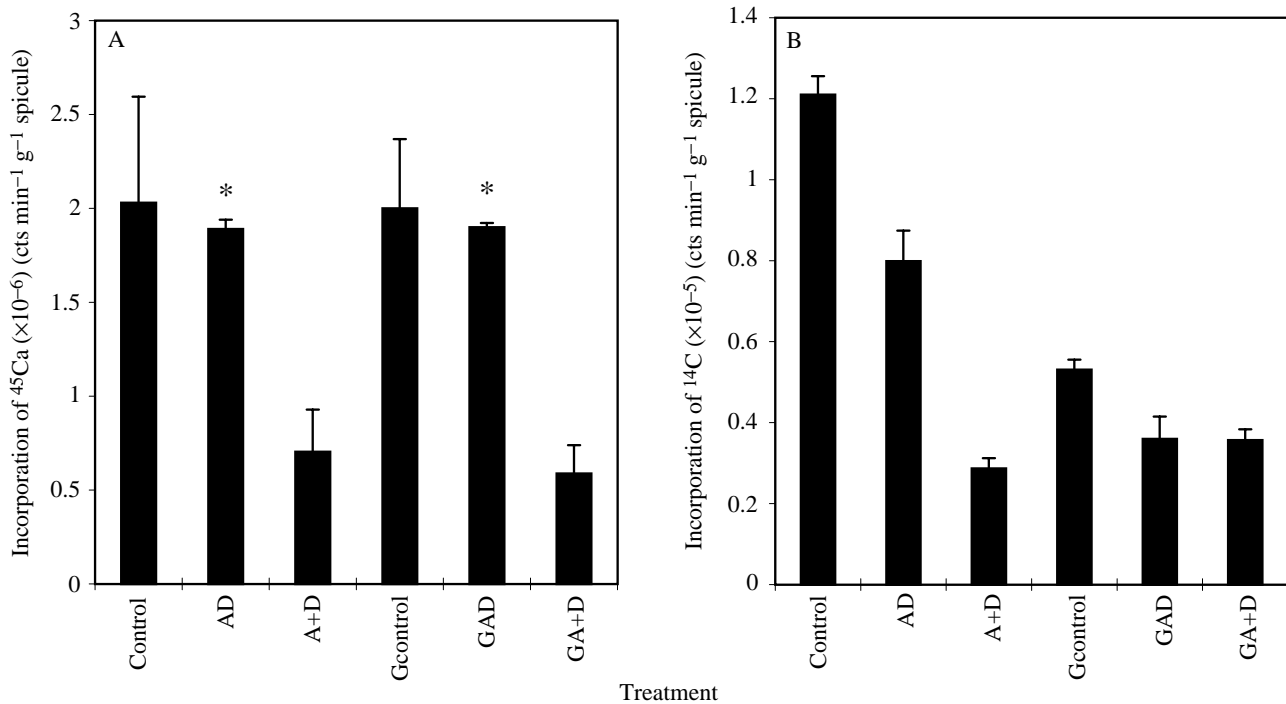
Fig. 5. (A) Comparison of ^{45}Ca incorporation in counts per minute per gram of spicules when acetazolamide and DIDS are added concurrently (AD) and when acetazolamide was added 12 h before DIDS (A+D) in the presence of ^{45}CaCl$_2$ and NaH^{14}CO$_3$. Similar results are obtained when [^{14}C]glucose is the carbon source: glucose control (Gcontrol), when acetazolamide and DIDS are added concurrently (GAD), and when they are added 12 h apart (GA+D). An asterisk indicates a value that is not significantly different from the control value. Values are given as mean + S.D., N=6. (B) Comparison of ^{14}C incorporation in counts per minute per gram of spicules when acetazolamide and DIDS are added concurrently (AD) and when acetazolamide was added 12 h before DIDS (A+D) in the presence of ^{45}CaCl$_2$ and NaH^{14}CO$_3$. Similar results are obtained when [^{14}C]glucose is the carbon source: glucose control (Gcontrol), when acetazolamide and DIDS are added concurrently (GAD), and when they are added 12 h apart (GA+D). Values are given as mean ± S.D., N=6. All treatments are significantly different from the control values (P<0.05).

formation of calcite would act as a buffer potentially maintaining a neutral to alkaline environment in which calcification would be supported. However, it remains to be determined exactly how carbonic anhydrase is involved in controlling the acid–base equilibrium affecting calcification and directly suppling HCO$_3^-$ for calcification.

The second inhibitor examined in this series of experiments was the anion transport inhibitor DIDS. DIDS is known to inhibit HCO$_3^-$ transport channels (Madshus, 1988). DIDS decreased calcium incorporation by only approximately 20 %. However, its effects on ^{14}C incorporation are much more dramatic. DIDS decreases the amount of carbon accumulation in sclerites from labeled NaHCO$_3$ by 47.2 %, but has no significant effect when glucose is the labeled carbon source. This pattern of inhibition indicates that DIDS is specific for plasma membrane anion transporters and therefore has no effect on the uptake of glucose or on the intracellular formation of HCO$_3^-$.

The final inhibitor applied to the growing tips of *Leptogorgia virgulata* was iodoacetic acid. Iodoacetic acid is a specific inhibitor of the enzyme glyceraldehyde-3-phosphate dehydrogenase (Webb, 1966). This enzyme is responsible for a crucial step in glycolysis, a step that occurs well before the

production of pyruvate and therefore effectively eliminates the production of CO$_2$ that occurs upon the entry of pyruvate into the citric acid cycle. In other words, iodoacetic acid prevents the breakdown of glucose into CO$_2$ and the concomitant production of ATP.

Tips incubated in iodoacetic acid show a decrease in calcium incorporation of approximately 30 %. The two most likely reasons for this decrease are (1) that calcium uptake and utilization are energy-requiring processes and that iodoacetic acid indirectly inhibits calcification by inhibiting the active transport of Ca^{2+}, and (2) that, under these conditions, the CO$_3^{2-}$ ion is the limiting ion in spiculogenesis and that metabolically derived CO$_2$ is normally a well-utilized substrate for carbonic anhydrase. These are not mutually exclusive explanations. It has been shown that calcium uptake and mobilization in *L. virgulata* are controlled, at least in part, by Ca^{2+}-ATPases (Kingsley and Watabe, 1984), which require sufficient ATP to operate. Iodoacetic acid decreased the incorporation of carbon from NaHCO$_3$ by 32.1 % and from glucose by 35.7 %. The percentage decrease in incorporation of carbon from glucose is significant but relatively low, indicating that iodoacetic acid may be taken up by coral cells only to a limited extent or does not completely inhibit

glycolysis. Another possibility is that *L. virgulata* is able to utilize available organic carbon in ways other than by glycolysis.

As a mechanism for elucidating further the pathways of carbon incorporation into sclerites, the above inhibitors were used in combination with each other. In almost all cases, combining inhibitors resulted in additive effects. When used in combination, acetazolamide and DIDS were expected to eliminate a large percentage of HCO_3^- utilization for calcite formation. In fact, this particular combination when added sequentially decreased calcium incorporation by almost 70 %. It is not known whether incorporation can ever be decreased by 100 %. Issues of isotopic exchange and the natural, nonenzymatic conversion of H_2O and CO_2 to H^+ and HCO_3^- make this seem unlikely. When the amount of carbon incorporated from $NaHCO_3$ is studied, the combination of acetazolamide and DIDS added sequentially decreases carbon incorporation by 76.3 %. However, the effect of this combination is very similar to the effect of acetazolamide alone on carbon incorporation from glucose, 39.9 %.

When acetazolamide is combined with iodoacetic acid, calcification decreases by approximately 47 %. This is essentially the same percentage decrease as is seen with acetazolamide alone. However, an additive effect occurs when carbon incorporation is examined. This additive effect is small when sodium bicarbonate is the labeled carbon source, 41.2 %, but is substantial when glucose is the source, 60.2 %. This particular combination essentially knocks out a large part of the metabolic CO_2 available for calcification. Even if glycolysis is not completely inhibited by iodoacetic acid, any CO_2 produced by the citric acid cycle will not be available as HCO_3^- since carbonic anhydrase will be inhibited. Once again, issues of isotopic exchange and the nonenzymatic conversion of carbon dioxide to bicarbonate probably prevent complete inhibition of this apparent calcification pathway.

The combination of DIDS and iodoacetic acid did not show any additive effects in comparison with these inhibitors used separately. Regardless of which isotope is examined and from which source it came, the decrease in calcium and carbon incorporation is approximately 30 %. This indicates that bicarbonate transport is minimally involved in providing anions for calcification and, therefore, that membrane-permeable CO_2 is probably a more important source of carbon for calcification in *Leptogorgia virgulata*.

As expected, the greatest decrease in calcium incorporation was caused by the addition of all three inhibitors: acetazolamide, DIDS and iodoacetic acid. An approximately 70 % decrease in calcium incorporation was observed. This value is matched by a decrease in carbon incorporation, 68.9 %, when $NaHCO_3$ is the labeled carbon source. However, when glucose is the radioactive carbon source, carbon incorporation is decreased by 59.8 %, a value very similar to the combined effects of acetazolamide and iodoacetic acid. Since DIDS should have no effect on glucose uptake or utilization, this is considered to be a reasonable result.

Fig. 2 presents a simple diagrammatic model of the potential sources and pathways for carbon uptake and subsequent accumulation as calcite in *Leptogorgia virgulata* scleroblasts. These inhibitor studies indicate that a substantial portion of carbon for calcification in *L. virgulata* is utilized through each of these pathways. While the contribution of metabolically generated carbon dioxide to calcification remains speculative, it appears that, under the conditions of these experiments, glycolysis and the citric acid cycle generate approximately 33 % of the carbon for spiculogenesis. The other 67 % comes from dissolved inorganic carbon.

In establishing the procedure used in this set of experiments, a number of difficulties were encountered. First, dimethyl sulfoxide and ethanol, both frequently used to dissolve acetazolamide and DIDS, proved problematic (data not shown). Tips incubated in these solvents had significantly higher than normal calcium incorporation. Presumably these chemicals perturbed the ability of *Leptogorgia virgulata* to regulate calcium uptake or accumulation, and ^{45}Ca was able to exchange directly with calcium in the calcitic spicule. Second, as stated in the Results, when acetazolamide and DIDS were added simultaneously, no effect was seen on calcium incorporation. Since this is contradictory to the effects of the individual inhibitors, we speculated that the order of addition of the inhibitors may be important. It was determined that acetazolamide had to be added first, since it must be taken up by the cells to be effective, followed 12 h later by DIDS, which acts extracellularly upon plasma membrane anion transporters. These two inhibitors have ring structures with multiple potentially reactive components. In all probability, under these conditions, some interaction occurred between the two, making each of them ineffective when added simultaneously.

While not unexpected, it is important to note that the amount of ^{45}Ca incorporated was not equal to the amount of ^{14}C incorporated into sclerites. A possible explanation for this is that the mechanisms for calcium uptake and transport to the site of calcification are faster than those for carbon accumulation. However, it has been suggested (Barnes and Crossland, 1977) that ^{14}C incorporation does not necessarily correlate with ^{45}Ca incorporation because there are multiple internal pools of carbon that act to dilute out newly absorbed ^{14}C. Presumably, dilution of labeled calcium with internal pools is not a significant factor affecting the incorporation of calcium.

The present study involved large amounts of data using a number of inhibitors and combinations of inhibitors, and two important conclusions can be drawn from this work. First, carbon for spiculogenisis can come both from dissolved inorganic carbon in the environment and from metabolically produced carbon dioxide. While the latter has been theorized, it has never been demonstrated in octocorals before. Second, regardless of the carbon source, the enzyme carbonic anhydrase plays a pivotal role in the physiology of spicule formation in *Leptogorgia virgulata*.

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