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Copper Toxicity towards *Saccharomyces cerevisiae*: Dependence on Plasma Membrane Fatty Acid Composition

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One major mechanism of copper toxicity towards microorganisms is disruption of plasma membrane integrity. In this study, the influence of plasma membrane fatty acid composition on the susceptibility of *Saccharomyces cerevisiae* to Cu^{2+} toxicity was investigated. Microbial fatty acid composition is highly variable, depending on both intrinsic and environmental factors. Manipulation was achieved in this study by growth in fatty acid-supplemented medium. Whereas cells grown under standard conditions contained only saturated and monounsaturated fatty acids, considerable incorporation of the diunsaturated fatty acid linoleate (18:2) (to more than 65% of the total fatty acids) was observed in both whole-cell homogenates and plasma membrane-enriched fractions from cells grown in linoleate-supplemented medium. Linoleate enrichment had no discernible effect on the growth of *S. cerevisiae*. However, linoleate-enriched cells were markedly more susceptible to copper-induced plasma membrane permeabilization. Thus, after addition of $Cu(NO_3)_2$, rates of cellular K^+ release (loss of membrane integrity) were at least twofold higher from linoleate-supplemented cells than from unsupplemented cells; this difference increased with reductions in the Cu^{2+} concentration supplied. Levels of cellular Cu accumulation were also higher in linoleate-supplemented cells. These results were correlated with a very marked dependence of whole-cell Cu^{2+} toxicity on cellular fatty acid unsaturation. For example, within 10 min of exposure to 5 μM Cu^{2+}, only 3% of linoleate-enriched cells remained viable (capable of colony formation). In contrast, 100% viability was maintained in cells previously grown in the absence of a fatty acid supplement. Cells displaying intermediate levels of linoleate incorporation showed intermediate Cu^{2+} sensitivity, while cells enriched with the triunsaturated fatty acid linolenate (18:3) were most sensitive to Cu^{2+}. These results demonstrate for the first time that changes in cellular and plasma membrane fatty acid compositions can dramatically alter microbial sensitivity to copper.

Metal-microbe interactions have been the subject of considerable research attention in recent years. Interest has arisen because of the biotechnological potential of microorganisms for metal removal and/or recovery, the possible transfer of accumulated metals to higher organisms in food chains, and the toxicity of heavy metals towards microbial metabolism and growth (13, 18). Metal toxicity towards microorganisms is of environmental concern because of possible inhibition of essential microbe-assisted processes (e.g., biogeochemical cycling). Furthermore, microorganisms may serve as useful models for laboratory-based metal toxicity studies (18). Toxic effects are generally related to the strong coordinating abilities of heavy metals, and they include blocking of functional groups and conformational modification of cellular macromolecules, displacement of essential ions, and disruption of cellular and organellar membrane integrity (13).

Copper is a potentially toxic metal which, at low concentrations, can act as an essential micronutrient for microbial growth, serving as a cofactor for certain enzymes and, as a result of its ability to undergo Cu(I)-to-Cu(II) transitions, playing a role in cellular redox reactions (8, 35). At toxic concentrations, Cu interacts with cellular nucleic acids and enzyme active sites, although a principal initial site of Cu action is considered to be at the plasma membrane (8, 29, 35). Thus, exposure of fungi and yeasts to elevated Cu concentrations can lead to a rapid decline in membrane integrity, which is generally manifested as leakage of mobile cellular solutes (e.g., K^+) and cell death (29). Similar effects reported in higher organisms have now been largely attributed to the redox-active nature of Cu and the ability of Cu to catalyze the generation of free radicals and promote membrane lipid peroxidation (16, 25, 35).

Extensive metal-induced disruption of membrane integrity inevitably leads to loss of cell viability. However, even relatively small alterations in the physical properties of biological membranes can elicit marked changes in the activities of many essential membrane-dependent functions, including transport protein activity (17, 22), phagocytosis (2), and ion impermeability (17). The physical properties of a membrane are largely determined by its lipid composition, and one important factor is the degree of fatty acid unsaturation. Microbial membrane fatty acid composition is highly variable and is influenced by both environmental and intrinsic factors. For example, the unsaturated fatty acid contents of microorganisms generally increase at low temperatures; the low melting temperatures and large physical volumes occupied by unsaturated fatty acids are thought to partially compensate for the lipid-ordering effect of chilling (9, 17, 27). In addition, some variation can be attributed to inherent differences in fatty acid composition between microbial groups. Indeed, microbial fatty acid profiles have proven to be useful taxonomic criteria (38, 40) and can be indirectly correlated with other phenotypic characteristics, including pathogenicity (34). However, despite the known effects of heavy metals on biological membranes, to date no studies have sought to investigate how variation in microbial mem-

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brane lipid composition might alter susceptibility to metal-induced plasma membrane disruption and whole-cell toxicity.

The purpose of the present investigation was to examine the relationship between plasma membrane fatty acid composition and copper toxicity in *Saccharomyces cerevisiae*. *S. cerevisiae* was selected as a model for the following reasons: (i) other aspects of metal-microbe interactions have been well characterized for this organism (13); (ii) studies of membrane biology in lower eukaryotes are most advanced for *S. cerevisiae* (39); and (iii) certain strains are highly amenable to cultural manipulation of fatty acid composition with no detrimental effects on their growth rates (6, 24). The most abundant fatty acids in *S. cerevisiae* are the saturated acids palmitate (16:0) and stearate (18:0) and the monounsaturated acids palmitoleate (16:1) and oleate (18:1) (6, 37, 39). Unlike other fungi, *S. cerevisiae* cannot synthesize polyunsaturated fatty acids. However, certain strains can readily take up and incorporate exogenous fatty acids. By growing *S. cerevisiae* in the presence or absence of a fatty acid supplement, we found that copper-induced plasma membrane permeabilization and whole-cell toxicity increase markedly in cells enriched with polyunsaturated fatty acids.

MATERIALS AND METHODS

Organism, medium, and growth conditions. *S. cerevisiae* NCYC 1383 was routinely maintained on solid YEPD medium, which contained (per liter) 20.0 g of neutralized bacteriological peptone, 10.0 g of yeast extract (Oxoid), 20.0 g of glucose, and 16.0 g of agar no. 3 (technical grade; Oxoid). For experimental purposes, *S. cerevisiae* was grown in 100 ml of liquid YEPD medium, which had the same composition as solid YEPD medium except that it lacked agar and was supplemented with 1% (wt/vol) tergitol (Nonidet P-40; Sigma), in 250-ml Erlenmeyer flasks (tergitol supplementation alone did not affect the fatty acid composition of *S. cerevisiae*). These flasks were incubated at 25°C with orbital shaking at 120 rpm. The experimental flasks were inoculated to an optical density at 550 nm of ~0.1 by using material from 48-h starter cultures. When indicated below, linoleic acid or linolenic acid was added to the desired concentration from filter-sterilized stock solutions solubilized with 5% (wt/vol) tergitol. Cell numbers were determined by using a modified Fuchs-Rosenthal hemocytometer slide after appropriate dilution with distilled water; more than 400 cells were counted in each sample.

Preparation of cell homogenates and plasma membrane isolation. Plasma membranes were purified from whole-cell homogenates by using a method adapted from that of Serrano (32). Cells were harvested during the late exponential or early stationary phase by centrifugation (1,200 × *g*, 5 min) and washed twice with distilled water at 4°C. All subsequent steps were performed at 4°C. Final pellets were resuspended in homogenization buffer (7.5 ml/liter of starting culture) containing 1.6 M sucrose (to aid separation of the plasma membrane from the cell wall), 0.4 M Tris (adjusted to pH 8.5 with HCl), 80 mM EDTA, and 8 mM phenylmethylsulfonyl fluoride. Cells were disrupted by shaking them with 0.5-mm-diameter glass beads (Sigma) for approximately 30 min with a homogenizer (Mickle Laboratories, Guildford, United Kingdom). A portion of the whole-cell homogenate was retained (see below), and the remainder was centrifuged at 700 × *g* for 10 min to remove large debris. The supernatant was centrifuged at 20,000 × *g* for 20 min, and the resulting pellet was resuspended in a solution containing approximately 4 ml of 20% glycerol and 400 μl of 0.2 M phenylmethylsulfonyl fluoride. The membrane suspension was applied to a discontinuous sucrose gradient comprising 2 parts of 43% (wt/vol) sucrose and 1 part of 53% (wt/vol) sucrose. After centrifugation for 6 h at 25,000 rpm in a Beckman type SW40 Ti rotor, the purified plasma membranes were recovered at the 43% sucrose–53% sucrose interface with a Pasteur pipette. After dilution with 4 volumes of water, the membranes were pelleted by centrifugation for 20 min at 21,000 rpm in a type SW40 Ti rotor. The purified plasma membranes were resuspended in 1 ml of buffer [25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) adjusted to pH 7.5 with bis-Tris propane, 0.33 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride] and immediately frozen in liquid nitrogen. Membranes were stored at −70°C and used within 1 month of isolation.

Determination of membrane purity. The purity of plasma membranes in fractions obtained after sucrose density gradient centrifugation and in whole-cell homogenates was determined by assaying for vanadate-sensitive (plasma membrane) and azide-sensitive (mitochondrial) ATPase activities by the methods described by Widell and Larsson (45). Optimal detergent activation of plasma membrane ATPase was achieved by using the procedure of Serrano (32). Protein concentration was determined by the method of Bradford (7).

Lipid extraction and analysis. Lipids were extracted from whole-cell homogenates and plasma membrane-enriched fractions by the method of Bligh and Dyer (5), as modified by Griffiths and Harwood (15). For fatty acid analysis, methyl esters were generated by acid-catalyzed methanolysis (2.5% [vol/vol] H_2SO_4 in methanol) at 70°C for 2 h. After extraction with petroleum ether (bp, 60 to 80°C), aliquots of methyl esters were analyzed by gas-liquid chromatography. Pentadecanoate was used as an internal standard. Separation was achieved by using 10% SP-2330 on 100/120 Chromosorb-WAW (Supelco, Saffron Walden, Essex, United Kingdom) packed into a stainless steel column (1.8 m by 0.3 mm [outside diameter]). Fatty acids were identified by comparison with authentic standards.

Potassium efflux and cell viability. Cells from the late exponential or early stationary growth phase were harvested by centrifugation (1,200 × *g*, 5 min) and washed twice with distilled water. Washed cells were suspended in 40 ml of distilled water to a final density of approximately 5×10^7 cells ml^{-1}, and the suspension was agitated by magnetic stirring. After 2 min, glucose was added to a final concentration of 1% (wt/vol), and after equilibration (5 min), $Cu(NO_3)_2$ was added to the desired concentration (2.5 to 40 μM) (copper nitrate was used throughout this study as NO_3^- is noncomplexing and does not influence the metabolism of *S. cerevisiae*). The extracellular K^+ concentration was measured continuously with a model EE-K K^+-selective electrode (EDT Instruments, Dover, United Kingdom) coupled to a Jenway model 3045 ion analyzer. Cu^{2+}-induced K^+ efflux measurements were corrected for small changes in extracellular K^+ concentration that occurred in control suspensions in the absence of $Cu(NO_3)_2$.

Cell viability was determined as the ability to produce CFU. At specified intervals after the addition of Cu^{2+}, aliquots were removed and, after appropriate dilution with sterile distilled water, plated onto OGYE agar (Oxoid). Colonies were enumerated after 3 days of incubation at 25°C.

Copper uptake. Cells from the late exponential or early stationary growth phase were harvested by centrifugation (1,200 × *g*, 5 min) and washed twice with 10 mM 3-(*N*-morpholino)ethanesulfonic acid (MES buffer), pH 5.5 (the pH was adjusted with 2 M HCl); pH 5.5 was selected as a pH value that was close to the value recorded during short-term K^+ efflux experiments and low enough to preclude extensive formation of hydroxo-metal complexes (19). Cells were suspended to a concentration of approximately 5×10^7 cells ml^{-1} in 50 ml of 10 mM MES buffer (pH 5.5) and were incubated with shaking at 120 rpm and 25°C. After 15 min and where indicated below, glucose was added to a final concentration of 1% (wt/vol). After an additional 15 min of equilibration, $Cu(NO_3)_2$ was added to a concentration of 10 μM. At specified intervals (up to 2 h), samples were removed, harvested by centrifugation, and washed twice with distilled deionized water. Final cell pellets were digested with 0.5 ml of 6 M HNO_3 at 100°C for 2 h. Cell debris was removed by centrifugation, and, after appropriate dilution with distilled deionized water, the copper concentrations of the supernatants were determined by inductively coupled plasma atomic emission spectroscopy, using an Instruments-SA model JY70C apparatus and appropriate standard solutions. Copper was analyzed with a polychromator.

RESULTS

Fatty acid unsaturation of *S. cerevisiae* during growth in unsupplemented medium and linoleate-supplemented medium. Incorporation of exogenous linoleate (18:2) was found to have no adverse effect on batch growth of *S. cerevisiae* (Fig. 1a). The cell doubling time during exponential division in both unsupplemented and linoleate (1 mM)-supplemented cultures was approximately 2.4 h. Cultures reached the stationary phase after approximately 16 h, when the cell density was approximately 1×10^8 cells ml^{-1}. Control cultures confirmed that the presence of tergitol (1%, wt/vol) in the media had no discernible effect on cell division (data not shown).

The cellular fatty acid composition was determined at different stages of growth in the absence and presence of linoleate. For clarity, the results in Fig. 1b are presented as whole-cell fatty acid unsaturation indices (average numbers of double bonds per fatty acid). The unsaturation index of the inoculum was low, 0.50. However, marked increases in fatty acid unsaturation occurred during exponential growth, and this was particularly evident for cells growing in linoleate-supplemented medium. Thus, after 16 h, when cultures were entering the stationary phase and displayed maximal cellular fatty acid unsaturation, the unsaturation indices of unsupplemented and linoleate-supplemented cultures were 0.76 and 1.44, respectively; the higher value was attributable to cellular incorporation of exogenous linoleate (Table 1). Subsequently, a small decline in the unsaturation index of linoleate-supplemented cells was evident, although the unsaturation index of cells in

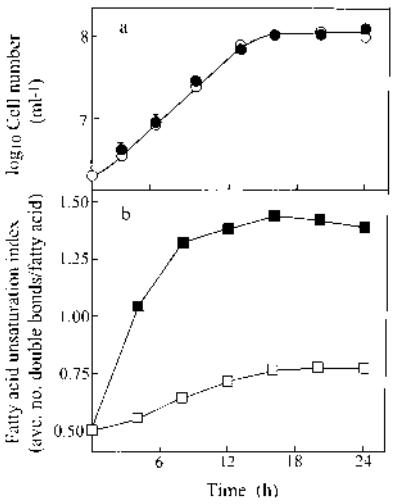

FIG. 1. Fatty acid unsaturation of *S. cerevisiae* during growth in unsupplemented medium and linoleate-supplemented medium. Cells from a 48-h starter culture (lacking fatty acid supplements) were inoculated into fresh medium to a concentration of approximately 2×10^6 cells ml^{-1}. Cell numbers (○ and ●) (a) and unsaturation indices (□ and ■) (b) were determined during growth at 25°C in unsupplemented medium (open symbols) and 1 mM linoleate-supplemented medium (solid symbols) (both media contained 1% [wt/vol] tergitol). The datum points are means from three replicate samples. Standard errors of the means are indicated where they exceed the dimensions of the symbols.

unsupplemented medium remained approximately constant (Fig. 1b).

Fatty acid compositions of whole-cell homogenates and plasma membrane-enriched fractions. The incorporation of exogenous linoleate was maximal after approximately 16 h (the late exponential or early stationary phase). Thus, it was decided to use cells from 16-h cultures for subsequent experiments. As the study was primarily concerned with metal toxicity exerted at the plasma membrane, it was initially necessary to confirm that plasma membrane incorporation of exogenous linoleate also occurred. Plasma membrane-enriched fractions were obtained by differential centrifugation of whole-cell homogenates (see Materials and Methods). The purity of plasma membranes was estimated by assaying for vanadate-sensitive ATPase (plasma membrane marker) and azide-sensitive ATPase (mitochondrial membrane marker) activities; mitochondrial ATPase activity was used as an indicator of nonplasma membrane contamination. The azide-sensitive ATPase activity was higher than the vanadate-sensitive activity in whole homogenates obtained from both unsupplemented and linoleate-supplemented cells (Table 2). However, the specific activity of the azide-sensitive ATPase was 45 to 65% lower in the pellet obtained after sucrose density gradient centrifugation. In contrast, the vanadate-sensitive ATPase activities were 15-fold higher (unsupplemented culture) and 26-fold higher (linoleate-supplemented culture) in the sucrose gradient fraction, indicating that considerable enrichment with plasma membranes occurred (Table 2). The specific activity of vanadate-sensitive ATPase in the plasma membrane-enriched fractions was similar to that observed in plasma membrane fractions isolated by Serrano (32).

The fatty acid compositions of whole-cell homogenates and plasma membrane-enriched fractions from cells grown for 16 h in unsupplemented and linoleate-supplemented media were similar (Table 1). In both cases, the proportions of the monounsaturated fatty acids palmitoleate (16:1) and oleate (18:1) were markedly lower in extracts from linoleate (18:2)-supplemented cells. The higher unsaturation indices of these cells (Fig. 1b) resulted from their very high proportion of linoleate (>65% of the total fatty acids). The incorporation of linoleate into (and the unsaturation index of) plasma membranes was slightly higher than the incorporation of linoleate into (and the unsaturation index of) whole cells. Conversely, in both unsupplemented and linoleate-supplemented 16-h cultures, the palmitoleate content of the plasma membranes was lower than that of whole cells. Linoleate supplementation had little effect on the relative proportions of palmitate (16:0) and stearate (18:0), which represented approximately 20 and 4.5%, respectively, of both the whole-cell and plasma membrane fatty acids (Table 1). Linoleate was not detectable in extracts from cells grown in unsupplemented medium.

A determination of the absolute cellular fatty acid content on a per-cell basis revealed an approximately 1.6-fold-higher content of total cellular fatty acids in linoleate-supplemented cells (601 pmol/10^6 cells) than in unsupplemented cells (986 pmol/10^6 cells). However, the total content of native fatty acids (palmitate, palmitoleate, stearate plus oleate) was reduced by approximately 50% in linoleate-supplemented cells. The linoleate content of these cells was greater than the total fatty acid content of unsupplemented cells (Table 1).

Copper-induced plasma membrane permeabilization in unsupplemented and linoleate-supplemented *S. cerevisiae* cultures. Cellular K^+ efflux was used as an indicator of plasma

TABLE 1. Whole-cell and plasma membrane fatty acid compositions of *S. cerevisiae* grown in the absence or presence of linoleate (18:2)[a]

Fatty acid[b]	Whole-cell homogenate[c]				Plasma membrane fraction[d]	
	Unsupplemented culture		Linoleate-supplemented culture		Unsupplemented culture (%)	Linoleate-supplemented culture (%)
	%	pmol 10^6 cells^{-1}	%	pmol 10^6 cells^{-1}		
16:0	20.2 ± 0.7[e]	121 ± 4	19.6 ± 0.3	193 ± 3	23.4 ± 1.1	17.7 ± 1.1
16:1	55.7 ± 1.4	335 ± 8	5.7 ± 1.0	56 ± 10	50.7 ± 0.8	3.9 ± 0.2
18:0	4.2 ± 0.9	25 ± 5	4.6 ± tr[f]	45 ± tr	5.3 ± 0.9	3.5 ± 0.1
18:1	19.9 ± 0.3	120 ± 2	2.4 ± tr	24 ± tr	20.7 ± 1.1	2.0 ± 0.1
18:2	ND[g]	ND	67.7 ± 0.8	668 ± 8	ND	72.9 ± 1.7

[a] Cells were grown for 16 h in the absence or presence of 1 mM linoleate (with 1% [wt/vol] tergitol in both cases) prior to analysis.
[b] Number of carbon atoms:number of double bonds.
[c] The unsaturation indices (average numbers of double bonds per fatty acid) for the unsupplemented and linoleate-supplemented cultures were 0.76 and 1.44, respectively.
[d] The unsaturation indices for the unsupplemented and linoleate-supplemented cultures were 0.71 and 1.52, respectively.
[e] Mean ± standard deviation from two determinations.
[f] tr, trace (<0.1% or <1.0 pmol 10^6 cells^{-1}).
[g] ND, not detectable.

TABLE 2. Isolation of plasma membrane-enriched fractions from whole-cell homogenates[a]

Culture	Sp act (μmol mg of protein^{-1} min^{-1})			
	Whole-cell homogenate		Plasma membrane fraction	
	Plasma membrane ATPase	Mitochondrial membrane ATPase	Plasma membrane ATPase	Mitochondrial membrane ATPase
Unsupplemented	0.055	0.146	0.799	0.053
Linoleate supplemented	0.026	0.076	0.679	0.042

[a] Homogenates and plasma membranes were prepared from cells previously grown for 16 h in the absence or presence of 1 mM linoleate. The plasma membrane ATPase is vanadate sensitive, and the mitochondrial membrane ATPase is azide sensitive. Typical results from one of several experiments are shown.

membrane permeabilization (13, 28, 29). K^+ efflux from cells previously grown for 16 h in either the absence or the presence of 1 mM linoleate was examined during exposure to a range of $Cu(NO_3)_2$ concentrations (Fig. 2). The initial rate of cellular K^+ release increased with Cu^{2+} concentration. However, at each concentration examined, the K^+ efflux was greater from cells with linoleate-rich plasma membranes than from unsupplemented cells displaying a lower degree of plasma membrane fatty acid unsaturation. This difference was particularly marked at low Cu^{2+} concentrations. Thus, at 2.5 μM Cu^{2+}, K^+ efflux from unsupplemented cells was not detectable, whereas approximately 0.45 nmol of K^+ per 10^6 cells was released from linoleate-enriched cells within 16 min (Fig. 2a). K^+ loss was evident from both unsupplemented and linoleate-enriched cells at 5 μM Cu^{2+}, although the initial rate of K^+ release was approximately fourfold greater in the latter cells (0.07 nmol of K^+ per 10^6 cells per min) (Fig. 2b). At 10 and 40 μM Cu^{2+}, the rates of K^+ release were initially high but declined rapidly, and the total amounts released after 16 min were only approximately 1.5-fold greater from cells with linoleate-enriched plasma membranes than the amount from unsupplemented cells (Fig. 2c and d). However, the initial rate of K^+ release was still approximately two- to threefold greater in linoleate-enriched cells.

Copper uptake by unsupplemented and linoleate-supplemented *S. cerevisiae* cultures. In order to confirm that K^+ efflux in the presence of Cu^{2+} was a result of Cu^{2+} toxicity and loss of plasma membrane integrity rather than physiological exchange of cellular K^+ for external Cu^{2+} (20), Cu^{2+} uptake was examined for 2 h in cells previously grown in the absence or presence of linoleate (Fig. 3). An initial phase of rapid Cu^{2+} uptake (to a concentration of approximately 0.2 nmol of Cu per 10^6 cells) occurred in both unsupplemented and linoleate-enriched cells within 10 min of exposure to 10 μM Cu^{2+} (Fig. 3a). A subsequent slower Cu^{2+} uptake phase was not evident in starved cells incubated in the absence of glucose (data not shown); the levels taken up by these cells were approximately the same as those observed after 10 min in the presence of glucose, did not change with prolonged incubation, and were similar for both unsupplemented and linoleate-supplemented cells (approximately 0.18 nmol of Cu per 10^6 cells). Cu^{2+} uptake by starved cells represented cell surface Cu binding (3), and the value obtained was subtracted from the values obtained for metabolizing cells (Fig. 3a) to give intracellular

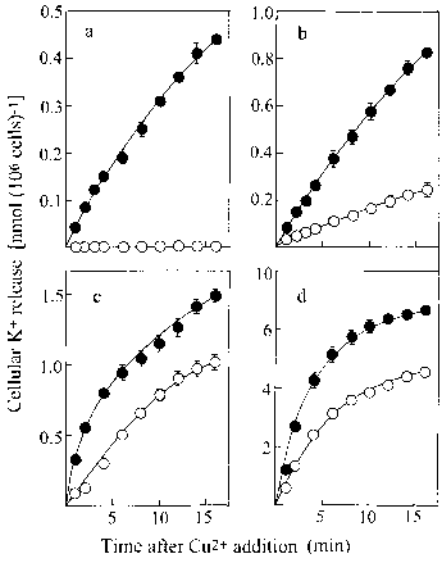

FIG. 2. Copper-induced potassium efflux from unsupplemented and linoleate-supplemented *S. cerevisiae* cultures. Cells were grown for 16 h in the absence (○) or in the presence (●) of 1 mM linoleate and then washed and suspended in distilled water at 22°C. After addition of glucose (1%, wt/vol), Cu^{2+} was added to final concentrations of 2.5 μM (a), 5 μM (b), 10 μM (c), and 40 μM (d). The datum points for K^+ release are means from three replicate experiments. Standard errors of the means are indicated where they exceed the dimensions of the symbols.

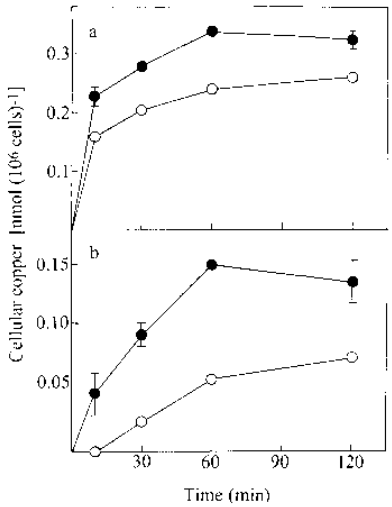

FIG. 3. Copper uptake by unsupplemented and linoleate-supplemented *S. cerevisiae* cultures. Cells were grown for 16 h in the absence (○) or in the presence (●) of 1 mM linoleate and then washed and suspended in 10 mM MES buffer (pH 5.5) at 22°C. Where specified, glucose was added to a concentration of 1% (wt/vol). Cu^{2+} uptake was initiated by adding 10 μM $Cu(NO_3)_2$ to the suspensions. (a) Total cellular Cu^{2+} uptake in the presence of glucose. (b) Intracellular Cu^{2+} accumulation in the presence of glucose, corrected for cell surface binding (determined with starved cells incubated without glucose). The datum points are means from three replicate experiments. Standard errors of the means are indicated where they exceed the dimensions of the symbols.

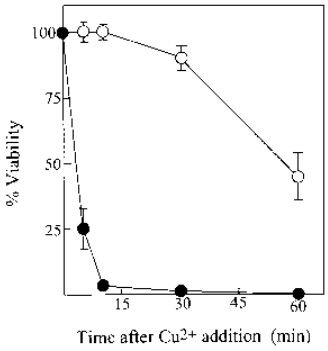

FIG. 4. Effect of copper exposure on viability of unsupplemented and linoleate-supplemented *S. cerevisiae* cultures. Cells were grown for 16 h in the absence (○) or in the presence (●) of 1 mM linoleate and then washed and suspended in distilled water at 22°C. After addition of glucose (1%, wt/vol), Cu^{2+} was added to a final concentration of 5 μM. Cell samples were aseptically removed at intervals, and viability was assessed by the ability of the samples to form colonies on agar. The datum points are means from three replicate samples. Standard errors of the means are indicated where they exceed the dimensions of the symbols.

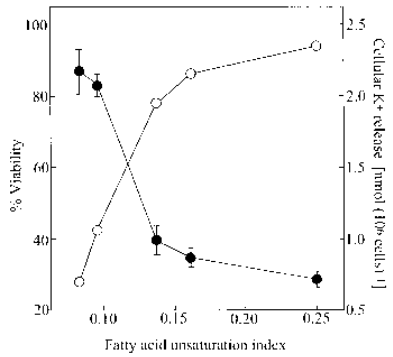

FIG. 5. Effect of copper exposure on *S. cerevisiae* cultures with different degrees of fatty acid unsaturation. Cells were grown for 16 h in the presence of different concentrations of linoleate or linolenate and then washed and suspended in 10 mM MES buffer (pH 5.5) at 22°C. After addition of glucose (1%, wt/vol), Cu^{2+} was added to a final concentration of 5 μM. The levels of extracellular K^+ (○) and viability (●) were determined after 30 min, as described in Materials and Methods. The datum points are means from three replicate samples. Standard errors of the means are indicated where they exceed the dimensions of the symbols.

Cu^{2+} accumulation values (Fig. 3b). The level of intracellular Cu^{2+} accumulated was higher in cells previously grown in the presence of linoleate. Thus, after 2 h of incubation in the presence of 10 μM Cu^{2+}, the intracellular Cu levels in unsupplemented and linoleate-enriched cells had increased to approximately 0.07 and 0.14 nmol 10^6 $cells^{-1}$, respectively (Fig. 3b). These levels were more than 10-fold lower than the levels of cellular K^+ released after only 16 min of incubation at the same Cu^{2+} concentration (Fig. 2c). Moreover, after 10 min, the levels of intracellular Cu^{2+} uptake in linoleate-enriched cells were approximately 30-fold lower than the levels of K^+ released.

Effect of copper exposure on viability of unsupplemented and linoleate-supplemented *S. cerevisiae* cultures. In order to determine whether differential susceptibility to Cu^{2+}-induced plasma membrane permeabilization was reflected in whole-cell toxicity, cell viability (defined as the ability to form colonies on agar) was monitored during incubation of cells in the presence of 5 μM Cu^{2+} (5 μM was selected as an intermediate Cu^{2+} concentration which gave large differences in K^+ efflux from linoleate-supplemented and unsupplemented cells). Cu^{2+}-induced reductions in cell viability were very markedly dependent on cellular fatty acid composition (Fig. 4). Within 10 min after exposure to 5 μM Cu^{2+}, only 3% of linoleate-enriched cells remained viable. In contrast, over the same period 100% viability was maintained by cells previously grown in the absence of linoleate. A decline in the viability of the latter cells was evident with longer exposure times. However, whereas approximately 45% of unsupplemented cells still retained the capacity to form colonies on agar after 60 min of incubation with Cu^{2+}, viable linoleate-enriched cells were not detected at this stage (Fig. 4).

Effect of copper exposure on *S. cerevisiae* cultures with different degrees of fatty acid unsaturation. Growth in the presence of different concentrations of linoleate or in the presence of the triunsaturated fatty acid linolenate (18:3) yielded cells with different degrees of fatty acid unsaturation (linolenate supplementation had no effect on the growth rate of *S. cerevisiae*). Thus, the unsaturation indices of 16-h cells from unsupplemented medium and from medium supplemented with 0.025, 0.1, and 1.0 mM linoleate and with 1.0 mM linolenate were approximately 0.08, 0.10, 0.14, 0.16, and 0.25, respectively. Increases in cellular fatty acid unsaturation indices were associated with increased cellular copper sensitivity (Fig. 5). Within 30 min after exposure to 5 μM Cu^{2+}, release of cellular K^+ was approximately 3.5-fold greater from linolenate-supplemented cells, which displayed the highest unsaturation index (~0.25), than from unsupplemented cells. Cu^{2+}-induced K^+ release did not increase linearly with fatty acid unsaturation. The dependence of K^+ loss on the degree of unsaturation was particularly marked between unsaturation indices of approximately 0.08 and 0.14. At the upper unsaturation index values tested, linolenate-enriched cells (unsaturation index, ~0.25) showed only an 8% greater K^+ release than linoleate-enriched cells (unsaturation index, ~0.16).

The K^+ efflux results were correlated with percentages of viability. Increases in the degree of cellular fatty acid unsaturation were associated with progressive increases in the susceptibility of *S. cerevisiae* to Cu^{2+} toxicity. Thus, the levels of viability after 30 min were approximately 87, 83, 40, 34, and 29% for cells with unsaturation indices of 0.08, 0.10, 0.14, 0.16, and 0.25, respectively. The most marked changes occurred at the lower unsaturation indices tested (Fig. 5).

DISCUSSION

Enrichment of *S. cerevisiae* with the polyunsaturated fatty acids linoleate and linolenate was found to markedly enhance the organisms susceptibility to copper toxicity. The *S. cerevisiae* strain used in this study readily incorporated the exogenous fatty acids tested, and can take up a range of other nonnative fatty acids (6), during simple aerobic growth in fatty acid-supplemented medium. The lower proportion of monounsaturated fatty acids in linoleate-supplemented cells than that in unsupplemented cells was consistent with the regulation of Δ9-desaturase activity in *S. cerevisiae* by microsomal membrane order (6, 24). In agreement with other reports (36), neither linoleate enrichment nor linolenate enrichment had an adverse effect on the growth rate. Thus, the system described in this paper is superior to other methods for manipulation of fatty acid composition, which may rely on anaerobic culturing (43), a low-temperature shift (9), or the use of cerulenin as an inhibitor of fatty acid synthesis (4). These blunt tools generally also affect growth parameters and often a range of other physiological characteristics, thus making it difficult to unequivocally attribute any incidental effects to altered fatty acid com-

position. The marked effect of linoleate supplementation on the cellular fatty acid unsaturation index was a result of the very high cellular incorporation of linoleate (>65% of the total fatty acids at a concentration of 1 mM). The comparable fatty acid compositions determined for whole-cell homogenates and plasma membrane-enriched fractions complement similar results obtained for microsomal (6) and mitochondrial (36) membranes of *S. cerevisiae* during growth in fatty acid-supplemented media. In conjunction with the alterations in absolute fatty acid content that were evident, the results suggest that a reorganization of cellular fatty acids occurs via a common fatty acid pool during growth of *S. cerevisiae* in the presence of linoleate and that the fatty acids are not selectively channelled to any particular cellular compartment(s).

Increases in the degree of fatty acid unsaturation in biological membranes under constant external physicochemical conditions can have profound effects on membrane order and stability. The introduction of a *cis* C-C double bond and a 30° bend into a fatty acyl chain results in the fatty acid occupying an expanded conformation, packing less compactly in a bilayer, and having a lower melting point (17). These properties generally confer a greater degree of disorder, or increased "fluidity," on biological membranes. In addition to effects on specific membrane-dependent functions, large changes in membrane lipid order (resulting from changes in temperature or fatty acid unsaturation) have been correlated with increased passive permeability of membranes to ions such as K^+ and Na^+ (17, 27). However, the loss of K^+ from linoleate-enriched *S. cerevisiae* under nonstressed conditions (in the absence of Cu) is small and is indistinguishable from the loss from unsupplemented cells under identical conditions (30a), which also indicates that *S. cerevisiae* can cope with large changes in membrane fatty acid composition with no obvious deleterious effects.

As has been reported elsewhere (13, 29), exposure of *S. cerevisiae* to elevated Cu^{2+} concentrations was associated with a rapid (within minutes) permeabilization of the *S. cerevisiae* plasma membrane, as detected by extensive loss of cellular K^+. Whereas electroneutral exchange of Cu^{2+} for intracellular K^+ can occur during uptake at nontoxic Cu^{2+} concentrations (11), the nonstoichiometry observed in this study (e.g., approximately 30 mol of K^+ released for each 1 mol of Cu^{2+} accumulated by linoleate-supplemented cells in the presence of 10 μM Cu^{2+}) indicates that there is a loss of membrane integrity. Less toxic metals (e.g., Zn, Co, and Mn) do not induce K^+ efflux of this nature (29). A reduction in the viability of *S. cerevisiae* was evident at 5 μM Cu^{2+}, suggesting that the strain used is at the sensitive end of the Cu toxicity range commonly observed in yeasts (8, 11, 29). However, such comparisons do not take into account the variations in the experimental conditions employed by different workers. The dependence of Cu^{2+}-induced plasma membrane permeabilization on membrane fatty acid composition was most obvious at low Cu^{2+} concentrations. It appears that any protection conferred on cells displaying a low initial degree of membrane fatty acid unsaturation is diminished at Cu^{2+} concentrations so high that a loss of plasma membrane integrity appears to be largely unavoidable.

The apparent biphasic nature of Cu^{2+} uptake by *S. cerevisiae* reported here is consistent with the results of other studies and probably results from rapid cell surface metal binding (or initial accumulation in an intermediate periplasmic Cu pool [23]), followed by intracellular accumulation (11, 13). The observed increase in intracellular Cu^{2+} uptake by linoleate-enriched cells probably resulted from increased passive metal diffusion into the cells with more extensively permeabilized membranes (the possibility that enhanced uptake resulted from membrane fluidity-dependent alterations in cation transporter activity [17, 22] can be discounted as the Cu^{2+} concentrations employed in this study were toxic).

The results of this study clearly revealed a marked dependence of Cu^{2+}-induced plasma membrane permeabilization on the degree of plasma membrane fatty acid unsaturation, which was also reflected in enhanced cellular Cu^{2+} permeation and a very rapid and marked decline in the viability of linoleate- and linolenate-enriched cells. The hypothesis that these results were unlikely to be attributable simply to the presence of nonnative fatty acids in the plasma membrane, irrespective of their degree of unsaturation, was supported by the observed greater Cu^{2+} sensitivity of linolenate (18:3)-enriched cells than linoleate (18:2)-enriched cells; these fatty acids are incorporated to similar extents by *S. cerevisiae* (17a).

Several possible mechanisms for metal-induced membrane permeabilization in microorganisms have been proposed (1, 17, 30, 35). The metabolism dependence of Cu^{2+}-induced membrane disruption observed in this study (data not shown) and elsewhere (29) suggests that the entry of some Cu^{2+} into the cell is a prerequisite for membrane disruption, which therefore cannot result solely from passive metal binding at the cell surface. The toxicity of copper towards higher organisms has been linked to the ability of copper to catalyze the formation of reactive oxygen species, with a resulting acceleration of membrane lipid peroxidation (16, 35). The presence of lipid peroxidation products in biological membranes is known to lead to marked alterations in membrane order and ultimately to a decline in membrane integrity and cell death (12, 21, 31, 35), effects which are similar, indeed, to those observed in this study in the presence of Cu^{2+}. The primary targets of free radicals in biological membranes are polyunsaturated fatty acids (16). Thus, metal-induced lipid peroxidation in higher organisms is often enhanced in cells displaying a high degree of fatty acid unsaturation (16, 26, 42). The protection against Cu toxicity in *S. cerevisiae* conferred by Cu,Zn-superoxide dismutase (10, 14) and anaerobiosis (14) has implied that reactive oxygen species play a role in Cu toxicity towards yeasts. The marked dependence of Cu toxicity on the degree of plasma membrane fatty acid unsaturation in *S. cerevisiae*, reported here for the first time, is fully consistent with and supports the hypothesis that lipid peroxidation plays a role in Cu toxicity towards yeasts.

Although Cu toxicity towards microorganisms is well documented, the precise molecular mechanism(s) of toxicity has yet to be clearly ascertained (8, 13). Similarly, while a number of characteristics which enhance resistance or tolerance to copper have been described (e.g., Cu^{2+} efflux activity, production of extracellular ligands, cell surface precipitation, metallothionein synthesis [8, 33]), microbial survival in polluted habitats can rely on intrinsic properties rather than adaptive changes, and in many cases these mechanisms are unknown. We have shown that copper toxicity towards *S. cerevisiae* increases considerably with increased plasma membrane fatty acid unsaturation. In view of the large inherent differences in membrane lipid composition between (and within) the major microbial taxonomic groups (40, 41, 44), as well as the variation attributable to environmental acclimation (17), it seems reasonable to speculate in light of our results that membrane fatty acid composition may be one important intrinsic characteristic that determines the differential susceptibility of individual microorganisms to copper toxicity.

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