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Viability and Formation of Conjugated Dienes in Plasma Membrane Lipids of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Rhodotorula glutinis* and *Candida albicans* Exposed to Hydrophilic, Amphiphilic and Hydrophobic Pro-oxidants

A. KRASOWSKA[a], L. CHMIELEWSKA[a], D. GAPA[a], A. PRESCHA[b], L. VÁCHOVÁ[c], K. SIGLER[c]

[a]*Institute of Microbiology, Wrocław University, 51 148 Wrocław, Poland*

e-mail aniak@microb.uni.wroc.pl

[b]*Department of Food Science and Nutrition, Medical University of Wrocław, 50 140 Wrocław, Poland*

[c]*Institute of Microbiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czechia*

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ABSTRACT. Effects of four lipid peroxidation-inducing pro-oxidants – amphiphilic *tert*-butyl hydroperoxide (TBHP), hydrophobic 1,1′-azobis(4-cyclohexanecarbonitrile) (ACHN), hydrophilic Fe^{II} and 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) – on cell growth and on generation of peroxidation products in isolated plasma membrane lipids were determined in four yeast species (*S. cerevisiae*, *S. pombe*, *R. glutinis* and *C. albicans*) differing in their plasma membrane lipid composition. TBHP and ACHN inhibited cell growth most strongly, Fe^{II} and AAPH exerted inhibitory action for about 2 h, with subsequent cell growth resumption. *S. cerevisiae* strain SP4 was doped during growth with unsaturated linoleic (18:2) and linolenic (18:3) acids to change its resistance to lipid peroxidation. Its plasma membranes then contained some 30 % of these acids as compared with some 1.3 % of 18:2 acid found in undoped *S. cerevisiae*, while the content of (16:1) and (18:1) acids was lower than in undoped *S. cerevisiae*. The presence of linoleic and linolenic acids in *S. cerevisiae* cells lowered cell survival and increased the sensitivity to pro-oxidants. Peroxidation-generated conjugated dienes (CD) were measured in pure TBHP- and ACHN-exposed fatty acids used as standards. The CD level depended on the extent of unsaturation and the pro-oxidant used. The TBHP-induced CD production in a mixture of oleic acid and its ester was somewhat lower than in free acid and ester alone. In lipids isolated from the yeast plasma membranes, the CD production was time-dependent and decreased after a 5–15-min pro-oxidant exposure. ACHN was less active than TBHP. The most oxidizable were lipids from *S. cerevisiae* plasma membranes doped with linoleic and linolenic acids and from *C. albicans* with indigenous linolenic acid.

Abbreviations

AAPH	2,2′-azobis(2-amidinopropane)dihydrochloride	PUFA	oligounsaturated fatty acids
ACHN	1,1′-azobis(4-cyclohexanecarbonitrile)	TBHP	*tert*-butyl hydroperoxide
CD	conjugated dienes	TBRS	thiobarbituric acid-reactive substances
FAME	fatty acid methyl esters	YEPD	yeast extract–peptone–dextrose

A characteristic feature of different microorganisms is the species-specific fatty acid composition of the plasma membrane (McElhaney 1976). The kind, quality and arrangement of fatty acids play an important role in determining the physico-chemical properties of the plasma membrane which, in addition to forming a relatively impermeable barrier for hydrophilic molecules, takes an active part in amino acid or sugar transport and ion translocation (Bisson *et al.* 1993; Serrano 1991).

Saturation of fatty acids is important in the regulation of plasma membrane fluidity (Subczyński and Wiśnicka 2000) and in determining its barrier function in the transport of small hydrophobic molecules (*e.g.*, free radicals) within the cells (Brown and London 1998). In membranes containing a high proportion of saturated fatty acids their ordering effect is very strong and fluidity is low.

Membrane functions may be damaged or disrupted by unfavorable changes in external conditions such as temperature (Suutari *et al.* 1990) or pH (Singh *et al.* 1990). An important factor influencing plasma membrane properties and function is free radicals (Howlett and Avery 1997; Sigler *et al.* 1999).

The presence of polar carotenoids (lutein, zeaxanthin) in the plasma membrane of certain microorganisms can increase membrane order, decrease its fluidity and pose a barrier for polar molecules, ions and small nonpolar molecules (Wiśniewska and Subczyński 1998; Subczyński *et al.* 1991). Nonpolar carotenoids, such as β-carotene, do not affect the physical properties of membranes (Wiśniewska and Subczyński 1998; Gruszecki 1999). On the other hand, β-carotene as a powerful antioxidant protects lipids from peroxidation (Tsuchihashi *et al.* 1995).

The commonly accepted mechanism of lipid peroxidation is as follows: Under a pro-oxidant attack a hydrogen atom is abstracted from a methylene group in PUFA, giving rise to lipid radicals and to oxidized fragments of fatty acids (Halliwell and Gutteridge 1999). As found recently, lipid peroxidation may be initiated also in monounsaturetd fatty acids, generating products similar to those generated in the presence of PUFA (Krasowska *et al.* 2000, 2001).

Determination of differences and similarities in lipid peroxidation in yeast species with different degrees of saturation is needed for complete understanding of this process in these organisms. Study of the effects of various kinds of pro-oxidants generating different free radicals on growing yeast cultures in comparison with peroxidative changes in isolated plasma membrane lipids may provide a useful information on cellular defenses against lipid peroxidation.

MATERIALS AND METHODS

Yeast strains and growth conditions. The study was performed on *Saccharomyces cerevisiae* SP4 (Leu^-, Arg^-) (Biliński *et al.* 1980), *Rhodotorula glutinis* (*Rhodosporidium toruloides* ATCC 26194), *Schizosaccharomyces pombe* 972h^- (NCYC 1824) and *Candida albicans* (isolated from hospital patients).

The yeast strains were grown in YEPD medium (in %, *W/W*; yeast extract 1, bacto peptone 2, glucose 2) at 28 °C under shaking for 20 h. For the incorporation of linoleic and linolenic acids into the membrane, *S. cerevisiae* SP4 was grown in YEPD medium supplemented with 1 % nonionic surfactant Tergitol (Nonidet P40) for fatty acid solubilization at 28 °C under shaking for 2 d. The flasks were inoculated to an absorbance of $A_{550} \approx 0.1$ from 2-d starter cultures. Linoleic (18 : 2) or linolenic (18 : 3) acid (final concentration 0.5 mmol/L) from filter-sterilized 20 mmol/L stock solution, solubilized with 5 % Tergitol, was added and the cultures were incubated at 28 °C with shaking for 20 h. Tergitol itself had no effect on cell growth.

Exposure to oxidants. The yeast cultures from YEPD or YEPD with PUFAs were diluted with sterilized water and added to 4.5 mL of GO medium (2 % yeast nitrogen base without amino acids, 2 % glucose, essential amino acids). These cell suspensions were supplied with 1 mmol/L different oxidants – TBHP, ACHN, AAPH and Fe^{II} – and incubated for 4 h at 28 °C with shaking. After each hour of incubation, the samples were diluted with fresh GO medium and plated in triplicate on GO-containing agar plates containing amino acids essential for *S. cerevisiae* SP4 strain.

Isolation of plasma membranes. The method was based on that used by Dufour *et al.* (1998) for *S. pombe*, with modifications. Briefly: A 20-h yeast culture (late exponential growth phase with *S. cerevisiae*, *S. pombe* and *C. albicans*, early exponential growth phase with *R. glutinis*) (A_{578} = 4.0) was spun down for 5 min at 1000 ***g***, resuspended in ice-cold distilled water, centrifuged again (5 min, 1000 ***g***) and resuspended in breaking medium (240 mmol/L sucrose, 50 mmol/L Tris-HCl, 5 mmol/L EDTA; pH adjusted to 7.5 with acetic acid) and supplied with proteinase inhibitors (4-aminobenzamidine and phenylmethanesulfonyl fluoride) in dimethylformamide. A proteinase inhibitor cocktail (chymostatin, antipain, leupeptin and pepstatin A) was used for *R. glutinis*. The cells were broken in a CO_2-cooled *Braun Melsungen* homogenizer with glass beads (six 10-s breaking runs interspersed with 5-s intermissions) or in smaller aliquots by vortexing under cooling with ice. The cell homogenate was centrifuged (5 min, 1200 ***g***; 1700 ***g*** for *R. glutinis*) and the supernatant was spun at 18 400 ***g*** (20 000 ***g*** for *R. glutinis*). The pellet was resuspended in a small volume of suspension medium (10 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L $MgSO_4$; pH 7.5), homogenized and acidified with acetic acid to pH 5.2–5.5. It was then spun on a microcentrifuge for 2 min at 13 000 ***g***, rapidly alkalinized to pH 7.5 and centrifuged at 45 000 ***g*** for 23 min. The pellet was homogenized, resuspended in MES buffer at pH 6.0, divided into suitable aliquots, frozen in liquid nitrogen and subsequently stored at –80 °C. The method was found to be applicable to *S. pombe*, *S. cerevisiae*, *R. glutinis* and *C. albicans*, yielding high-purity membrane preparations.

Plasma membrane lipids were isolated by a method adapted from Allen and Good (1971) with modifications. The plasma membranes were suspended in chloroform–methanol (1 : 2, *V/V*) and stirred vigorously. A 0.9 % NaCl solution was added to the samples under vigorous stirring and, after a 12-h incubation at room temperature, the chloroform layer was concentrated on a rotary vacuum evaporator.

The *fatty acid composition* of the fatty acids isolated from the yeast plasma membranes was examined by gas chromatography. Methyl esters of the fatty acids (FAME) were obtained by esterification of fat samples by a modified method of Prescha *et al.* (2001). This method is suitable for small lipid quantities with large amounts of unsaponifiables. Fifty mg of lipids was saponified at 70 °C for 1 h with 1 mL of 2 mol/L KOH in 75 % aqueous methanol. The unsaponifiable material was extracted two times with petroleum ether and then with 1 mL 1.25 mol/L HCl for ½ h (both solutions in anhydrous methanol). The FAME were extracted with hexane. The methyl ester mixture was separated on a CP-Sil 88 Chrompack capillary column (50 m × 0.25 mm). Helium was used as carrier gas and the separation was carried out at a temperature programmed from 150 °C (for 6 min) to 235 °C; the temperature increased at a rate of 6 K/min. Particular fatty acids were identified by comparison with external standards.

Determination of conjugated dienes as indicators of lipid peroxidation in pure fatty acids and in plasma membranes. The final concentration of the oxidation inducers TBHP and ACHN in all samples was 1 mmol/L. All samples contained 1 mg lipids. The time of exposure to oxidants was 5, 15 and 30 min. The concentration of conjugated dienes was determined as described by Pryor and Castle (1984).

RESULTS AND DISCUSSION

Four yeast species differing in their plasma membrane lipid composition (Table I) were treated with four kinds of oxidants. These species were previously found to incorporate readily exogenous PUFA from the growth medium to a level of approximately 65 % (according to the external concentration of PUFA) of total fatty acids (Howlett and Avery 1997). Using this technique, *S. cerevisiae* strain SP4 was doped with linoleic and linolenic acids to change its resistance to lipid peroxidation. Among the oxidants used, the most active ones were amphiphilic (TBHP) and hydrophobic (ACHN) (Figs 1 and 2). Hydrophilic oxidants (Fe^{II} and AAPH) were found to exert an intense action for about 2 h, with subsequent cell growth resumption. The presence of linoleic and linolenic acids in *S. cerevisiae* cells lowered the colony-forming ability of the cells and increased the sensitivity to pro-oxidants (Fig. 2). In addition, linoleic and/or linolenic acid-doped control cells seemed to be more sensitive to aeration during their culture in GO-mineral medium. *S. pombe* and *C. albicans* were the most resistant species (Fig. 1B, 1D). The relative fatty acid composition in plasma membranes of the tested species mostly overlapped with the data given by others authors (Marinho *et al.* 1997; Bjornstedt *et al.* 1995). The little differences found in this study correspond to strain-specific features.

The monounsaturated fatty acids in all four yeast species were palmitoleic (16:1), *cis*-10-heptadecenoic (17:1) and oleic (18:1). Indigenous PUFA were found in *C. albicans* (13.8 % linoleic and 4.2 % linolenic acid) and *R. glutinis* (5.9 % and 1.9 %, respectively) (Table I). In contrast to the other species, *S. cerevisiae* contained a great deal (33.3 %) of palmitoleic acid and a little quantity of 17:1 acid and, in addition, a 20:1 fatty acid.

Plasma membranes of *S. cerevisiae* cells grown in PUFA-supplemented media contained 35.2 % of linoleic (18:2) and 36.1 % of linolenic (18:3) acid out of total fatty acids as compared with some 1.3 % of 18:2 and nil 18:3 found in *S. cerevisiae* grown in unsupplemented medium. In contrast, their contents of palmitoleic (16:1) and oleic (18:1) acids were lower than those in *S. cerevisiae* grown in unsupplemented medium (Table I).

To obtain basic information on the peroxidating effect of our pro-oxidants on pure fatty acids, the level of CD was measured in pro-oxidant-exposed fatty acid standards, both unsaturated and saturated (palmitic acid, 16:0). Only amphiphilic (TBHP) and hydrophobic (ACHN) reagents were used for technical reasons (the measurements were done in cyclohexane). Production of CD was observed with all kinds of fatty acids except the saturated palmitic acid (Fig. 3), and depended on the extent of unsaturation and the pro-oxidant used. After a 30-h incubation, ACHN induced in all cases about three times less CD than TBHP did, the quantity of CD increasing with increasing unsaturation.

In order to find out if the CD production in the monounsaturated oleic acid depends on its esterification, we incubated samples of the acid, its methyl ester, and 1 : 1 mixtures of the two for up to 30 min with 1 mmol/L TBHP. While free acid and ester exhibited about the same autoxidation-induced CD level (*see* Fig. 3), their mixture contained somewhat lower CD concentration (*data not shown*).

In lipids isolated from plasma membranes, the CD production was time-dependent and decreased after a 5-min incubation with pro-oxidants (after 15 min for *C. albicans* and ACHN) (Fig. 4). ACHN was less productive than TBHP. In both kinds of pro-oxidants, the most oxidizable were lipids from *S. cerevisiae* plasma membranes doped with linolenic and linoleic acids and from *C. albicans* with indigenous linolenic acid. The CD production in lipids from *R. glutinis* receded more rapidly than in the other species.

Table I. Fatty acid composition[a] of different yeast species

Species	C_{10}	C_{12}	C_{14}	C_{15}	$C_{16:0}$	$C_{16:1}$	$C_{17:0}$	$C_{17:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	$C_{20:1}$	Others
S. cerevisiae	0.3 ± 0.1	0.8 ± 0.06	2.2 ± 0.3	0.4 ± 0.02	20.6 ± 0.1	33.3 ± 0.4	0.4 ± 0.01	0.2 ± 0.06	6.0 ± 0.7	29.0 ± 0.3	1.3 ± 0.3	–	–	0.5 ± 0.1	3.0 ± 0.1
S. cerevisiae + linoleic acid	0.6 ± 0.06	1.6 ± 0.1	3.4 ± 0.1	0.3 ± 0.01	24.7 ± 0.2	6.3 ± 0.2	0.5 ± 0.02	3.2 ± 0.2	16.8 ± 0.1	4.5 ± 0.01	35.2 ± 0.4	–	–	–	2.2 ± 0.3
S. cerevisiae + linolenic acid	0.1 ± 0.1	0.9 ± 0.1	3.6 ± 0.2	0.1 ± 0.01	22.4 ± 0.3	1.2 ± 0.01	0.7 ± 0.05	–	23.7 ± 0.2	1.8 ± 0.03	1.1 ± 0.2	36.1 ± 0.1	0.3 + 0.08	0.6 ± 0.03	2.0 ± 0.1
S. pombe	0.6 ± 0.1	1.1 ± 0.01	3.8 ± 0.03	0.8 ± 0.04	21.5 ± 0.4	4.3 ± 0.06	0.9 ± 0.06	9.0 ± 0.3	25.0 ± 0.4	25.5 ± 0.4	–	–	0.4 ± 0.01	–	3.2 ± 1
R. glutinis	0.8 ± 0.1	–	3.2 ± 0.4	0.8 ± 0.01	22.2 ± 0.9	3.9 ± 0.5	1.0 ± 0.2	2.9 ± 0.2	15.6 ± 0.4	35.9 ± 0.2	5.9 ± 0.2	1.9 ± 0.4	0.5 ± 0.1	–	4.4 ± 0.2
C. albicans	–	0.5 ± 0.1	3.4 ± 0.01	0.5 ± 0.07	22.3 ± 0.5	6.8 ± 0.01	0.7 ± 0.01	0.3 ± 0.02	21.7 ± 0.1	13.8 ± 0.3	13.8 ± 0.1	4.2 ± 0.1	0.4 ± 0.07	–	3.2 ± 0.2

[a]Relative peak area (%) ± SD, $n = 2$.

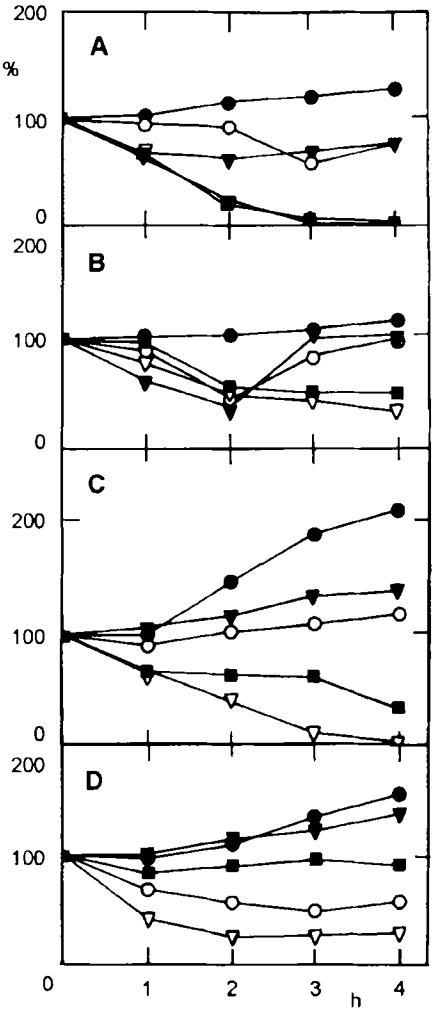

Fig. 1. Changes in the colony-forming ability of *R. glutinis* (**A**), *S. pombe* (**B**), *S. cerevisiae* (**C**) and *C. albicans* (**D**) cells exposed to the pro-oxidants AAPH (*open cirles*), Fe^{II} (*closed triangles*), TBHP (*open triangles*) and ACHN (*closed squares*) as determined by plating (control – *closed circles*); for experimental conditions and further details *see Materials and Methods*.

Although the susceptibility of different cell components to free radical attack and their repair have been well characterized (Halliwell and Gutteridge 1999), information about correlations between *in vitro* and *in vivo* processes is scarce. Lipid peroxidation gives rise to fatty acid hydroperoxides, and linoleic and linolenic acid hydroperoxides play a major role in this process. However, some data indicate that lipid peroxidation can take place not only with PUFA but also with monounsaturated acids (Krasowska *et al.* 2000, 2001).

In yeast cells many enzymes (*e.g.*, phospholipases, glutathione peroxidase, thioredoxin reductase) are involved in the breakdown of lipid hydroperoxides (Marinho *et al.* 1997; Bjornstedt *et al.* 1995). In general, free radicals generated by hydrophilic pro-oxidants (Fe^{II}, AAPH) seem to be more

readily eliminated than those produced by hydrophobic and amphiphilic ones (Fig. 1). On the other hand, incorporation of hydrophilic oxidants into membranes is less intense than with their amphiphilic or hydrophobic counterparts.

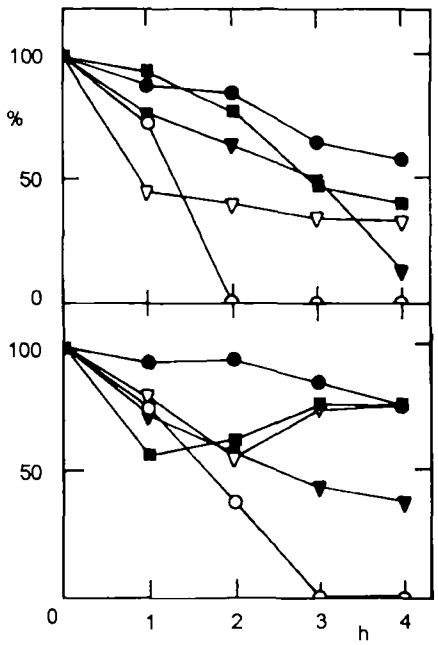

Fig. 2. Changes in the colony-forming ability of *S. cerevisiae* strain SP4 doped with linoleic (*above*) and linolenic (*below*) acid during growth and then exposed to the pro-oxidants AAPH (*open triangles*), Fe^{II} (*closed squares*), TBHP (*open circles*) and ACHN (*closed triangles*) as determined by plating (control – *closed circles*); for experimental conditions and further details *see Materials and Methods*.

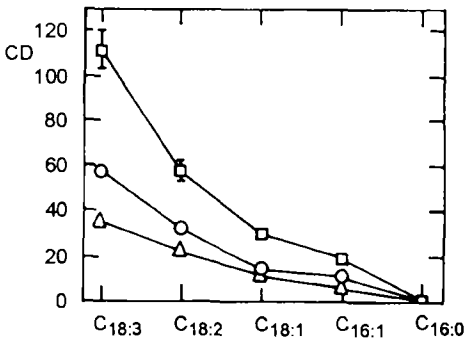

Fig. 3. Levels of conjugated dienes (CD, mmol/g lipid) in standard fatty acid methyl esters (1 g/L) after treatment with 1 mmol/L TBHP (*squares*) and ACHN (*circles*); *triangles* – control (autoxidation); mean ± SD ($n = 3$).

S. cerevisiae cells can adapt to lipid hydroperoxides when they are pretreated with a sublethal dose of linoleic acid hydroperoxide (50 μmol/L, 1 h) (Evans *et al.* 1998). The cell adaptation can be more efficient if the penetration of lipid hydroperoxides produced by hydrophilic oxidants into membranes is more difficult and slower (Fig. 1). The species most sensitive in our experiments, *R. glutinis*, contains in its plasma membrane, in addition to PUFA, also carotenoids which can induce free radicals during their antioxidative function (Halliwell and Gutteridge 1999). *S. pombe*, whose membranes contain hardly any PUFA

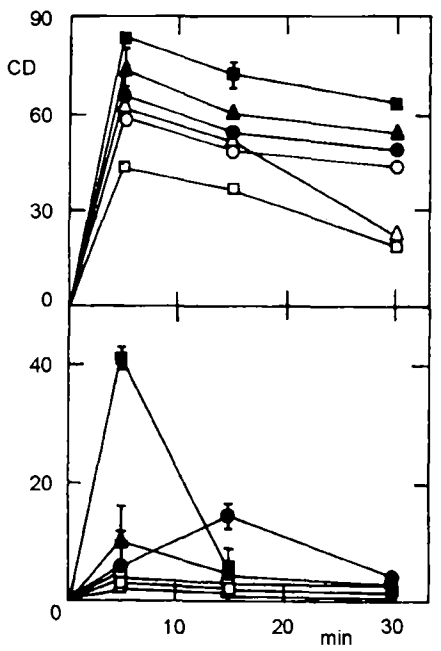

Fig. 4. Dependence of conjugated diene level (CD, mmol/g lipid) on the length of incubation (30 °C) of plasma membrane lipids (1 g/L) from different yeast species after TBHP (*above*) and ACHN (*below*) (1 mmol/L) treatment; mean ± SD ($n = 3$); *closed triangles* – *S. cerevisiae* doped with linoleic acid, *closed squares* – *S. cerevisiae* doped with linolenic acid, *open circles* – *S. cerevisiae*, *open squares* – *S. pombe*, *open triangles* – *R. glutinis*, *closed circles* – *C. albicans*.

(Table I), was found to be most resistant to pro-oxidant attack (Fig. 1B). Surprisingly, *C. albicans*, with its indigenous linoleic and linolenic acids seems to be also resistant to the influence of pro-oxidants (Fig. 1D). This may be ascribed, *e.g.*, to the presence of domains with decreased detergent solubility, such as those observed in detergent resistant membranes (Brown and London 1995), or it may reflect a slower motion of small hydrophobic molecules or free-radical fragments, as was observed in unsaturated membranes (Subczyński *et al.* 1991; Yin and Subczyński 1996). However, a detailed analysis of lipid domains in *C. albicans* plasma membranes is lacking.

A strong destructive effect of pro-oxidants is clearly seen in *S. cerevisiae* cells supplemented with linoleic and linolenic acids (Fig. 2). The cells were sensitive to aeration during growth in the mineral GO medium but not when YPG medium was used (Howlett and Avery 1997). The observed decrease in the relative proportions of monounsaturated fatty acids in linoleate- and linolenate-enriched cells (Table I) is caused by the repression of Δ-9 fatty acid desaturase activity (Bossie and Martin 1989).

Under a pro-oxidant attack, a hydrogen atom is abstracted from a methylene group in PUFAs to give rise to CD. These are thus the first free radicals that arise during lipid peroxidation and, on reacting with oxygen, produce lipid hydroperoxides. In our model experiments (Fig. 3), low amounts of CD were formed from monounsaturated fatty acids whereas, as expected, none were formed from saturated palmitic acid. The low action of the hydrophobic pro-oxidant ACHN can reflect the fact that, at the temperature of 30 °C used in our experiments, yeast growth is optimal but the radical-producing ACHN thermolysis is low.

The CD production was found to be highly time-dependent. The drop in the CD level after the initial rise obviously corresponds to the conversion of CD to further products – hydroperoxides and TBRS (Fig. 4). The quantity of generated CD also depends on the fatty acid type and composition. Lipids from *C. albicans* plasma membranes with 13.8 % 18 : 2 and 4.2 % 18 : 3 PUFA (Table I and Fig. 4) undergo stronger peroxidation than lipids from other species. The delayed CD production in lipid emulsions from *C. albicans* plasma membranes exposed to ACHN may also reflect specific features of the lipid composition (Fig. 4B).

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