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Inorganic Carbon Supply to Symbiont Photosynthesis of the Sea Anemone, *Anemonia viridis*: Role of the Oral Epithelial Layers

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Abstract

The role of the oral epithelial cell layers of the symbiotic sea anemone, *Anemonia viridis*, in dissolved inorganic carbon (DIC) supply for the photosynthesis of the symbiotic dinoflagellates (*Symbiodinium* sp.) was investigated. For this purpose, we used sections of isolated tentacles prepared as small "bags" either normally oriented or everted (inside-out). Preliminary experiments showed that the dark respiration rate cannot supply enough CO_2 for dinoflagellate photosynthesis and that DIC must be obtained from seawater. Experiments with bicarbonate-free seawater showed that while the major source of DIC is the external medium, about one third is absorbed from the internal space. Both the ectoderm and the endoderm seem to be able to absorb DIC. Calculation of spontaneous dehydration of HCO_3^- into CO_2 indicates that HCO_3^- absorption by the host cells is involved in DIC supply to dinoflagellate photosynthesis. Further, the permeability of the oral epithelial layers to HCO_3^- and CO_2 appears to be insufficient to maintain the optimal photosynthetic rate. The utilization of an anion carrier inhibitor (400 μM DIDS) showed that HCO_3^- uptake is likely operative on both ectodermal and endodermal cells. The other sources of DIC come from the dehydration of bicarbonate into carbon dioxide as suggested by the sensitivity to inhibitors of

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carbonic anhydrase (Diamox) and of H^+-ATPase (DES). Our results suggest the involvement of the ectodermal cell layer in the supply of inorganic carbon for photosynthesis of zooxanthella located inside the endodermal cells.

Keywords: Sea anemone, dinoflagellate, photosynthesis, epithelium, HCO_3^- transport

1. Introduction

Endosymbiotic relationships between dinoflagellates and anthozoans play a dominant role in their respective physiology. Symbiotic dinoflagellate cells, commonly called zooxanthellae, are generally located in the endodermal cell layer of the anthozoan oral epithelia (Taylor, 1973; Trench, 1981). They are isolated from the cytoplasm of the animal cell by a perisymbiotic membrane (Rands et al., 1992). Oral epithelia of sea anemones consist of two layers of cells, the ectoderm and the endoderm, separated from each other by a thin connective layer, the mesoglea. This epithelial structure delimits a cavity, the coelenteron. The ectoderm faces the external seawater whereas the endoderm faces the coelenteron. In contrast to free-living algae, symbiotic dinoflagellates are then not directly in contact with seawater, consequently the inorganic carbon required for photosynthesis must be absorbed from the host animal cell. The origin of DIC can be the animal respiration and/or the external seawater and/or the coelenteric fluid. If DIC comes from the coelenteric fluid, it must diffuse from the fluid surrounding the endodermal cells to the zooxanthellae across at least four different membranes (endodermal cell plasma membrane, perisymbiotic membrane, zooxanthella plasma membrane and chloroplast membrane) to reach the photosynthetic site. If DIC comes from the external seawater, in addition to this pathway, it has also to diffuse from seawater across the oral ectodermal cells and mesoglea to the fluid surrounding the endodermal cells.

Depending on physico-chemical parameters, DIC is present in seawater in several forms. In the atmosphere, the average CO_2 level is 340 ppmv (Sundquist, 1985). At this atmospheric level, dissolved CO_2 concentration is approximately 10 μM when in equilibrium with the atmosphere, but even if the level of dissolved CO_2 in water is similar to that in the air, CO_2 diffuses about 1000 times slower in water (Raven, 1970) and so CO_2 is less available for the "organism's" requirements. In seawater, dissolved CO_2 is hydrated, forming H_2CO_3, which equilibrates with HCO_3^- and CO_3^{2-} as shown below:

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \Leftrightarrow CO_3^{2-} + 2H^+$$

In seawater, at pH 8.2, the predominant form of DIC is HCO_3^- with a concentration of about 2 mM. Under these conditions, DIC could be limiting, because the movement of HCO_3^- across unstirred boundary layers and several animal and algal membranes can be very slow (Kerby and Raven, 1985; Gutknecht et al., 1977; Walker et al., 1980). HCO_3^- can however be transported by facilitated diffusion (Na^+-independent Cl^-/HCO_3^- exchange) or by active mechanisms (HCO_3^- stimulated ATPases, Na^+-dependent transport, H^+/HCO_3^- symport) (Lucas, 1983; Smith, 1988). HCO_3^- can also be converted in the extracellular medium into CO_2 according to its equilibrium constant, to the temperature and environmental pH and then it may diffuse across the membrane. This can be enhanced by carbonic anhydrase (CA), an enzyme responsible for the conversion between CO_2 and HCO_3^- (see for a recent review Badger and Price, 1994). Finally, it has also been suggested that HCO_3^- may be actively dehydrated by a proton-translocating ATPase (Ferrier, 1980; Lucas, 1985; Walker et al., 1980).

The objective of the present study is to determine i) the permeability properties of the oral epithelial layers to DIC and the origin of DIC (animal respiration and/or external seawater and/or coelenteric fluid), ii) the form of DIC transported from seawater to zooxanthellae, and iii) the mechanism of transport. For this purpose we used the temperate actinian *Anemonia viridis*, whose tentacles can be easily manipulated. A specially made biological preparation was set up. It consists of little bags made of pieces of tentacles. The intraluminal medium of these bags was filled with different media by perifusing the tentacles, so that both external or internal media could be modified. In some experiments, we also used everted bags (i.e. inside-out tentacles). The rate of net O_2 production was used to measure the rate of photosynthesis. After determining photosynthesis-irradiance relationships, DIC absorption was studied by using bicarbonate-free seawater and inhibitors of inorganic carbon transport pathways.

2. Materials and Methods

Biological material

Specimens of the Mediterranean sea anemone, *Anemonia viridis* (Forsskål), were collected in Villefranche-sur-mer, France, and were maintained in an open-circuit seawater aquarium. The seawater was pumped from the Mediterranean sea at a depth of 50 m, 100 m away from the coast. Light was provided by a metal halide lamp (HQI-T 400 W, Philips), with a photosynthetic photon flux density of 125 µmol photons.$m^{-2}.s^{-1}$ and a 12:12

photoperiod. Tentacles were removed from the animals by cutting them at the base and placing them in a beaker containing seawater.

Preparation of Anemonia viridis tentacles

One catheter (external diameter of 1.57 mm) was introduced at the base of the tentacle and another catheter (external diameter of 1.22 mm) was introduced at the apex of the tentacle and ligatures were made on both catheters. The tentacle was either filled using a syringe containing the appropriate medium or perifused. In the former, once the tentacles were filled with appropriate medium, ligatures were made in order to obtain small bags. In the latter, the perifused tentacle was simultaneously perifused by placing it in a closed tube with holes for the catheters which were connected to a peristaltic pump (Ismatec-MV-GE pump system; Fig. 1). The perifusion volume in the tube was 10 ml and the perifusion flow was 2 $ml.min^{-1}$. After the perifusion, small bags were obtained as described above. The tentacle bag with the ectoderm outside and the endoderm inside was called a "normal" tentacle. In another kind of experiment, a catheter (external diameter of 0.96 mm) was introduced at the cut apex of the tentacle and through the whole tentacle. A ligature was made and the tentacle was everted along the catheter. The ligature was cut and the catheter removed. This "inside-out" tentacle was used in the same way as the normal tentacle in order to obtain small bags. In all

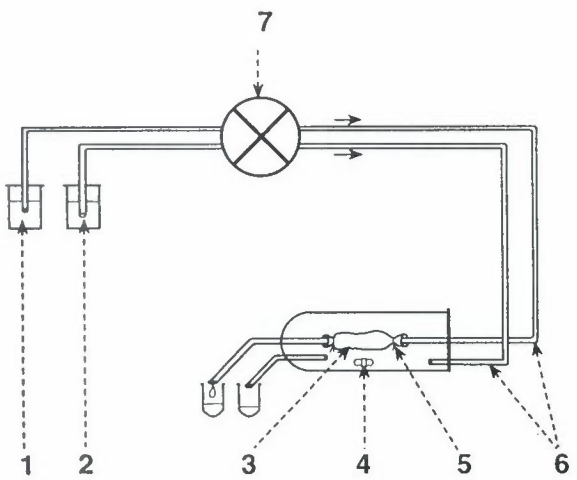

Figure 1. Experimental system used for the simultaneous perifusion and perifusion of isolated tentacles of *Anemonia viridis*.
1 = Internal perifusion medium; 2 = external perifusion medium; 3 = tentacle; 4 = magnetic bar; 5 = ligature; 6 = catheters; and 7 = peristaltic pump.

cases, the medium inside the bag was called "internal medium" whereas the medium outside the bag was called "external medium".

Measurement of photosynthetic rate

The rate of photosynthesis was measured by the rate of net O_2 production. Irradiance was measured using a 4 π light sensor (Biospherical Instruments Inc. QSL-100). Illumination was provided by a metal halide lamp (HQI TS 250W, Osram). Oxygen flux was measured using micro-respirometers (Rank Brothers, Ltd, Cambridge, England) comprising a double-walled cylindrical perspex chamber (4–6 ml volume) and a Clark oxygen electrode coupled to a chart recorder and a magnetic stirrer. The chamber was closed by a stopper which prevented any exchange with the atmosphere. Temperature was maintained at 16 ± 0.5°C with a recirculating water bath (Lauda RM20) and O_2 stratification was avoided using a magnetic stirring bar. In each of the three chambers used, one tentacle bag was put on a plastic grid. The O_2 electrode was calibrated before each measurement in oxygen-free seawater (2% sodium sulphite) and in air-saturated seawater (247.75 nmol.ml^{-1} at 16°C, Green and Carritt, 1967).

Seawater preparation

Filtered seawater (FSW) was obtained by filtering Mediterranean seawater through a 0.45 µm Millipore membrane. The composition of artificial seawater (ASW) was: NaCl 490 mM, $CaCl_2$ 10 mM, $MgCl_2$ 27 mM, $MgSO_4$ 29 mM, $NaHCO_3$ 2 mM and KCl 10 mM in distilled water, pH was adjusted to 8.2 by 0.1 N NaOH (Allemand et al., 1984). Bicarbonate-free seawater (0HCO_3^- FSW) was prepared by adjusting the pH of filtered seawater to 4.5 using 1 N HCl to convert all DIC to CO_2. The CO_2 was removed by bubbling nitrogen through the seawater for at least 30 min. The seawater was stirred throughout this procedure. The pH was then adjusted to 8.2 with CO_2-free NaOH prepared by making a saturated NaOH solution to precipitate any sodium carbonate from solution (Yellowlees et al., 1993).

The composition of sodium-free artificial seawater (0Na^+-ASW) was: choline chloride 490 mM, $CaCl_2$ 10 mM, $MgSO_4$ 29 mM, $MgCl_2$ 27 mM, KCl 10 mM, choline bicarbonate 2 mM, Tris 0.5 mM and pH was adjusted to 8.2 with NaOH 0.1 N.

All chemicals were obtained from Sigma or Merck.

Photosynthesis-irradiance curve

The photosynthesis-irradiance (PI) curve was established by measuring O_2 flux at various light intensities from 0 to 1200 µmol photons.m^{-2}.s^{-1}. Different irradiances were provided by varying the distance between the metal halide lamp and the micro-respirometer. In all cases, O_2 flux was measured for at least 10 min.

Pharmacology

DIDS (4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid), an anion carrier inhibitor, was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution of 100 mM. Except where otherwise stated, the final concentration in FSW was 400 µM. Acetazolamide, N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl]-acetamide (Diamox), a carbonic anhydrase inhibitor, was dissolved in DMSO to a concentration of 60 mM and buffered with 1 M Tris to a pH of 8.2. The final concentration in FSW was 600 µM. The H^+-pump inhibitor diethylstilbestrol (DES) was dissolved in absolute ethanol to a concentration of 50 mM and buffered with 1 M Tris to a pH of 8.2. The final concentration in FSW was 100 µM.

While preliminary experiments demonstrated that concentrations of DMSO and ethanol up to 1% (v/v) caused no significant effect on net O_2 flux (results not shown), in this study we never used DMSO or ethanol at a concentration over 0.5%. Inhibitors were added through a small hole in the stopper of the micro-respirometer or when specified, they were introduced in the perifusion and/or perifusion medium for 20 min.

Presentation of results

Results are presented as net O_2 flux (expressed in nmol O_2.min^{-1}.mg^{-1} protein) or as percentage of inhibition of the net O_2 production in relation to the control experiments. Protein determination was carried out according to the method of Lowry et al. (1951) with bovine serum albumin as a standard using an autoanalyzer (Alliance Instruments) on tentacle sonicated for 10 s in 1 ml of distilled water.

Statistical analysis and curve fitting

Curves were fitted by an hyperbolic tangent function using the Igor 1.27 (Wave Metrics, Inc.) program. Three replicate incubations were conducted for each experiment and the results are presented as mean ± standard deviation.

3. Results

Photosynthesis-Irradiance curves

Fig. 2 shows that zooxanthellae in the tentacle of the Mediterranean sea anemone *Anemonia viridis* exhibit typical PI curve fitted by an hyperbolic tangent function (Crossland and Barnes, 1977). The maximum net O_2 production was reached at about 200 µmol photons.$m^{-2}.s^{-1}$ and had a value of 5.39±0.43 nmol $O_2.min^{-1}.mg^{-1}$ protein. The compensation irradiance (Ic) at which the net O_2 flux is 0, was approximately 30 µmol photons.$m^{-2}.s^{-1}$. No photoinhibition occurred up to 1200 µmol photons.$m^{-2}.s^{-1}$. All subsequent experiments have been set at the saturating light intensity of 300 µmol photons.$m^{-2}.s^{-1}$.

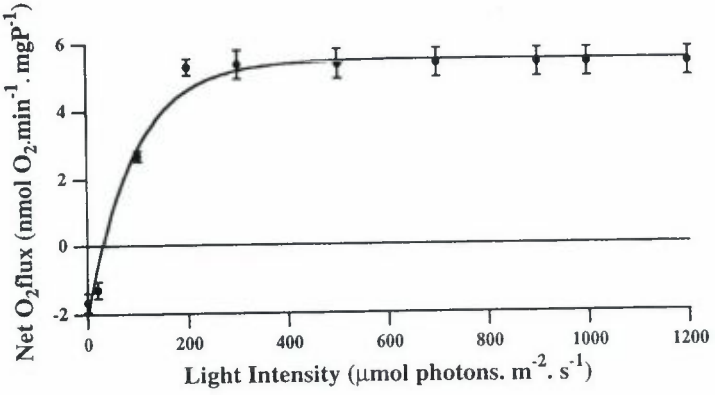

Figure 2. Photosynthesis-irradiance curve of bags from normal tentacle of *Anemonia viridis*.

The respiration rate measured in darkness was 1.67±0.27 nmol $O_2.min^{-1}.mg^{-1}$ protein. According to the hypothesis of Harland and Davies (1995), the respiratory rate measured in the light should be higher than the respiratory rate measured in the dark. Since we measured this rate just after a light period, it can be suggested that this value was the best estimate of the real respiration rate in light. Assuming that a close stoichiometry exists between oxygen evolution and inorganic carbon uptake (Cook et al., 1988), the rate of respiration is about 3 times lower than the rate of photosynthesis suggesting that zooxanthellae have to use another source of inorganic carbon than respiratory CO_2 to achieve photosynthesis.

Inorganic carbon-dependent variations in photosynthesis

Fig. 3 shows the results of experiments on the normal tentacle in bicarbonate-free seawater. Four experimental conditions are shown depending on the presence of bicarbonate outside and/or inside the tentacle. When bicarbonate was omitted both inside and outside the tentacle, net O_2 production was totally inhibited, i.e. the compensation point was reached. When bicarbonate was only absent from the external medium, the inhibition was of 67±8% whereas it was of 37±6% when it was only absent from the internal medium. The same set of experiments was performed on the inside-out tentacle (Fig. 4). The rate of net O_2 production of inside-out control tentacle was not modified when compared to control normal tentacle demonstrating the viability of our preparation (normal: 5.39±0.43 nmol $O_2.min^{-1}.mg^{-1}$ protein; inside-out: 6.41±0.71 nmol $O_2.min^{-1}.mg^{-1}$ protein). Surprisingly, it appeared that approximately the same results as observed for normal tentacle were obtained with inside-out tentacle with a greater inhibition when bicarbonate was omitted in the external medium (60±7% of inhibition vs. 27±5% of inhibition when only bicarbonate was absent from the internal medium). Whatever the orientation of the tentacle, the rate of net photosynthesis was maintained constant for at least 30 min when bicarbonate was present in the external medium, while it was constant only during about 6 min before decreasing to reach the compensation point after 20 min when it was present only in the internal medium (results not shown).

Pharmacology

In all the cases where the inhibitors were added through the hole of the stopper of the micro-respirometer, the effects were observed in less than one minute.

Anion carrier inhibitor

We tested the effect of a band 3 anion exchange inhibitor, DIDS (Gillies and Martinez-Zaguilan, 1991), added in the external medium on net O_2 flux. First a dose-response curve determined the concentration of DIDS needed to inhibit photosynthesis. The inhibition by DIDS as shown in Fig. 5 followed a saturable function. Maximum inhibition was obtained by the addition of about 200 µM DIDS, the apparent half inhibition constant (IC_{50}) was about 70 µM of DIDS. Approximately 70% of net O_2 production appeared to be DIDS-insensitive. The effect of DIDS on the net O_2 production of the normal tentacle is shown in Fig. 6. The tentacle was perifused 20 min in absence or in presence of 400 µM DIDS either outside or inside the tentacle or both outside and inside. Then the photosynthetic rate of the tentacle was measured. It can be seen that

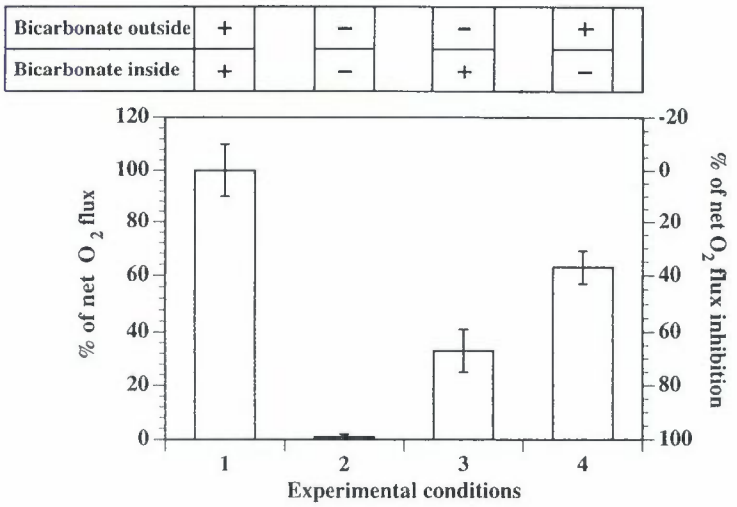

Figure 3. Effect of bicarbonate-free artificial seawater on the photosynthesis of bags from normal tentacles of *Anemonia viridis*.
1 = Control experiment: ASW in the external medium and in the internal medium;
2 = 0HCO_3^- ASW in the external medium and in the internal medium;
3 = 0HCO_3^- ASW in the external medium, ASW in the internal medium;
4 = ASW in the external medium, 0HCO_3^- ASW in the internal medium.

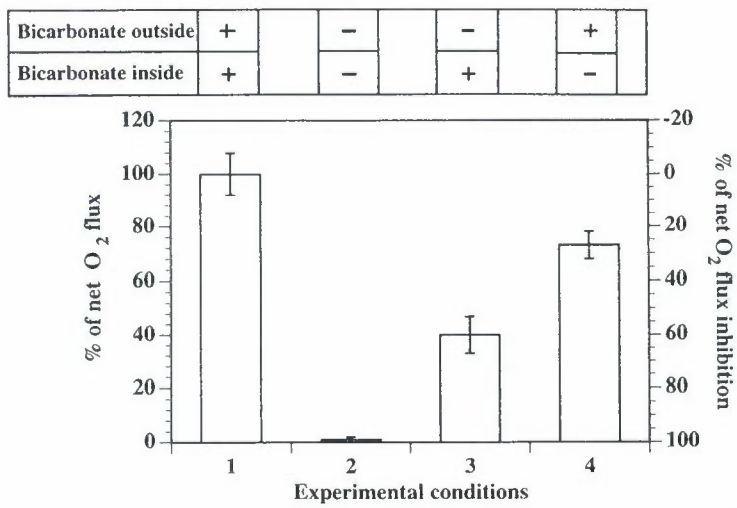

Figure 4. Effect of bicarbonate-free artificial seawater on the photosynthesis of bags from inside out tentacles of *Anemonia viridis*.
1 = Control experiment: ASW in the external medium and in the internal medium;
2 = 0HCO_3^- ASW in the external medium and in the internal medium;
3 = 0HCO_3^- ASW in the external medium, ASW in the internal medium;
4 = ASW in the external medium, 0HCO_3^- ASW in the internal medium.

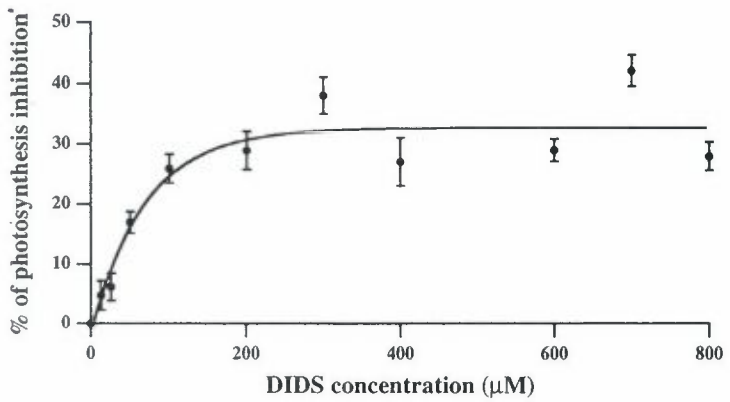

Figure 5. Dose-response curve of the effect of the anion carrier inhibitor, DIDS, added in the external medium on photosynthesis of bags from normal tentacle of *Anemonia viridis*.

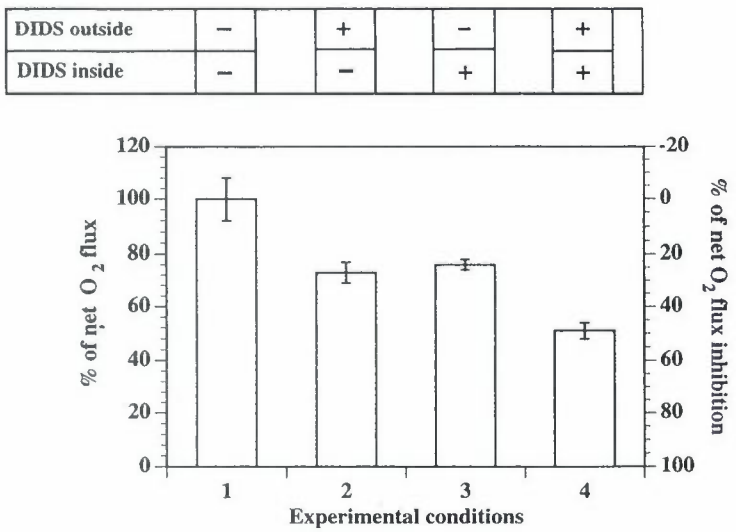

Figure 6. Effect of external and/or internal 400 μM DIDS on the photosynthetic activity of bags from normal tentacle of *Anemonia viridis*.

DIDS led to a similar percentage of inhibition when it was perifused either outside or inside the tentacle (DIDS outside: 27±4% inhibition; DIDS inside: 24±2% inhibition). The effect was additive when it was perifused both inside and outside (i.e. 49±3%).

Carbonic anhydrase inhibitor

Diamox (600 μM), a carbonic anhydrase inhibitor, was used in order to check whether carbonic anhydrase (CA) played a role in inorganic carbon supply for zooxanthella photosynthesis (Fig. 7). When Diamox was added in the external medium, it inhibited 30±5% of photosynthesis, while an inhibition of 62±4% was observed when the inhibitor was perifused in the tentacle. The effect of Diamox was not additive when it was added simultaneously outside and inside the tentacle.

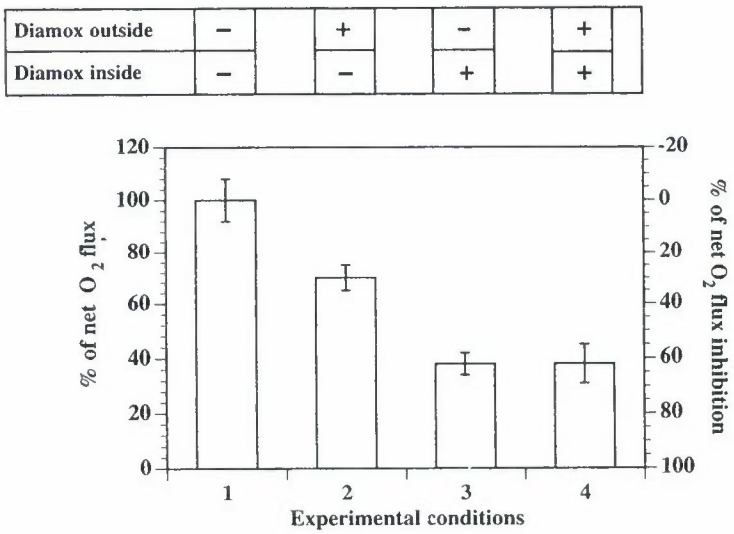

Figure 7. Effect of external and/or internal carbonic anhydrase inhibitor Diamox (acetazolamide) 600 μM, on the photosynthetic activity of bags from normal tentacle of *Anemonia viridis*.

Proton ATPase inhibitor

Bicarbonate transport by aquatic plants is often associated with proton translocating ATPases (Lucas, 1983). We used diethylstilbestrol (DES 100 μM), a specific inhibitor of E_1-E_2 type ATPase (Al-Awqati, 1986) to test the possible role of plasma-membrane bound H^+-ATPase. Fig. 8 shows that addition of DES in the external medium inhibited 30±2% of net O_2 production, while an inhibition of 18±3% was observed when the inhibitor was perifused in the tentacle. The effect of DES was almost additive when it was added simultaneously outside and inside the tentacle.

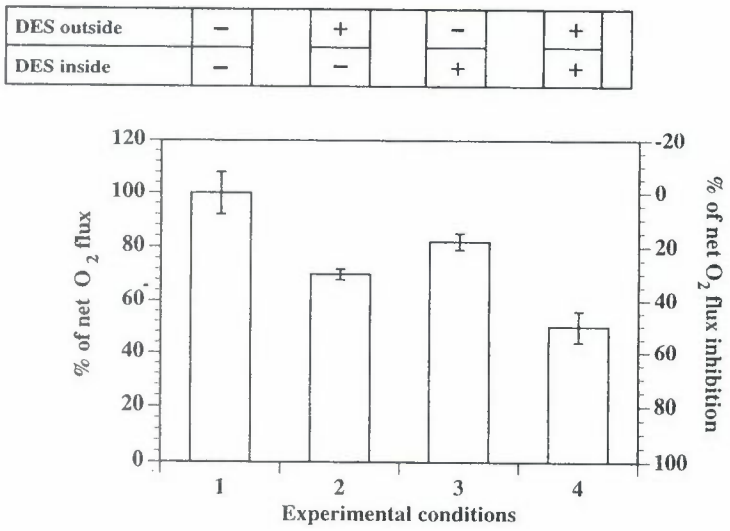

Figure 8. Effect of external and/or internal H^+-ATPase inhibitor Diethylstilbestrol (DES) 100 µM, on the photosynthetic activity of bags from normal tentacle of *Anemonia viridis*.

Effects of inhibitor combinations

The effect of the simultaneous addition in the external medium of different inhibitors was also tested. The effect was nearly additive in every case (see Table 1) suggesting parallel pathways. When the three inhibitors were added simultaneously, net O_2 production was almost totally inhibited and the compensation point was reached.

Table 1. Effect of combination of inhibitors added in the external medium on net photosynthesis of bags from normal tentacles of *Anemonia viridis*. Results are expressed as % of net O_2 flux ± standard deviation (N = 3).

	DIDS (400 µM)	Diamox (600 µM)	DES (100 µM)
DIDS (400 µM)	73 ± 4	30 ± 9	49 ± 7
Diamox (600 µM)	30 ± 9	65 ± 5	34 ± 6
DES (100 µM)	49 ± 7	34 ± 6	70 ± 2

Effect of external Na^+

Several types of bicarbonate transport systems are known to be Na^+-dependent. To test the sodium dependency, experiments were performed in sodium-free artificial seawater. The absence of sodium in the external medium did not induce any significant modification of net O_2 production (result not shown). This result shows that photosynthesis is strictly Na^+-independent.

4. Discussion

The supply of inorganic carbon by cnidarians to endosymbiotic dinoflagellates is essential for the maintenance of the symbiotic relationship. This report attempts to demonstrate how the oral epithelial layers of the sea anemone *Anemonia viridis* can maintain this supply in order to ensure optimum photosynthetic rates exhibited by dinoflagellates. For this purpose, we used short sections of sea anemone tentacles in form of a bag, either normally oriented or everted (i.e. inside-out).

Source of DIC

In a first set of experiments, these bags were filled and bathed with either normal or bicarbonate-free seawater. The experiments set with normal or inside-out tentacles were made in order to suppress the effect of the difference of volume existing between the external (4 ml) and the internal (200 µl) medium since in our experimental conditions the DIC pool of the external medium is twenty times larger than that of the internal medium. In both normal and inside-out tentacles, the omission of bicarbonate in the external medium alone led to similar inhibition of net photosynthesis (respectively 67% and 60%). This result shows that whatever the orientation of the epithelial layers, the major source of DIC comes from the external medium. However, since the omission of bicarbonate in the internal fluid alone led also to a significant inhibition of photosynthesis (37% in normal and 27% in inside-out tentacles), it can be concluded that the supply from the external medium is not sufficient to ensure a normal rate of photosynthesis. Moreover, this observation suggests that the permeability of the oral epithelial layers for DIC is not high enough to maintain the photosynthetic rate of zooxanthellae.

Three important conclusions can be drawn from these experiments: 1) while the external medium represents the main DIC source (about 60–70%), both ectoderm and endoderm are able to perform DIC absorption, 2) the DIC absorption capacity is quite similar for the two epithelial layers, and 3) the

diffusional permeability of DIC is not sufficient to allow an optimal photosynthetic rate of zooxanthellae.

Since *in vivo* tentacles are bathed with a large volume of external medium and a small volume of internal medium, it can be concluded that a transcellular transport of DIC across the ectodermal cells is likely operative to supply zooxanthella photosynthesis in endodermal cells.

Chemical form of DIC absorbed by the epithelial layers

It is known that the spontaneous dehydration of bicarbonate into carbon dioxide may supply photosynthesis (Miller and Colman, 1980; Colman and Gehl, 1983; Cook et al., 1986, 1988; Burns and Beardall, 1987; Rotatore and Colman, 1991; Goiran et al., in press). Does the DIC absorbed by the oral epithelial layers result from such a phenomenon? To answer this question we calculated the maximum rate of spontaneous dehydration of HCO_3^- to form CO_2 according to the method of Miller and Colman (1980) with values of apparent dissociation constants of carbonic acid in seawater given by Johnson (1982). So, in the internal medium (200 µl), for a DIC concentration of about 2 mM, the rate of spontaneous CO_2 production is about 46.6 $nmol.ml^{-1}.min^{-1}$, i.e. 9.3 $nmol.ml^{-1}$. This rate is less than half of the total net photosynthesis measured when only bicarbonate is present in the internal medium (about 25 nmol $O_2.min^{-1}$ in our experimental conditions). This result demonstrates that spontaneous dehydration inside the tentacle (due to the small internal volume) cannot supply enough CO_2 to support photosynthesis, suggesting that bicarbonate absorption by the endoderm (and ectoderm in experimental inside-out conditions) is involved in the DIC supply for zooxanthella photosynthesis. However, pharmacological data suggested that no membrane-bound carbonic anhydrase was present on the endodermal cells, thus invalidating the hypothesis of an active dehydration process. The spontaneous dehydration in the external medium (4 ml) led to a production of about 180 µmol $O_2.min^{-1}$, i.e. about 3 fold more than enough to ensure 100% of net photosynthesis. Nevertheless, a 30 to 40% of inhibition was observed when bicarbonate was only present in the external medium. In this case, whereas the volume of the external medium is large enough to supply DIC by spontaneous dehydration, there is still an inhibition of photosynthesis, suggesting that the CO_2 diffusion across the epithelial layer is too low.

This set of experiments indicates: 1) that at both the ectodermal and endodermal layer level, not only CO_2 is involved in DIC supply to endodermal zooxanthella photosynthesis but also HCO_3^-, 2) that the permeability of the oral epithelial layers for carbon dioxide is likely to be low. A limitation of gas diffusion through oral tissues of sea anemones was previously reported by

Brafield and Chapman (1983), who showed that the mesoglea of *Calliactis parasitica* behaves as a significant barrier to the diffusion of O_2 between the two cell layers. This relatively low diffusion of O_2 was recently confirmed by Harland and Davies (1995).

Mechanism of bicarbonate transport

A pharmacological approach was used in order to determine the pathway of DIC transport from the seawater pool to the endodermal cells. Stilbene derivatives like DIDS are known to interact competitively with Cl^- (Cabantchik and Greger, 1992) leading to the inhibition of band 3 protein-mediated anion exchange which facilitates the electroneutral exchange of HCO_3^- and Cl^- (Madshus, 1988). DIDS-sensitive anion carriers have been shown to be involved in DIC-concentrating mechanism of some marine algae (Rybova et al., 1991; Drechsler and Beer, 1991; Drechsler et al., 1993; Sharkia et al., 1994). Recently, such carriers have also been implied in DIC transport for zooxanthella photosynthesis in corals (Al-Moghrabi et al., in press). In the present study the sensitivity of photosynthesis to DIDS suggests that an anion carrier is required for inorganic carbon. External DIDS inhibited 27% of photosynthesis and internal DIDS inhibited 24% of photosynthesis (Fig. 6). Since these effects are cumulative, it can be concluded that DIDS does not cross the oral epithelia. It is indeed generally assumed that these kind of drugs are largely impermeant (Cabantchik and Greger, 1992). This result suggests that DIDS-sensitive carriers are equally distributed on both ectodermal and endodermal cells. Several types of HCO_3^- transport systems have been described in animal cells: Na^+-dependent and Na^+-independent Cl^-/HCO_3^- exchanger, Na^+/HCO_3^- cotransporter, $Na^+/CO_3^{2-}/HCO_3^-$ cotransporter (Calonge et al., 1992; Ilundain, 1992; Orsenigo et al., 1991; Soleimani et al., 1991). Since photosynthesis was Na^+-independent, it can be concluded that the DIDS-sensitive carrier present in *A. viridis* cells is a Na^+-independant Cl^-/HCO_3^- exchanger. The inhibition constant (IC_{50} 70 µM) of DIDS fits well with inhibitory coefficients reported in the literature for DIDS inhibition of anion transport by vertebrate cells, which lie between 2 to 100 µM (Cabantchik and Greger, 1992). Similar values were also found for microcolonies of the coral *Galaxea fascicularis* and its cultured zooxanthellae by Al-Moghrabi et al. (in press). Nevertheless, in microcolonies of the coral *G. fascicularis*, DIDS led to a complete inhibition of net photosynthesis (Al-Moghrabi et al., in press) suggesting that Anthozoa harbour different mechanisms of DIC absorption.

The involvement of carbonic anhydrase in the photosynthetic process is well documented in marine algae and cyanobacteria (Aizawa and Miyachi, 1986; Badger and Price, 1994). Weis et al. (1989) found that carbonic anhydrase

activity in the animal tissue was 29 times higher in symbiotic species than in non-symbiotic species. Weis (1993) localized by immunocytochemistry the CA, in the endodermal cells of *Aiptasia pulchella*, on or near the vacuolar membranes surrounding the zooxanthellae but did not detect any CA activity in the ectoderm. In our experiments, a different effect of Diamox was observed when the inhibitor was added in the external or in the internal medium. Since it is generally assumed that Diamox is only weakly permeable (Palmqvist et al., 1988), our results suggest that a diamox-sensitive process was present in both ectoderm and endoderm. However, since the effect on both sides was not additive, it can be concluded that the carbonic anhydrase present in the endodermal side is in series rather that in parallel with that present in the ectodermal side. It is therefore suggested that a carbonic anhydrase is present on the plasma membrane of ectodermal cells, facilitating the dehydration of HCO_3^- to CO_2 and then its diffusion into the cells. A second isoform, probably located at the interface of the two symbionts as previously suggested by Weis (1993), could provide an unidirectional flux of CO_2 by dehydrating HCO_3^- into CO_2. Recently, Al-Moghrabi et al. (in press) showed by immunolocalization that CA is uniformly distributed around zooxanthellae freshly isolated from the coral *Galaxea fascicularis*.

Finally, experiments carried out with DES, a specific inhibitor of the E_1-E_2 type of ATPase (Al-Awqati, 1986) suggest that a H^+-ATPases may play a role in HCO_3^- absorption. The fact that addition of this inhibitor both inside and outside was cumulative suggests that the proton pump was present on both sides of the oral epithelium. When a combination of the inhibitors is made, it can be seen that the effects are always almost additive. This quite surprising result suggests that these three inhibitors act on parallel bicarbonate uptake pathways. We can thus suggest the following model of bicarbonate transepithelial supply: carbonic anhydrase- and proton pump-mediated dehydration of bicarbonate from the external medium leads to an increase in carbon dioxide gradient between the seawater and the ectodermal cell. The carbon dioxide can then diffuse into the cell. A Cl^-/HCO_3^- exchanger also participates in bicarbonate uptake either at the apical or basal side of the ectodermal cell. The endodermal cells are also able to absorb bicarbonate, although their supply is restricted by the low surrounding bicarbonate pool. These cells should also present H^+-ATPases and Cl^-/HCO_3^- exchangers. A likely intracellular carbonic anhydrase at the vicinity of the zooxanthellae (as previously demonstrated by Weis, 1993 and more recently by Al-Moghrabi et al., in press) could finally provide an unidirectional flux of CO_2 by dehydration of HCO_3.

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