Oxygen toxicity, respiration and behavioural responses to oxygen in free-living anaerobic ciliates

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Three species of marine, anaerobic free-living ciliates (*Metopus contortus, Plagiopyla frontata* and *Parablepharisma collare*) were studied with respect to their relation to O_2. Survival was adversely affected at O_2 tensions exceeding 1–2% of atmospheric (air) saturation (atm. sat.): *Parablepharisma collare* survived for about 1 h at 100% atm. sat., while the other two species survived the treatment for up to 2 d. Survival in the presence of O_2 depended on the composition of the medium; cells survived longer in clean seawater than in culture medium which, when exposed to O_2, became toxic. This is probably related to peroxide production mediated by solutes in the culture medium: addition of catalase prolonged survival. The ciliates did not contain catalase. Two of the species harbour endosymbiotic methanogens; at O_2 tensions exceeding 2% atm. sat. the bacteria were inactivated, but they remained viable even after more than 5 h exposure to atmospheric O_2 tension. *Metopus contortus* and *Plagiopyla frontata* respired O_2 at rates similar to those of aerobic species; this O_2 uptake is not coupled to energy conservation since the ciliates do not contain cytochromes, and low O_2 tensions do not stimulate growth. O_2 consumption is probably a detoxification mechanism which can maintain an intracellular anaerobic environment at a low ambient O_2 tension. *Metopus contortus* and *Plagiopyla frontata* showed chemosensory behaviour in response to O_2; this allowed them to find and remain within anaerobic microhabitats.

Introduction

Most anaerobic environments harbour unicellular eukaryotes with an O_2-independent energy metabolism. Among these, the symbiotic forms living in the intestinal or urogenital tract of animals have so far attracted most attention (Müller, 1988, and references therein). Anaerobic protozoa also occur in aquatic sediments and in the water column of stratified lakes and anoxic marine basins, as well as in anaerobic sewage and in landfill sites (Fenchel, 1969; Fenchel *et al.*, 1977, 1990; Finlay, 1980; van Bruggen *et al.*, 1986; Wagener & Pfennig, 1987; Goosen *et al.*, 1988; Finlay *et al.*, 1988). Among the free-living forms, most is known about the anaerobic ciliates. All the species so far studied possess organelles which morphologically resemble mitochondria, but do not contain cytochromes. In some species these organelles contain a membrane-bound hydrogenase and are then referred to as hydrogenosomes. They are capable of oxidizing pyruvate completely to acetate, CO_2 and H_2 (Zwart *et al.*, 1988; Finlay & Fenchel, 1989). H_2 excretion has previously been demonstrated in several

urogenital, intestinal and rumen protozoa (Yarlett *et al.*, 1984; Müller, 1988). Among the hydrogenosome-containing free-living ciliates, many also harbour endosymbiotic methanogenic bacteria which presumably depend on the H_2 production of the host (van Bruggen *et al.*, 1986; Wagener & Pfennig, 1987; Goosen *et al.*, 1988; Finlay & Fenchel, 1989). The mechanism of energy metabolism of anaerobic ciliates which do not possess a hydrogenase is not known in any detail, but it must also be fermentative. Growth yields and growth rates are consistent with this assumption (authors' unpublished observations).

Among the parasitic anaerobic protozoa several forms (e.g. the trichomonad flagellates) are quite tolerant of O_2, whereas the ciliates from the rumen tolerate only trace amounts of this gas (Lloyd *et al.*, 1982; Müller, 1988). In all cases studied the organisms are capable of O_2 respiration and the affinity for O_2 is comparable to that of aerobic protozoa. The uptake of O_2 by rumen ciliates is considered to be important for the maintenance of a low O_2 tension in the rumen (Ellis *et al.*, 1989). The terminal oxidase has not been identified with certainty in

any case, but it is not cytochrome oxidase, and the O_2 uptake is generally unaffected by respiratory inhibitors (Müller, 1988).

With regard to free-living forms, very little is known about their relationship to O_2. In nature they occur only in reducing environments where O_2 is undetectable. On the other hand, chemical gradients in sediments can be very steep, ranging from atmospheric O_2 tension to complete anoxia over a few millimetres. In addition, chemoclines may move vertically or be destroyed rapidly due to changes in the intensity of oxygenic photosynthesis at the sediment surface or to increased water turbulence resulting in oxidation of the sediment to a considerable depth (Fenchel, 1969). Therefore in some sediments the anaerobes are likely to be periodically exposed to O_2. It also seems likely that isolated anaerobic niches may become colonized by anaerobic protozoa via aerobic environments. In this paper we describe the responses to O_2 in three species of free-living anaerobic ciliates.

Methods

Isolation and cultivation of the ciliates. Metopus contortus Kahl, Plagiopyla frontata Kahl and Parablepharisma collare Kahl were all isolated from anaerobic sediments collected in Nivå Bay about 15 km south of Helsingør, Denmark. They were grown in 125 ml Hypo-Vials capped with butyl stoppers and aluminium seals. The medium was 100 ml seawater (salinity 32 parts per thousand) diluted with distilled water to a salinity of 18 parts per thousand, boiled with dried grass (400 mg l^{-1}) and filtered. Two boiled wheat grains and a few drops of resazurin solution (as a redox indicator) were added finally. The medium was then bubbled with purified N_2 gas (<5 p.p.m. O_2 according to the supplier) and reduced with a few drops of a 10 mM-sulphide solution prepared by dissolving Na_2S in deoxygenated water and neutralizing with HCl. The vials were then capped and inoculated with 1 ml containing 50–100 protozoan cells (together with a mixed community of bacteria to serve as food) through the butyl stoppers with a hypodermic syringe. During the growth phase of the protozoa, the pH remained at around 7 and the medium contained from 0·7 to 4 mM-sulphide after a few days of growth. At 20 °C Metopus has a doubling time of about 60 h and the two other species have a doubling time of 20–25 h; the eventual yield was 100–200 cells ml^{-1}. Plagiopyla and Metopus both have a hydrogenase and harbour methanogen symbionts. The mitochondria of Parablepharisma do not contain hydrogenase and the ciliate does not harbour methanogens; the cells are, however, densely covered by ectosymbiotic bacteria of unknown identity and function (Fenchel et al., 1977; T. Fenchel, unpublished observations).

Experimental methods. For experiments on the survival or behaviour of cells exposed to different O_2 tensions, 50 µl drops of cell suspensions were placed in the bottom of test tubes flushed with N_2. The tubes were then closed with butyl stoppers and the desired amount of air injected into the tube with a hypodermic syringe. Surviving cells were counted directly with a dissection microscope and swimming behaviour recorded with a video camera (Sony DXC-101P) connected to a recorder (Panasonic AG-6100). When the recordings were viewed frame by frame (time interval 40 ms) the position of individual cells was plotted on a transparent overlay on the monitor. Some survival experiments were also made using Hypo-Vials, with a 10 mm layer of

ciliate suspension in the bottom and with different O_2 tensions in the head space. During the experiment the vials were gently agitated on a rotating table to secure equilibrium between the gas and the liquid phase. For counting cells, samples were withdrawn with a hypodermic syringe. Behaviour in O_2 gradients was studied using microslide tubes (Camlab, Cambridge, UK) with internal dimensions of 4 × 0·4 mm. The capillaries were filled with an anoxic ciliate suspension (in a N_2 atmosphere) and placed in a stoppered test tube in which the oxygen content of the gas phase was controlled through hypodermic needles penetrating the stoppers.

O_2 uptake was measured in a 0·5 ml thermostatically controlled respiration chamber with a stirring magnet and an O_2 electrode (Radiometer, Copenhagen). Suspensions of washed cells (3000–5000 cells ml^{-1}) in sterile seawater with initial O_2 tensions of 10–20% atm. sat. were used and the reading of the O_2 meter was monitored until all O_2 was consumed (Fig. 5). All experimental results refer to a temperature of 20 °C. O_2 tension is throughout given as a percentage of atmospheric (air) saturation (% atm. sat.). With the salinity and temperature of the experiment 100% atm. sat. corresponds to 0·26 mM-O_2.

Endosymbiont methanogens were quantified microscopically using the autofluorescence of coenzyme F_{420} characteristic of these bacteria (Zehnder, 1988). Ciliates were fixed in 2% (v/v) formaldehyde (final concentration) in seawater and concentrated on black Nuclepore filters. The filters were mounted with immersion oil under a coverslip and viewed using an epifluorescence microscope (Olympus) with violet excitation.

Results

Effect of O_2 on survival

O_2 adversely affected the survival of all three species (Figs 1–4), but the species differed in their sensitivities. Survival time for a given O_2 tension also depended on the medium. Cells which were washed (by centrifugation) and placed in clean, filtered seawater survived longest; at atmospheric O_2 tension some Plagiopyla and Metopus
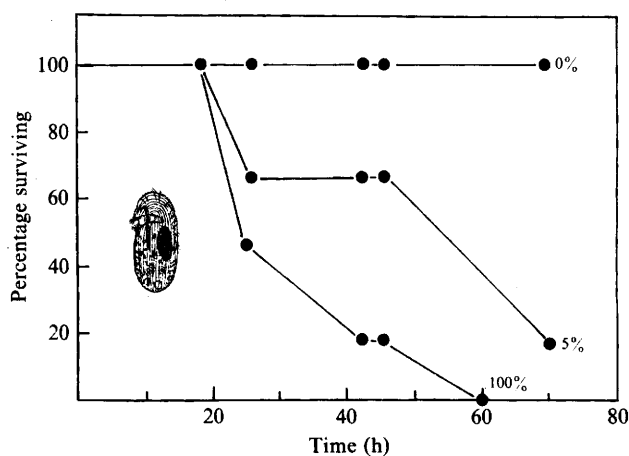

Fig. 1. Survival of Plagiopyla frontata in drops of sterile, clean seawater, the headspace being N_2 plus different percentages of atmospheric air. There were initially about 20 cells for each treatment.
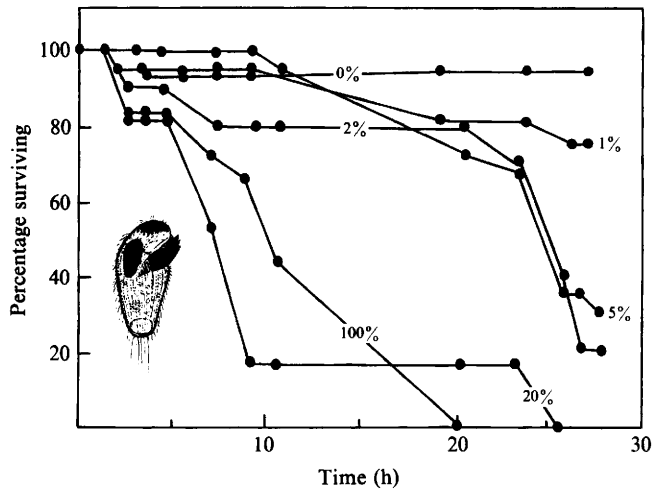
Fig. 2. Survival of *Metopus contortus* in drops of sterile, clean seawater, the headspace being N_2 plus different percentages of atmospheric air. There were initially about 20 cells for each treatment.
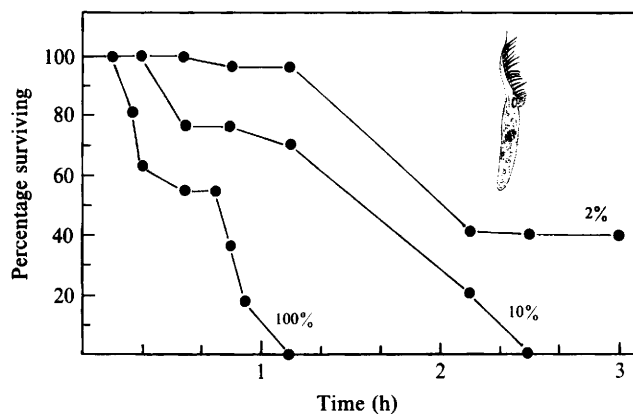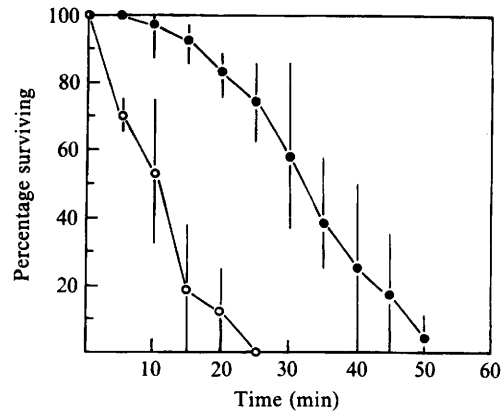

Fig. 4. Survival of *Parablepharisma collare* in drops of culture medium exposed to atmospheric air, with (●) and without (○) added catalase (0·1%). Three experiments were performed for each treatment and the mean and total range for each set of counts are shown. Each drop initially contained about 20 cells.

Fig. 3. Survival of *Parablepharisma collare* in Hypo-Vials containing about 10 ml sterile clean seawater and initially about 100 cells ml⁻¹, the headspace being N_2 with different percentages of atmospheric air. Each sample was 0·5 ml. No vial with 0% O_2 was employed in this experiment.

cells survived for several days, whereas *Parablepharisma* cells lived for only about 1 h. At lower O_2 tensions survival increased in all species (Figs 1–3). It is possible that *Metopus* and *Plagiopyla* can survive very low O_2 tensions indefinitely. They can be grown in open, unstirred hypovials, but anoxia at the bottom or within aggregates of bacteria is likely to occur. In one experiment, cultures of these two species were bubbled with N_2 plus 2% atmospheric air. The cells survived for more than 200 h, but they did not grow. In a control vial bubbled with pure N_2 *Metopus* (but not *Plagiopyla*) did increase in numbers for several generations.

If the cells remained in the culture medium when exposed to O_2, survival was much poorer: in one experiment maximum survival time for *Metopus* was 5·5 h, and for *Parablepharisma*, less than 30 min. The presence of 0·1% catalase (bovine liver, Sigma) restored survival time to that characteristic for clean seawater (Fig. 4). Catalase had no significant effect on survival in clean, oxygenated seawater. Part of the greater O_2 toxicity of the medium was due to the presence of the redox dye resazurin; when this dye was added to clean seawater (final concentration 2 p.p.m.) survival time decreased in the presence of O_2, but this effect was ameliorated by catalase. Adding sulphide to oxygenated water did not decrease survival. Addition of 0·1% superoxide dismutase (from bovine erythrocytes, Sigma) either had no effect or decreased survival in the presence of O_2. Thus *Metopus* cells in culture medium with superoxide dismutase all died between 3 and 6 h when exposed to air while 75% of the cells were alive after 6 h in a control experiment without superoxide dismutase.

When 2–3 drops (corresponding to 80–120 μl) of a 3% (v/v) H_2O_2 solution was added to 1 ml of a sufficiently dense suspension of aerobic ciliates, bubble formation was clearly evident. This effect was not observed with any of the three anaerobic ciliates studied, indicating the absence of catalase activity. Survival was 100% for an aerobic ciliate (*Euplotes* sp.) exposed to 5 μM-H_2O_2 and 40% after 6 h in 25 μM-H_2O_2. In contrast, both solutions killed 100% of *Metopus* cells within 40–60 min.

When the ciliates had been exposed to O_2 there was a time lag before they resumed cell division following re-incubation in anaerobic culture medium. The duration of this lag depended on the duration of the exposure to O_2:

Table 1. *Quenching of methanogen fluorescence as function of O_2 tension and exposure time*

Organism	Exposure (h)	Fluorescence* at O_2 tension (atm. sat.) shown:					
		0%	1%	2%	5%	10%	20%
Metopus	2	+	+	+	+	+	+
•	23	+	+	+	−	−	−
	40	+	+	+	−	−	−
	64	+	+	+	−	−	−
Plagiopyla	2	+	+	+	+	+	+
	22	+	+	+	±	−	−
	51	+	+	±	−	−	−

* +, Fluorescence normal; −, fluorescence absent; ±, only a small fraction of the cells showed fluorescence.

thus *Plagiopyla* cells exposed to 100% atm. sat. for 5 or 10 min had a lag of 10–12 h, cells exposed for 20 min or 1 h had lag times of 20 and 22 h, respectively, and cells exposed for 5 h did not resume exponential growth until after about 160 h in an anaerobic environment.

Effects of O_2 on methanogenic endosymbionts

When *Plagiopyla* and *Metopus* cells were exposed to O_2 for a sufficiently long time the autofluorescence of the endosymbiont methanogens disappeared (Table 1). This was due to the destruction of the F_{420}-hydrogenase enzyme complex (Zehnder, 1988) and it presumably reflects inactivation of methanogenesis. At an O_2 tension of 1–2% atm. sat., fluorescence was apparently sustained indefinitely. At 100% atm. sat., fluorescence was destroyed within 5 min in *Metopus* and after somewhat longer in *Plagiopyla*. In all cases fluorescence reappeared within a few hours if the ciliates were reincubated in an anaerobic medium. In *Plagiopyla* cells kept for 5 h at atmospheric O_2 tension, the methanogens first regained fluorescence after 15–20 h in an anoxic environment. During subsequent cell divisions of the ciliate hosts the bacterial numbers per cell remained almost constant; therefore the viability of the methanogens was unaffected even after a relatively long exposure to atmospheric O_2 tension.

O_2 uptake

It was not possible to measure O_2 uptake in *Parablepharisma* due to its poor survival under aerobic conditions. *Metopus* and *Plagiopyla* showed very similar uptake patterns. At O_2 tensions exceeding about 5% atm. sat., uptake was saturated at 0·1–0·15 nl O_2 h^{-1} per cell. The
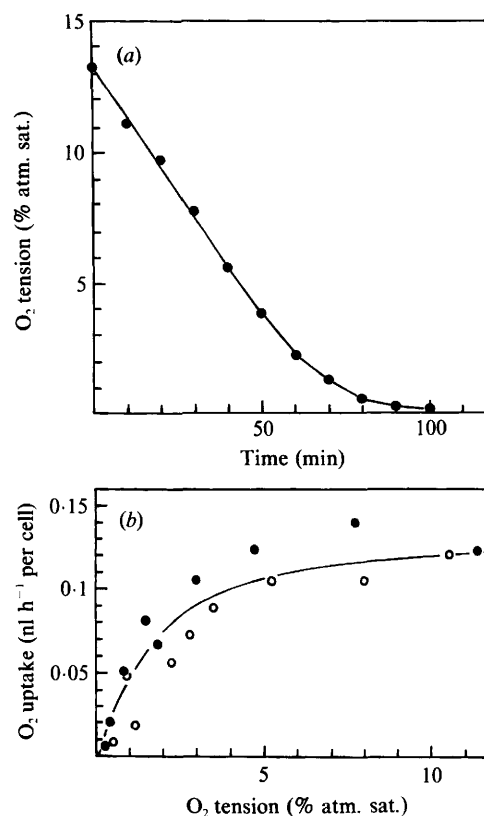

Fig. 5. (a) O_2 tension as a function of time in a respiration chamber with *Metopus* cells (5000 cells ml^{-1}). (b) O_2 uptake of *Metopus* cells as a function of O_2 tension; results from two experiments such as those shown above are given (●, ○).

apparent half-saturation constant was about or slightly more than 1% atm. sat. or, at the ambient temperature and salinity, about 2·6 μM (Fig. 5).

Chemosensory responses to O_2

The fact that the anaerobic ciliates showed chemosensory behaviour to O_2 tension and they were capable of respiration was most easily illustrated by adding a sufficiently dense cell suspension of *Metopus* or *Plagiopyla* to oxic water. The ciliates rapidly clumped together in response to the layer of decreased O_2 tension surrounding neighbouring cells (Fig. 6). One mechanism which partly explains this effect is a kinetic response, that is, that the motility of the cells is at a minimum under optimum conditions, so they tend to accumulate there. Fig. 7 shows the swimming velocity of *Metopus* cells exposed to different O_2 tensions; it is seen that the cells responded by increasing their swimming velocity at O_2 tensions exceeding 1% atm. sat. or less.

Cells also showed a phobic response in O_2 gradients; that is, they reacted with ciliary reversals (tumbling)

Fig. 6. *Plagiopyla frontata* cells clumping together after being immersed in oxygenated water. Bar, 0·5 mm.

Fig. 7. Swimming velocity of *Metopus contortus* at different O_2 tensions. Two experiments are shown. In one experiment (●) the cells were returned to anoxia between each recording to allow them to recover the low swimming velocity characteristic of anoxia. In the other experiment (–●–) O_2 tension was increased after each recording and 10 min of acclimatization was allowed after each change in O_2 tension. Each data point represents the mean of ten swimming tracks and the standard deviation.

when swimming from an anoxic zone into one containing oxygen. This was easily observed in experiments such as that shown in Fig. 8. A microslide tube open at one end
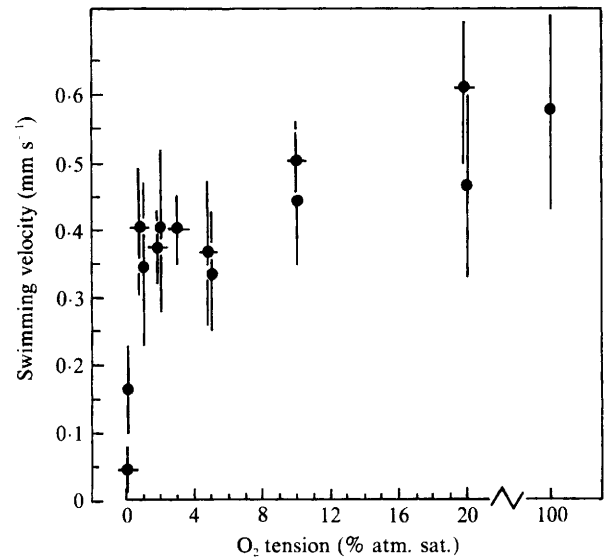
and filled with an anaerobic ciliate suspension was placed in a test tube in which the atmospheric composition could be controlled by hypodermic needles which penetrated the butyl stopper. At an O_2 tension as low as 1 % atm. sat. the *Metopus* cells retreated from the meniscus while the microaerophilic *Euplotes* sp. (see Fenchel *et al.*, 1989) congregated there.
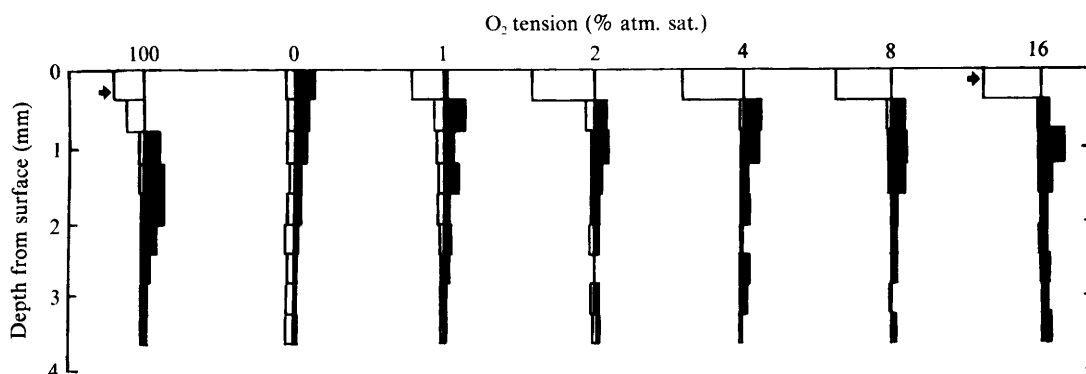

Fig. 8. Distribution of *Metopus contortus* (■) and the microaerophilic *Euplotes* sp. (▢) in a microslide tube in atmospheres with different O_2 tensions. Block size is proportional to cell numbers. Most of the contents of the capillary remained anoxic due to the respiration of the cells (about 100 of each species). At the highest external O_2 tension the *Euplotes* also retreats from the meniscus since it prefers an O_2 tension of 5% atm. sat. (see Fenchel *et al.*, 1989). Thus the arrow, which indicates the maximum density of *Euplotes*, also indicates an O_2 tension of 5% atm. sat. There was an interval of 15 min between recordings of the position of the cells after changing the atmospheric composition.

Discussion

Our results suggest that anaerobic protozoa vary with respect to O_2 sensitivity. We could not find evidence that any of the species we studied can grow at low O_2 tensions. Survival experiments and observations of behaviour suggest that the organisms certainly have a preference for a $pO_2 < 1\%$ atm. sat. Goosen *et al.* (1988, 1990) found that the limnic *Plagiopyla nasuta* and *Trimyema compressum* could grow with O_2 tensions in the gas phase up to about 5% atm. sat. In neither case, however, was there evidence to suggest that O_2 uptake was coupled to energy conservation.

O_2 toxicity is a complex phenomenon (e.g. Morris, 1979). One important element of toxicity is the oxygen radicals which are produced when reduced compounds are oxidized, either biologically or spontaneously, by O_2. Our findings suggest that peroxide formation in oxygenated water, in conjunction with the absence of cellular catalase in anaerobic protozoa, is one reason for the toxic effect of O_2 on these organisms. The observation that the presence of superoxide dismutase in the medium tends to augment rather than to decrease O_2 toxicity suggests that the superoxide radical is the lesser peril compared to peroxide. It is apparent, however, that peroxide is not the only critical component of O_2 toxicity since catalase has no life-prolonging effect in clean oxygenated seawater. The formation of peroxide and superoxide following oxygenation of anaerobic growth media has previously been demonstrated by Carlsson *et al.* (1978).

In *Metopus* and in *Plagiopyla* there are three obvious targets for O_2 toxicity: the hydrogenase, the pyruvate:ferrodoxin oxidoreductase, and the endosymbiont methanogens, although the latter are not essential for the survival of the hosts (Wagener & Pfennig, 1987; authors' unpublished results). It is therefore surprising that of the three ciliates studied, *Parablepharisma*, which has neither a hydrogenase nor methanogen symbionts (T. Fenchel, unpublished results), is by far the most O_2 sensitive. We have no explanation for this. Some unusual features of the biochemistry of *Parablepharisma* may be responsible. Alternatively, this ciliate may lack most of the molecular mechanisms offering defence against O_2 toxicity (e.g. Frank & Massaro, 1980). It is unlikely to be completely devoid of defence mechanisms since it will, like all ciliates in anaerobic and low-O_2 environments (Finlay *et al.*, 1986), be periodically exposed to elevated levels of O_2.

Chemosensory responses to O_2 allow the ciliates to orient themselves in O_2 gradients and to seek out anaerobic micropatches, providing some defence against O_2 toxicity. Another aspect of their defence against O_2 toxicity is their respiration. At low O_2 tensions ($<1-2\%$ atm. sat.), where uptake is almost linearly proportional to

O_2 tension, uptake is probably diffusion-limited and the cells are capable of maintaining an anaerobic environment inside the cell. This is consistent with the observation that fluorescence of the methanogens is unaffected even after long exposure to such low O_2 tensions. At higher O_2 tensions, when O_2 uptake becomes saturated, the intracellular environment must become oxic. It must then be assumed that the ciliate hydrogenases as well as the methanogens become inactivated. Using O_2 as an electron sink may then be a substitute for H_2 production in the maintenance of a fermentation redox balance (cf. Müller, 1988). Most methanogens are extremely sensitive to oxygen (Zehnder, 1988). It is therefore noteworthy that the endosymbiotic methanogens remain viable and are capable of growth when returned to anoxic conditions even after prolonged exposure to O_2.

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