Oxygen induction of a novel fatty acid $n-6$ desaturase in the soil protozoon, *Acanthamoeba castellanii*

Andrew J. RUTTER*, Katie L. THOMAS*, Derek HERBERT*, R. James HENDERSON†, David LLOYD* and John L. HARWOOD*[1]

*School of Biosciences, Cardiff University, Cardiff CF10 3US, Wales, U.K., and †NERC Unit of Aquatic Biochemistry, University of Stirling, Stirling FK9 4LA, Scotland, U.K.

Induction of fatty acid desaturation is very important for the temperature adaptation of poikilotherms. However, in oxygen-limited late-exponential-phase *Acanthamoeba castellanii* cultures, oxygen alone was able to induce increased activity of a fatty acid desaturase that converts oleate into linoleate and which has been implicated in the temperature adaptation of this organism. Experiments with Δ^{10}-nonadecenoate showed that the enzyme is an $n-6$ desaturase rather than a Δ^{12}-desaturase. It also used preferentially 1-acyl-2-oleoyl-phosphatidylcholine as substrate and NAD(P)H as electron donor. The involvement of cytochrome b_5 as an intermediate electron carrier was shown by difference spectra measurements and anti-(cytochrome b_5) antibody experiments. Of the three protein components of the desaturase complex, oxygen only increased the activity of the terminal (cyanide-sensitive) protein during $n-6$ desaturase induction. The induction of this terminal protein paralleled well the increase in overall oleate $n-6$ desaturation. The ability of oxygen to induce oleate desaturase independently of temperature in this lower eukaryotic animal model is of novel intrinsic interest, as well as being important for the design of future experiments to determine the molecular mechanism of temperature adaptation in poikilotherms.

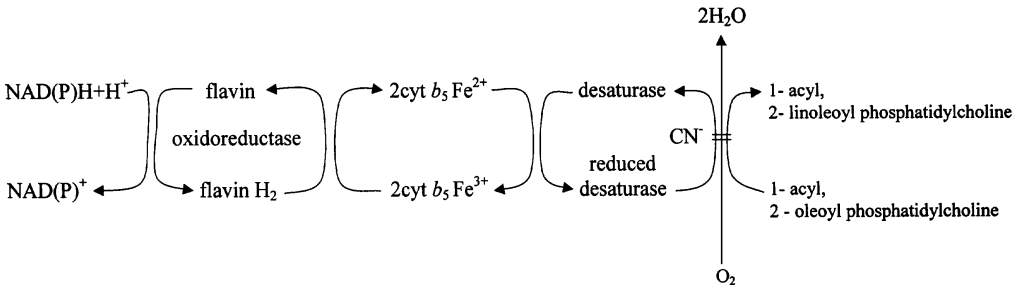
Key words: *Acanthamoeba castellanii*, $n-6$ fatty acid desaturation, oleate desaturation, oxygen induction, soil protozoa.

INTRODUCTION

Perhaps the most common adaptive mechanism for ensuring functional membrane dynamic order in poikilotherms that experience a decrease in their environmental temperature is an increase in fatty acid unsaturation [1]. To achieve this rise in unsaturation, fatty acid desaturase activity is increased, and the molecular mechanism for this has been studied in several cases. Activity can be enhanced by activation of pre-existing enzyme [2], by increased gene transcription [3–6] and, possibly, by enhanced availability of substrates [7].

We have been studying fatty acid desaturase activity in *Acanthamoeba castellanii*, which is a small, free-living amoeba found widely in soils and fresh water. In its natural environment *A. castellanii* is subject to marked spatial and temporal variations in temperature. The organism must adapt rapidly to such changes in its environment, particularly so that its plasma membrane order is homoeodynamically maintained and its functions, such as phagocytosis [8], can continue. We have found that the most rapid change in the membrane lipid composition of *A. castellanii* in response to a shift to lower growth temperatures was an increase in linoleate accompanied by a commensurate fall in oleate [9]. Linoleate proportions in microsomal membrane fractions isolated from this amoeba double within the first 1 h after a shift in growth temperature from 30 °C to 15 °C [10]. The ability of *A. castellanii* to desaturate oleate to linoleate is interesting, because animals are regarded generally as being unable to synthesize linoleate *de novo*. However, another protozoon, *Tetrahymena pyriformis*, is also able to produce linoleate (by Δ^{12} desaturation of oleoyl-CoA), although this process does not appear to be important for the temperature adaptation of that organism [11].

We studied the desaturation of oleate to linoleate further in *A. castellanii* using microsomal fractions. We could detect increased



Scheme 1 Diagrammatic representation of the $n-6$ desaturase system in *Acanthamoeba castellanii*

The system utilizes NADH or NADPH as the electron donor, and a cytochrome b_5 protein as the intermediate electron carrier. The terminal 'desaturase' protein, which inserts the $n-6$ double bond into 1-acyl-2-oleoyl-phosphatidylcholine, is often referred to as the 'cyanide-sensitive protein' in desaturase systems because of its inhibition by this reagent. The mechanism of H^+ transfer from reduced flavin to the desaturase protein is unknown.

Abbreviations used: U.I., unsaturation index (average number of double bonds per fatty acid).

[1] To whom correspondence should be addressed (e-mail Harwood@Cardiff.ac.uk).

oleate desaturation in microsomes from cells subjected to as little as 10 min of chilling from 30 °C to 15 °C [12]. The increase in activity was found to be mainly due to fresh protein synthesis, and took place with 1-acyl-2-oleoyl-phosphatidylcholine as the major substrate [12]. Moreover, induction of Δ^{12} ($n-6$) desaturation was shown to be an essential part of the adaptation of *Acanthamoeba* to lower growth temperatures when we observed a sequence of changes: desaturase induction, increased membrane unsaturation, restoration of membrane 'fluidity' and, finally, renewed phagocytotic activity [13,14]. The question of whether the desaturase counted from the methyl or carboxy end of the acyl chain (i.e. is it a Δ^{12} or a $n-6$ desaturase?) was, however, not resolved. Both types of desaturase are found in different organisms.

Although Δ^{12} ($n-6$) desaturase induction has been shown clearly to be stimulated by shifts to low temperatures [12], *A. castellanii* also displays marked changes in desaturase activity and membrane lipid composition during growth in batch culture under isothermal conditions, at 30 °C [15]. The temperature-independence of these changes suggested that temperature is not the sole trigger for desaturase induction. One alternative possible determinant is oxygen, which acts as the terminal electron acceptor for aerobic desaturations [16]. Moreover, oxygen had been suggested to influence fatty acid desaturase activity significantly in nitrogen-purged plant tissue preparations [17]. Therefore the influence of dissolved oxygen on the synthesis and activity of the $n-6$ desaturase of *A. castellanii* was investigated, and it was shown clearly, for the first time, that oxygen availability (independently of temperature) can regulate *de novo* synthesis of the desaturase [18,19]. It was also noted that oxygen availability can limit the activity of pre-existing Δ^{12} ($n-6$) desaturase in *A. castellanii* [18].

Because of the importance of the above observations for temperature studies of organisms in a low oxygen environment (such as protozoa like *Acanthamoeba castellanii* with a high oxygen demand), we undertook a study of the detailed characteristics of oxygen-induced oleate desaturase activity. The results of these experiments are now reported, and show that the desaturase is an $n-6$ type which shares characteristics in its properties with the low-temperature-induced desaturase activity. We also show that it is only the terminal (cyanide-sensitive) protein of the desaturase complex (see Scheme 1) whose formation is induced by oxygen.

MATERIALS AND METHODS

Materials

Acanthamoeba castellanii (Neff strain) was originally obtained from Dr K. M. G. Adam (Department of Zoology, University of Edinburgh, Scotland, U.K.). It was grown in PGY medium containing (%, w/v): Difco proteose peptone, 0.75; Oxoid yeast extract, 0.75; glucose, 1.5. Cultures were grown either in 200 ml of medium (in 500 ml Erlenmeyer flasks) or in 800 ml of medium (in a 1 litre fermenter; LH Fermentation). Flask cultures were incubated at 30 °C with reciprocal shaking at 160 strokes/min. The fermenter vessel was temperature-controlled (30 °C), stirred (165 rev./min) and the gas flow was constant (140 ml/min). It was also equipped with a sampling port and a Teflon-membrane-covered oxygen electrode. Fermenter cultures were sparged with air throughout growth (0–48 h).

Cell numbers were routinely determined using a Fuchs–Rosenthal haemocytometer slide, after appropriate dilution with distilled water: more than 400 cells were counted in each sample. Membrane dry weights were determined using tared foil cups, dried to a constant weight at approx. 100 °C. Cells for lipid analysis were harvested by centrifugation for 1 min at 750 ***g***.

Viability of cultures

The viability of cultures was measured by flow cytometry [20]. Samples (1 ml) were taken from cultures, oxonol was added at a concentration of 2 μg/ml and incubations were carried out for 5 min. Oxonol (bis-1,3-dibutylbarbituric acid trimethine oxonol) is an anionic fluorescent dye that is only taken up by damaged *A. castellanii* cells with depolarized plasma membranes, a condition showing loss of viability [21,22]. Control samples consisted of live (non-sparged) and dead (fixed in 30 % ethanol) cells. Samples were analysed with a Skatron Argus flow cytometer (Skatron, Tranby, Norway) [23]. Photomultiplier voltages were 200 and 440 V, and gain settings were 1.41 and 5.65, for forward angle light scatter and fluorescence respectively. Signals were collected using an FITC filter block. Thresholds were set for forward light angle scatter channels so that only data for cell-associated fluorescence were presented. Typical histograms contained data from approx. 5000 cells.

Radiolabel incorporation studies

For acetate labelling studies, culture samples (12 ml) were transferred to universal bottles and placed in a water bath. Agitation was achieved by sparging suspensions with defined air/nitrogen mixtures. The oxygen concentration of the gas phase was varied by mixing air or oxygen and nitrogen (containing < 3 p.p.m. of oxygen) from separate cylinders. The accuracy of mixing was confirmed by measuring the oxygen concentration of gas-saturated distilled water, using an oxygen electrode (see below) set to zero after equilibration with N_2-saturated medium. O_2 concentrations were calculated using published solubility data [24]. After a 10 min equilibration, approx. 110 Bq of [1-^{14}C]acetate (1.85–2.2 GBq/mmol; Nycomed Amersham plc, Amersham, Bucks., U.K.) was added directly to the suspensions. After the appropriate period of incubation, incorporation was stopped by the addition of organic solvents used for lipid extraction (see below).

$n-6$ (Δ^{12}) fatty acid desaturase assay

A. castellanii microsomal membranes were harvested from whole-cell homogenates by differential centrifugation as previously described [10]. Analysis of membrane purity revealed that > 80 % of total choline phosphotransferase (used as a marker enzyme for the endoplasmic reticulum) activity was recovered in the microsomal fractions. Furthermore, the specific activity of this enzyme (per mg of protein) was enriched on average 6.4-fold compared with the homogenate, emphasizing the enrichment of endoplasmic reticulum in the microsomal fraction. Microsomal $n-6$ (Δ^{12}) desaturase activity was determined by measurement of the conversion of oleate into linoleate in isolated microsomal membranes. [1-^{14}C]Oleoyl-CoA (1.85–2.2 GBq/nmol; Nycomed Amersham plc) was included as a radiolabelled precursor (this is rapidly incorporated into 1-acyl-2-oleoyl-phosphatidylcholine, the desaturase substrate), as described previously [12]. It should be noted that, because of dilution by endogenous substrate, actual desaturation rates are some 10–12 times the rates calculated with exogenously added radiolabelled substrate [12]. After the incubation period, lipid extraction and analysis was carried out as described below. When inhibitors were used, they were added to the total incubation system (minus NADH) and preincubations were carried out for 10 min before starting the reaction by the addition of NADH.

Inhibition of protein synthesis

Inhibition of protein synthesis by anisomycin (0.1 mg/ml) was measured by [^{14}C]leucine incorporation as described previously [12].

Lipid analyses

Lipids were extracted using the method of Bligh and Dyer [25] as modified by Griffiths and Harwood [26]. Total lipids were separated by TLC on silica gel G plates (E. Merck, Darmstadt, Germany) using a solvent of chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Lipid bands were routinely revealed by spraying with 0.05% (w/v) 1-anilino-4-naphthosulphonic acid in methanol and viewing under UV light. Routine identification was by comparison with markers, but full identification of major bands had been carried out by chemical degradation and differential colour staining [27].

For fatty acid analysis, methyl esters were generated by acid-catalysed methanolysis [2.5% (v/v) H_2SO_4 in methanol] at 70 °C for 2 h. After extraction with light petroleum (b.p. 60–80 °C), aliquots of methyl esters were analysed by (radio) GLC. Pentadecanoate was used as an internal standard. Separations were usually carried out using 10% SP-2330 on 100/120 Supelcoport (Supelco UK, Poole, Dorset, U.K.) packed into a glass column (1.5 m × 4 mm internal diameter). Fatty acids were identified by comparison with authentic standards, but had been fully identified previously [9]. Radiolabelled acids were quantified with a gas flow proportional counter after catalytic conversion into $^{14}CO_2$, and using integrating Rachel software (LabLogic, Sheffield, U.K.). Masses were calculated in relation to the internal standard using the method of Carroll [28] or with the integrating software.

Induction of $n-6$ desaturation

For the time-course experiment of desaturase induction, *A. castellanii* cells were cultured at 30 °C under standard conditions [15] until the late exponential state (48 h). They were then subjected to sparging for 20 min at increasing partial pressures (20, 30, 40, 45, 50, 55 and 60 kPa) of oxygen. Samples were removed at the end of each sparging period, and total lipids extracted and analysed as above.

Assay of component proteins of the fatty acid desaturase

Assay of NADH:cytochrome b_5 oxidoreductase activity was based on the methods for microsomal electron transport chain activities in yeast, *T. pyriformis* and *A. castellanii* [29–31]. Cytochrome b_5 levels were measured using a dual-wavelength spectrophotometer (Hitachi–Perkin Elmer; model 557). Oxidation was achieved with ammonium persulphate and reduction with sodium dithionite, and the difference between absorbances in the range 558–575 nm (molar difference absorbance coefficient $\Delta\epsilon_{558-575} = 21\ mM^{-1} \cdot cm^{-1}$) was measured [32]. The activity of the terminal (cyanide-sensitive) desaturase protein was quantified by measuring the re-oxidation of cytochrome b_5 following addition of oleoyl-CoA (see [11]). The re-oxidation occurred with no detectable lag phase, showing that transfer of oleate to the main desaturase substrate, phosphatidylcholine (see Results), was not rate-limiting.

Desaturation of nonadecenoate

cis-Δ^{10}-Nonadecenoic acid and *cis*-$\Delta^{10,13}$-nonadecadienoic acid were synthesized from oleic acid and linoleic acid respectively, as described elsewhere [33]. *A. castellanii* were grown at 30 °C in the presence of 50 μg of oleate or *cis*-Δ^{10}-nonadecenoate (ammonium salts). A control flask with no added fatty acids was also used. The growth and morphology (assessed by transmission electron microscopy) were not affected by these additions. After 40 h, 10 ml of cells from each flask was incubated with 111 kBq of [1-^{14}C]acetate at 30 °C for 2 h with constant shaking. Cells were pelleted and lipids were extracted and analysed as described above.

Microsomal incubations were carried out under normal conditions (as above) and with oleate or nonadecenoate present (45 μM final concn) complexed to fatty acid-free BSA (0.4 mg/ml). Lipids were extracted and fatty acid methyl esters were separated by silver nitrate silica gel G TLC [34] using hexane/diethyl ether (9:1, v/v) as solvent. Following detection using aqueous rhodamine 6G (0.01%, w/v) spray, the bands were scraped, silver ions were removed [34] and the fatty acid methyl esters were analysed by GLC.

For examination of the products of nonadecenoate desaturation, the total lipids extracted from cells grown in the presence of this fatty acid were separated into component lipid classes by HPTLC using methyl acetate/propan-2-ol/chloroform/methanol/0.25% (w/v) KC1 (25:25:25:10:9, by vol.) as the developing solvent. After visualization by spraying with 0.1% (w/v) 2,7-dichlorofluorescein and viewing under UV light, the band of adsorbent containing phosphatidylcholine was scraped from the glass plate and subjected to acid-catalysed *trans*-esterification. A portion of the resulting fatty acid methyl esters was analysed by GC using a capillary column (50 m × 0.32 mm internal diam.) coated with CP Wax 52 CB (Chrompack). Hydrogen was used as the carrier gas and the oven temperature was programmed to rise from 50 °C to 225 °C during the course of each analysis. The remainder of the fatty acid methyl ester sample was separated by silver nitrate TLC as above [34]. The band of adsorbent containing the dienoic components was removed from the plate and eluted with hexane/diethyl ether (1:1, v/v). The organic solvent was washed with 2% NaCl to remove silver ions and the solvent was evaporated to recover the fatty acid methyl esters, which were analysed as above by capillary gas chromatography. The identity of nonadecadienoic acid in chromatograms was confirmed by reference to the retention time of authentic, synthesized *cis*-$\Delta^{10,13}$-nonadecadienoic acid. To establish the position of the double bonds within the nonadecadienoic acid formed by the desaturation of nonadecanoate, the fatty acid methyl esters of the phosphatidylcholine were converted into the diethylamide derivatives of the fatty acids [35] and subjected to GC/MS. This was carried out using a Carlo Erba 8000 gas chromatograph coupled to a MD 800 mass spectrometer (Fisons Instruments, Crawley, U.K.) operating in the electron ionization mode at 70 eV. The gas chromatograph was equipped with a fused silica capillary column (15 m × 0.25 mm internal diam.) coated with DB 5MS (J&W Scientific, Folsom, CA, U.S.A.), and helium was used as carrier gas. Samples were applied by on-column injection with the oven temperature programmed to rise from 50 to 225 °C.

Analysis of electron transport components

Electron transport components likely to be associated with the fatty acid desaturase were analysed by reduced−oxidized difference spectra of sedimented microsomal preparations resuspended in 2 vol. of 0.8 M mannitol, at liquid N_2 temperatures [36], using a Unicam SP1800 spectrophotometer. Cuvettes with a path length of 2 mm, constructed of lucite, were held in a specially constructed Dewar vessel held as closely as possible to the photomultiplier tube. The band width was 1 nm

and the scanning speed was 1 nm/s. Both sample and reference suspensions were oxidized in the cuvettes by mixing with air immediately before freezing to obtain a baseline before adding oxidant and reductant.

RESULTS

Oxygen levels correlate with increased unsaturation

We showed previously that oxygen was able to increase the activity of an oleate desaturase [18], at least in part by causing biosynthesis of new enzyme [19]. A key observation that led to these experiments was that low temperatures could only induce increased desaturase synthesis (and activity) at certain stages of cell growth – in particular towards the end of the exponential phase, when respiration was so vigorous that oxygen levels in the medium became depleted [18].

The oxygen concentration of a culture growing in the fermenter had decreased after 27 h of growth to a level below the lower limit of detection of the oxygen electrode (approx. 0.1 μM; results not shown). After 48 h, at a cell concentration of 2.2×10^6 cells per ml, the partial pressure of the O_2 supply was increased stepwise from 20 to 60 kPa (i.e. 20 % to 60 % O_2 in N_2) with sparging for 20 min at each increment. Dissolved O_2 measured < 0.1 μM at 20 or 30 kPa in the mobile gas phase; however, as the O_2 supply was increased from 40 to 45 kPa, dissolved O_2 in the culture rose from 3.5 to 7.0 μM O_2 (Figure 1). Increasing the partial pressure in the mobile phase further led to dissolved O_2 concentrations of 74, 180 and 330 μM for 50, 55 and 60 kPa O_2 supplied respectively (Figure 1).

The unsaturation index (U.I.), or average number of double bonds per fatty acid, was determined in samples removed at each O_2 concentration. The U.I. value increased from 1.64 when O_2 was below the lower limit of detection to 2.03 at > 300 μM dissolved O_2 (Figure 1). (Without oxygen sparging, the U.I. value did not change; results not shown.) It was particularly noticeable that the first statistically significant change in unsaturation occurred at 40 kPa, when the dissolved O_2 levels were detectable for the first time. The lipid analysis shown in Figure 1 is for whole cells, but we know from previous studies on temperature adaptation in *A. castellanii* [10] that fatty acid changes in, for example, the plasma membrane are observed within a few minutes. Furthermore, experiments on the induction of oleate desaturase activity showed that this was increased maximally by only a 10 min exposure to increased oxygen levels for oxygen-limited (late exponential) cultures [19].

When the details of fatty acid changes are examined (Table 1), it can be seen that, at oxygen concentrations below detectable levels (20 or 30 kPa O_2 supplied), oleate was the major unsaturated fatty acid, with approx. 10 % linoleate. *cis*-ω9,12-Eicosadienoate and arachidonate were both present at higher levels than linoleate, at approx. 14 % and 15 % respectively. As soon as the dissolved oxygen reached detectable levels (3.5 μM at 40 kPa applied O_2) then the level of linoleate started to increase significantly. For 50–60 kPa applied oxygen, linoleate concentrations had risen to approx. 30 %, with a commensurate decline in the percentage of oleate (Table 1). There were also some smaller changes in the C_{20} fatty acids, which were consistent with an increase in $n-6$ fatty acid desaturation. Thus some $n-9$,12-eicosadienoate appeared to be converted into $n-6$,9,12-eicosatrienoate, and the level of arachidonate was increased, presumably as a result of the extra availability of its metabolic precursor (dihomo-γ-linolenic acid) [37]. These changes, which were induced by oxygen alone, mimicked very closely those induced by low temperature [9,10], and could be explained fully by induction of $n-6$ fatty acid desaturase activity.

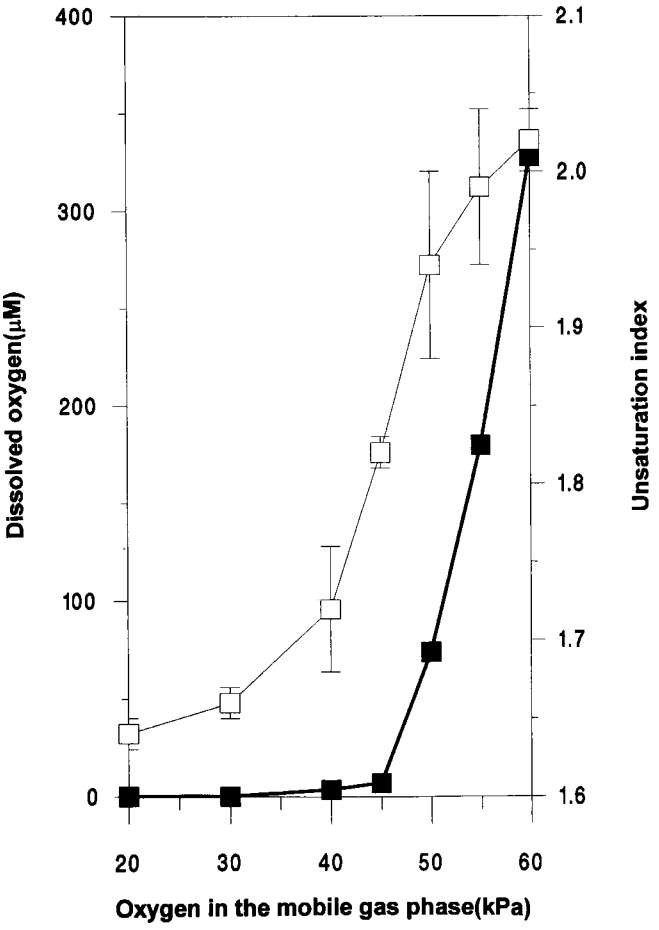


Figure 1 Dissolved oxygen levels influence the amount of fatty acid unsaturation in late-exponential cultures of *Acanthamoeba castellanii*

Dissolved oxygen levels (■) were measured with an oxygen electrode, and the U.I. (□) is the average number of double bonds per fatty acid in the total acyl lipids. Means ± S.D. are shown ($n = 4$). For oxygen measurements, the S.D.s were less than the size of the symbols.

The results shown in Figure 1 and Table 1 were from experiments where vigorous sparging was necessary in order to increase the dissolved O_2 in rapidly respiring cultures. *A. castellanii* lacks a cell wall and is a somewhat fragile organism. Thus it was necessary to determine if vigorous sparging had any detrimental effects on the integrity of the plasma membrane, and hence cell viability. To this end, the cellular uptake of oxonol, a fluorescent viability stain, was measured in flasks bubbled for 1 h, and compared with that of control (non-sparged) and dead (fixed in 30 % ethanol) cells. Oxonol is only taken up by *A. castellanii* cells with depolarized plasma membranes, i.e. non-viable cells [20–22]. The results are presented as dual-parameter histograms, with forward angle light scatter (an indicator of cell size) against oxonol fluorescence: the results in Figure 2 show clear differences in cell-associated fluorescence between dead (Figure 2A) and live (control) (Figure 2B) cells. Cells from either air-sparged (Figure 2C) or N_2-sparged (Figure 2D) flasks showed no uptake of oxonol, indicating no decrease in viability over 1 h. These results also show the advantage of using flow cytometry to provide a rapid and accurate method for measuring the distribution of viable organisms in a population [14]. Oxonol has been shown to be a good indicator of plasma membrane integrity in protozoa [21], as has been demonstrated previously in bacteria [22].

Table 1 Oxygen induces an increase specifically in $n-6$ unsaturation in oxygen-limited *Acanthamoeba castellanii* cultures

A. castellanii were in the late exponential state (30 °C, 48 h [15]) and were subjected to sparging for 20 min at each partial pressure. Samples were removed, and total lipids were extracted and analysed. Values are means ± S.D. of percentage fatty acids of four independent determinations. $C_{18:1}$ is oleate, $C_{18:2}$ is linoleate, $C_{20:2}$ is *cis*-$\Delta^{8,11}$-eicosadienoate, $C_{20:3}$ is $\Delta^{8,11,14}$-eicosatrienoate and $C_{20:4}$ is arachidonate. The U.I. is the average number of double bonds per fatty acid. n.d., none detected; tr, trace ($< 0.05\%$).

Partial pressure of O_2 (kPa)	Dissolved O_2 (μM)	Fatty acid (% of total)										
		$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:2}$	$C_{20:3}$	$C_{20:4}$	U.I.
20	n.d.	10.6 ± 0.4	4.1 ± 0.1	1.9 ± 0.2	2.3 ± tr	35.9 ± 1.9	9.7 ± 0.2	1.5 ± 0.4	13.8 ± 0.2	4.9 ± 0.6	15.0 ± 0.2	1.6 ± tr
30	n.d.	10.6 ± 0.5	4.0 ± 0.2	1.8 ± 0.1	2.1 ± 0.3	36.2 ± 0.1	9.7 ± 0.5	1.9 ± tr	13.6 ± 0.2	5.1 ± 0.2	15.0 ± 0.3	1.7 ± tr
40	3.5	8.6 ± 1.6	3.9 ± 0.2	2.6 ± 0.3	1.4 ± 0.4	35.2 ± 1.7	13.4 ± tr	1.5 ± 0.4	12.5 ± tr	5.6 ± tr	15.3 ± 0.8	1.7 ± tr
45	7.0	8.6 ± 1.2	3.1 ± 0.7	2.8 ± 0.2	1.3 ± 0.1	27.3 ± 0.8	22.7 ± 1.5	1.3 ± 0.1	11.8 ± 0.5	5.9 ± 0.4	15.2 ± 1.5	1.8 ± tr
50	73.9	7.7 ± 0.3	3.5 ± 1.3	3.5 ± 0.3	1.4 ± 0.6	20.6 ± 0.2	27.7 ± 0.8	1.3 ± 0.3	10.3 ± 0.2	6.7 ± 0.4	17.3 ± 0.5	1.9 ± 0.1
55	179.6	7.2 ± 0.9	2.7 ± 0.1	3.6 ± 0.2	2.0 ± 0.8	17.5 ± 0.2	30.9 ± 1.0	1.3 ± tr	9.9 ± 0.1	7.1 ± 0.8	17.7 ± 0.2	2.0 ± 0.1
60	327.6	7.1 ± tr	2.9 ± 0.1	3.8 ± 0.1	2.3 ± 0.4	15.2 ± 0.2	31.5 ± 0.6	1.5 ± 0.1	9.7 ± 0.2	7.3 ± 0.5	18.7 ± 0.2	2.0 ± tr

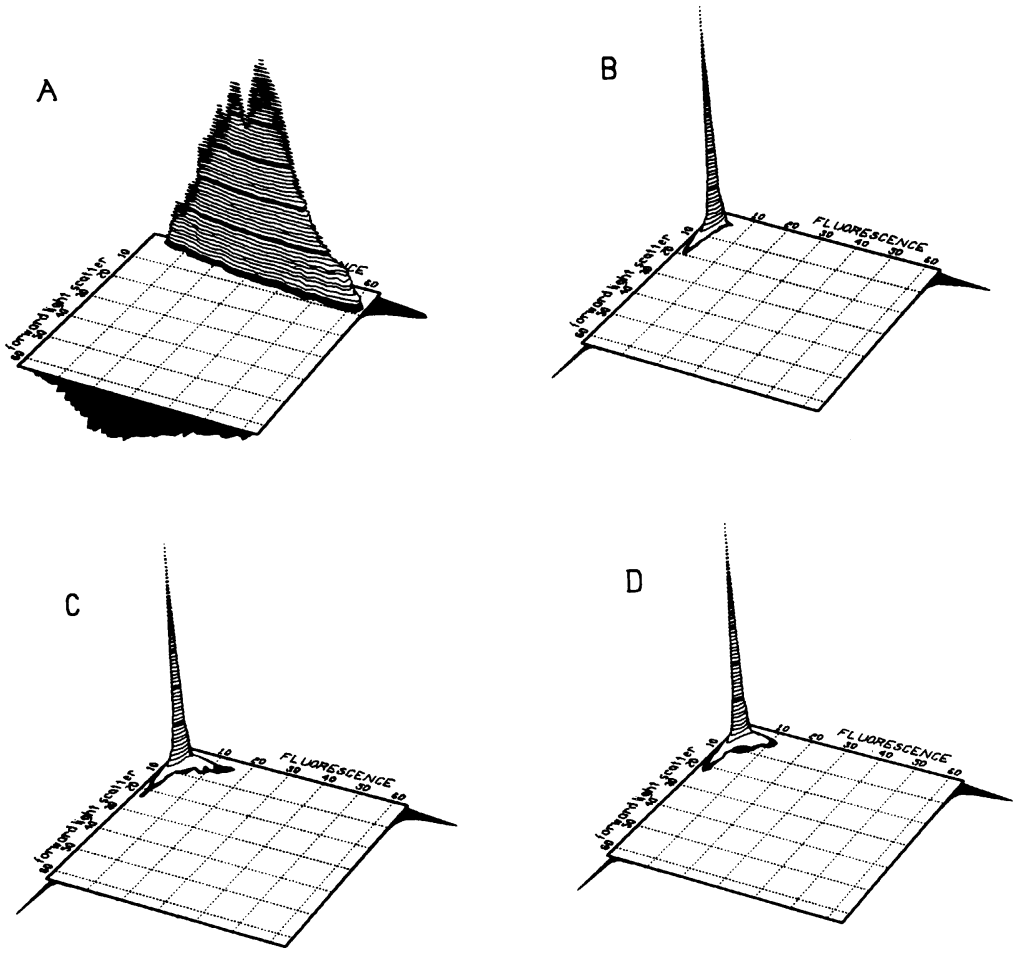


Figure 2 Effect of sparging on cell viability

Dual-parameter histograms show forward angle light scatter (an indication of cell size) against fluorescence. The uptake in late exponential *A. castellanii* cultures of oxonol, a fluorescent viability stain, was compared in (**A**) dead (ethanol-fixed) cells; (**B**) live (non-sparged) cells; (**C**) cells sparged with air for 1 h; and (**D**) cells sparged with N_2 for 1 h. Each histogram contains data from approx. 5000 cells.

Is the desaturase an $n-6$ or a Δ^{12} enzyme?

For the conversion of oleate into linoleate, a desaturase specific for the $n-6$ or the Δ^{12} position is needed. For the oxygen-induced enzyme, we found in experiments similar to those carried out previously for the low-temperature-induced desaturase ([12]; results not shown) that the major substrate was 1-acyl-2-oleoyl-phosphatidylcholine. Therefore one might predict that the

Table 2 Changes in the fatty acid composition of *A. castellanii* cells grown in the presence of exogenous fatty acids

A. castellanii was grown at 30 °C in the presence of 50 μg of oleate ($C_{18:1}$) or nonadecenoate ($C_{19:1}$). After 40 h of growth, lipids were extracted and analysed (see the Materials and methods section). Results are given as the percentage of total fatty acids, and are means ± S.D. ($n = 2$). tr, trace (< 0.05%); n.d., none detected.

	Content (%)		
Fatty acid	Control	+ $C_{18:1}$	+ $C_{19:1}$
$C_{14:0}$	6.7 ± 0.4	6.5 ± 0.4	4.2 ± 0.6
$C_{16:0}$	7.2 ± 0.4	6.9 ± 1.0	9.9 ± 1.0
$C_{16:1}$	0.9 ± 0.4	0.8 ± 0.2	0.5 ± 0.4
$C_{18:0}$	5.0 ± 1.0	4.7 ± 1.4	5.5 ± 1.0
$C_{18:1}$	21.2 ± 1.4	26.4 ± 1.8	21.0 ± 2.0
$C_{18:2}$	18.3 ± 0.6	17.1 ± 1.2	17.7 ± 1.2
$C_{18:3}$	0.5 ± 0.2	0.4 ± tr	0.7 ± tr
$C_{19:1}$	n.d.	n.d.	10.4 ± 1.0
$C_{20:2}$	15.7 ± 1.4	14.8 ± 3.2	11.2 ± 2.4
$C_{20:3}$	7.2 ± 1.4	6.8 ± 1.6	6.0 ± 1.0
$C_{20:4}$	17.3 ± 2.6	15.7 ± 3.0	13.0 ± 2.0

Table 3 Growth in the presence of exogenous nonadecenoate alters the quality, but not the quantity, of fatty acids labelled from [1-^{14}C]acetate by *A. castellanii*

Incubations were with 10 ml of cells and 111 kBq of [1-^{14}C]acetate at 30 °C for 2 h (see the Materials and methods section). Values are means ± S.D. ($n = 3$).

	Content (% of total recovered label)		
Fatty acid labelled	Control	+ $C_{18:1}$	+ $C_{19:1}$
$C_{14:0}$	9.9 ± 1.8	12.2 ± 4.4	31.0 ± 4.0
$C_{16:0}$	18.6 ± 3.2	19.2 ± 4.4	17.9 ± 1.8
$C_{18:0}$	15.8 ± 3.6	19.8 ± 1.6	14.9 ± 3.8
$C_{18:1}$	31.7 ± 3.4	28.2 ± 1.4	22.7 ± 2.7
$C_{18:2}$	23.9 ± 4.2	20.5 ± 0.8	13.5 ± 3.4
10^{-3} × Total label (d.p.m.)	20.0 ± 1.8	18.5 ± 1.7	22.2 ± 2.0

enzyme would insert the second double bond by numbering from the methyl end (i.e. $n-6$) because it was using an intact lipid substrate as opposed to, for example, the oleoyl-CoA substrate which is used by insect Δ^{12}-fatty acid desaturases [38]. Nevertheless, desaturases forming polyunsaturated fatty acids on complex lipid substrates, such as those in the cyanobacterium *Synechocystis* PCC 6803 [39], have been found to number from both the methyl and the carboxy ends of the chain.

In order to resolve whether the oleate desaturase induced by oxygen is an $n-6$ or a Δ^{12} enzyme, we used an odd-chain substrate, *cis*-Δ^{10}-nonadecenoate. *A. castellanii* was grown at 30 °C in the presence of exogenous fatty acid, with exogenous oleate as a control. There was no difference in the growth rate of the amoebae in the presence of either exogenous fatty acid compared with control cells (results not shown), but the overall fatty acid composition was changed by the uptake and incorporation of oleate or nonadecenoate into lipids (Table 2). The presence of exogenous oleic acid led to an increase in the proportion of this fatty acid in the cellular lipid, but did not affect greatly the relative amounts of the other constituent fatty acids. Lipid extracted from cells grown in the presence of nonadecenoate contained 10.4% of this odd-chain fatty acid and had substantially lower levels of $C_{20:2}$, $C_{20:3}$ and $C_{20:4}$ than lipid from control cells or those grown with oleic acid. (This may reflect competition between the incorporated nonadecenoic acid with oleic acid for conversion into C_{20} fatty acids.) These results showed that nonadecenoate, as a potential $n-6$ desaturase substrate, was incorporated successfully into total *A. castellanii* lipids. However, in the total lipid fraction, any desaturation of nonadecenoate to nonadecadienoate was too small to detect (Table 2).

Because nonadecenoate had been rapidly taken up by *A. castellanii*, we tested whether such incorporation changed the pattern of radioactive labelling from [1-^{14}C]acetate. The latter precursor has been shown to be useful for labelling studies in *A. castellanii*; moreover, the labelling of oleate and linoleate from [^{14}C]acetate has been shown to be altered by low temperature manipulation [9] as oleate desaturase activity was induced. When incubations were carried out for 2 h, control cells incorporated label into fatty acid synthase products (myristate, palmitate, stearate) and the unsaturated fatty acids oleate and linoleate rapidly (Table 3). This pattern was similar to that obtained previously [9]. When exogenous oleate was included, there was a slight decrease in the labelling of linoleate (as expected because of dilution of specific radioactivity of the oleate substrate) and a small increase in that of fatty acid synthase products such as stearate (perhaps by feedback inhibition).

Nonadecenoate also decreased linoleate labelling (Table 3), as would be expected if it competed with oleate for a binding site on the desaturase. However, instead of causing feedback inhibition and an increase in radioactive stearate, radioactivity in myristate was mainly affected. This unexpected result makes a straightforward interpretation of the increased labelling of myristate problematic.

In view of the difficulties in detecting nonadecadienoate in total lipid samples, isolation of the main desaturase substrate, phosphatidylcholine, was undertaken in order to check for evidence of desaturation of nonadecenoate to nonadecadienoate. When the fatty acid pattern of phosphatidylcholine from *A. castellanii* grown in the presence of nonadecenoate was examined, a small amount of nonadecadienoate was detected (0.6%), unlike in that of the total lipid extracts (Table 2), where its presence was diluted by all the other acyl lipids present. Methyl esters of fatty acids from the phosphatidylcholine fraction were prepared and separated by capillary GLC. MS analysis of the eluted peaks confirmed the double band position in the substrate nonadecenoate as Δ^{10} ($n-9$), with fragments of mass 212 and 224 (results not shown). Nonadecadienoate accounted for 2% of the fatty acids in the dienoic fraction of phosphatidylcholine. When this dienoic fraction was subjected to MS analysis, the nonadecadienoate gave the fragmentation pattern presented in Figure 3. According to the criteria set out for the interpretation of the mass spectra of fatty acid diethylamides [35], the gap of 12 mass units between 212 and 224 indicates a double bond between carbons 10 and 11, while the similar gap between 252 and 264 denotes a double bond between carbons 13 and 14. In short, the fragment with 12 mass units corresponds to the carbon of the double bond which is nearest to the C-terminus. The spectrum was identical with that obtained with the diethylamide derivative of synthesized $\Delta^{10,13}$-nonadecadienoic acid [33]. The mass fragmentation patterns confirm the desaturation product of Δ^{10}-nonadecanoic acid in *A. castellanii* to be $\Delta^{10,13}$ ($n-6,9$)-nonadecadienoic acid. This showed that the second desaturation had taken place at the $n-6$ position. Also in agreement with our assignment of the enzyme as an $n-6$ desaturase is the increased conversion of $n-9,12$-eicosadienoate into $n-6,9,12$-eicosatrienoate by oxygen (Table 1) or low temperature treatment [10].

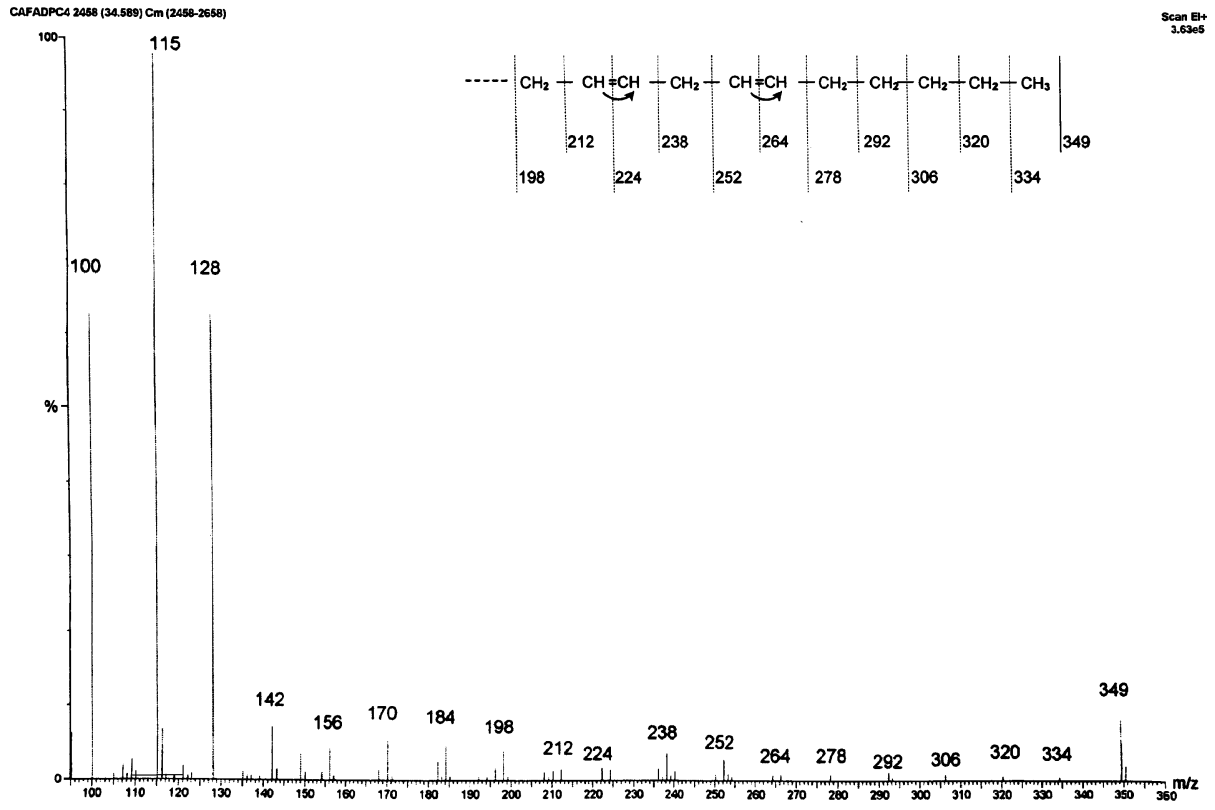


Figure 3 Mass spectrum of nonadecadienoate from phosphatidylcholine in *A. castellanii* indicates the presence of an $n-6$ fatty acid desaturase

The incubation conditions for *A. castellanii* and the methods for lipid extraction, preparation and analysis of samples are given in the Materials and methods section. The spectrum shows the fragmentation pattern for the nonadecadienoate peak, with double bond postions at *m/z* values at 212/224 and 252/264 which correspond to $n-9$ (Δ^{10}) and $n-6$ (Δ^{13}) respectively.

Characteristics of the oxygen-induced $n-6$ desaturase

We evaluated the properties of the oxygen-induced $n-6$ fatty acid desaturase and compared them, where the information was available, with those of the low-temperature-induced enzyme [12]. As mentioned above, we showed that the main substrate for desaturation was 1-acyl-2-oleoyl-phosphatidylcholine, as before [12]. Briefly, incubation of microsomes with [^{14}C]oleoyl-CoA in the absence of NADH resulted in a rapid transfer of [^{14}C]oleate on to complex lipids (mainly the *sn*-2 position of phosphatidylcholine) at rates indistinguishable from those found in the presence of NADH. If NADH was then added to allow desaturation to take place in the former incubations, [^{14}C]linoleate was formed rapidly at the *sn*-2 position of phosphatidylcholine (results not shown). Moreover, as in the experiments for low-temperature-induced desaturation [12], linoleate formation was linear over 60 min, whereas oleoyl-CoA was undetectable after 30 min. Thus the $n-6$ fatty acid desaturase induced by oxygen appears to use a complex lipid (phosphatidylcholine) substrate.

The desaturase showed an apparent K_m for NADH of approx. 50 μM, and could also use NADPH, although with less efficiency. A K_m for O_2 of approx. 15 μM was found, which was similar to that obtained for microsomes from low-temperature-exposed *A. castellanii* [18]. The enzyme was slightly inhibited by SH-reagents such as *N*-ethylmaleimide (2 mM; 81% of control) or *p*-chloromercuribenzoate (2 mM; 42% of control), but not significantly by iodoacetate (2 mM). Iron chelators such as 8-hydroxyquinoline (1 mM; 62% of control) or 1,10-phenanthroline (1 mM; 69% of control) also lowered its activity (results not shown).

Oleate desaturase activity from another protozoon is known to require an electron-transport chain involving cytochrome b_5 as well as an NADH oxidoreductase [2]. We investigated the possible involvement of cytochrome b_5 in the $n-6$ fatty acid desaturase activity in two ways. First, we looked at difference spectra for microsomes incubated with the usual assay requirements minus NADH or after its addition. Reduction of a component with a peak at 558 nm, which was assigned to cytochrome b_5 [40], was seen (Figure 4). In addition, we had an antibody (raised to cauliflower cytochrome b_5) available to us. This antibody was shown by Western blotting to cross-react with a single band of approx. 13 kDa in solubilized microsomal membranes (results not shown) and was assumed, therefore, to form a complex with *Acanthamoeba* cytochrome b_5. The antibody was included in assays for $n-6$ fatty acid desaturase, and inhibited desaturation by up to 52% (Table 4). These data agree with our assumption [12] that the desaturase uses cytochrome b_5 as an intermediate electron carrier.

Which desaturase component is increased by oxygen?

By analogy with other animal desaturase systems and from our own data (above), there are most probably three components in the overall $n-6$ oleate desaturase complex, i.e. NADH:cytochrome b_5 oxidoreductase, cytochrome b_5 and the terminal (cyanide-sensitive) desaturase protein. We showed previously [19] that induction of desaturase activity by oxygen required protein synthesis. Therefore one can ask the question: which of the three components is increased? To do this we carried out two types of experiment.

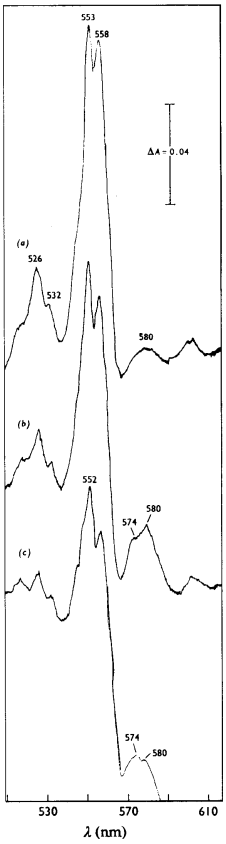


Figure 4 Difference spectra recorded at 77 K of microsomal membranes from *A. castellanii*

A microsomal suspension (18.5 mg/ml protein) in 0.8 M mannitol was reduced using (**a**) excess $Na_2S_2O_4$, (**b**) NADPH or (**c**) NADH. The contents of the reference cuvettes were oxidized with $K_3Fe(CN)_6$. The absorption maximum at 558 nm corresponds to that of cytochrome b_5.

Table 4 Inhibition of $n-6$ fatty acid desaturase activity by an anti-(cytochrome b_5) antibody

The antibody was raised to purified cauliflower cytochrome b_5 and was shown to cross-react with a single protein of approx. 13 kDa in solubilized microsomal membranes from *Acanthamoeba castellanii*. The antibody was preincubated in the complete assay system minus NADH for 10 min before the reaction was started by the addition of NADH. IgG preparations were used and purified as described in [57]. Results show duplicate values from experiments with three separate microsomal preparations. Approx. 2 μg of microsomal protein was used in each assay. n.m., not measured.

	$n-6$ desaturase activity (nmol/h per mg of protein)		
Conditions	Expt 1	Expt 2	Expt 3
Control (no addition)	3.7, 3.9	6.0, 6.4	5.4, 5.4
+ Anti-(cytochrome b_5)			
0.1 μg	n.m.	3.4, 3.6	3.9, 3.9
0.3 μg	n.m.	2.6, 3.0	3.4, 4.0
1.0 μg	2.4, 2.8	2.8, 3.2	n.m.
+1 μg of pre-immune serum	4.0, 4.1	6.4, 6.6	5.5, 5.7

First, we induced desaturase activity by sparging late-exponential phase cells for 1 h with air or nitrogen, and then isolated a microsomal fraction. This procedure has already been shown [19] to induce significant desaturase activity following air sparging. For the experiments shown (Table 5), $n-6$ desaturase activity more than doubled on sparging with air, while nitrogen sparging decreased this activity, as observed previously [19]. In the three separate preparations, the activity of NADH: cytochrome b_5 oxidoreductase, the level of cytochrome b_5 and the rate of re-oxidation of cytochrome b_5 (by oleoyl-CoA; a measure of the terminal desaturase) were measured. There were no significant changes in the first two components, but the rate of re-oxidation of cytochrome b_5 changed approximately in proportion with the changes in desaturation activity measured with [^{14}C]oleoyl-CoA. Similarly, nitrogen sparging decreased the re-oxidation of cytochrome b_5 and overall desaturase activity by a similar amount, but did not alter the other components significantly. These data indicate that it was the synthesis of the terminal desaturase protein that was induced by oxygen, and that it is turnover of this component that controls overall desaturase activity.

In a second experiment, we monitored the levels of the three components in microsomal membranes isolated from cells grown to late exponential stage and then sparged with different levels of oxygen. The results (Table 6) showed, again, that the increased rates of desaturation induced by oxygen were only associated with increases in the terminal desaturase protein, as evaluated by the re-oxidation of cytochrome b_5 [4,32].

DISCUSSION

Although oxygen is a required substrate (terminal acceptor) for fatty acid desaturation in animals, its ability to increase desaturase activity [19] was unexpected. *In vivo*, in the experiment shown in Figure 1, the increase in fatty acid unsaturation seen once the applied O_2 was 40 kPa or above corresponded to dissolved oxygen levels of 7.5 μM or more. In agreement with these results, measurements of the $n-6$ desaturase *in vitro* have previously shown that dissolved oxygen levels of 7 μM or above were sufficient to induce desaturase activity [19]. In view of the apparent K_m for the oleate desaturase that we determined (approx. 15 μM), this may mean that the rate of the reaction catalysed by any desaturase present was also limited by substrate supply.

The desaturase induced by oxygen was clearly an oleate ($n-6$ or Δ^{12}) desaturase, as shown by the changes in fatty acid composition (Table 1), which were virtually identical to the alterations induced by low temperatures [10]. The small changes observed in the proportions of C_{20} fatty acids with increasing oxygen concentration are consistent with the oxygen-induced desaturase in *A. castellanii* being specific for the $n-6$ position rather than the Δ^{12} position. Thus the decrease in the proportion of $\Delta^{8,11}$-$C_{20:2}$ and the increase in that of $\Delta^{8,11,14}$-$C_{20:3}$ can be attributed to the operation of an $n-6$ desaturase which inserts a double bond at the Δ14 or $n-6$ carbon of the dienoic fatty acid. The subsequent action of a Δ^5 desaturase would then give rise to arachidonate ($\Delta^{5,8,11,14}$-$C_{20:4}$).

Direct evidence for the oxygen-induced desaturase being an $n-6$ position desaturase came from the experiments on the desaturation of Δ^{10}-nonadecenoic acid by *Acanthamoeba* (Figure 3). With oleic acid as substrate, desaturation by an $n-6$ or a Δ^{12} desaturase both yield the same product, $\Delta^{9,12}$-octadecadienoic acid (linoleic acid), and the two desaturases cannot be distinguished by analysis of the product. The presence of an extra carbon in nonadecenoate means that the actions of an $n-6$ desaturase and a Δ^{12} desaturase on this substrate can be separated. The double bonds in the nonadecadienoate formed from nonadecenoate were located in the Δ^{10} ($n-9$) and Δ^{13} ($n-6$) positions, with no evidence for a double bond at the Δ^{12} position. The

Table 5 Oxygen induction of $n-6$ fatty desaturase activity only increases the activity of the terminal cyanide-sensitive protein

A. castellanii cultures from late exponential phase (48 h) were sparged for 1 h prior to cells being harvested and microsomes prepared. The viability of cells following sparging was confirmed by flow cytometry (see the Materials and methods section). Data are means ± S.D. ($n = 3$). As discussed in [12], $n-6$ desaturation is an underestimate because of the dilution of radiolabelled oleate with endogenous substrate. Abbreviations: cyt. b_5, cytochrome b_5; tr, trace.

Treatment	NADH:cyt. b_5 oxidoreductase (nmol/min per mg of protein)	Cyt. b_5 (pmol/mg of protein)	Re-oxidation of cyt. b_5 (nmol/h per mg of protein)	$n-6$ desaturation (nmol/h per mg of protein)
Nitrogen sparge	1.02 ± tr	90 ± 7	9.0 ± 0.5	2.4 ± 0.3
None	1.02 ± tr	77 ± 7	38.4 ± 2.4	8.4 ± 0.4
Air sparge	1.02 ± tr	95 ± tr	77.0 ± 4.0	18.8 ± 0.4

Table 6 Influence of dissolved oxygen concentration on $n-6$ desaturase activity and its component proteins in *A. castellanii* cultures

A. castellanii were grown to late exponential stage (48 h) at 30 °C. Cells were then sparged with different concentrations of oxygen for 1 h at 30 °C, harvested and microsomal membranes isolated. Assays were carried out as described in the Materials and methods section. Data are means ± S.D. ($n = 3$). Abbreviation: cyt. b_5, cytochrome b_5.

Dissolved O_2 (μM)	NADH:cyt. b_5 oxidoreductase (nmol/min per mg of protein)	Cyt. b_5 (pmol/mg of protein)	Re-oxidation of cyt. b_5 (nmol/h per mg of protein)	Desaturation ($C_{18:2}$ formed) (nmol/h per mg of protein)
0	0.90 ± 0.5	100 ± 3	12.6 ± 0.6	7.1 ± 0.6
23	0.90 ± 0.5	106 ± 3	18.0 ± 0.4	9.5 ± 1.5
118	1.02 ± 0.8	100 ± 2	37.8 ± 2.1	19.1 ± 1.4

finding that the oxygen-induced desaturase of *A. castellanii* inserts a double bond at the $n-6$, rather that the Δ^{12}, position of a fatty acid is in keeping with the fact that the major substrate for this enzyme is a complex lipid, 1-acyl-2-oleoyl-phosphatidylcholine. Thus, for physical/chemical reasons, the desaturase may align itself with respect to the methyl terminal of the oleoyl moiety, rather than with the carboxy end, which is esterified to the glycerol backbone of the phosphatidylcholine.

Therefore we conclude from our experiments that the oleate desaturase in *Acanthamoeba* functions as an $\omega-6$ (or $n-6$) desaturase (rather than a Δ^{12} enzyme). The technique that we used to obtain evidence on this point is similar to experiments carried out by Cahoon et al. [41] in elucidating the pathway for petoselinic acid formation in *Coriandrum sativum*. However, these experiments do not exclude the possibility that the desaturase inserts the new double bond three carbons from the existing double bond and towards the methyl end, i.e. it measures from the existing double bond rather than accurately from the methyl carbon. Nevertheless, what is clear is that the *Acanthamoeba* oleate desaturase is not a Δ^{12}-desaturase.

The oxygen-induced $n-6$ oleate desaturase used 1-acyl-2-oleoyl-phosphatidylcholine as its main substrate, in agreement with the conclusion for the low-temperature-induced desaturase. Other characteristics, such as K_m values for substrates, were also similar, so it is possible that temperature and oxygen act independently (see [18]) by increasing transcription of the same gene. It is also of note that, whereas the insect oleate desaturase is thought to use oleoyl-CoA as substrate [38], that from another protozoon, *Tetrahymena pyriformis*, was proposed to use complex lipids [42].

The involvement of cytochrome b_5 in the reaction of the $n-6$ oleate desaturase agrees with this cytochrome acting as an electron carrier for many fatty acid desaturases [37,43]. However, because only a single low-molecular-mass (13 kDa) *Acanthamoeba castellanii* protein reacted with the anti-(cytochrome b_5) antibody, and because cytochrome b_5 levels were unaffected by oxygen induction (when desaturation was increased), then we have no evidence for a fused cytochrome b_5–desaturase protein, as reported for a number of systems recently [44,45]. Indeed, such fused desaturases appear to insert double bonds into fatty acids towards their C-termini, and have been termed front-end desaturases (see [45,46]). Based on the characteristics discussed above, the *Acanthamoeba castellanii* $n-6$ fatty acid desaturase is envisaged to operate in the complex depicted in Scheme 1.

When $n-6$ oleate desaturase activity was induced by oxygen, only the activity of the terminal desaturase protein (see Scheme 1) appeared to increase (Tables 5 and 6). This agrees with previous conclusions for the temperature-induced fatty acid desaturase activity [11,12], and also with the fact that the activity of the terminal desaturase protein was low (Tables 5 and 6). In contrast, in *Tetrahymena pyriformis* [47], electron transport also appeared to change, although the main effect of low temperature was still thought to be through the cyanide-sensitive desaturase protein [11]. Inhibition of the oxygen induction of the $n-6$ desaturase by anisomycin [19] does not distinguish between increased gene transcription and mRNA stability effects. Thus the gene for the terminal desaturase protein needs to be identified in *Acanthamoeba* in order to clarify the situation. Unfortunately, our attempts to isolate an *Acanthamoeba* $n-6$ desaturase gene, using probes to other oleate desaturases, have not proven successful so far. For the Δ^9-desaturase of *Tetrahymena thermophila*, effects on gene expression and mRNA stability have both been implied [4].

The ability of oxygen alone to induce $n-6$ oleate desaturase activity is an important observation, especially in view of increased oxygen solubility at low temperatures. These facts must be borne in mind for future experiments on low temperature adaptation. However, oxygen may only be of importance in organisms, such as *Acanthamoeba castellanii*, which respire at high rates, or for organisms living in low-oxygen environments. Certainly, for one classic model of temperature adaptation – the cyanobacterium *Synechocystis* PCC 6803 [48,49] – which evolves oxygen during photosynthesis, this gas is present in excess. Oxygen has been implicated at the level of transcription in the induction of genes in yeast [50] and in *Escherichia coli* [51]. The data for yeast include the observation that *OLEI* expression (for the Δ^9-desaturase) is controlled by cellular oxygen levels [52] via a low-oxygen response element [53]. Moreover, limitation of

membrane functions in *A. castellanii* by low environmental O_2 concentrations [54] has also been shown to elicit respiratory adaptation [55] and the morphogenetic differentiation necessary for encystation [56]. The ability of oxygen to specifically induce a fatty acid desaturase protein is another important fact to add to these previous observations with *Acanthamoeba*.

We thank Dr Mark Smith (then at the School of Biology, University of Bristol, U.K.) for the gift of an anti-(cytochrome b_5) antibody. The financial support of the NERC (GR 3/8456) and the BBSRC (H 96294) is gratefully acknowledged.

REFERENCES

1 Cossins, A. R. (ed.) (1994) The Temperature Adaptation of Biological Membranes, Portland Press, Colchester
2 Kates, M., Pugh, E. L. and Ferrante, G. (1984) Biomembranes **12**, 379–395
3 Cossins, A. R. and Raynar, R. S. (1987) Adaptive responses of animal cell membranes to temperature. In Temperature and Animal Cells (Bowler, K. and Fuller, B. J., eds.), pp. 95–111, Company of Biologists, Cambridge
4 Nakashima, S., Zhao, Y. and Nozawa, Y. (1996) Molecular cloning of delta 9 fatty acid desaturase for the protozoan *Tetrahymena thermophila* and its mRNA expression during thermal membrane adaptation. Biochem. J. **317**, 29–34
5 Wada, H., Gombos, Z. and Murata, N. (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature (London) **347**, 200–203
6 Ishizakini-Shizawa, D., Fujii, T., Azuma, M., Sekiguchi, K. and Murata, N. (1996) Low-temperature resistance of higher plants is significantly enhanced by a non-specific cyanobacterial desaturase. Nat. Biotechnol. **14**, 1003–1006
7 Harwood, J. L., Jones, A. L., Perry, H. J., Rutter, A. J., Smith, K. L. and Williams, M. (1994) Changes in membrane lipids during temperature adaptation. In Temperature Adaptation of Biological Membranes (Cossins, A. R., ed.), pp. 107–118, Portland Press, London
8 Avery, S. V., Lloyd, D. and Harwood, J. L. (1995) Temperature-dependent changes in plasma-membrane lipid order and the phagocytotic activity of the amoeba *Acanthamoeba castellanii* are closely correlated. Biochem. J. **312**, 811–816
9 Jones, A. L., Pruitt, N. L., Lloyd, D. and Harwood, J. L. (1991) Temperature-induced changes in the synthesis of unsaturated fatty acids by *Acanthamoeba castellanii*. J. Protozool. **38**, 532–536
10 Jones, A. L., Hann, A. C., Harwood, J. L. and Lloyd, D. (1993) Temperature-induced membrane-lipid adaptation in *Acanthamoeba castellanii*. Biochem. J. **290**, 273–278
11 Thompson, Jr, G. A. and Nozawa, Y. (1984) The regulation of membrane fluidity in *Tetrahymena*. In Membrane Fluidity (Kates, M. and Manson, L. A., eds.), pp. 397–432, Plenum Press, New York
12 Jones, A. L., Lloyd, D. and Harwood, J. L. (1993) Rapid induction of microsomal Δ^{12} (ω6)-desaturase activity in chilled *Acanthamoeba castellanii*. Biochem. J. **296**, 183–188
13 Avery, S. V., Harwood, J. L. and Lloyd, D. (1994) Low-temperature-induced alterations in fatty acid metabolism of *Acanthamoeba castellanii* cultures of different ages: relationship to changes in cell division, oxygen uptake and phagocytotic activity. Microbiology **140**, 2425–2431
14 Avery, S. V., Harwood, J. L. and Lloyd, D. (1995) Quantification and characterization of phagocytosis in the soil amoeba *Acanthamoeba castellanii* by flow cytometry. Appl. Environ. Microbiol. **61**, 1124–1132
15 Avery, S. V., Lloyd, D. and Harwood, J. L. (1994) Changes in membrane fatty acid composition and Δ12-desaturase activity during growth of *Acanthamoeba castellanii* in batch culture. J. Eukaryotic Microbiol. **41**, 396–401
16 Hazel, J. R. and Williams, E. E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog. Lipid Res. **29**, 167–227
17 Harris, P. and James, A. T. (1969) Effect of low temperature on fatty acid biosynthesis in seeds. Biochim. Biophys. Acta **187**, 13–18
18 Avery, S. V., Rutter, A. J., Harwood, J. L. and Lloyd, D. (1996) Oxygen-dependent low-temperature Δ12 (n6)-desaturase induction and alteration of fatty acid composition in *Acanthamoeba castellanii*. Microbiology **142**, 2213–2221
19 Thomas, K., Rutter, A. J., Suller, M., Harwood, J. L. and Lloyd, D. (1998) Oxygen induces fatty acid (n-6) desaturation independently of temperature in *Acanthamoeba castellanii*. FEBS Lett. **425**, 171–174
20 Mason, J., Allman, R., Stark, J. M. and Lloyd, D. (1994) Rapid estimation of bacterial antibiotic susceptibility with flow cytometry. J. Microsc. **176**, 8–16
21 Khunkitti, W., Avery, S. V., Lloyd, D., Furr, J. R. and Russell, A. D. (1997) Effects of biocides on *Acanthamoeba castellanii* as measured by flow cytometry and plaque assay. J. Antimicrob. Chemother. **40**, 227–233
22 Lloyd, D. and Hayes, A. J. (1995) Vigour, vitality and viability of micro-organisms. FEMS Microbiol. Lett. **133**, 1–7
23 Boye, E. and Steen, H. B. (1993) The physical and biological basis for flow cytometry of *Escherichia coli*. In Flow Cytometry in Microbiology (Lloyd, D., ed.), pp. 11–26, Springer-Verlag, Berlin
24 Wilhelm, E., Battino, R. and Wilcock, R. J. (1977) Low-pressure solubility of gases in liquid water. Chem. Rev. **77**, 219–262
25 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917
26 Griffiths, G. and Harwood, J. L. (1991) The regulation of triacylglycerol biosynthesis in cocoa (*Theobroma cacao* L.). Planta **184**, 279–284
27 Kates, M. (1972) Techniques of Lipidology, Elsevier, Amsterdam
28 Carroll, N. K. (1961) Separation of lipid classes by chromatography on florisil. J. Lipid Res. **2**, 135–141
29 Cartledge, T. G. and Lloyd, D. (1972) Subcellular fractionation by zonal centrifugation of anaerobically-grown glucose repressed *Saccharomyces carlsbergensis*. Biochem. J. **127**, 693–703
30 Poole, R. K., Nicholl, W. G., Howells, L. and Lloyd, D. (1971) The "microsomal fraction" from *Tetrahymena pyriformis*, strain ST; characterisation and subfractionation. J. Gen. Microbiol. **68**, 283–294
31 Edwards, S. W., Chagla, A. H., Griffiths, A. J. and Lloyd, D. (1977) The cytochromes of *Acanthamoeba castellanii*. Biochem. J. **168**, 113–121
32 Smith, M. A., Cross, A. R., Jones, O. T. G., Griffiths, T. W., Stymne, S. and Stobart, A. K. (1990) Electron-transport components of the 1-acyl-2-oleoyl-glycerol-3-phosphocholine Δ^{12}-desaturase in microsomal preparations from developing safflower. Biochem. J. **272**, 23–29
33 Rodriguez, C., Henderson, R. J., Porter, A. E. A. and Dick, J. R. (1997) Modification of odd-chain length unsaturated fatty acids by hepatocytes of rainbow trout (*Onchorhyncus mykiss*) fed diet containing fish oil or olive oil. Lipids **32**, 611–619
34 Christie, W. W. (1982) Lipid Analysis, 2nd edn, Pergamon Press, Oxford
35 Nilsson, R. and Liljenberg, C. (1991) The determination of double-bond positions in polyunsaturated fatty acids – *g*as-chromatography mass-spectrometry of the diethylamide derivative. Phytochem. Anal. **2**, 253–259
36 Chance, B. (1957) Techniques for the assay of the respiratory enzymes. Methods Enzymol. **4**, 273–329
37 Cook, H. W. (1996) Fatty acid desaturation and elongation in eukaryotes. In Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D. E. and Vance, J., eds.), pp. 129–152, Elsevier, Amsterdam
38 Cripps, E., Borgeson, C., Blomqvist, G. J. and de Renobales, M. (1990) The delta 12-desaturase from the house cricket, *Acheta domesticus* (Orthoptera, Gryllidae): characterisation and form of the substrate. Arch. Biochem. Biophys. **278**, 46–51
39 Murata, N. (1994) Genetic and temperature-induced modulation of cyanobacterial membrane lipids. In Temperature Adaptation of Biological Membranes (Cossins, A. R., ed.), pp. 155–162, Portland Press, London
40 Estabrook, R. W. and Cohen, B. (1969) Cytochrome b_5. In Microsomal Electron Transport in Microsomes and Drug Oxidations (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fous, J. R. and Mannering, G. J., eds.), pp. 95–109, Academic Press, New York
41 Cahoon, E. B., Dormann, P. and Ohlrogge, J. G. (1994) Petroselenic acid biosynthesis and production in transgenic plants. Prog. Lipid Res. **33**, 155–163
42 Kameyama, Y., Yoshioka, S. and Nozawa, Y. (1980) The occurrence of direct desaturation of phospholipid acyl chain in *Tetrahymena pyriformis*. Thermal adaptation of membrane phospholipid. Biochim. Biophys. Acta **618**, 214–222
43 Murphy, D. J. and Piffanelli, P. (1998) Fatty acid desaturases: structure, mechanism and regulation. In Plant Lipid Biosynthesis: Fundamentals and Agricultural Applications (Harwood, J. L., ed.), pp. 95–130, Cambridge University Press, Cambridge
44 Mitchell, A. G. and Martin, C. E. (1997) Fah1p, a *Saccharomyces cerevisiae* cytochrome b_5 fusion protein, and its *Arabiopsis thaliana* homolog that lacks the cytochrome b_5 domain both function in the α-hydroxylation of sphingolipid-associated very long chain fatty acids. J. Biol. Chem. **272**, 28281–28288
45 Sperling, P. and Heinz, E. (2001) Desaturases fused to their electron donor. Eur. J. Lipid Sci. Technol. **103**, 158–180
46 Napier, J. A., Sayanova, O., Sperling, P. and Heinz, E. (1999) A growing family of cytochrone b_5 fusion desaturases. Trends Plant Sci. **4**, 2–5
47 Fukushima, H., Umeki, S., Watanabe, T. and Nozawa, Y. (1982) Purification and partial characterization of cytochrome b_5 from *Tetrahymena pyriformis*. Biochem. Biophys. Res. Commun. **105**, 502–508
48 Murata, N. and Wada, H. (1995) Acyl lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. Biochem. J. **308**, 1–8
49 Vigh, L., Los, D. A., Horvath, I. and Murata, N. (1993) The primary signal in the biological perception of temperature – Pd-catalysed hydrogenation of membrane lipids stimulated the expression of the *DesA* gene in *Synechocystis* PCC 6803. Proc. Natl. Acad. Sci. U.S.A. **90**, 9090–9094
50 Guarente, L. and Mason, T. (1983) Heme regulates transcription of the CYC1 gene of *S. cerevisiae* via an upstream activation site. Cell **32**, 1279–1285

51 Zheng, M., Aslund, F. and Storz, G. (1998) Activation of the Oxy R transcription factor by reversible disulphide bond formation. Science **279**, 1718–1721
52 Kwast, K. E., Burke, P. V. and Poyton, R. O. (1998) Oxygen sensing and the transcriptional regulation of oxygen-responsive genes in yeast J. Exp. Biol. **201**, 1177–1195
53 Vasconcelles, M. J., Jiang, Y., McDaid, K., Gilooly, L., Wretzel, S., Porter, D. L., Martin, C. E. and Goldberg, M. A. (2001) Identification and characterization of a low oxygen response element involved in the hypoxic induction of a family of *Saccharomyces cerevisiae* genes. Implications for the conservation of oxygen sensing in eukaryotes J. Biol. Chem. **276**, 14371–14384
54 Lloyd, D., Mellor, H. and Williams, J. L. (1983) Oxygen affinity of the respiratory chain of *Acanthamoeba castellanii*. Biochem. J. **214**, 47–51
55 Lloyd, D., Protheroe, R., Williams, T. N. and Williams, J. L. (1983) Adaptation of the respiratory system of *Acanthamoeba castellanii* to anaerobiosis. FEMS Microbiol. Lett. **17**, 143–146
56 Turner, N., Biagini, G. A. and Lloyd, D. (1997) Anaerobiosis-induced differentiation of *Acanthamoeba castellanii*. FEMS Microbiol. Lett. **157**, 149–153
57 Smith, M. A., Jonsson, L., Stymne, S. and Stobart, A. K. (1992) Evidence for cytochrome b_5 as an electron donor in ricinoleic acid biosynthesis in microsomal preparations from developing castor bean. Biochem. J. **278**, 141–144

Received 31 January 2002/23 July 2002; accepted 2 August 2002
Published as BJ Immediate Publication 2 August 2002, DOI 10.1042/BJ20020189