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OXYGEN RADICAL PRODUCTION IN THE SEA ANEMONE *ANTHOPLEURA ELEGANTISSIMA* AND ITS ENDOSYMBIOTIC ALGAE

BY JAMES A. DYKENS[1,*], J. MALCOLM SHICK[2], CRAIG BENOIT[3], GARRY R. BUETTNER[4] AND GARY W. WINSTON[3]

[1]*Department of Biology, Grinnell College, Grinnell, IA 50112, USA,* [2]*Department of Zoology, University of Maine, Orono, ME 04469, USA,* [3]*Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803, USA* and [4]*ESR Center, College of Medicine, University of Iowa, Iowa City, IA 52242, USA*

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Summary

Host animals in algal–invertebrate endosymbiotic associations are exposed to photosynthetically generated hyperoxia while in sunlight, conditions conducive to photodynamic excitations and production of cytotoxic oxygen-derived radicals such as the superoxide anion ($O_2^{\cdot-}$) and the hydroxyl radical ($^{\cdot}OH$). All previous evidence of oxyradical production in symbiotic associations has been circumstantial. We here present direct evidence, from electron paramagnetic resonance studies on tissue homogenates of the photosymbiont-containing sea anemone *Anthopleura elegantissima* (Brandt), of substantial light-dependent $^{\cdot}OH$ and $O_2^{\cdot-}$ production that is abolished by dichlorophenyldimethylurea (DCMU), an inhibitor of photosynthesis. Shade-adapted *A. elegantissima* lacking endosymbiotic algae likewise show $^{\cdot}OH$ production upon illumination. The latter flux is not dependent on photosynthesis, and DCMU has no effect. Rather, $^{\cdot}OH$ production in apozooxanthellate anemones is *via* direct photoexcitations. The selective reaction of dimethyl sulfoxide (DMSO) with $^{\cdot}OH$ to form methane sulfinic acid allows quantification of $^{\cdot}OH$ produced *in vivo*. Such *in vivo* measurements confirm the production of $^{\cdot}OH$ in both host and algae in illuminated zooxanthellate anemones, where the amount of $^{\cdot}OH$ in the zooxanthellae is disproportionately large relative to their fractional contribution to the biomass of the symbiosis. *In vivo* studies using DMSO also suggest a photochemical production of $^{\cdot}OH$ in apozooxanthellate anemones exposed to simulated sunlight enriched in ultraviolet (UV) wavelengths, and the enhancement by UV light of $^{\cdot}OH$ production in zooxanthellate individuals. Such chronic radical exposure necessitates defenses

* Present address: Immunopathology, Parke-Davis Pharmaceutical Research, 2800 Plymouth Road, Ann Arbor, MI 48106, USA.

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against photooxidative stress, a cost that is seldom considered in these mutualistic symbioses.

Introduction

A wide variety of symbiotic associations exists between animal hosts, ranging from cnidarians to chordates, and photosynthetic endosymbionts such as cyanobacteria, prochlorophytes and dinoflagellates (zooxanthellae) (Smith and Douglas, 1987). Much of the recent research on such symbioses has focused on refining our understanding of the various interactions between the partners of the association. The nutritional benefits to the host from the endosymbionts, inorganic exchange between the partners and host regulation of endosymbiont population have all received attention. Less extensively studied, however, are the potentially deleterious consequences of simultaneous exposure to hyperoxia and sunlight experienced by these associations.

Regardless of their specific constitution, in sunlight these symbiotic associations typically produce more O_2 than they consume, so that the host animal is subjected to high fluxes of photosynthetically generated O_2. The P_{O_2} experienced by host tissues depends on the localization of the photosymbionts (intra- or extracellular), the photosynthetic capacity of the endosymbionts, the solar irradiance and the extent to which O_2 can be consumed or removed from its site of production (Shick and Dykens, 1985; Shick, 1990; Newton and Atkinson, 1991). For example, in the zooxanthellate sea anemone *Anthopleura elegantissima*, light intensities less than 0.33 normal sunlight elevate tissue P_{O_2} to approximately 47 kPa (Dykens and Shick, 1982). Although net O_2 production by symbioses has long been recognized (e.g. Yonge *et al.* 1932), it has generally been viewed in the context of photosynthesis:respiration ratios and assessment of nutritional exchanges (e.g. Muscatine *et al.* 1981; Gattuso and Jaubert, 1990) and in ameliorating the effects of environmental hypoxia or stagnation (Shick and Brown, 1977; Rinkevich and Loya, 1984; Shick, 1990).

However, D'Aoust *et al.* (1976) suggested that corals might require defenses against the hyperoxia imposed by their zooxanthellae. Hyperoxia is injurious to most tissues because of the formation of reactive oxygen-centered radicals, molecules with one or more unpaired electrons. Because of orbital spin restrictions, molecular O_2 preferentially undergoes univalent reductions, the first product of which is the superoxide anion radical, $O_2^{\cdot-}$. Although the superoxide radical is capable of depolymerizing hyaluronate, inducing membrane lipid peroxidation and inactivating enzymes (Fucci *et al.* 1983; McCord and Russell, 1988; Halliwell and Gutteridge, 1989), the proximate mediator of oxygen toxicity is probably not the superoxide radical, but rather an ensuing flux of hydroxyl radicals ($^{\cdot}OH$) formed *via* the Fenton reaction involving heterolytic H_2O_2 cleavage catalyzed by iron and other transition metals (reviewed by Halliwell and Gutteridge, 1989). Hydroxyl radicals are the most reactive radical species found in biological systems: they have rate constants of the order of 10^9–

$10^{10}(\text{mol}\,l^{-1})^{-1}\,s^{-1}$ (Buxton *et al.* 1988) and react within less than five molecular radii of the site of their production. In view of the ubiquitous availability of transition metals in biological systems, it is likely that $^{\cdot}OH$ is the proximal cause of most biological oxygen toxicity. In any event, if intracellular levels of $O_2^{\cdot-}$ are minimized, so also will be the ensuing flux of $^{\cdot}OH$ and damage from either radical. All aerotolerant organisms contain at least one of three forms of the metalloenzyme superoxide dismutase (SOD) that scavenge $O_2^{\cdot-}$ and thereby moderate oxygen toxicity.

In addition to the direct toxicity of oxygen-centered radicals, photosensitizing compounds such as chlorophylls, flavins and porphyrins also injure cells by the production not only of singlet oxygen (see Foote, 1976), a non-radical but highly reactive form of dioxygen, but also $O_2^{\cdot-}$, which may be formed directly from photodynamic events and/or secondarily from singlet oxygen (Saito *et al.* 1980; Peters and Rodgers, 1980; Ueda *et al.* 1988) (see below). In these photoexcitations, energetic wavelengths of sunlight from ultraviolet to the visible blue region excite a sensitizer that passes the energy to O_2 to form singlet oxygen and/or superoxide radical. Such radical-mediated photodynamic damage is synergistically exacerbated by hyperoxia (see Jamieson *et al.* 1986), and there is growing awareness that solar UV radiation also poses a threat to symbioses (Jokiel and York, 1982; Dykens and Shick, 1984; Shick and Dykens, 1984; Dunlap *et al.* 1986), perhaps by means of forms of active oxygen (Lesser *et al.* 1990; Shick *et al.* 1991).

Sea anemones, like other algal–invertebrate symbioses examined thus far, exhibit behavioral, biochemical and enzymic responses to light-induced hyperoxia that serve to moderate photodynamic action and oxyradical toxicity (reviewed by Shick, 1991). In the last case, the animal tissue in *A. elegantissima* maintains SOD and catalase activities in direct proportion to its endosymbionts' chlorophyll content, an index of O_2 production capacity (Dykens and Shick, 1982), and the antioxidant enzymes are concentrated in those tissues that contain the highest density of endosymbionts (Dykens, 1984; Dykens and Shick, 1984). Similar correlations between chlorophyll content and antioxidant enzyme activities are shown by 36 species of symbiotic invertebrates in four phyla collected from Australia's Great Barrier Reef (Shick and Dykens, 1985). Moreover, the activities of antioxidant enzymes in zooxanthellae of *Aiptasia pallida* are greater in specimens from brightly lit habitats than in those from dimly illuminated areas, a difference that is reversed by reciprocal transplantation of the anemones (Lesser and Shick, 1989*a*). Finally, experimental manipulation of symbioses and of cultured zooxanthellae results in higher activities of antioxidant enzymes under conditions that promote photooxidative stress (Dykens and Shick, 1984; Lesser and Shick, 1989*b*; Lesser *et al.* 1990; Matta and Trench, 1991; Shick *et al.* 1991). Antioxidant defenses are particularly robust in the zooxanthellae (Lesser and Shick, 1989*b*; Matta and Trench, 1991; Shick *et al.* 1991), which would be expected from their high concentration of oxygen and photosensitizing chlorophyll.

The available evidence indicates that photooxidative stress is a valid concern in

studying these associations, but the evidence is all circumstantial; no direct observations of free radical production in any algal–animal symbioses have been reported. Accordingly, we have used electron paramagnetic resonance spectrometry (EPR) to examine directly whether free radicals are indeed generated upon illumination of the sea anemone *Anthopleura elegantissima*. However, EPR is not amenable to rigorous quantification of radical production in intact tissues, nor can it localize radical production to the host or algal partner. Therefore, it would also be desirable to use a relatively innocuous molecular probe capable of reacting with oxyradicals *in vivo* and forming a stable and quantifiable product. Dimethyl sulfoxide (DMSO) has proved to be such a molecule, being tolerated by animals in concentrations of up to $1\,mol\,l^{-1}$, and reacting selectively with the hydroxyl radical to form methane sulfinic acid, a stable, non-radical product that can be quantified colorimetrically (Babbs and Steiner, 1990). Because the EPR and DMSO observations provide independent but mutually supportive assessments of radical flux, we have combined in this paper results from what were initially two separate examinations of free radical production in the *A. elegantissima* symbiosis.

Materials and methods

Electron paramagnetic resonance studies

Specimens of *Anthopleura elegantissima* were collected from two clones at the same intertidal height from Cattle Point on San Juan Island, Washington, and shipped to Iowa where they were held in marine aquaria at 15 °C for less than a week prior to use. Apozooxanthellate specimens lacking zooxanthellae (for terminology, see Schumacher and Zibrowius, 1985) and not normally exposed to sunlight were collected from deep recesses in the south jetty at Bodega Bay, California, shipped to Orono, Maine, where they were held in darkness at 15 °C and subsequently shipped to Iowa where they were held under similar conditions for 2 days before use.

Anemone tissues were gently homogenized in $15\,mmol\,l^{-1}$ Tris-buffered Ca^{2+}- and Mg^{2+}-free artificial sea water (ASW; formulated as in Cavanaugh, 1956) using a Teckmar Tissuemizer followed by a hand-driven Teflon–glass Potter-Elvehjem homogenizer. The diameter of the Teflon pestle had been reduced using a hand drill and fine sandpaper to increase the side clearance, which serves to keep cells intact. The resulting homogenate was centrifuged at 10 °C and $300\,g$ for 5 min to pellet large clumps of undisrupted tissue. The supernatant was removed and centrifuged at $3000\,g$ and the resulting pellet was resuspended in divalent-ion-free ASW to remove viscous material. This was repeated three times, and the final pellet of intact cells and small clumps of cells was resuspended in ASW containing Ca^{2+} and Mg^{2+} (seawater formula no. 4 in Cavanaugh, 1956) and kept on ice.

Zooxanthellae were isolated following homogenization as above except that a tighter-fitting Teflon pestle was used. The crude tissue homogenate was washed

several times in Ca^{2+}- and Mg^{2+}-free ASW (Cavanaugh, 1956). The resulting suspension was placed on a 10 %–20 %–30 % discontinuous sucrose gradient and spun at 4000 *g* for 20 min. The isolated zooxanthellae were removed from the 10 %–20 % interface, washed and recentrifuged in the sucrose gradient. Alternatively, zooxanthellae were isolated with a 30 min spin at 15 000 *g* in Percoll self-generating density gel. Although repeated washings and sucrose density centrifugations provided similar purity of isolated zooxanthellae, as assessed by phase contrast microscopy, isolation with Percoll was easier and faster. The resulting isolate was washed and resuspended in potassium-enriched ASW (K^+ increased to 18 mmol l^{-1}, Na^+ correspondingly reduced). The photosynthetic integrity of isolated zooxanthellae was assessed using fiber-optic illumination (215 μmol photons $m^{-2} s^{-1}$, photosynthetically active radiation, PAR) and a YSI model 5300 oxygen meter with a YSI model 5331 oxygen sensor in a YSI model 5301 water bath. Lettuce chloroplasts were isolated using similar procedures except that the leaves were homogenized and the resulting slurry was filtered through four layers of cheesecloth and spun at 500 *g* for 5 min prior to final centrifugation for 10 min at 3000 *g* and resuspension.

Electron paramagnetic resonance studies were performed at room temperature using a Varian E-4 spectrometer equipped with a TM_{110} cavity and quartz flat cell. Spin trap dimethyl 5,5-dimethyl-pyrroline-*N*-oxide (DMPO; Aldrich Chemical Co.), cleared of impurities using neutral charcoal filtration (Buettner, 1982), was present at 100 mmol l^{-1} final concentration. The sample in the EPR cavity was illuminated using a 150 W tungsten microscope lamp with tube lens, which provided approximately 667 μmol photons $m^{-2} s^{-1}$ as measured with a YSI 65A Radiometer and model 6551 probe. The light was focused into the chamber through an 80 mm water infrared filter, the effectiveness of which was verified using a thermocouple inserted into a sample in the EPR cavity. The interior of the cavity is highly reflective, resulting in nearly uniform illumination of the sample in the flat cell, and the window into the cavity passes 75 % of incident light. Superoxide dismutase, catalase and the metal chelator diethylenetriaminepentaacetic acid (DETAPAC), all from Sigma Chemical, were dissolved in distilled water previously treated with Chelex resin (Bio-Rad) to remove adventitious iron and other transition metals (Buettner, 1988).

When indicated, 3-3,4-dichlorophenyl-1,1-dimethylurea (DCMU) was present at 10^{-7} mol l^{-1}. DCMU was made up to 1 mmol l^{-1} by dissolving in a small volume of absolute ethanol prior to dilution in 15 ml of ASW. DCMU was further diluted by addition to samples to yield the indicated final concentration. The amount of ethanol, a potential $^{\cdot}OH$ sink, in the EPR cell was, therefore, too low to compete substantially with the 100 mmol l^{-1} spin trap for free $^{\cdot}OH$. It should also be noted in this context that, because its aqueous solubility coefficient is 42 p.p.m. at 25 °C (Budavari, 1989), it is unlikely that any aqueous DCMU concentration exceeds 0.2 mmol l^{-1}. Nevertheless, DCMU was present in quantities sufficient to abolish O_2 production from isolated zooxanthellae and lettuce chloroplasts as well as free radical flux in zooxanthella-containing samples (see Results).

DMSO studies

Zooxanthellate and limited numbers of apozooxanthellate specimens of *Anthopleura elegantissima* were collected from the south jetty at Bodega Bay, California, in July 1990 and shipped to Orono. Anemones were placed in large fingerbowls of 30‰ artificial sea water at 15°C to which dimethyl sulfoxide (DMSO; Sigma Chemical Company) was added to a final concentration of 250 mmol l^{-1}. The anemones became flaccid but remained responsive to touch during 72 h maintenance in DMSO in the dark, after which individual groups of five anemones were exposed to various treatments for 1 h. The treatments were: zooxanthellate anemones kept in the dark; zooxanthellate anemones exposed to an irradiance of 800 μmol photons m^{-2} s^{-1} (400–700 nm, PAR) with UV-A and UV-B supplementation (see below); zooxanthellate anemones exposed to PAR but shielded from UV light; apozooxanthellate anemones maintained in the dark; apozooxanthellate anemones exposed to PAR, in the presence of UV light; and apozooxanthellate anemones exposed to PAR+UV and maintained under hyperoxia (P_{O_2}=47–53 kPa) by bubbling with O_2. Illumination was provided by a Kratos SS1000X xenon arc solar simulator fitted with an airmass 1 filter, which produces a spectrum approximating that at sea level at noon, and was supplemented with fluorescent UV-A and UV-B lamps (NEC T10, 20 W blacklight, 362 nm peak emission; Westinghouse FS20, 20 W sunlamp, 312 nm peak emission, respectively) situated 25 cm above the anemones. Plexiglas (cut-off approximately 375 nm) was used to shield some anemones from UV light. Anemones were blotted and weighed, then frozen in liquid nitrogen immediately after the various exposures and stored at −80°C prior to biochemical analysis.

Additional zooxanthellate anemones were collected from the harbor at Santa Barbara, California, in January 1990 and brought to Orono. Following maintenance in 250 mmol l^{-1} DMSO in ASW for 72 h in the dark, large anemones were exposed to the same irradiance under the beam of the solar simulator, and supplemented with UV light, as in the previous experiment. After 1 h of illumination, anemones were bisected longitudinally and one half of each anemone was immediately weighed, then frozen and stored at −80°C; the second half of each specimen was homogenized (Brinkman Polytron) in ice-cold, Ca^{2+}-free ASW containing 0.02% sodium dodecyl sulfate, and the freshly isolated zooxanthellae (FIZ; prepared as described in Lesser and Shick, 1989*b*) were recovered quantitatively. All procedures were conducted in dim green light. Microscopic examination revealed clean zooxanthellae with a minimum of contamination by cnidae. The FIZ were weighed, then frozen and stored at −80°C. FIZ and anemones from this and the previous experiment were shipped on dry ice to Baton Rouge for assay of methane sulfinic acid.

Methane sulfinic acid assay

Methane sulfinic acid (MSA), the primary product of the reaction of DMSO with the hydroxyl radical, was quantified in duplicate samples by the spectrophoto-

metric method of Babbs and Steiner (1990), with the following modifications. Because of the high concentration of lipids and other interfering substances in sea anemone homogenates, the effluent of the first Supelclean C_{18} column in the published procedure was placed onto a second pre-eluted column, the effluent reacidified with HCl, and Fast Blue BB dye added again prior to a second toluene:butanol extraction and wash with butanol-saturated water. The final sample was fixed with pyridine:glacial acetic acid and MSA was measured (as the diazosulfone dye) at 425 nm. Also, homogenates of sea anemones and FIZ unexposed to DMSO and weighing approximately the same as the experimental tissues were used to correct for background absorbance not due to MSA.

Results

Electron paramagnetic resonance studies

In the absence of light, EPR reveals no radicals in tissue suspensions of the endosymbiont-containing sea anemone *Anthopleura elegantissima* (Fig. 1A). However, immediately upon illumination, DMPO–hydroxyl radical spin adducts (DMPO/·OH) are detected (Fig. 1B) ($a^N=a^H=14.9$ G; Buettner, 1987). The signal builds throughout the 4 min required to complete the scan, as shown by the discrepancy between the size of the two outer wing lines (they would be the same size if radical production were not increasing). Radical flux continues to increase in illuminated tissue suspensions as shown by the continued growth of the peaks in Fig. 1C, taken immediately after scan B, and it continues to increase for at least 12 min after illumination (not shown). This ·OH production is fully dependent on light; the signal begins to collapse immediately after illumination ceases and is no longer detectable within 4 min.

When SOD is inhibited with 50 μmol l^{-1} nitroprusside, an inhibitor of all three morphs of SOD (Misra, 1984), light-induced ·OH production is augmented as shown by faster growth and the stronger DMPO/·OH signal (Fig. 2A); the DMPO/·OH signal is 1.6 times larger when SOD is inhibited compared with identical conditions when SOD is unimpaired. When SOD is inhibited, the DMPO/·OH spectrum becomes symmetrical after 8–12 min of illumination, indicating equilibrium between spin-adduct formation and decay. Conversely, addition of SOD (30 units ml^{-1}; McCord and Fridovich, 1969) reduces the DMPO/·OH signal by 40% (compare Fig. 2B with Fig. 1C). Addition of 926 units ml^{-1} (Beers and Sizer, 1952) catalase and 2 mmol l^{-1} of the chelator DETAPAC to block Fenton reactions fails to diminish the DMPO/·OH signal, which suggests that alternative sources of DMPO/·OH, such as superoxide radicals, may contribute to the hydroxyl radical signal (see Discussion).

Chemical inhibition of photosynthetic O_2 production in endosymbiont-containing tissues using DCMU totally abolishes all light-induced DMPO EPR signals (Fig. 3). The absence of any signals from illuminated ASW containing DMPO and DCMU (Fig. 3C), or spin trap plus catalase, SOD and DETAPAC (not shown), eliminates artifactual photochemical excitations as a potential radical source.

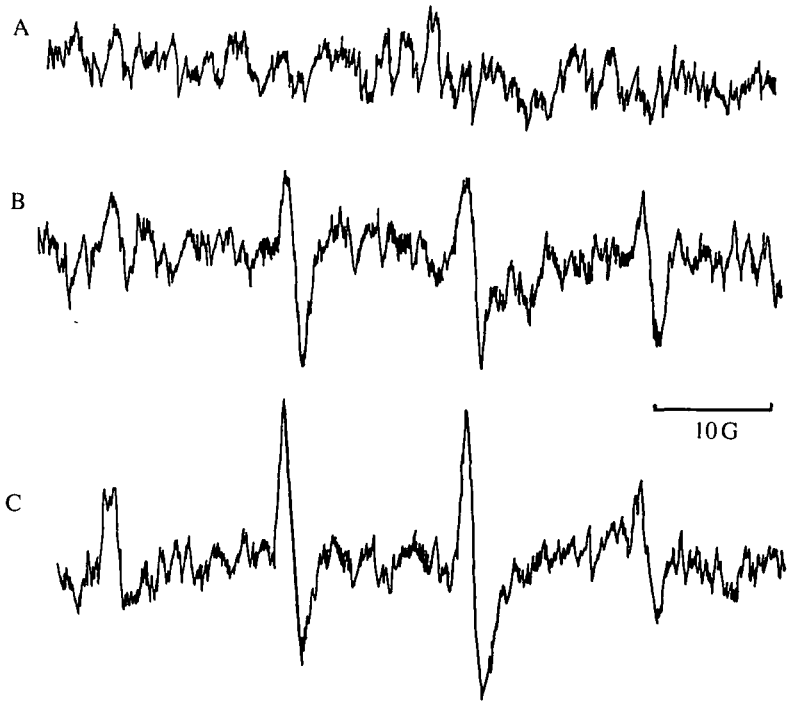

Fig. 1. Electron paramagnetic resonance spectra from tissue suspensions of zooxanthellate specimens of *Anthopleura elegantissima*. No radical signals are detected in the dark (A). However, DMPO/·OH adducts are detected immediately upon illumination; scan B was begun when the light was turned on and required 4 min to complete. Four equally spaced lines 14.9 G apart in an amplitude ratio of 1:2:2:1 are characteristic of the DMPO/·OH adduct. The DMPO/·OH signal continues to accumulate during the next 4 min (C), and does not reach equilibrium during the 16 min of observations (not shown). Radical production depends on the presence of light; the signal fades within seconds when the light is turned off. Receiver gain 2.5×10^4, scan range 100 G, modulation amplitude 1.0 G, time constant 1 s, microwave power 20 mW, 4 min scan.

DMPO/·OH signals are also detected upon illumination of tissue suspensions of anemones totally lacking endosymbionts (Fig. 4A,B). As might therefore be expected, ·OH flux in these apozooxanthellate individuals is unaffected by addition of the photosynthetic inhibitor DCMU (Fig. 4C). Although this is a reasonable finding given the apparent absence of zooxanthellae, failure of DCMU to inhibit ·OH production in apozooxanthellate anemones could have been due to the presence of a few algae combined with the failure of DCMU to block photosynthesis. Accordingly, a sample of zooxanthellate tissue was immediately put into the spectrometer whereupon the usual ·OH signal was fully abolished by the same concentration and solution of DCMU that failed to block ·OH production in the apozooxanthellate anemones. Moreover, no chlorophyll could be detected spectrophotometrically in these apozooxanthellate homogenates following two 12-h acetone extractions (method of Jeffrey and Humphrey, 1975).

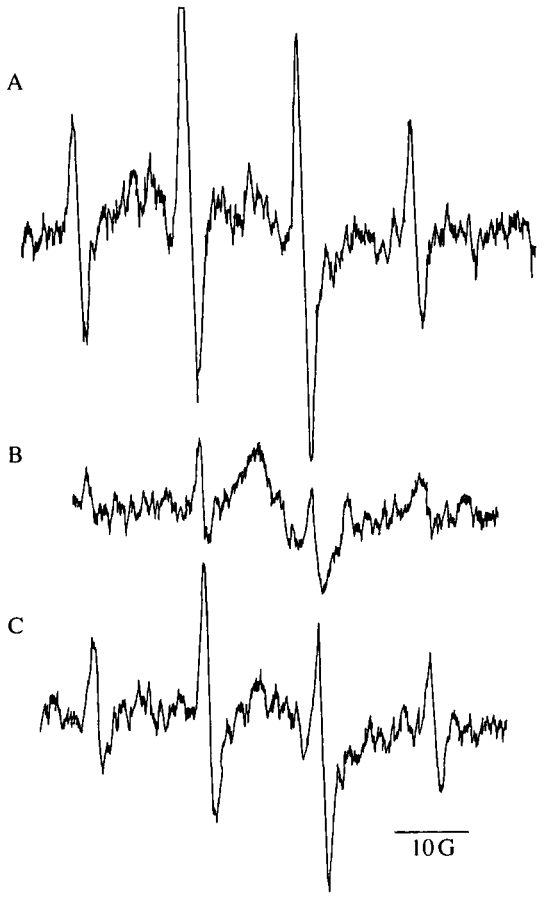

Fig. 2. Light-induced DMPO/·OH EPR signal from tissue suspensions of zooxanthellate specimens of *Anthopleura elegantissima* increases 1.6-fold when superoxide dismutase (SOD) is inhibited by addition of 50 μmol l^{-1} nitroprusside to the medium (A) and declines by 40 % when SOD (30 units ml^{-1}) is added (B) (compare to Fig. 1C). Similarly, addition of catalase (926 units ml^{-1}) and a chelator (2 mmol l^{-1} DETAPAC), to block Fenton reactions, fails to abolish the DMPO/·OH signal from illuminated tissue (C). Peak heights of figures are not directly comparable as spectrometer gain was 4×10^4 for A compared with 2.5×10^4 for B and C (and Fig. 1). The 1.6-fold increase reported above takes into account receiver gain. All other spectrometer settings as in Fig. 1.

In addition to the previously observed DMPO/·OH adduct, a DMPO adduct of a carbon-centered radical is also occasionally detected in illuminated apozooxanthellate *Anthopleura elegantissima* tissue (10^{-7} mol l^{-1} DCMU had been added as a precaution against possible algal radical production) (Fig. 5). The hyperfine splitting characteristics (a^N=16.25 G, a^H=23.25 G) suggest it is a ·CH_3 radical (Buettner, 1987), and the lines are labeled to distinguish it from the concomitant ·OH signal (Fig. 5). After illumination ceases, this ·C radical persists for longer

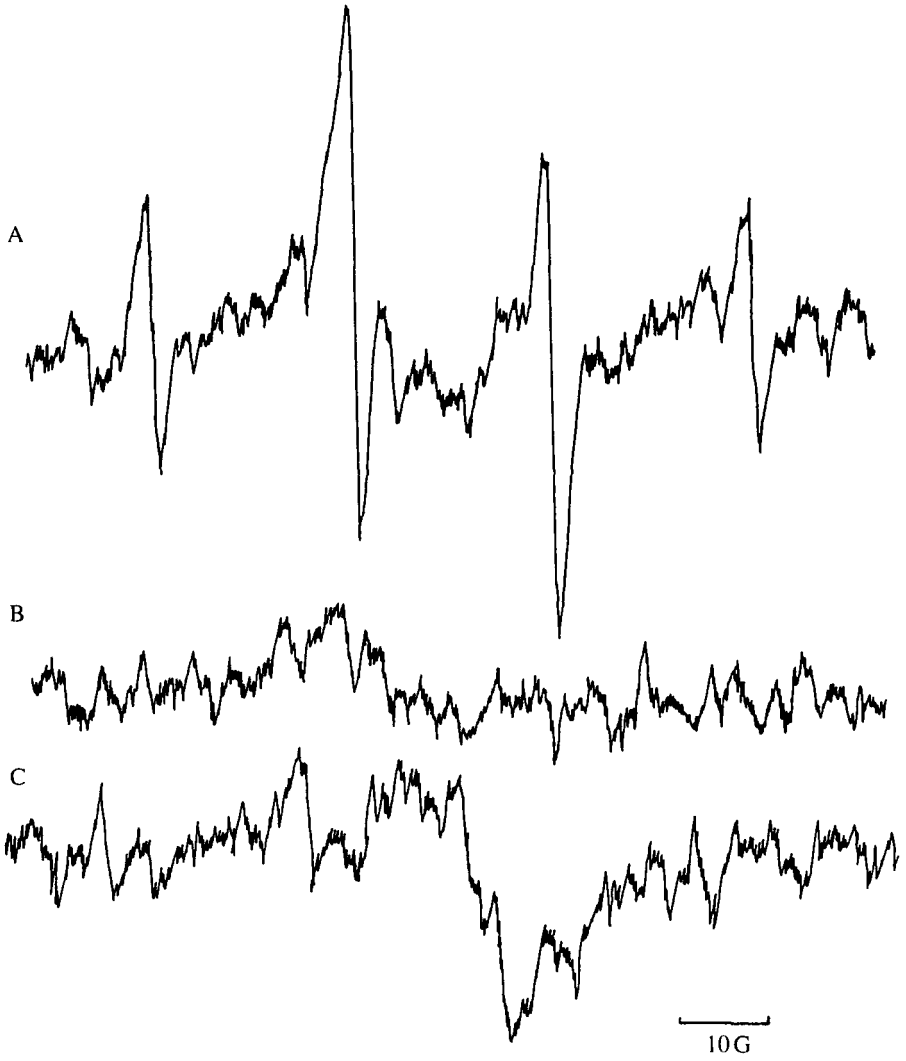

Fig. 3. The DMPO/·OH signal from illuminated tissue suspensions of the zooxanthellate *Anthopleura elegantissima* (A) is lost when photosynthetic oxygen production and electron transport are inhibited by DCMU (C). Note the centered signal (peak-to-peak width approximately 10 G) due to semiplastoquinone in the illuminated DCMU treatment (C). This signal causes the vertical displacement of the central pair of DMPO/·OH lines in A, and in many of the other spectra (for example, see Figs 4A and 5A). No adduct is detected in the absence of light (B). Spectrometer settings as in Fig. 1, except receiver gain 5×10^4, microwave power 40 mW.

than the ·OH adduct and even grows as the DMPO/·OH signal collapses during the first 4 min after illumination ceases (Fig. 5B).

DMPO/·OH spin adducts are also detected when suspensions of isolated zooxanthellae are illuminated. The signal is abolished by 10^{-7} mol l^{-1} DCMU (Fig. 6). This is different from the response of isolated vascular plant (lettuce)

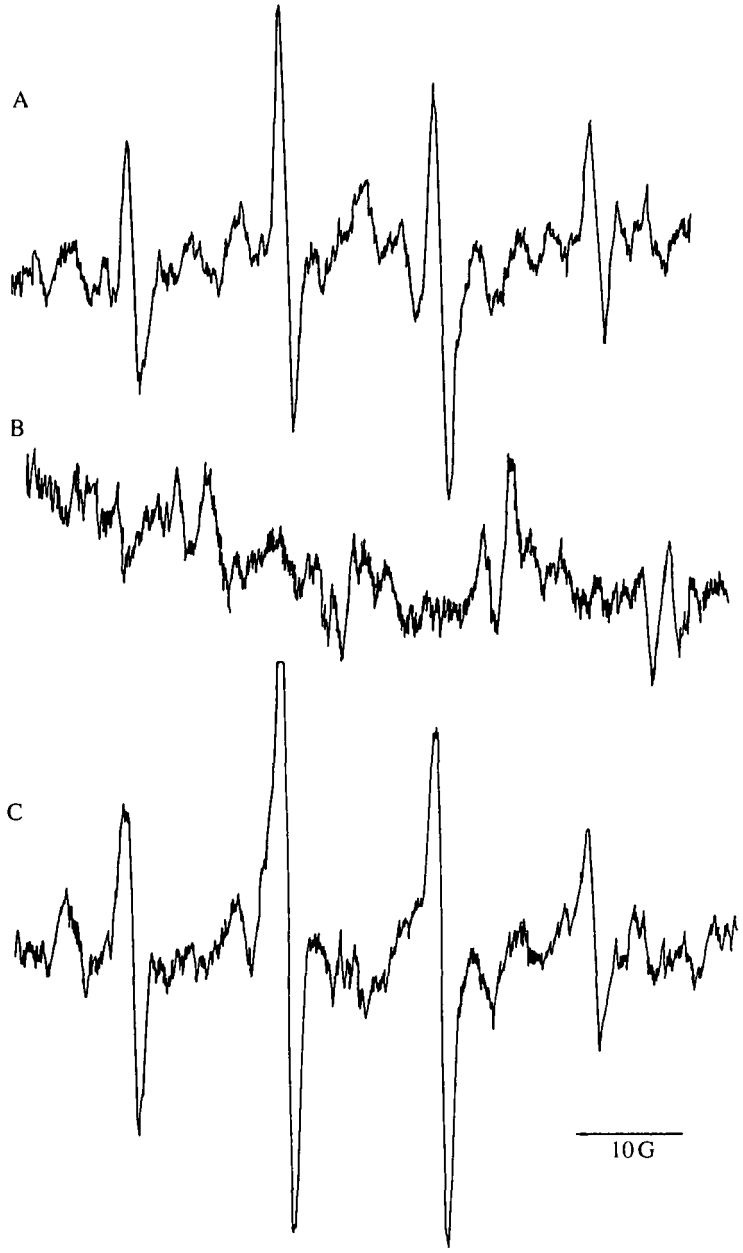

Fig. 4. Electron paramagnetic resonance spectra from tissue suspensions of naturally occurring apozooxanthellate specimens of *Anthopleura elegantissima*. Despite the absence of zooxanthellae, DMPO/·OH signals are detected when apozooxanthellate anemones are illuminated (A), compared to unilluminated sample (B). This radical production is clearly not dependent on photosynthetically induced hyperoxia and, as expected, DCMU has no effect (C). The larger peaks and increased baseline noise compared with other figures are due to increased receiver gain (8×10^4), whereas larger peak heights in C compared with A probably result from there being more biomass in the flat cell. Spectrometer settings as in Fig. 3, except 8 min scan.

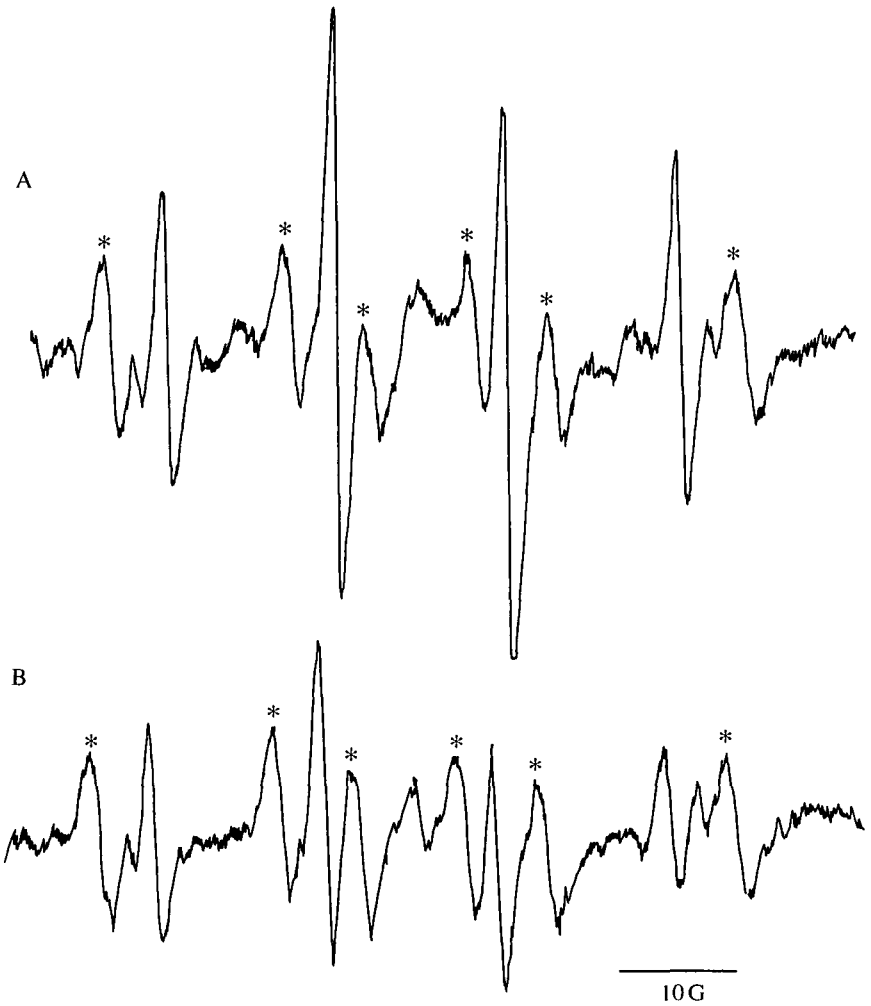

Fig. 5. A carbon-centered radical in addition to the previously observed DMPO/·OH is detected upon illumination of apozooxanthellate *Anthopleura elegantissima* tissues in the presence of 10^{-7} mol l^{-1} DCMU (A) (·C lines indicated by asterisks). The hyperfine splittings are consistent with a $\cdot CH_3$ radical (Buettner, 1987). Spectrum B, obtained immediately after ceasing illumination of A, shows that carbon-centered radicals accumulate as the DMPO/·OH signal fades as a result of radical-mediated carbon reduction. Spectrometer settings as in Fig. 4.

chloroplasts, where superoxide radical adducts were readily detected under identical circumstances (spectra not shown), but ·OH adducts were never seen. Lettuce chloroplasts were used as a positive control in the EPR studies and to verify the efficacy of DCMU. Attempts to isolate intact zooxanthella chloroplasts were not successful, as reflected by much lower O_2 production per unit chlorophyll compared to lettuce chloroplasts under similar illumination: EPR was therefore not attempted.

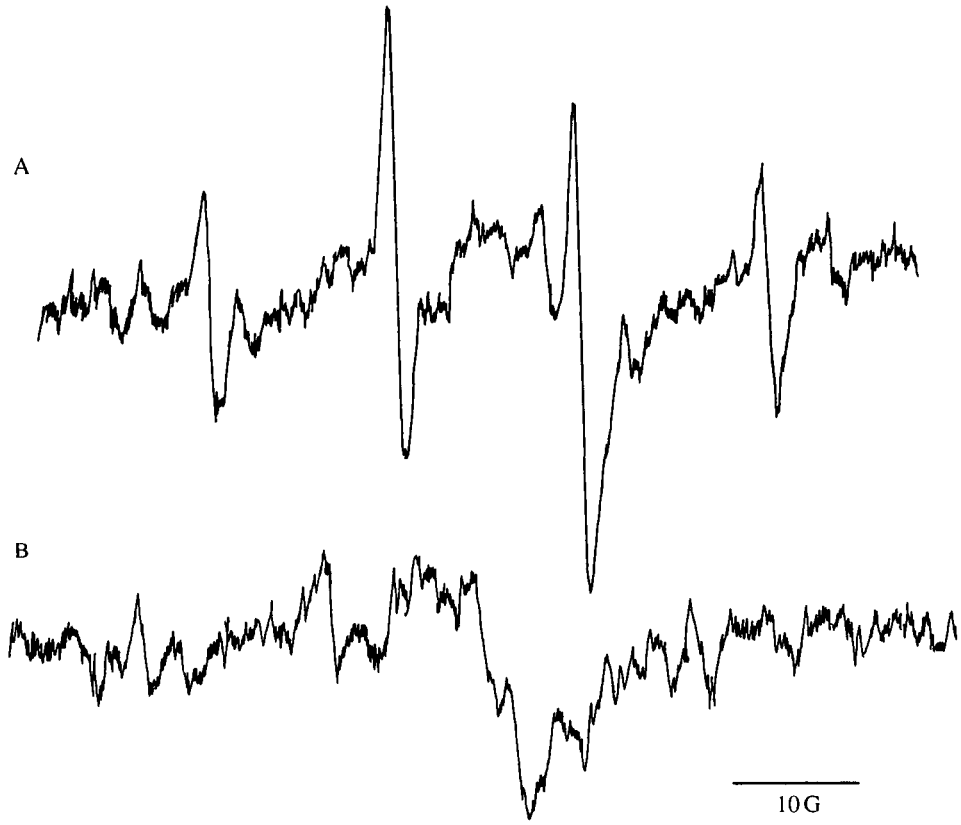

Fig. 6. The DMPO/·OH spectrum is detected upon illumination of zooxanthellae freshly isolated from *Anthopleura elegantissima* (A). The signal is abolished when photosynthesis is chemically inhibited with DCMU (B). Note the centered asymmetry in the inner two lines of the ·OH spectra due to underlying semiplastoquinone (and no doubt to a lesser extent to mitochondrial semiquinone). Spectrometer settings as above, except receiver gain 6.3×10^4.

Finally, regardless of the presence or absence of light, an EPR signal from a non-DMPO adduct is detected in many of the spectra (for example, see Figs 3C and 6B). This signal underlies the center of the DMPO/·OH spectra and is seen as an asymmetry between the heights of the two center DMPO/·OH lines (for example, see Figs 2A,C, 4A, 6A). Its characteristics (peak to peak approximately 10 G) coincide with those of a membrane-bound semiquinone (Klimov *et al.* 1980; O'Malley and Babcock, 1984; Hoff, 1987; Baker *et al.* 1988); i.e. it is either mitochondrial semiquinone or chloroplast semiplastoquinone (PQ·) in the zooxanthellae. In endosymbiont-containing samples and algal isolates it is impossible to distinguish between the various possible sources (chloroplast, algal and animal mitochondria) since the apparent signal is derived from the average of all sources. However, the width of the semiquinone signal from the apozooxanthellate samples, which, in the absence of chlorophyll, can reasonably be ascribed solely to

animal mitochondrial semiquinone, is 2–3 G narrower than that seen in isolated zooxanthellae or endosymbiont-containing tissues. The zooxanthella PQ$^{\cdot}$ signal waxes and wanes depending totally on illumination, and it is not abolished by DCMU, which blocks PSII electron transport at a site after PQ$^{\cdot}$ (Mathis and Rutherford, 1987). When the sample is frozen in liquid N_2 and maintained at 77 K in the spectrometer (frozen samples were jacketed in a Dewar flask with liquid N_2 while spectra were obtained), the PQ$^{\cdot}$ and semiquinone spectra both increase three times faster and dissipate almost four times more slowly after illumination ceases.

DMSO studies

The amount of methane sulfinic acid (MSA) produced (which is directly proportional to the amount of hydroxyl radical generated) during 1 h of exposure to the various treatments in the Bodega Bay anemones is shown in Fig. 7. One-factor analysis of variance (ANOVA) (StatView II software, Abacus Concepts, Berkeley, CA) indicates a highly significant effect of treatment (F=6.78, d.f.=29, P=0.0005). Duncan's multiple-comparison test reveals significant differences (at P=0.01) in MSA production between the zooxanthellate anemones exposed to UV light and all other groups, but not between any other groups (almost certainly because of the large variance in the data, some of which derives from the assay method). Reproducibility of duplicate samples was ±29 %, in part because of the high concentrations of interfering substances that must be removed and also

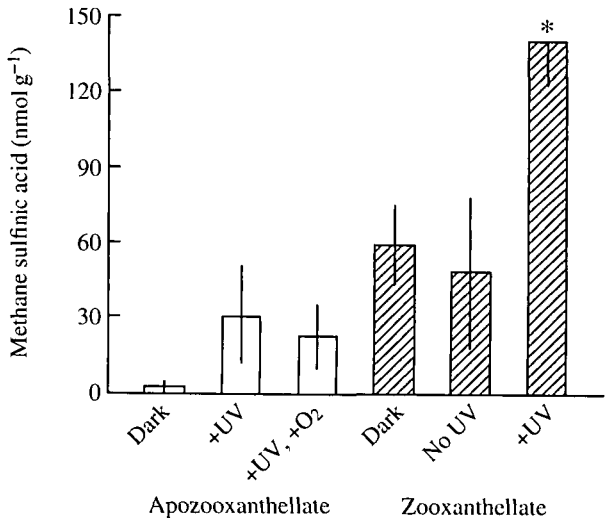

Fig. 7. Production of methane sulfinic acid (MSA) in apozooxanthellate (open columns) and zooxanthellate (hatched columns) specimens of *Anthopleura elegantissima* under the various conditions of illumination and hyperoxia described in Materials and methods. Anemones were pre-exposed to 250 mmol l^{-1} DMSO in sea water for 72 h and then subjected to experimental treatments for 1 h. N=5 anemones in each group; vertical bars indicate ±1 S.E.

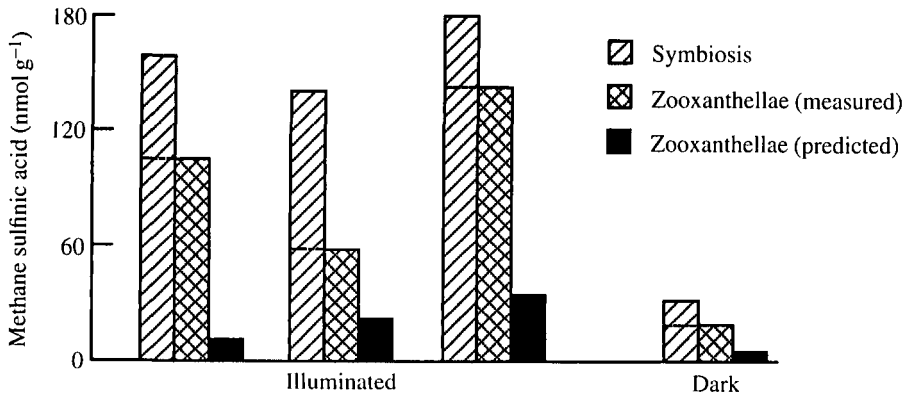

Fig. 8. Production of MSA in the *Anthopleura elegantissima* symbiosis and in the zooxanthellae isolated from it. 'Measured' indicates the MSA actually measured in isolated zooxanthellae, and 'predicted' denotes the amount predicted strictly from the fractional representation of the zooxanthellae in the biomass of the symbiosis. MSA in the host animal tissues is calculated from the difference between that in the whole symbiosis and that in the zooxanthellae, in each of three illuminated specimens, and is the area in the 'Symbiosis' histograms above the broken horizontal line. MSA production in a zooxanthellate specimen maintained in the dark is shown for reference.

because of the necessity of using a single background correction for individual anemones that varied slightly in their color. Nevertheless, the data are ordered in general agreement with our expectations: zooxanthellate anemones exposed to UV light have the highest hydroxyl radical production and apozooxanthellate anemones kept in the dark the lowest (only one of five specimens of the latter had any detectable MSA); exposure of apozooxanthellate anemones to UV light apparently results in additional $^{\cdot}OH$ production; and $^{\cdot}OH$ production in all groups of zooxanthellate anemones tends to be greater than that in apozooxanthellate specimens (Fig. 7). Insufficient numbers of apozooxanthellate specimens precluded an additional treatment of illumination with light lacking UV.

By measuring total MSA production in the symbiosis (host plus zooxanthellae) and in the isolated zooxanthellae quantitatively recovered from exactly half of the same anemone, it is possible to partition MSA (and $^{\cdot}OH$) production between the animal and algal moieties, as shown in Fig. 8. In all cases, $^{\cdot}OH$ production in the zooxanthellae is greater than would be predicted simply on the basis of their fractional representation of the biomass of the symbiosis. Zooxanthellae constitute 7.0, 14.8 and 19.5 % (mean=13.8 %) of the biomass of the three specimens so analyzed, but contain a mean of 63.0% (67.4, 41.5 and 80.1 %) of the MSA produced in the whole anemone (Fig. 8). MSA produced in the host and algae of a specimen kept in the dark is shown for reference.

MSA production in zooxanthellate anemones kept in the dark was surprisingly high (Fig. 7). We suspect that this resulted not from the reaction of DMSO with hydroxyl radicals, but from the oxidation of DMSO by ascorbate in the algae during storage (see Babbs and Steiner, 1990). Three observations support this

supposition: most MSA in darkened zooxanthellate anemones is localized in the algae (Fig. 8); dark production of MSA is all but undetectable in darkened apozooxanthellate anemones (Fig. 7); and the EPR studies indicate no ·OH production in homogenates of zooxanthellate anemones in the dark (Figs 1 and 2). To the extent that dark MSA production occurred as we suggest, the production of hydroxyl radicals in zooxanthellate anemones, as calculated from MSA production, must be lowered accordingly, but this does not alter the conclusion that the flux of ·OH is highest in UV-exposed zooxanthellate anemones, or that most of it occurs in the zooxanthellae.

Discussion

Despite a wealth of circumstantial evidence for their presence (see references in Introduction), free radical fluxes in algal–invertebrate symbioses have not previously been observed directly. The EPR data presented in this report provide the first unambiguous evidence of free radical production in an algal–animal symbiosis. The concordance of the circumstantial evidence (e.g. SOD activity) with the direct also justifies the use of the former as an indicator for the occurrence of photooxidative stress in studies where spin trapping may not be feasible.

Hydroxyl radical EPR spin-trap signals are observed immediately upon illumination of tissue suspensions of zooxanthellate *A. elegantissima*. This radical flux requires light since the DMPO/·OH adduct is detected only upon illumination and the signal begins to dissipate as soon as illumination ceases. Radical production also requires unimpeded photosynthesis in the zooxanthellae; blocking photosynthetic oxygen production and electron transport with DCMU abolishes the DMPO/·OH signal in homogenates of zooxanthellate anemones. These observations suggest that radical production requires both light and high concentrations of O_2, or some other product such as H_2O_2 or $O_2^{\cdot-}$ (see below), derived from algal photosynthesis.

Interpretation of EPR evidence is confounded by the complex chemistry shown by nitrone spin traps in biological samples. For example, both the superoxide radical and its protonated form, the hydroperoxyl radical (·OOH; pKa=4.88), form an unstable organic hydroperoxide upon DMPO addition (DMPO/·OOH), which is susceptible not only to biological oxidation to the alcohol by peroxidases in the sample but also to metal-catalyzed Fenton reactions that yield ·OH (Finkelstein *et al.* 1980; Buettner and Mason, 1990). Indeed, although the DMPO/·OH adduct was the only oxyradical signal directly detected in the present experiments, at least four lines of evidence indicate that, in addition to ·OH, $O_2^{\cdot-}$ is also produced in illuminated tissues containing endosymbionts.

First, if the DMPO/·OH signal arises directly from reaction with free ·OH, addition of an ·OH scavenger will compete with the spin trap for available ·OH and correspondingly diminish the DMPO adduct signal (Buettner, 1982; Buettner and Mason, 1990). In the present experiments, addition of equimolar amounts (100 mmol l^{-1}) of the ·OH scavenger mannitol reduced the apparent DMPO/·OH

signal by only 30 %. This is less than the 50 % reduction predicted assuming that all spin adduct was derived from reaction with free ·OH and given the roughly equivalent rate constants for the reactions of DMPO and mannitol with ·OH (Buxton *et al.* 1988).

Second, the presence of catalase and/or chelators, such as deferroxamine or DETAPAC which block transition metal reactivity, sharply curtails ·OH flux from Fenton reactions (Halliwell and Gutteridge, 1989). However, the intensity of the observed DMPO/·OH signal in illuminated endosymbiotic tissues was not moderated by addition of catalase and DETAPAC either singly or together, suggesting that Fenton reactions are not responsible for the observed DMPO/·OH signal (although the possibility of direct ·OH production independent of H_2O_2 remains).

A third observation supporting the contention that $O_2^{\cdot-}$ is generated in illuminated zooxanthellate tissues is the appearance of a three-line nitroxyl radical (a^N=15.0 G; spectrum not shown) when the SOD inhibitor nitroprusside is present in excess (25 mmol l^{-1}). This radical spectrum appears to be identical to the one described by Misra (1984), who convincingly demonstrated that it results from univalent nitroprusside reduction by $O_2^{\cdot-}$, but not ·OH. In our hands, production of the nitroprusside nitroxyl radical signal requires uninhibited photosynthesis (DCMU treatment abolishes it), and the signal dissipates upon illumination of the tissue and recovers after the light is turned off. This is not surprising given the photolabile nature of nitroprusside (Ivankovich *et al.* 1978) and the substantial availability of reducing potential in the form of radicals and/or ascorbate, which can also reduce nitroprusside to the nitroxyl radical (Misra, 1984), in endosymbiont-containing tissues even in the dark (see DMSO data below). Finally, light-induced DMPO/·OH signals from cell suspensions of endosymbiotic anemone tissues increase when SOD, which scavenges $O_2^{\cdot-}$ but not ·OH, is inhibited by addition of 50 μmol l^{-1} nitroprusside to the medium. Superoxide radical production, therefore, apparently fuels DMPO/·OH formation, perhaps because the DMPO/·OOH adduct is enzymatically oxidized to yield DMPO/·OH (Buettner and Mason, 1990). Taken together, these four lines of evidence indicate that both $O_2^{\cdot-}$ and ·OH are produced in illuminated endosymbiont-containing tissues when photosynthesis is unimpaired, and that the observed DMPO/·OH signals arise not only from ·OH production, but also in large part from the superoxide radical DMPO adduct which is subsequently modified, probably by endogenous peroxidase activity, to form the hydroxyl radical adduct.

Despite the absence of algae, DMPO/·OH signals are also detected when apozooxanthellate anemones are illuminated with visible light. Because these anemones lack endosymbionts, this radical production is clearly not dependent on photosynthetically induced hyperoxia and, as expected, DCMU has no effect on this radical production. We therefore infer that this radical flux arises from photodynamic excitations within the tissues of these anemones, which are not normally exposed to direct illumination in their natural habitat. Such a flux was inferred from our earlier experiments, where a 1-week exposure of apozooxan-

thellate anemones to sunlight from which UV light had been filtered resulted in a fourfold elevation in SOD activity compared with unexposed controls (Dykens and Shick, 1984); in this case, superoxide radicals might have been generated by blue light, as is known to occur in other organisms (Ueda *et al.* 1988). Exposure of apozooxanthellate anemones to the additional UV component of sunlight resulted in a sixfold increase in SOD activity (Dykens and Shick, 1984). Damage by UV light was also inferred from the contraction shown by aposymbiotic anemones in response to UV exposure at midday (Shick and Dykens, 1984). Results of the present DMSO experiments also suggest a photodynamic production of hydroxyl radical related to UV exposure (Fig. 7; discussed below).

Such a photochemically generated radical flux is not seen in illuminated homogenates of zooxanthellate anemones when photosynthesis is inhibited (Fig. 3C). This may be related to the generally higher pigmentation and SOD activities in the sun-adapted zooxanthellate anemones (Dykens and Shick, 1982, 1984) and in the zooxanthellae themselves (Lesser and Shick, 1989*a*,*b*; Shick *et al.* 1991) as well as to the likelihood of greater concentrations of other antioxidants, such as carotenoids and ascorbate, in the zooxanthellate anemones and their algae.

Results of the DMSO experiments indicate that much of the hydroxyl radical flux in intact zooxanthellate anemones is primarily an effect of UV light. Recall that we attribute most of the MSA production in darkened zooxanthellate anemones to oxidation of DMSO by ascorbate in the algae (see Results); if this background MSA production is subtracted from the total MSA produced in zooxanthellate anemones exposed to bright visible light but no UV, we must conclude that the latter condition results in no measurable hydroxyl radical flux, even though hyperoxic conditions due to photosynthesis prevail. High fluxes of ·OH in zooxanthellate anemones are obtained only when UV light is present. This does not contradict the EPR studies, where ·OH is spin-trapped in the absence of UV light because much of this apparent ·OH flux probably results from $O_2^{\cdot-}$ and/or H_2O_2 production, neither of which are detected by the DMSO assay. Similarly, the conclusion that ·OH production in zooxanthellate anemones is particularly a consequence of UV exposure is consistent with the failure of light lacking a UV component to cause ·OH production in photosynthetically inhibited zooxanthellate anemones in the EPR studies [although the possibility remains that such illumination produces intracellular ·OH where, because of its extreme reactivity (see below), it is unavailable to the extracellular EPR spin trap]. Also, the conclusion from the DMSO experiment on zooxanthellate anemones that photosynthetically generated hyperoxia alone does not enhance ·OH production is consistent with DMSO studies of apozooxanthellate anemones, where imposed hyperoxia in the presence of UV light does not elevate ·OH production above that seen with UV alone. Two obvious experiments that would clarify the separate and interacting effects of hyperoxia and UV light were not done: it was not feasible to illuminate the EPR sample chamber with UV light, and preliminary tests indicated that the photosynthetic inhibitor DCMU interferes with the MSA assay.

Isolated zooxanthellae also produce a light-dependent ·OH flux that is abolished by DCMU. It has long been recognized that chloroplasts isolated from vascular plants have a light-dependent production of superoxide radicals and ·OH (reviewed by Asada and Takahashi, 1987). In studies where the algae and their chloroplasts remain intact, any ·OH produced in the chloroplast would have to diffuse through the thylakoid matrix and across several membranes before encountering the extracellular DMPO spin trap (see below), an unlikely possibility in view of the high reactivity of this radical *in situ*.

Radical toxicity *in vivo* reflects a combination of reactivity and site of production; less reactive radicals and H_2O_2 can diffuse away from the site of production and undergo damaging reactions elsewhere, whereas highly reactive radicals react shortly after production and are therefore effectively confined to the micro-domain of their production (Slater and Cheeseman, 1988). In this context, algal–animal associations are extraordinarily complex. Numerous intracellular compartments must be considered as potential sources and sinks of $O_2^{\cdot-}$ and ·OH: the algal chloroplast, the algal and animal cytosols, each with a wealth of subcompartments, and even the intercellular matrix. It is not possible to distinguish between radical production in one compartment and that in another when using disrupted cell suspensions or when using the hydrophilic spin trap DMPO, which does not readily traverse membranes; the radicals spin-trapped in these experiments were already outside either the cell or the organelle that produced them. With second-order rate constants of the order of 10^9–$10^{10}(\text{mol l}^{-1})^{-1}\text{s}^{-1}$, it is highly unlikely that the spin-trapped ·OH had negotiated the membrane surrounding its intracellular or intraorganellar site of formation.

Therefore, a more reasonable model to account for the present observations of DMPO/·OH in suspensions of intact zooxanthellae and zooxanthelate tissues is that the algae generate a flux of $O_2^{\cdot-}$ and H_2O_2, derived from $O_2^{\cdot-}$ dismutation and other sources in the algal cytoplasm, which diffuses out of the algae where $O_2^{\cdot-}$ (*via* enzymatic peroxidation or hydrolysis of DMPO/·OOH) and H_2O_2 (*via* Fenton chemistry or direct photolysis) yield the ·OH that is spin-trapped. The observation that inhibition of SOD, which increases $O_2^{\cdot-}$ and hence H_2O_2 levels, also increases DMPO/·OH concentration, supports this scenario. That oxygen reactivity presents a problem to the zooxanthellae is also implied by their high levels of antioxidant enzymes (Lesser and Shick, 1989*a*,*b*; Matta and Trench, 1991) and compounds protective against UV light (Shick *et al.* 1991).

DCMU blocks electron transfer from univalently reduced, membrane-bound quinone (Q_A) to protein B (Q_B), which, following divalent reduction, passes two electrons to soluble plastoquinone (reviewed by Mathis and Rutherford, 1987). Because this blockage occurs at a site after Q_A reduction, the semiquinone EPR spectrum should not be abolished by DCMU, and this is what is observed (cf. Figs 3 and 6). Likewise, impeding lateral diffusion of the semiquinone within the membrane by freezing should correspondingly impede electron transfer and stabilize the light-induced semiquinone EPR signal; the semiquinone signal is

indeed larger and persists longer after illumination ceases when the tissues are frozen at 77 K while illuminated in the spectrometer (see also O'Malley and Babcock, 1984).

Partitioning of *in vivo* ·OH production between host animal and algal endosymbionts is possible with DMSO, which readily enters cells, where it reacts with ·OH to form a stable product that can subsequently be quantified in the isolated partners. Experiments on three zooxanthellate specimens of *A. elegantissima* reveal a total MSA (·OH) production of about 150 nmol g^{-1} fresh mass of the whole symbiosis (Fig. 8), the same as in the earlier experiment where the anemones were exposed to UV light (Fig. 7). The zooxanthellae contain 63 % of the total MSA, which is much greater than would be predicted simply on the basis of their constituting about 14 % of the biomass of the symbiosis. The result is not surprising, since zooxanthellae contain high concentrations of chlorophyll and flavins capable of initiating various photosensitized reactions and experience the highest intracellular concentrations of molecular oxygen. Such relationships probably underlie the higher SOD activity seen in the zooxanthellae than in the host (Dykens, 1984; Shick *et al.* 1991).

In absolute terms, the production of 60–150 nmol MSA g^{-1} in the zooxanthellae during 1 h of exposure to bright PAR+UV is similar to that in paraquat+DMSO-treated vascular plants (Babbs *et al.* 1989). The difference between MSA concentration in the intact symbiosis and that in the zooxanthellae is a measure of MSA in the host animal. By comparison with the specimen kept in the dark, it is evident that illumination of the anemones increases the production of hydroxyl radical not only in the zooxanthellae but also in the host tissue (Fig. 8). Moreover, the mean MSA concentration in the animal tissue of the illuminated zooxanthellate anemones is about twice that in the illuminated apozooxanthellate anemones, which supports our suggestion that active oxygen species may be exported from the algae.

Algal–animal associations are generally regarded as mutualistic, no doubt because most research on these symbioses has focused on mutually beneficial nutritional interactions. However, chronic exposure of the host animal to a flux of reactive radicals during illumination necessitates host maintenance of robust oxidative defenses, while host avoidance behavior that reduces photosynthesis during the brightest portion of the day limits algal productivity (Shick and Dykens, 1984; Shick, 1991). Moreover, chronic exposure of the host to oxyradicals and photodynamic events, which are synergistically exacerbated by hyperoxia, suggests that algal–animal symbioses may not be wholly mutualistic. Direct observations of oxyradical flux in a symbiosis not only confirm the earlier circumstantial evidence suggesting free radical production but they also suggest that the maintenance of defenses against such radicals is a real, but rarely considered, cost to the host of harboring photoautotrophic endosymbionts.

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