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Induction of Lipid Peroxidation during Heavy Metal Stress in *Saccharomyces cerevisiae* and Influence of Plasma Membrane Fatty Acid Unsaturation

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The degree of plasma membrane fatty acid unsaturation and the copper sensitivity of *Saccharomyces cerevisiae* are closely correlated. Our objective was to determine whether these effects could be accounted for by differential metal induction of lipid peroxidation. *S. cerevisiae* S150-2B was enriched with the polyunsaturated fatty acids (PUFAs) linoleate (18:2) and linolenate (18:3) by growth in 18:2- or 18:3-supplemented medium. Potassium efflux and colony count data indicated that sensitivity to both copper (redox active) and cadmium (redox inactive) was increased in 18:2-supplemented cells and particularly in 18:3-supplemented cells. Copper- and cadmium-induced lipid peroxidation was rapid and associated with a decline in plasma membrane lipid order, detected by fluorescence depolarization measurements with the membrane probe trimethylammonium diphenylhexatriene. Levels of thiobarbituric acid-reactive substances (lipid peroxidation products) were up to twofold higher in 18:2-supplemented cells than in unsupplemented cells following metal addition, although this difference was reduced with prolonged incubation up to 3 h. Conjugated-diene levels in metal-exposed cells also increased with both the concentration of copper or cadmium and the degree of cellular fatty acid unsaturation; maximal levels were evident in 18:3-supplemented cells. The results demonstrate heavy metal-induced lipid peroxidation in a microorganism for the first time and indicate that the metal sensitivity of PUFA-enriched *S. cerevisiae* may be attributable to elevated levels of lipid peroxidation in these cells.

The toxicity of heavy metals to microorganisms has attracted considerable research attention in recent years, particularly as a result of the continuing anthropogenic mobilization of metals in the environment. Most heavy metal ions have a strong affinity for ligands such as phosphates, purines, pteridines, porphyrins, and cysteinyl and histidyl side chains of proteins, which are all abundant in microbial cells. Thus, the toxic effects of metals can be attributed partly to the multiplicity of coordination complexes and clusters that they can form (16). For example, cadmium can react with polythiol groups on cellular macromolecules and substitute for zinc in Zn-containing enzymes, e.g., carboxypeptidases (30) and metallothioneins (24). Among other mechanisms of toxicity, copper may interact with cellular nucleic acids and enzyme active sites (9). As a primary mechanism of toxicity, both cadmium and copper, along with other transition metals, cause disruption of cellular and organellar membranes, resulting in rapid impairment of membrane function and loss of membrane integrity. Cadmium- and copper-induced plasma membrane permeabilization, with associated cellular K^+ efflux, has been extensively reported for the yeast *Saccharomyces cerevisiae* (4, 16, 27).

Our recent studies with *S. cerevisiae* have demonstrated that susceptibility to heavy metal-induced plasma membrane permeabilization and toxicity is markedly dependent on plasma membrane fatty acid composition (4). Both cadmium-induced (22) and copper-induced (4) plasma membrane disruptions were markedly accelerated in *S. cerevisiae* enriched with polyunsaturated fatty acids (PUFAs). These results are of pertinence to metal toxicity in the natural environment, where the fatty acid composition of microorganisms shows considerable variation (21, 28, 36). The mechanism underlying the metal sensitivity of PUFA-enriched cells was not elucidated. However, one general cause for loss of membrane integrity in biological systems, in response to a variety of stimuli, can arise through oxy-radical-mediated lipid peroxidation (20). The accumulation of lipid peroxidation products, such as lipid hydroperoxides, within the hydrophobic core of plasma membranes can result in disturbances in the arrangement of phospholipid moieties and impairment of membrane function (32); this may be manifested as K^+ loss. The fact that the susceptibility of fatty acids to lipid peroxidation increases with the degree of fatty acyl chain unsaturation (29) indicates a possible link with our results. Furthermore, redox-active metals, such as copper, are known to be capable of inducing free-radical production and promoting oxidative stress through their redox-cycling activity. Non-redox-active metals, such as cadmium, can indirectly enhance oxidative stress by depleting free-radical scavengers such as glutathione and protein-bound sulfhydryl groups (31). The metal hypersensitivity of various mutants of *S. cerevisiae* defective in components of the oxidative stress response supports a role of oxygen free radicals in microbial metal toxicity (23). For example, *sod1* mutants deficient in copper/zinc superoxide dismutase activity are hypersensitive to copper (19) (these observations may be complicated, however, by the involvement of this protein in copper buffering [12]). An important role for lipid peroxidation in the toxicity of heavy metal ions to higher organisms has been implicated from recent studies (31). To date, no studies have investigated such a relationship in microorganisms.

In the present study, we demonstrate heavy metal-induced lipid peroxidation in a microorganism for the first time. Furthermore, the increased susceptibility of PUFA-enriched *S. cerevisiae* to cadmium- and copper-induced plasma membrane perturbation and toxicity is shown here to correlate with elevated levels of lipid peroxidation in these cells.

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MATERIALS AND METHODS

Organism and culture conditions. *S. cerevisiae* S150-2B (kindly provided by D. J. Jamieson, University of Dundee, Dundee, United Kingdom) was routinely maintained on solid YEPD medium, comprising 2% (wt/vol) neutralized bacteriological peptone, 1% (wt/vol) yeast extract (Oxoid), 2% (wt/vol) glucose, and 1.6% (wt/vol) agar (no. 3, technical; Oxoid). For experimental purposes, *S. cerevisiae* was grown in 100 ml of YEPD broth of the same composition but lacking agar and supplemented with 1% (wt/vol) tergitol (Nonidet P-40; Sigma) for fatty acid solubilization, in 250-ml Erlenmeyer flasks (tergitol supplementation alone was found to have no effect on the fatty acid composition or growth of *S. cerevisiae*). Experimental flasks were inoculated to an optical density at 550 nm of ~0.1 from 48-h starter cultures and incubated at 25°C with orbital shaking at 120 rpm. Where indicated, linoleate (18:2) or linolenate (18:3) (final concentration, 1 mM) from filter-sterilized 20 mM stock solutions, solubilized with 5% (wt/vol) tergitol, was added. Cell numbers were determined by using a modified Fuchs-Rosenthal hemocytometer slide after appropriate dilution with distilled deionized water; more than 400 cells were counted in each sample.

Preparation of cell homogenates. Cells were harvested by centrifugation at 1,500 × *g* for 5 min and washed twice with distilled deionized water at 4°C to restrict further lipid metabolism. The cells were disrupted by being shaken with 0.5-mm-diameter glass beads (Sigma) for 15 min at 4°C with a homogenizer (Mickle Laboratories, Guildford, United Kingdom). The beads were removed by vacuum filtration through a glass-sintered filter, and the filter and beads were washed with distilled deionized water. The filtrate (whole-cell homogenate) was used for lipid extraction.

Lipid extraction and fatty acid analysis. Lipids were extracted from whole-cell homogenates by the method of Bligh and Dyer (6). For fatty acid analysis, methyl esters were generated by acid-catalyzed esterification (2.5% [vol/vol] H_2SO_4 in dry methanol) at 70°C for 2 h. Fatty acid methyl esters were extracted with redistilled petroleum spirit (bp, 60 to 80°C) and subsequently analyzed by gas-liquid chromatography. Pentadecanoate was used as an internal standard. Separations were routinely achieved by using 10% SP-2330 on 100/120 Chromosorb-WAW (Supelco, Saffron Waldon, Essex, United Kingdom) packed into a stainless steel column (1.8 m by 0.3 mm [outer diameter]). Fatty acids were identified by comparison with standards.

Preparation of cell suspensions for metal toxicity experiments. Cells from the late exponential phase (13 h) were harvested by centrifugation at 1,500 × *g* for 5 min and washed twice with distilled deionized water. The washed cells were suspended to a density of approximately 10^8 ml^{-1} in 50 ml of 10 mM morpholineethanesulfonic acid (MES) buffer (pH 5.5)–1% (wt/vol) glucose and incubated at room temperature with orbital shaking at 120 rpm. After 10 min of equilibration, $Cd(NO_3)_2$ or $Cu(NO_3)_2$ was added to the desired concentration.

Cell viability. Cell viability was determined as the ability to produce CFU. At certain intervals after the addition of $Cd(NO_3)_2$ or $Cu(NO_3)_2$ to cell suspensions, aliquots were removed and, after appropriate dilution with sterile distilled deionized water, plated on OGYE agar (Lab M). OGYE is a yeast-selective agar containing 0.1% (wt/vol) oxytetracycline hydrochloride (Sigma). Colonies were enumerated after incubation at 25°C for 4 days, after which time no further colonies appeared.

Potassium efflux. At certain intervals after the addition of $Cd(NO_3)_2$ or $Cu(NO_3)_2$ to cell suspensions, 0.5-ml aliquots were removed and microcentrifuged for 3 min, and the supernatant was then diluted with 4 volumes of distilled deionized water. Extracellular K^+ was measured with a Corning 410 flame photometer, with reference to appropriate standard KCl solutions.

Evaluation of lipid peroxidation. Lipid peroxidation was quantified by two methods: determination of thiobarbituric acid (TBA)-reactive substances (TBARS) and determination of conjugated dienes. TBARS were determined by a method adapted from that described by Aust (2). At certain intervals (up to 3 h), after the addition of $Cd(NO_3)_2$ or $Cu(NO_3)_2$, 0.5-ml samples of cell suspension were removed and added to 1 ml of TBA reagent (0.25 M HCl, 15% [wt/vol] trichloroacetic acid, 0.375% [wt/vol] TBA). Addition of the reagent terminated lipid peroxidation and initiated the assay. Samples were heated for 15 min in a boiling water bath and, after cooling, were centrifuged at 1,000 × *g* for 5 min in order to remove cell debris. Absorbances of the samples at 535 nm were then measured by using a Shimadzu UV-120-02 spectrophotometer, against a reference solution comprising 1 ml of TBA reagent with the sample replaced by an equal volume (0.5 ml) of distilled deionized water. The concentrations of TBARS in samples were calculated by reference to a standard curve prepared by using 1,1,3,3,-tetramethoxypropane.

For the determination of conjugated dienes (18), 10-ml samples were removed from cell suspensions and lipid extracts were prepared from whole-cell homogenates as described above. After evaporation of chloroform under nitrogen, the dried lipids were resuspended in 3 ml of cyclohexane. The UV spectra of lipid solutions between 215 and 280 nm were recorded by using a Perkin-Elmer lambda-7 UV/visible spectrophotometer. Conjugated dienes absorb light between approximately 215 and 255 nm, with a peak at 233 nm (10). Peak absorbances at 233 nm were normalized against absorbance values at 260 nm, and relative conjugated-diene content was expressed as A_{233}/A_{260}.

Fluorescence depolarization. Plasma membrane lipid order was determined by measuring steady-state fluorescence anisotropy in whole cells labeled with trimethylammonium diphenylhexatriene (TMA-DPH). The cationic probe TMA-DPH becomes anchored primarily at the plasma membrane of intact cells (7, 17). TMA-DPH was added to *S. cerevisiae* cell suspensions (5×10^7 cells ml^{-1}; see above) from a 1 mM stock solution, prepared in concentrated dimethylformamide, to give a final concentration of 1 μM. After 30 min of equilibration, when measurements had stabilized, $Cd(NO_3)_2$ was added to the cell suspensions. Triplicate 3-ml samples of suspension were removed at intervals, transferred to 10-mm-pathlength quartz cuvettes, and analyzed by using a Perkin-Elmer LS5 fluorescence spectrometer with a polarization accessory. TMA-DPH was excited with vertically polarized light at 360 nm, and the vertical and horizontal vectors of emitted light were measured at 450 nm. Membrane order was expressed as the order parameter *S*, which reflects the orderliness of membrane phospholipids: $S = (r/r_0)^{0.5}$ (17), where r_0 is the theoretical limiting anisotropy (0.395 for TMA-DPH) in the absence of rotational motion and *r* is the steady-state anisotropy measured in the membrane. The effects of Cd^{2+} on membrane order were determined by measuring *S* at intervals over 60 min. Light scatter, determined in the absence of TMA-DPH, was found to account for less than 5% of the total emitted light, and therefore no corrections were employed (3).

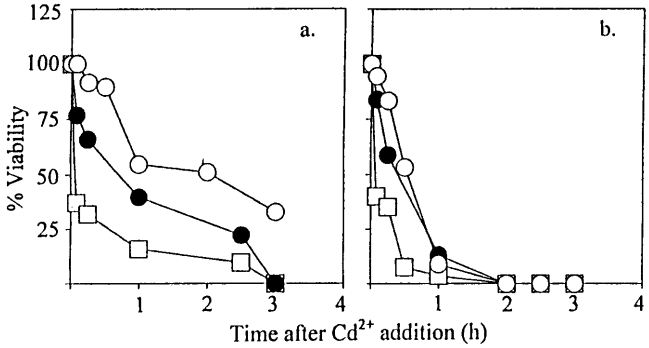

FIG. 1. Effect of cadmium exposure on the viability of unsupplemented and PUFA-supplemented *S. cerevisiae* cells. Cells were grown for 13 h in unsupplemented (○), linoleate-supplemented (●), or linolenate-supplemented (□) medium and then harvested, washed, and resuspended in 10 mM MES (pH 5.5)–1% (wt/vol) glucose. $Cd(NO_3)_2$ was added to a final concentration of 50 (a) or 200 (b) μM. Typical results from one of at least two experiments are shown. Points represent means of three replicate samples from single experimental flasks. Standard errors of the means (SEM) are smaller than the dimensions of the symbols.

RESULTS

In a previous study (4), we demonstrated that *S. cerevisiae* NCYC 1383 readily incorporated exogenous PUFAs (linoleate and linolenate) from its growth medium to greater than 65% of the total fatty acids. *S. cerevisiae* S150-2B was selected for this study because of its well-characterized responses to oxidative stress (23). *S. cerevisiae* S150-2B was also observed to incorporate exogenous PUFAs; levels of incorporation were very similar to those described previously (results not shown). Increases in cellular PUFA content, as a proportion of total fatty acids, were contemporaneous with decreases in the monounsaturated fatty acids palmitoleate (16:1) and oleate (18:1). Cellular fatty acid unsaturation indices (average number of double bonds per fatty acid) decreased in the order 18:3-supplemented cells (2.28) > 18:2-supplemented cells (1.48) > unsupplemented cells (0.82). The fatty acid compositions of plasma membrane-enriched fractions from *S. cerevisiae* match those of their corresponding whole-cell homogenates very closely (4).

Effects of cadmium and copper on the viability of unsupplemented and PUFA-supplemented *S. cerevisiae*. *S. cerevisiae* S150-2B cells previously grown in PUFA-supplemented media were markedly more susceptible to Cd^{2+} (Fig. 1) and Cu^{2+} (Table 1) toxicity than cells grown in unsupplemented medium. For example, after 1 h of incubation in the presence of 50 μM $Cd(NO_3)_2$, only 40 and 16% of cells previously grown in 18:2- and 18:3-supplemented media, respectively, were still viable

TABLE 1. Effect of $Cu(NO_3)_2$ on viability of *S. cerevisiae* cells previously grown in unsupplemented and PUFA-supplemented media

$[Cu(NO_3)_2]$ (μM)	% Viability of the indicated cell type[a]		
	Unsupplemented	18:2 supplemented	18:3 supplemented
5[b]	88 ± 1	70 ± 5	63 ± 3
40[c]	11.0 ± 1.1	1.4 ± 1.4	0

[a] Values are means ± SEM derived from three replicate determinations.
[b] Determined after 60 min.
[c] Determined after 5 min.

(capable of colony formation) (Fig. 1a), whereas 55% viability was maintained in cells from unsupplemented medium. Similar trends were evident with 200 μM $Cd(NO_3)_2$, albeit over a shorter time scale. No viable cells were detected after 2 h of incubation in the presence of 200 μM Cd^{2+}. This was also the case after 5-min exposure of 18:3-supplemented cells to 40 μM $Cu(NO_3)_2$, although some capacity for colony formation was retained by Cu^{2+}-exposed unsupplemented and 18:2-supplemented cells (Table 1). Incubation for 60 min in the presence of 5 μM $Cu(NO_3)_2$ had far less marked effects on cell viability, although here again the percent viability decreased in the order unsupplemented cells > 18:2-supplemented cells > 18:3-supplemented cells.

Cadmium- and copper-induced K^+ efflux from unsupplemented and PUFA-supplemented *S. cerevisiae*. Release of intracellular K^+ was used as an indicator of plasma membrane permeabilization (4). Following heavy metal addition, K^+ efflux was initially very rapid but slowed considerably after 15 min (Fig. 2). The level of metal-induced K^+ release from *S. cerevisiae* previously grown in PUFA-supplemented media was markedly higher than that from cells grown in unsupplemented medium. For example, the amounts of K^+ released from 18:2- and 18:3-enriched cells were approximately equal at 5.6 nmol 10^6 cells^{-1} after 15-min exposure to 50 μM $Cd(NO_3)_2$, whereas that from cells grown in unsupplemented medium was only 3.1 nmol 10^6 cells^{-1} (Fig. 2a). K^+ loss continued after 15 min, and the amounts released from 18:3-supplemented cells after 3 h were approximately 17% larger than those from 18:2-supplemented cells. K^+ efflux was greater with 200 μM $Cd(NO_3)_2$ than with 50 μM $Cd(NO_3)_2$, and differences between 18:2- and 18:3-supplemented cells were more marked. Thus, the amounts of K^+ released after 1-h exposure to 200 μM Cd^{2+} from cells previously grown in unsupplemented and 18:2- and 18:3-supplemented media were approximately 4.8, 7.6, and 8.4 nmol 10^6 cells^{-1}, respectively (Fig. 2b). Similar trends were apparent during copper exposure (Table 2). The amounts of K^+ released from 18:2- and 18:3-enriched cells incubated in the presence of 5 μM $Cu(NO_3)_2$ were similar but were approximately 100 and 84% higher after 30 and 210 min, respectively, than those from unsupplemented cells under the same conditions. At 40 μM $Cu(NO_3)_2$, a greater relative permeabilization of the plasma membrane of 18:3-supplemented cells than of 18:2-supplemented cells also became apparent (Table 2).

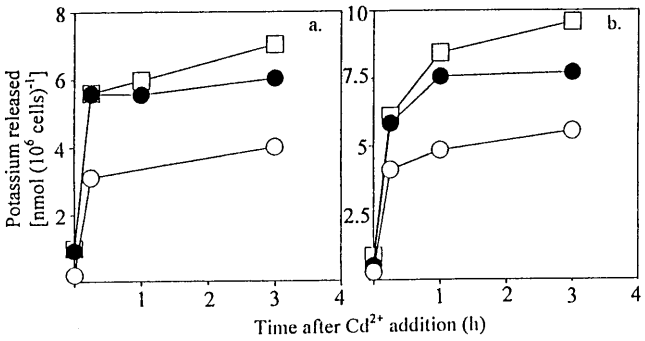

FIG. 2. Cadmium-induced K^+ efflux in unsupplemented and PUFA-supplemented *S. cerevisiae* cells. Cells were grown for 13 h in unsupplemented (○), linoleate-supplemented (●), or linolenate-supplemented (□) medium and then harvested, washed, and resuspended in 10 mM MES (pH 5.5)–1% (wt/vol) glucose. $Cd(NO_3)_2$ was added to a final concentration of 50 (a) or 100 (b) μM. Typical results from one of at least two experiments are shown. Values for K^+ release are means from three replicate determinations. SEM are smaller than the dimensions of the symbols.

TABLE 2. Copper-induced K^+ release from *S. cerevisiae* cells grown previously in unsupplemented and PUFA-supplemented media

$[Cu(NO_3)_2]$ (μM)	Exposure time (min)	K^+ release from the indicated cell type[a]		
		Unsupplemented	18:2 supplemented	18:3 supplemented
5	30	0.55 ± 0.04	1.18 ± 0.03	1.10 ± 0.05
	210	0.62 ± 0.03	1.14 ± 0.06	1.14 ± 0.10
40	30	3.4 ± 0.1	5.0 ± 0.1	5.7 ± 0.1
	210	4.1 ± 0.1	5.0 ± 0.1	6.1 ± 0.1

[a] Nanomoles of K^+ released per 10^6 cells. Values are means ± SEM derived from three replicate determinations.

Cadmium- and copper-induced lipid peroxidation in unsupplemented and linoleate-supplemented *S. cerevisiae*. Lipid peroxidation was evaluated as TBARS production and conjugated-diene formation. Two analytical methods were used to assess lipid-localized oxidative stress, since no single method adequately measures the range of possible reactions (11). Furthermore, the TBARS assay alone was not suitable for direct comparison of linolenate-enriched cells with the other cell types, since malondialdehyde, to which the assay is particularly sensitive, is formed upon the breakdown of only linolenate and not linoleate or monounsaturated fatty acids (2).

Difficulties related to altered membrane density and partitioning behavior were encountered with the purification of plasma membranes from cells following metal exposure. Thus, the values for whole-cell lipid peroxidation (given) include contributions from organellar membranes. Because the plasma membrane is a primary target for metal toxicity (16, 27) and because its fatty acid composition is similar to that of organellar membranes (4), it can be inferred that lipid peroxidation per unit plasma membrane was at least equal to (and probably greater than) that of organellar membranes.

Prior to the addition of metal, the amounts of TBARS were generally approximately 25 to 55% larger in linoleate-enriched cells than in cells grown previously in unsupplemented medium (Fig. 3; Table 3). Marked increases in TBARS formation were evident following metal addition. Control experiments confirmed that no detectable rise in TBARS levels occurred during 3-h incubation in buffer in the absence of cadmium or copper (results not shown). Metal-induced increases were particularly large during the first minutes of metal exposure, although in certain cases TBARS formation continued up to 3 h (Fig. 3). The level of metal-induced TBARS production was considerably higher in *S. cerevisiae* previously grown in 18:2-supplemented medium than in cells grown in unsupplemented medium. For example, after 1-h exposure to 50 μM $Cd(NO_3)_2$,

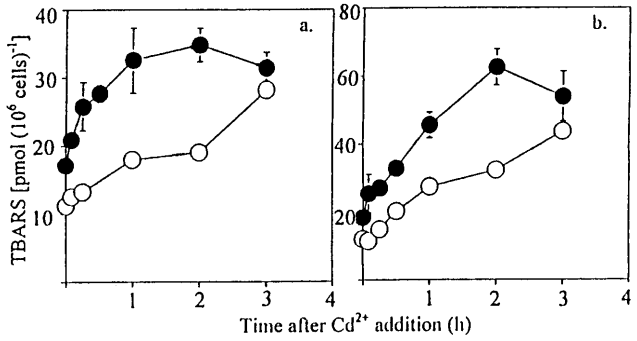

FIG. 3. Cadmium-induced TBARS production in unsupplemented and linoleate-supplemented *S. cerevisiae* cells. Cells were grown for 13 h in unsupplemented (○) and linoleate-supplemented (●) medium and then harvested, washed, and resuspended in 10 mM MES (pH 5.5)–1% (wt/vol) glucose. $Cd(NO_3)_2$ was added to a final concentration of 50 (a) or 100 (b) μM. Typical results from one of at least two experiments are shown. Values for TBARS are means of three replicate determinations from single experimental flasks, ± SEM where these values exceed the dimensions of the symbols.

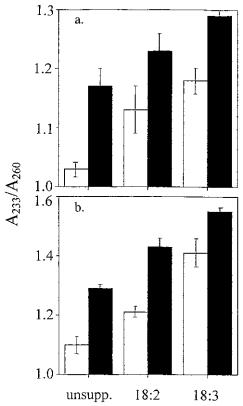

FIG. 4. Cadmium- and copper-induced conjugated-diene formation in unsupplemented and PUFA-supplemented medium. Cells were grown for 13 h in unsupplemented or linoleate- or linolenate-supplemented medium and then harvested, washed, and resuspended in 10 mM MES (pH 5.5)–1% (wt/vol) glucose. (a) Cd^{2+} was added to a final concentration of 50 (□) or 200 (■) μM. (b) Cu^{2+} was added to a final concentration of 5 (□) or 20 (■) μM. Values are means for three individual lipid extracts ± SEM. unsupp., unsupplemented.

the level of TBARS in 18:2-enriched cells was approximately 33 pmol 10^6 cells^{-1}, whereas that from cells grown in unsupplemented medium was only 18 pmol 10^6 cells^{-1} (Fig. 3a). The level of TBARS production was higher at 200 μM $Cd(NO_3)_2$ than at 50 μM $Cd(NO_3)_2$. Up to 2 h, levels of TBARS were between 1.6- and 2-fold greater in 18:2-supplemented cells than in unsupplemented cells at either $Cd(NO_3)_2$ concentration (Fig. 3). A reduction in cellular TBARS levels between 2 and 3 h of incubation with cadmium was evident only in 18:2-supplemented cells, whereas lipid peroxidation continued to rise in unsupplemented cells during this period. Thus, after 3 h, the levels of TBARS measured in 18:2-supplemented cells were only approximately 1.2-fold higher than those of unsupplemented cells (Fig. 3). Similar trends were observed for copper exposure, with which TBARS production was also associated. Again, lipid peroxidation increased with metal concentration, and, after 1 h, levels of Cu-induced TBARS were between 20 and 40% higher in 18:2-enriched cells than in unsupplemented cells. However, between 1 and 3 h, TBARS levels in 18:2-supplemented cells either increased only slightly (5 μM Cu^{2+}) or declined (40 μM Cu^{2+}). In contrast, those of unsupplemented cells continued to increase and, at 3 h, exceeded TBARS levels evident in 18:2-enriched cells (Table 3).

Lipid peroxidation was also evaluated by measuring conjugated dienes, which are early intermediates in the lipid peroxidative chain (18). Relative levels of conjugated dienes were determined after 1-h exposure to Cd and Cu and expressed as A_{233}/A_{260}. At 50 μM $Cd(NO_3)_2$, A_{233}/A_{260} values for cells grown previously in unsupplemented and 18:2- and 18:3-supplemented media were 1.03, 1.12, and 1.18, respectively, indicating increased Cd-induced conjugated-diene formation in cells displaying elevated fatty acid unsaturation indices (Fig. 4a). A similar trend was evident at 200 μM $Cd(NO_3)_2$, although the A_{233}/A_{260} values were higher in all cases at this increased cadmium concentration. Conjugated-diene levels also increased with the unsaturation index in copper-exposed cells. A_{233}/A_{260} values after 1 h at 5 μM $Cu(NO_3)_2$ for cells grown previously in unsupplemented and 18:2- and 18:3-supplemented media were 1.10, 1.21, and 1.41, respectively (Fig. 4b). Conjugated-diene formation was greater at 20 than at 5 μM $Cu(NO_3)_2$, although again the A_{233}/A_{260} values were highest for cells that had previously been grown in the presence of linoleate and particularly linolenate. Interestingly, despite the lower copper concentrations employed, and in contrast to TBARS results, copper-exposed cells generally displayed higher conjugated-diene levels than cadmium-exposed cells. The elevated levels of lipid peroxidation evident (from both sets of data) in PUFA-enriched *S. cerevisiae* cells were correlated with the increased susceptibility of these cells to Cd^{2+}- and Cu^{2+}-induced plasma membrane permeabilization and whole-cell toxicity.

TABLE 3. Cu^{2+}-induced TBARS production in *S. cerevisiae* cells grown previously in unsupplemented and linoleate-supplemented media

$[Cu(NO_3)_2]$ (μM)	Exposure time (min)	TBARS produced by the indicated cell type[a]	
		Unsupplemented	18:2 supplemented
5	0	5.7 ± 1.1	7.3 ± 0.9
	60	8.9 ± 1.8	12.4 ± 1.5
	180	14.3 ± 0.4	14.1 ± 3.1
40	0	5.4 ± 1.7	7.0 ± 0.4
	60	14.2 ± 0.2	17.5 ± 1.1
	180	17.2 ± 1.0	16.0 ± 0.7

[a] Picomoles of TBARS produced per 10^6 cells. Values are means ± SEM derived from three replicate determinations.

Effect of cadmium on plasma membrane order of unsupplemented and PUFA-supplemented *S. cerevisiae*. In order to assess metal-induced changes in plasma membrane physical properties, plasma membrane lipid order was monitored (by fluorescence depolarization with TMA-DPH-labeled cells) for 50 min following the addition of 50 μM $Cd(NO_3)_2$ (Fig. 5). Prior to the addition of metal, *S* values for cells previously grown in unsupplemented and 18:2- and 18:3-supplemented media were 0.74, 0.71, and 0.69, respectively, indicating decreased orderliness in plasma membranes enriched with PUFAs. After the addition of metal, decreases in plasma membrane order in all three types of cell were clearly evident, and they continued for at least 50 min. The Cd^{2+}-induced change in *S* was slightly greater for cells previously grown in unsupplemented medium than for 18:2- and 18:3-enriched cells. However, after 50 min of incubation in the presence of Cd^{2+}, values for *S* still decreased in the order unsupplemented cells >

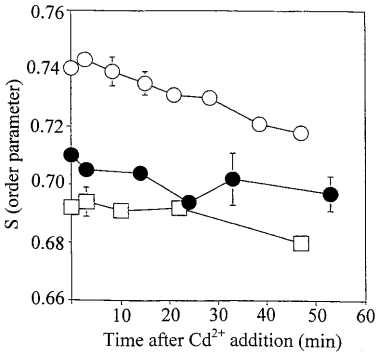

FIG. 5. Effect of cadmium on membrane order in unsupplemented and PUFA-supplemented *S. cerevisiae* cells. Cells were grown for 13 h in unsupplemented (○), linoleate-supplemented (●), or linolenate-supplemented (□) medium and then harvested, washed, and resuspended in 10 mM MES (pH 5.5)–1% (wt/vol) glucose. TMA-DPH was added to a final concentration of 1 μM, and 30 min later, Cd^{2+} was added to a final concentration of 50 μM. Values for *S* are means for three replicate samples from single experimental flasks, ± SEM where these values exceed the dimensions of the symbols.

18:2-supplemented cells > 18:3-supplemented cells. The order parameter for plasma membranes of Cd^{2+}-exposed unsupplemented cells did not decrease below the values for PUFA-enriched cells determined prior to cadmium addition (Fig. 5).

DISCUSSION

Metal-induced plasma membrane permeabilization and whole-cell toxicity in *S. cerevisiae* S150-2B (a strain for which various components of the oxidative stress response have been well characterized [23]) were initially confirmed to be greater in cells enriched with the PUFAs linoleate and linolenate. Similar effects of plasma membrane fatty acid composition on copper toxicity, albeit more marked, have also been observed in *S. cerevisiae* NCYC 1383 (4). Hence, this effect is clearly not strain specific. Our results also suggest that the ability of *S. cerevisiae* to incorporate exogenous PUFAs with little effect on growth rate may be widespread; previous reports of this characteristic were restricted to *S. cerevisiae* NCYC 1383 (4, 8). The consequent experimental advantages of *S. cerevisiae* for modeling membrane-dependent effects have been highlighted elsewhere (4).

As in previous reports (4, 5, 27), extensive metal-induced potassium release was associated with a loss of viability of *S. cerevisiae*. Whereas a stoichiometric relationship between physiological metal uptake and potassium release can occur at nontoxic metal concentrations (14), our previous studies have confirmed nonstoichiometry at the toxic metal concentrations used here (4, 22). Thus, potassium release can be attributed to plasma membrane permeabilization and toxicity (4, 27). As is usually the case among yeast and fungi (16), copper was more toxic than cadmium for *S. cerevisiae*. However, copper exposure resulted in less K^+ release than that observed during cadmium exposure at the concentrations used. Thus, it can be inferred that effects other than membrane permeabilization, e.g., direct interaction with nucleic acids or misincorporation into metallothioneins (9), may make a more important contribution in copper toxicity than cadmium toxicity to loss of *S. cerevisiae* viability. An important role of copper-induced membrane effects was nevertheless implicated by the observed influence of membrane fatty acid composition.

A role of lipid-targeted oxy-radical attack in the toxicity of heavy metals to higher organisms has been inferred from observations of elevated levels of lipid peroxidation in metal-exposed organisms (20, 31). In this study, metal-induced lipid peroxidation in a microorganism was demonstrated for the first time.

The integration of different analytical methods for the measurement of lipid peroxidation was necessary (11). Results from conjugated-diene and TBARS analyses suggested contrasting pathways of cadmium- and copper-induced lipid peroxidation. Unlike TBARS, conjugated dienes are primary products in the lipid peroxidative chain, arising through oxy-radical-mediated abstraction of hydrogen atoms from methylene groups separating PUFA double bonds (18). Therefore, the higher relative levels of conjugated dienes in Cu-exposed cells suggest that initiation of lipid peroxidation occurs at a greater rate in the presence of copper than cadmium in *S. cerevisiae*; such a conclusion is consistent with the respective redox activities and inactivities of these metals (31). The mechanism of lipid peroxidation initiation is unlikely to differ, since the principal initiating species generated by both copper (direct catalysis of the Fenton reaction) and cadmium (indirect promotion of the Fenton reaction by glutathione depletion) is probably the hydroxyl radical (15, 31). However, observed differences in initial TBARS levels, prior to metal addition, do suggest the possibility of additional initiation via preformed lipid hydroperoxides (15).

The TBARS assay detects primarily products arising from the decomposition of lipid hydroperoxides (2). During normal cellular metabolism, the formation of high levels of TBARS can be precluded by glutathione peroxidase activity. Glutathione peroxidase converts lipid hydroperoxides to their corresponding hydroxy fatty acids (13). However, glutathione is a principal cellular target or sequestration site of cadmium (25, 31). Thus, the higher relative levels of TBARS in Cd-exposed cells than in Cu-exposed cells may reflect glutathione depletion and hence a reduced capacity of the former to repair lipid peroxidation damage.

The convergence of TBARS levels in unsupplemented and linoleate-supplemented *S. cerevisiae* after prolonged metal exposure may reflect a greater induction of antioxidant activity in the latter cells; the oxidative stress response of *S. cerevisiae*, which includes the induction of glutathione, catalase, the superoxide dismutases, and glutathione peroxidase, increases with the extent of initial insult (23, 26).

Exposure to neither metal was associated with a decline in the degree of cellular fatty acid unsaturation within 1 h (results not shown). However, longer-term reductions in the proportion of unsaturated fatty acids were previously noted during growth of *S. cerevisiae* in cadmium-supplemented medium (22). This supports the view that reduced polyunsaturated fatty acid content can be a late marker of lipid peroxidation (34).

The similarities between the short initiation time scales (noting that TBARS are not immediate products of lipid peroxidation), and between the relative extents, of lipid peroxidation and K^+ release observed here were consistent with the former process being associated with a deterioration of membrane integrity (10, 32). Loss of membrane impermeability during lipid peroxidation may arise through covalent bond formation between adjacent acyl radicals, resulting in increased membrane rigidity, or through the incorporation of short-chain and/or polar oxidation products, resulting in decreased membrane order (15, 26). The small cadmium-induced reductions in plasma membrane order reported here were indicative of a predominance of the latter type of effect and were in agreement with the reported effects of cadmium on *Schizosaccharomyces pombe* plasma membranes (1). Unlike K^+ efflux, the effect of Cd^{2+} on plasma membrane order was apparently no more pronounced in PUFA-enriched cells than in unsupple-

mented cells. Thus, a threshold value for membrane order, below which K^+ efflux occurred, was not discernible. It appears that values for the membrane order parameter (*S*) do not provide a clear linear index of the state of a membrane in relation to either its integrity or its content of lipid peroxidation products. Nevertheless, values for plasma membrane order determined after metal exposure decreased in the same order (unsupplemented cells > linoleate-supplemented cells > linolenate-supplemented cells) as that predicted from metal toxicity and lipid peroxidation experiments. Note that this is also the order to be expected on the basis of differences in initial fatty acid composition, irrespective of the extent of lipid peroxidation (3). In addition to the rapid gross effects on membrane permeability observed at the high metal concentrations used here, smaller changes in membrane physical properties at lower, nontoxic metal concentrations might still elicit changes in cellular activity; the functions of many membrane-bound enzymes are known to be sensitive to the enzymes' lipid environment (21).

The accentuated levels of lipid peroxidation and heavy metal toxicity in linoleate- and linolenate-enriched *S. cerevisiae* cells were consistent with the known susceptibility of PUFAs to oxidation (15, 29). The possibility that the results might also be partly attributable to differences in the physical properties of PUFA-enriched plasma membranes (33) cannot be discounted; in common with studies of higher organisms (34, 35), the fatty acid unsaturation index of *S. cerevisiae* (1.5-fold higher for linolenate- than linoleate-enriched cells) was not linearly related to susceptibility to lipid peroxidation. Sensitivity may relate more closely to initial plasma membrane order.

The potentially major role of membrane lipid peroxidation in heavy metal toxicity to yeast implicated from the present results suggests that changes in a number of highly variable cellular properties, including antioxidant status (e.g., the induction of antioxidant defenses during respiratory adaptation [23, 26]) and membrane fatty acid composition (e.g., changes occurring during environmental acclimation [21]), could seriously alter the cells' ability to cope with heavy metal stress. As well as being of relevance to the stress biochemistry of yeast, these findings may have significant implications for metal-microbe interactions in the natural environment.

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