The microaerophilic flagellate *Giardia intestinalis*: oxygen and its reaction products collapse membrane potential and cause cytotoxicity

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Trophozoites of the microaerophilic flagellate parasitic protozoon *Giardia intestinalis* have only a limited capacity to detoxify O₂. Thus, when exposed to controlled concentrations of dissolved O₂ >8 µM, they gradually lose their ability to scavenge O₂. In a washed cell suspension stirred under 10% air in N₂ (equivalent to 25 µM O₂), inactivation of the O₂-consuming system was complete after 3.5 h; during this period accumulation of H₂O₂ (3 µmol per 10⁶ organisms) and oxidation of cellular thiols to 16% of their initial level occurred. Under 20% air (50 µM O₂), respiratory inactivation was complete after 1.5 h, and under air (258 µM O₂), after 50 min. Loss of O₂-consuming capacity was accompanied by loss of motility. Use of the fluorogen 2,7-dichlorodihydrofluorescein acetate indicated that intracellular H₂O₂ is produced at extranuclear sites. Flow cytometric estimation of the plasma membrane electrochemical potentials using bis(1,3-dibutylbarbituric acid) trimethine oxonol, DiBAC₄(3), showed that values declined from –134 mV to –20 mV after 4.5 h aeration. Incubation of organisms with 60 µM H₂O₂ for 10 min gave partial collapse of plasma membrane potential and complete loss of O₂ uptake capacity; motility and viability as assessed by DiBAC₄(3) exclusion were completely lost after 1 h. Inactivation of the O₂-consuming system and loss of viability were also observed on exposure to singlet oxygen photochemically generated from rose bengal or toluidine blue.

Keywords: hydrogen peroxide, oxidative stress, reactive oxygen species

INTRODUCTION

The flagellate protozoon *Giardia intestinalis* is a world-wide cause of intestinal infection and diarrhoea, and especially in infants and the elderly, morbidity (Ortega & Adam, 1997) and mortality (Shukry *et al.*, 1986). The trophozoite infects the upper jejunum by attachment to the mucosal surface. Waterborne transmission occurs by ingestion of cysts (Craun, 1990), which survive for periods of months in freshwater ecosystems at low temperatures. Control of contamination of fresh water and supplies of potable water is achieved only with difficulty, due to resistance of cysts to chemical agents

(Winiacka-Krusnell & Linder, 1998), and the probable existence of animal reservoirs (Smith *et al.*, 1995).

The upper intestinal lining is well supplied with capillaries, and O₂ concentration there has been measured at 60 µM (Atkinson, 1980). *G. intestinalis* is usually regarded as an 'anaerobic' protozoon, and grown and studied as such in laboratory culture (Meyer, 1976). However, several observations indicate that this organism is a microaerophile (Paget *et al.*, 1989, 1993). Measurement of O₂ consumption as a function of dissolved O₂ indicates that at low levels (0–50 µM), the organism is capable of scavenging O₂ (apparent K_m for O₂ 6.4 µM for the trophozoite). Above a threshold of 80 µM O₂, O₂ inhibits its own consumption. Changes in the balance of major fermentation products occur as O₂ concentration is increased. Paget *et al.* (1990) found that anaerobically, alanine and ethanol were formed in

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazonol; DCCD, *N,N'*-dicyclohexylcarbodiimide; DiBAC₄(3), bis(1,3-dibutylbarbituric acid) trimethine oxonol.

roughly equimolar amounts, ethanol production occurred maximally at $1.0 \mu\text{M O}_2$, whereas acetate accumulation continued to increase to at least $46 \mu\text{M O}_2$. The intracellular redox state, as indicated by *in vivo* measurement of NADH, and the integrity of an EPR-detectable iron-sulphur centre are both highly sensitive to traces ($< 0.1 \mu\text{M}$) of O_2 (Paget *et al.*, 1993; Ellis *et al.*, 1993). Here we aim to define the limits of O_2 tolerance of this microaerophilic organism, and to characterize the nature of the structural and functional consequences of accumulation of reactive O_2 species.

METHODS

Organisms and cultures. *Giardia intestinalis*, Portland 1 strain ATCC 30888, originally described by Meyer (1976), was obtained from the Queensland Institute of Medical Research. Trophozoites were grown axenically at 37°C , using TYI-S-33 medium (Keister, 1983; Edwards *et al.*, 1989) but with complement-inactivated new-born calf serum at 10% (v/v). Cultures were initiated by inoculating 15 ml medium in polypropylene centrifuge tubes with 4×10^4 organisms ml^{-1} . A 0.5 ml air headspace was routinely employed. Growth was for 2 d and final cell numbers reached about 10^7 per tube.

Harvesting. Tubes were chilled on ice for 20 min and then shaken gently to dislodge adhered trophozoites. Cell numbers were measured by counting on a haemocytometer slide. Cell suspensions were centrifuged at $1000 g$ (3000 r.p.m.) for 4 min at room temperature in a bench centrifuge at room temperature. After washing once with phosphate-buffered saline (pH 7.1, PBS) and recentrifugation, organisms were finally resuspended in PBS or in 0.31 M mannitol solution (as indicated) and kept at 4°C .

Measurements of O_2 consumption rates. Cell suspensions (2.2×10^{-6} organisms ml^{-1}), 2 ml total volume (1:1) in PBS were incubated at 37°C in a 'closed' O_2 electrode vessel (Rank) with magnetic stirring (200 r.p.m.). Where incubations were performed at low O_2 concentrations, a stainless-steel open O_2 electrode system fitted with a Teflon-membrane-covered O_2 electrode (Radiometer) (Lloyd *et al.*, 1979) was employed. A digital gas mixer (Lundsgaard & Degn, 1973) was used to mix 1% or 5% O_2 with N_2 ; after humidification by passage over moist filter paper, the gas mixture was passed over the surface of the stirred liquid vortex (stirring at 500 r.p.m.), enabling the O_2 tension to be maintained at desired levels. Addition of substrates (e.g. glucose) or of inhibitors were made through a septum. To obtain the transfer constant, k , the value of $t_{1/2}$ for equilibration of buffer upon switching the gas phase from 1% O_2 to N_2 was determined, then k was calculated from the relationship $k = \log_e 2/t_{1/2}$.

Respiration rates (V_r) were calculated from $V_r = k(T_g - T_l)$, where T_g is the O_2 concentration in the gas phase, and T_l that in the liquid phase.

O_2 concentrations in PBS were calculated from the air-saturation value for air at 37°C ($250 \mu\text{M O}_2$).

Measurement of cellular swelling. Changes in cell volume were monitored by following the time course of absorbance change at 550 nm.

Confocal laser-scanning microscopy. A Bio-Rad MRC confocal system attached to a research microscope (1024-Leica DMRB) was used with an argon-krypton air-cooled laser at 448 nm. Images were obtained with a $\times 63$ oil-immersion objective (NA 1.38). Section thickness was $5.5 \mu\text{m}$. The 0.3 W

laser was used at 10% power to minimize photobleaching. Unless otherwise stated, organisms were washed and resuspended in 0.31 M mannitol before observation using 'FITC' filters. Images were acquired on Zip disc and printed using an Epson 750 colour printer.

Flow cytometry. Forward narrow-angle light scatter, side-scatter and fluorescence were measured using a flow cytometer (Multi-Laser Sorter, MoFlo Cytomation) fitted with a Cicero or a Cyclops Summit version 2 operating software. Fluorescence of bis(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC₄(3)] was measured using a water-cooled 200 mW 488 nm argon-ion laser (Coherent I-90). An interference filter (D530/540) was used for emitted wavelengths (Chrome Technology), together with a Hammamatsu Hybrid Photodetector (no. 957-06). The characteristics of 20000 organisms were accumulated routinely.

Flow cytometric measurement of plasma membrane potential. Plasma membrane potential was measured by flow cytometry (Krasznai *et al.*, 1995), using the negatively charged fluorescent membrane potential indicator dye DiBAC₄(3). This dye distributes across the cytoplasmic membrane, according to the Nernst equation.

A calibration curve of fluorescence intensity (i.e. channel number) measured from stained cells vs extracellular dye concentration allows evaluation of membrane potential in mV using live cells by comparison with those in depolarized state. Rather than employing fixed organisms (Emri *et al.*, 1998), heat treatment ($> 80^\circ\text{C}$) for 3 min was employed in order to produce a suspension with zero plasma membrane potential.

Transmission electron microscopy. Washed *G. intestinalis* cells were fixed at 4°C for 1 h in 0.1 M cacodylate buffer (pH 6.9) containing 1% paraformaldehyde and 2% glutaraldehyde. They were post-fixed with phosphate-buffered 1% OsO_4 at 4°C for 1 h, then dehydrated with successive washes of ethanol: 50%, 70%, 90% at 4°C and two washes of 100% at room temperature. The cells were embedded in Spurr resin. Ultrathin sections were obtained with an LKB Ultratome III and mounted onto 0.5% Pioloform (in chloroform) coated copper grids. The sections were stained with aqueous uranyl acetate and lead citrate. Grids were analysed using a JEOL 1210V transmission electron microscope.

Assay methods. Thiols were estimated after reaction with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) at 412 nm ($\epsilon_{412} = 14700 \text{ l mol}^{-1} \text{ cm}^{-1}$) (Ellman, 1959). Hydrogen peroxide was measured in a closed O_2 electrode using catalase.

Materials. Toluidine blue and rose bengal (tetraiodotetra-chlorofluorescein) were gifts from Mr Till Böcking and Dr Kevin Barrow.

RESULTS

Survival of organisms in the presence of O_2

When washed suspensions of *G. intestinalis* in PBS (pH 7.1) were stirred under a gas phase of air, O_2 demand decreased until inactivation of the O_2 consumption system was complete after 50 min (Fig. 1a), when dissolved O_2 reached the air-saturated value. Accumulation of H_2O_2 continued for the duration of the experiment (195 min), to reach $4 \mu\text{mol per } 10^6$ organisms. Under 4% O_2 in N_2 , inactivation took 75 min (Fig. 1b), whereas under 2% O_2 in N_2 it took 140 min; under 1% O_2 in N_2 , O_2 uptake was still measurable

Fig. 1. Changes in dissolved O₂ during the inactivation of *G. intestinalis* (10⁶ cells ml⁻¹) by exposure to O₂. Balances between O₂ supply and demand resulted in increased levels as inactivation occurred until dissolved O₂ became equivalent to that in the gas phase. (a) Dissolved O₂ (◇) and H₂O₂ (■) accumulation under air. (b) Dissolved O₂ under 4% O₂ in N₂ (◇), 2% O₂ in N₂ (----) and 1% O₂ in N₂ (—). (c) H₂O₂ accumulation (■) and depletion of cellular thiols (○) under 2% O₂ in N₂ %.

after 4 h. Fig. 1(c) shows the accumulation of H₂O₂ and the decrease in total cellular thiols (conversion from thiol to disulphide forms) when the organisms were stirred under 2% O₂ in N₂. Cessation of O₂ consumption under all conditions of exposure to inhibitory O₂ concentrations preceded loss of membrane potential (see below), flagellar movement and cell motility. These changes were irreversible; restoration of anaerobiosis did not lead to recovery of cellular function.

Structural changes under O₂

Fig. 2(a) shows a bright-field image of a population of washed *G. intestinalis* trophozoites in the presence of 1 μM DiBAC₄(3). The same field examined for fluorescence (excitation 490 nm, emission 519 nm; Fig. 2b) indicates that almost all the organisms exclude the

fluorophore; those that do not are damaged. After exposure of organisms to O₂ almost all the cells still excluded the fluorophore; again those that did not were damaged (not shown). Storage at 4 °C for 18 h (Fig. 2c) gave a population that was completely DiBAC₄(3) permeable. Organisms stirred under 5% air remained impermeable to DiBAC₄(3) for more than 6 h, whereas under 10% air after 4.5 h many organisms became fluorescent. Confocal laser-scanning microscopy (Fig. 2d) indicated that DiBAC₄(3) had permeated the plasma membrane and the flagella. Under 100% air, DiBAC₄(3) penetration was rapid, occurring in less than 1 h. Heat-killed organisms also became completely DiBAC₄(3) permeable (not shown). Exposure to air for 10 min (Fig. 2e, f) or incubation with 60 μM H₂O₂ for 10 min (Fig. 2g) gave organisms in which 2,7-dichlorodihydrofluorescein became oxidized to give fluorescence in the cytosol. The distribution of the fluorescence suggested that externally applied H₂O₂ also gave fluorophore oxidation in peripheral vesicles, which was not the case when organisms were oxidatively stressed in air.

Flow cytometry of organisms exposed to O₂

Measurement of fluorescence of DiBAC₄(3)-treated organisms showed that the population behaved uniformly towards agents that depolarized the plasma membrane potential and that oxidative stress, produced by exposure to air or by externally added H₂O₂, also had this effect. Fig. 3 shows two typical experiments. Live cells did not take up the anionic fluorophore, whereas heat-killed organisms were highly DiBAC₄(3) permeable and hence were counted in channels indicative of high fluorescence intensities. Other treatments known to affect ion transport processes and hence lead to partial collapse of the potential across the plasma membrane resulted in fluorescence emission values intermediate between those of active organisms and the completely depolarized dead cells. Cells aerated for 1 h (Fig. 3a, panel 5) and those treated with 60 μM H₂O₂ for 5 min (Fig. 3b, panel 2) also showed increased permeability to DiBAC₄(3). Plots of forward light scatter (a measure of cell size) vs side scatter showed that as cells became damaged by O₂, they also progressively increased in volume (data not shown). Heat-killed organisms gave lower forward scatter signals than live ones. Pulse-width measurements showed that cell doublets or aggregates were rare for live, damaged or dead organisms.

Calibration of fluorescence intensity (channel no.) against DiBAC₄(3) concentration provides a means of measuring plasma membrane potential (Krasznai *et al.*, 1995; Emri *et al.*, 1998). Heat-killed organisms (with zero membrane potential, used as a control) were taken to have an intracellular fluorescence equal to that of dye in the suspending medium. Fig. 4 shows that live organisms when suspended in 0.31 M mannitol had a plasma membrane potential of -134 ± 3 mV ($n = 6$). Exposure to 5% air for 4.5 h decreased this value to -100 mV, whereas under 100% air, *G. intestinalis* became almost completely depolarized in 4.5 h. Effects

Fig. 2. For legend see facing page.

of some inhibitors are also shown: the most effective (albeit at high concentration, 100 μM), was the proton-pumping ATPase inhibitor DCCD. Gramicidin (1 μM),

an ionophore acting to increase membrane permeability to H^+ , K^+ and Na^+ , or the protonophore CCCP (10 μM) were both highly efficient at diminishing potentials.

Fig. 2. Micrographs of *G. intestinalis* incubated with 1 μ M DiBAC₄(3) (a–d) or with 2',7'-dichlorodihydrofluorescein diacetate (e–g). (a) Bright-field illumination. (b) Fluorescence (same field as in a). (c) Fluorescence after storage at 4 °C for 18 h. (d) Confocal laser-scanning fluorescence image of an organism exposed to air for 4.5 h. (e–g) Organisms incubated for 10 min in air (e, f; ventral and dorsal aspects respectively), or in 60 μ M H₂O₂ (g).

Oxygen consumption measurements

The effects of O₂ exposure could be mimicked by addition of 60 μ M H₂O₂ to washed cell suspensions in PBS: Fig. 5(a) shows its immediate inhibitory effect on O₂ consumption. That $\cdot$ OH is produced when *G. intestinalis* is treated with H₂O₂ is indicated by protective effects of $\cdot$ OH radical quenchers (50 mM sodium benzoate or 50 mM mannitol); ferric nitrilotriacetic acid accentuates H₂O₂ inhibition. Similar results were obtained with a well-washed non-proliferating suspension of *G. intestinalis* in a reactor open for gas flow, under an atmosphere of defined low O₂ value (Fig. 5b).

When the organism was illuminated (400 μ mol m⁻² s⁻¹) during incubation with 2.5 μ M toluidine blue or 10 mM rose bengal under 5% air in N₂ (= 12.5 μ M O₂), almost complete inactivation of the O₂-consuming system was observed within 45 min; after this time organisms were completely immobilized, and for rose bengal, they also became dye-permeable. Similar incubations under anaerobiosis in the light gave no cytotoxic effects.

Electron microscopy

All of the main distinguishing features of *G. intestinalis* can be seen in the controls shown in Fig. 6(a, b), i.e. the ventral disc composed of numerous microtubules, dorsal ribbons and cross-links with its rigid lateral crest, overlying ventrolateral flange and supporting marginal

plates or striated bodies. The two equivalent nuclei lie lateral to the kinetosomal complex consisting of eight kinetosomes in four pairs. In the horizontal cross-section (Fig. 6b) the ventral and posteriolateral axonemes are visible along with the caudal axonemes. The anterior axonemes cannot be seen at this level but the anterior flagella can be seen external to the cell. All axonemes and flagella show the typical 9+2 microtubule arrangement. The vertical section (Fig. 6a) shows how the anterior axonemes course anteriorly in the cell before crossing and moving posteriorly to emerge at the cell's widest point. These axonemes are accompanied by the striated bodies. The microtubules of the median bodies can be seen in their disorganized array. Numerous peripheral vacuoles lie below the plasmalemma. The cytoplasm stains dark owing to the presence of numerous stored granules, e.g. glycogen.

Incubation with 60 μ M H₂O₂ for 30 min brought about rapid morphological change and cell breakdown (Fig. 6c, d). Most of the cells were 'ghostlike', with no dark-staining material in the cytoplasm and condensation within the nucleus. Numerous peripheral vacuoles scattered beneath the plasmalemma on the dorsal surface were visible. These appeared to converge and burst the cell. The ventral disc, axonemes and kinetosomes remained intact, indicating that oxidative stress has no evident impact on microtubule structure.

After 1 h aeration, the bulk of the cells maintained the pyriform structure, but some cells became swollen and

Fig. 3. Flow cytometric analyses of DiBAC₄(3)-treated *G. intestinalis*. (a) 1, Control (live organisms). 2–6, Treated cells: 2, 10 μ M CCCP; 3, 1 μ M gramicidin; 4, 100 μ M DCCD; 5, aeration for 1 h; 6, heat-killed ($> 80^{\circ}\text{C}$ for 5 min). Incubations were with 0.31 M mannitol/1 μ M DiBAC₄(3) for 30 min. (b) 1, Control (live cells). 2–4, Treated cells: 2, 60 μ M H₂O₂ for 5 min; 3, 1 μ M gramicidin; 4, heat-killed ($> 80^{\circ}\text{C}$ for 5 min). Organisms were incubated in 0.31 M mannitol/0.5 μ M DiBAC₄(3) for 30 min. FL1 and FL2 represent the fluorescence emission in channel 2, at 530–540 nm.

misshapen (Fig. 6e). In all cells the ventral disc, its rigid lateral crest and ventrolateral flange were intact; no fragmentation of the disc was visible. The nuclei were no longer spheroid but appeared to have several cytoplasmic projections, and some had associated multilamellar bodies (Fig. 6f). The axonemes were intact and were still located centrally within the cell. The misshapen swollen cells had large cytoplasmic vacuoles and

the dark granule-filled cytoplasm was now very sparse. They appeared 'ghostlike'. In these cells the ventral disc was still intact.

After 2 h aeration the number of swollen and misshapen cells had increased, yet many cells still remained pyriform in structure (Fig. 6g, h, i). Many nuclei had associated multilamellar bodies (Fig. 6h). The ventral discs, lateral crests and ventrolateral flanges were still

Fig. 4. Plasma membrane potentials of *G. intestinalis* as determined by DiBAC₄(3) permeability. Determinations were made over a range (0.25–2.0 µM) of external DiBAC₄(3) concentrations.

Fig. 5. O₂ consumption by *G. intestinalis*; inhibition by added H₂O₂. (a) O₂ depletion in a closed vessel fitted with an O₂ electrode. Total reaction volume 1.1 ml, stirring at 200 r.p.m., 2.2 × 10⁶ organisms ml⁻¹. Numbers on the trace are nmol min⁻¹ per 10⁶ organisms. (b) Steady-state dissolved O₂ in a vessel open for gas flow. Total reaction volume 6 ml, gas phase, 0.5% O₂ in N₂ stirring rate 200 r.p.m., 10⁶ organisms ml⁻¹.

intact. The cytoplasm was less dense and did not stain as darkly as previously. The number of peripheral vacuoles had increased.

After 3 h aeration only a few cells remained in the intact pyriform shape. Many cells had completely broken down and cellular debris abounded. The cytoplasm stained only weakly, with very few dark-staining granules remaining. Most cells were not recognizable as *Giardia* (Fig. 6j). The nuclei had broken down and their chromatin had condensed. The ventral disc and axonemes were still intact, suggesting again that oxidative stress does not affect the microtubule structure within the cell.

DISCUSSION

G. intestinalis has previously been shown to have a limited capability for withstanding exposure to dissolved O₂ (Paget *et al.*, 1989). In those experiments where the O₂ dependence of O₂ uptake were investigated, the concentration of dissolved O₂ was increased from an undetectable level to 5% air saturation in a series of steps of 20 min each. Those data provide indications on apparent K_m values and of a threshold above which O₂ became inhibitory. In the present investigation, we have systematically investigated the time- and concentration-dependence of the O₂ effects during long-term maintenance of steady-state levels. Oxidative damage was shown, not only to the electron-transport systems responsible for O₂ reduction, but to cell functions across a wide range. Thus damage to the plasma membrane, as indicated by the progressive loss of ability to exclude the anionic voltage-sensitive dye DiBAC₄(3) (Lloyd & Hayes, 1995), led to swelling of organisms and loss of the regulatory volume control process, described in fully functional organisms (Park *et al.*, 1995). Sites known to be O₂-inhibited in *G. intestinalis* include a (4Fe-4S) iron-sulphur cluster (Ellis *et al.*, 1993) and redox-active thiols involved in volume regulation (Park *et al.*, 1995). The fact that these effects could also be produced almost immediately by 60 µM H₂O₂ suggests that exposure to inhibitory levels of O₂ results in accumulation of this partial reduction product. The Fenton Haber-Weiss reaction is then free to produce [•]OH from H₂O₂ and [•]O₂⁻, especially as this organism contains no detectable superoxide dismutase (Smith *et al.*, 1988), peroxidase or catalase (Brown *et al.*, 1995). Enhanced loss of functional activity in the presence of ferric nitrotri-acetic acid (Aruoma *et al.*, 1989) and diminished inactivation in the presence of the [•]OH radical scavengers, sodium benzoate or mannitol (Halliwell, 1978) suggests that [•]OH, a radical known for its extremely damaging reactivity (Halliwell & Gutteridge, 1989) may be an important intracellular oxidant in these experiments. The O₂-dependent production of photo-emissive species in *Giardia lamblia* has been demonstrated (T. Paget & D. Lloyd, unpublished data). These species, observed as low-level chemiluminescence in organisms metabolizing glucose, could be greatly enhanced in the presence of the redox-cycling naphthoquinone menadione. The fact that the oxygen-consuming redox chains of organisms can be damaged by ¹O₂-generating systems, the photodynamically acti-

Fig. 6. For legend see facing page.

Fig. 6. Electron micrographs of *G. intestinalis* Portland-1. (a) Vertical section through the ventral surface of a control trophozoite. (b) Horizontal section at the level of the nuclei through a control trophozoite. (c) Vertical section through the ventral surface of a trophozoite incubated with 60 μ M H₂O₂ for 30 min. (d) Horizontal section through a trophozoite incubated with H₂O₂ for 30 min. (e, f) Sections through trophozoites that have been aerated for 1 h. (g, h, i) Sections through trophozoites that have been aerated for 2 h. (j) Section through a trophozoite that has been aerated for 3 h. N, nuclei; P, peripheral vesicles; S, striated bodies; D, ventral disc; K, kinetosomal complex; F, funis, M, median body microtubules; A, axonemes; C, lateral crest; V, ventrolateral flange; AF, anterior flagella; MLB, multilamellar body; Cy, cytoplasm.

vated dyes toluidine blue and rose bengal (Krinsky, 1979), supports the suggestion (Lamberts & Neckers, 1985) that the menadione sensitivity of *G. lamblia* can also be accounted for by free-radical damage by reactive oxygen species. Thus the anti-giardial effects of a redox-cycling naphthoquinone are produced by an amplification of the effects of O₂ damage. The anti-giardial effects both of menadione, and of H₂O₂ reported here, warrant further investigation as a means of control of this important human pathogen.

ACKNOWLEDGEMENTS

We are grateful to Dr Kevin Barrow and Mr Till Bocking for advice on singlet oxygen generation. The work was in part supported by a travel grant from the Royal Society to D.L.

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Received 7 June 2000; revised 7 August 2000; accepted 21 August 2000.