COMPOSITION, STRUCTURE AND PHASE TRANSITION IN YEAST FATTY ACID AUXOTROPH MEMBRANES: SPIN LABELS AND FREEZE-FRACTURE

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1. Fatty acid desaturase mutant yeast cells have been enriched with 2 fatty acids having substantially different physical properties.
2. Differences in fatty acid composition are reflected in the ESR determined phase transitions and the freeze-fracture morphology of the tonoplast.
3. In these cells, fatty acid composition, rather than the position of the phase transition relative to growth temperature, appears to be the more important variable in determining membrane morphology.
4. The freezing process used in the freeze-fracture technique does not appear to cause a demixing of the lipid components of the membrane.

1. INTRODUCTION

One approach to studying the organization of membranes is to modify their composition and study the effect of this modification on the structural and functional characteristics of the membrane. Membranes of varying fatty acid composition have been produced in yeast (1,2), Mycoplasma (3) and *Escherichia coli* (4,5). The effect of fatty acid composition on the physical state and structure of these membranes has been studied by a variety of techniques, including electron spin resonance (ESR) (2,6), x-ray diffraction (7,8), differential scanning calorimetry (9,10) and electron microscopy (6,11). A finding common to many of these studies is the detection of a phase transition in membranes when their composition is sufficiently enriched in a particular fatty acid.

The present study attempts to: 1) produce yeast membranes with a high enrichment in 2 fatty acids with different physical properties, 2) characterize the phase transitions of these membrane using ESR probes, 3) analyze the mor-

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phology of the membranes by freeze-fracture electron microscopy and 4) determine whether any changes in freeze-fracture morphology are the direct result of the altered fatty acid composition or the result of the altered phase transition.

Stearolic acid ($18:1^{\Xi 9}$) and linolenic acid ($18:3^{9,12,15}$) were chosen as fatty acid enrichments because their physical properties differ substantially. Stearolic acid has a melting point of about 45°, a thin-layer chromatographic (TLC) Rf of 0.30 on Silica Gel-G using chloroform as a moving phase, and a relative retention time of 4.0 relative to palmitic acid ($18:1^{\Xi 9}$ time/16:0 time) by gas liquid chromatography (GLC). Linolenic acid has a melting point of -10°, an Rf of 0.8 using the same TLC conditions and a relative GLC retention time of about 2.0. Consequently, it was expected that enrichment in either of these fatty acids would result in yeast cells whose membranes would have very different properties.

2. METHODS

(a) *Organism*

A general characterization of the yeast fatty acid desaturation mutant *ole-1* (KD115) has been carried out (1,12,13) and will not be described here. Aliquots were removed from liquid cultures at stationary phase and plated onto growth-supporting media. The resulting colonies were replica plated onto minimal plates to determine if any of these were revertants.

(b) *Chemicals*

Stearolic acid, 9-octadecynoic acid ($18:1^{\Xi 9}$), was purchased from Lachat Chemicals, Inc., Chicago Heights, Ill. It was 99% pure or better as determined by gas-liquid chromatography of the methyl ester. All *cis*,9,12,15-octadecatrienoic acid ($18:3^{9,12,15}$ all *cis*) was a product of the Hormel Institute, Austin, Minn. The purity was described as 99% or better and was not further tested. Tergitol NP-40, a non-ionic detergent, was obtained from Union Carbide Corp., Institute, W. Va.

(c) *Cultures*

The desaturation mutant *ole-1* was grown in 40 ml or 200 ml of medium in side-arm Erlenmeyer flasks aerated by shaking. Three basic media, all with 1% Tergitol NP-40 were used: 1) YNBD (0.67% yeast nitrogen base and 2% dextrose); 2) YNBL (0.67% yeast nitrogen base and 1% DL-sodium lactate, pH 5.8-6.5 adjusted with K_2HPO_4); 3) YEPD (1% yeast extract, 2% peptone and 2% dextrose). These media were supplemented with 2×10^{-4} M stearolic or linolenic acid to produce enriched cells. (Exception: with the YEPD medium, 10^{-3} M stearolic acid was required for enrichment.) Flasks were innoculated with about 10^5 cells per ml and incubated at 30°. At various points up to mid log phase some flasks were shifted to 18°. Cells were harvested by centrifugation at various times from mid log phase to late stationary phase. The cells were washed once with 1% Tergitol and once with distilled water and then used for fatty acid analysis, ESR spectroscopy or freeze-etching. During harvest and until starting ESR or freezing the organisms were maintained at their growth temperature.

(d) *Electron spin resonance*

Molecular motion is reported as τ_o an imperical molecular motion parameter which was calculated from the expression

$$\tau_o = KW_o\left[\left(\frac{h_o}{h_{-1}}\right)^{1/2} - 1\right]$$

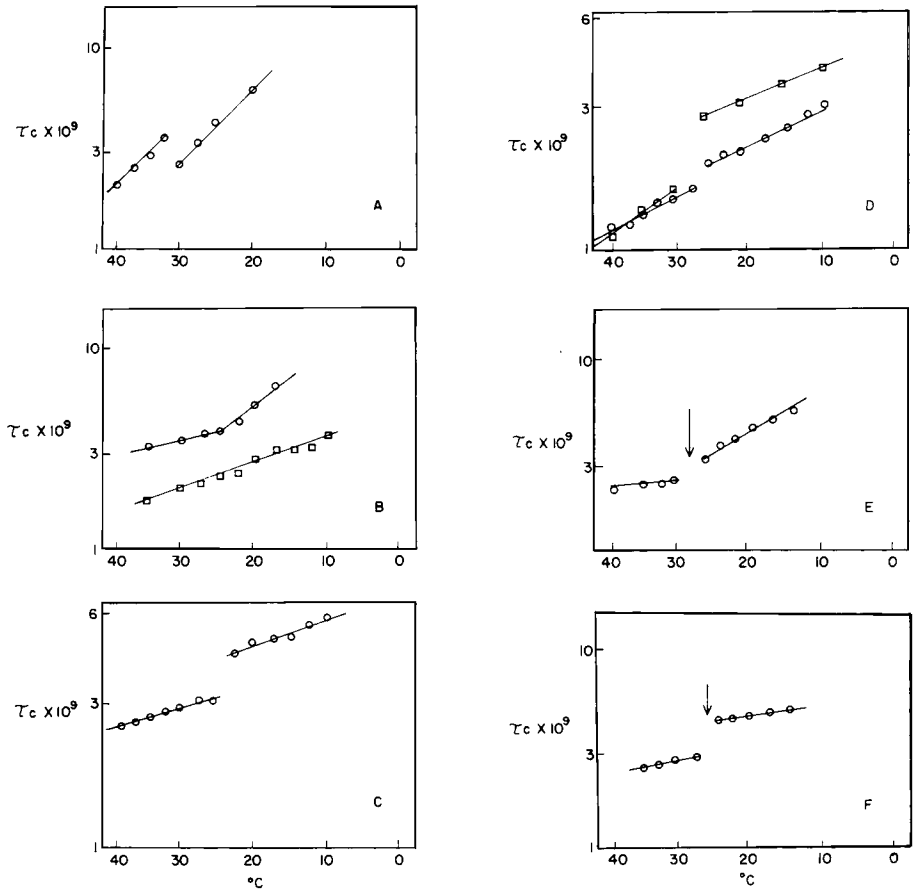


Fig. 1. *Arrhenius plots of the ESR data of 12NS labeled* ole-1 *mutants supplemented with sterolic acid (19:1$^{\Delta 9}$).* A-F represent data from several experiments. The growth conditions and fatty acid analysis for each experiment is given in Table 1, A-F.

where the numerical value of K is unimportant for relative values, W is line width, h is line height and 1, o, and -1 refer to low, mid, and high field lines. The derivation of the equation employs the spectral parameters of Griffith *et al.* (14) and Lorentzian line shapes and is based on work by Kivelson (15). The derivation is identical to that reported by Henry and Keith (2).

A Varian model 4500 electron paramagnetic resonance spectrometer was used. This instrument was equipped with a Varian temperature accessory calibrated with an iron constantan thermocouple accurate to approximately ±1.5°. A detectable destruction of the spin label immediately after labeling indicates that the methyl ester of 12NS penetrates the cell quickly allowing incorporation into all membranes.

(e) *Chromatography*

Gas liquid chromatography was performed on the methyl esters of the isolated fatty acids employing a Varian Aerograph Model 600-D analytical instrument as reported elsewhere (1).

(f) *Freeze-fracture and E.M.*

Samples from the washed pellets were pipetted onto 3 mm cardboard discs. The discs were then quenched in liquid freon cooled by liquid N_2, and stored in liquid N_2. Samples were freeze-fractured on a Balzers apparatus at -110° with no etching (16,18). Replicas obtained in this way were serially treated with 20, 40 and 70% sulfuric acid to remove cellular debris, washed, and then viewed in a Siemens Elmskop 1 using direct magnifications of 4,000 to 24,000. All fracture faces are convex surfaces and shadows are from bottom to top.

3. RESULTS

(a) *ESR and fatty acid enrichment*

ESR and chromatographic determinations were carried out on 23 cultures supplemented with stearolic acid. Fig. 1 shows representative examples of Arrhenius plots of 8 cultures which were *in vitro* spin-labeled with the methyl ester of 12NS. The growth conditions and fatty acid composition of these cultures are shown in Table 1, A-F. The fatty acid composition (Table 1) shows considerable variation as is reflected in the Arrhenius plots (Fig. 1). These show variation in the absolute τ_0 values and in the Arrhenius discontinuities which varied between 22° and 30°. Arrhenius plots shown in Fig. 1 A,C,D,E, and F all have the same general shape, and represent samples having a stearolic acid composition greater than 50%. The type of culture whose Arrhenius plot is shown in Fig. 1B (○), occurred 4 times out of 23 and had stearolate enrichments ranging from 12% to 45% with a relatively high proportion of palmitic and/or stearic acid. The type of culture shown in Fig. 1B (□), occurred twice and tests showed them to be revertants. The fatty acid composition (Table 1B, □) shows relatively high concentrations of palmitoleic and oleic acids which are reflected in the linearity of the Arrhenius plot. The highest enrichment is in sample D (□), and was obtained by stearolate supplementation of wild type. This high enrichment is reflected in the discontinuity of the Arrhenius plot (Fig. 1D, □). Neither the type of growth medium nor the growth temperature appeared to influence the fatty acid composition or the Arrhenius plots of stearolate supplemented cells.

ESR and chromatographic determinations were carried out on 25 cultures supplemented with linolenic acid. Fig. 2 shows representative examples of Arrhenius plots of 7 cultures which were *in vitro* spin-labeled with the methyl ester of 12NS. The growth conditions and fatty acid composition of these cultures are shown in Table 1, G-L. As in the case of stearolate supplemented cultures, there is variation in the fatty acid composition (Table 1, G-L), and this is reflected in the variation of the discontinuities in Arrhenius plots (8°-12°); however, there is not as much variation in the absolute τ_0 values. Arrhenius plots in Figs. 2G, H, J, K and L all have the same general shape and represent samples with linolenate representing greater than 50% of the total fatty acids. Fig. 2I was the only case of the 25 linolenate enriched preparations analyzed where the Arrhenius line has a lower activation energy for temperatures below the intercept. The cells from this experiment also had a high proportion of palmitate and a low proportion of linolenate (Table 1). Neither the type of growth medium nor the growth temperature appeared to influence the fatty acid composition or the Arrhenius plots of linolenate supplemented cells.

In general, cultures with high enrichment in the fatty acid supplement show an Arrhenius plot line shape which is characteristic of that particular supplement. Cultures which show poor enrichment have Arrhenius plots of distinctly different shape.

Preliminary ESR experiments were performed to determine the effect of rapid freezing on the structure of a 2 component system because Phillips *et al.* (17) have suggested that freezing of specimens may cause a demixing of

TABLE 1

Growth conditions and fatty acid analysis of ole-1 *cultures*

Fig.	Medium	Growth phase	Fatty acid supplement	Growth temperature	Fatty acid composition						
					16:0	16:1	18:0	18:1	18:2	18:3	$18:1^{\Xi 9}$
1A	YNBD	early stationary	$18:1^{\Xi 9}$	30°	32	2	8	2	2	--	54
1B (○)	YEPD	early stationary	"	30°	36	1	11	3	2	--	46
1B (□)	YEPD	late log	"	30°	34	25	12	7	--	--	22
1C	YEPD	early stationary	"	30°	36	2	8	2	1	--	51
1D (○)	YNBD	late log	"	18°	32	1	8	2	--	--	57
1D (□)	YNBD	early stationary	"	30°	3	5	2	4	--	--	85
1E*	YEPD	late log	"	30°	38	2	5	2	--	--	53
1F*	YEPD	late Log	"	18°	18	1	3	2	--	--	76
2G	YNBD	late log	18:3	30°	24	3	10	7	--	56	--
2H	YEPD	early stationary	"	18°	19	2	8	2	--	69	--
2I	YEPD	early stationary	"	30°	60	--	12	2	--	26	--
2J	YEPD	mid log	"	30°	21	3	7	6	--	63	--
2K*	YEPD	late log	"	30°	21	--	6	1	--	72	--
2L*	YEPD	late log	"	18°	20	--	5	2	--	73	--

* Freeze-fractured cells shown in Figs. 3-6.

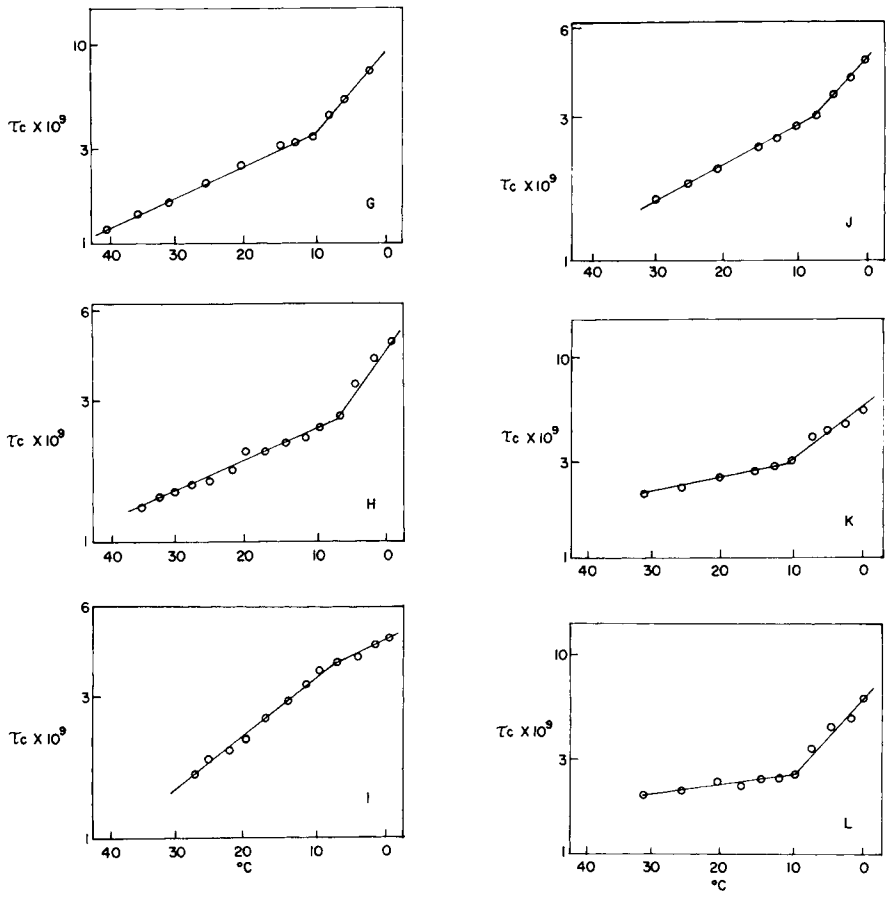
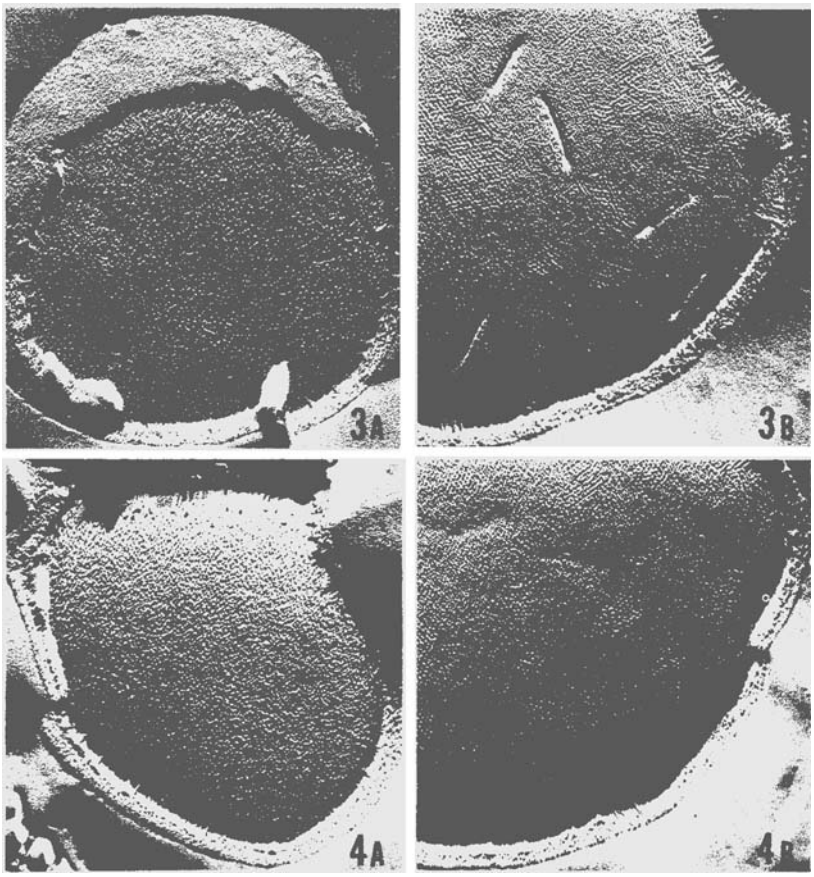


Fig. 2. *Arrhenius plots of the ESR data of 12NS labeled* ole-1 *yeast mutants supplemented with linolenic acid (18:3^{9,12,15} all* cis*).* G-L represent data from several experiments. The growth conditions and fatty acid analysis for each experiment is given in Table 1, G-L.

components as each passes through its separate phase transition (liquid to crystalline). One mmole 12NS in Hormel stearic acid (18:0, 99% pure) gives a clear line signal above the melting point of 18:0. At temperatures below the melting point of 18:0, the 12NS shows drastic exchange broadening indicating that some 12NS molecules have collected in impurity pools. When a similar sample above the phase transition of 18:0 was rapidly cooled to -80° under conditions similar to those employed in the freeze-fracture technique, the 12NS signal was highly immobilized but not exchange broadened. Subsequent warming of this sample to 40° resulted in the gradual exchange broadening of the 12NS signal over about 15 min. These results confirm that heterogeneous lipid mixtures tend to separate during slow cooling. However, rapid cooling of mixtures under conditions employed in freeze-fracture does not appear to result in a separation of components.

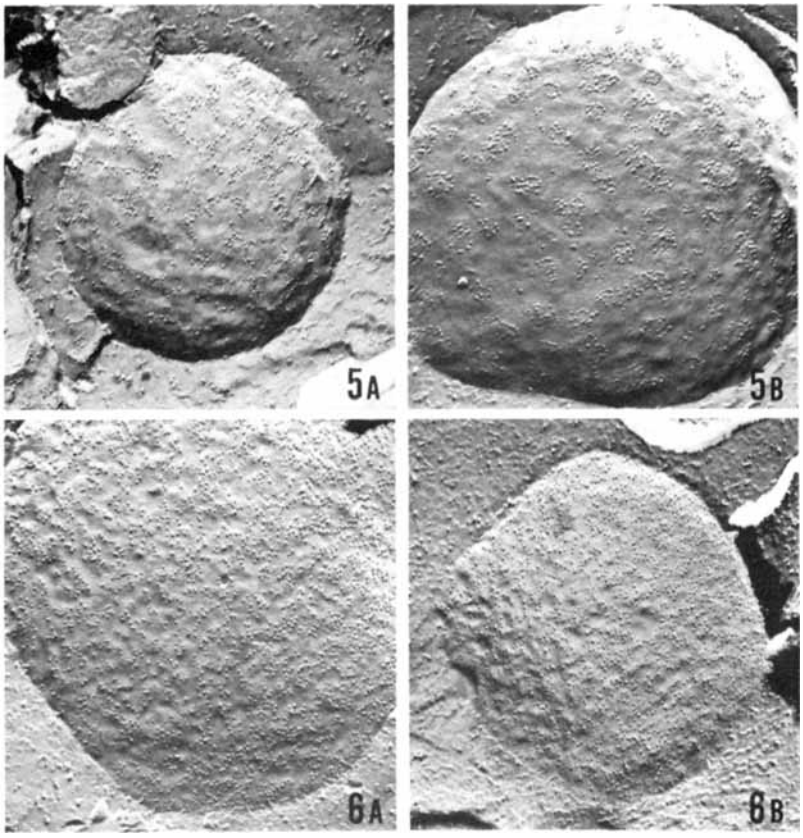


Figs. 3 and 4. *Freeze-fractured plasmalemmas of whole yeast cells enriched in stearolate (Fig. 3) and linolenate (Fig. 4).* A) Cells grown at 18°; B) cells grown at 30°.

(b) *Freeze-fracture*

Freeze-fracture of whole yeast cells enriched with stearolate or linolenate, and grown at 30° and 18°, revealed detectable and clear-cut differences in both the plasmalemma and the tonoplast. Except as noted, the general morphology of the freeze-fractured whole yeast cells was similar to that reported by other workers (18-20) and is therefore not described.

Figs. 3 and 4 show freeze-fractured whole yeast cells enriched with stearolate (Fig. 3) and linolenate (Fig. 4). In each case, the cell wall has been fractured away, and the plasmalemma has fractured so as to reveal the membrane face characteristic of the freeze-fracture technique (16,21). Table 2 summarizes the features of the plasmalemmas shown in Figs. 3 and 4, the ESR data in Figs. 1 and 2, and the fatty acid enrichment in Table 1. Table 2, Figs. 3 and 4 show that growth temperature determines the morphology of the plasmalemma; equally important is the observation that plasmalemmas from cells grown above and below their phase transition show no detectable freeze-fracture differences. They also show that at 30° the fatty acid composition has a minor effect on plasmalemma morphology.



Figs. 5 and 6. *Freeze-fractured tonoplasts of whole yeast cells enriched in stearolate (Fig. 5) and linolenate (Fig. 6).* A) Cells grown at 18°; B) cells grown at 30°.

Figs. 5 and 6 show fracture faces of tonoplasts in yeast cells enriched with stearolate (Fig. 5) and linolenate (Fig. 6). In each case the vacuole shown is contained within a whole yeast cell and has fractured so as to reveal the membrane face characteristic of the freeze-fracture technique (16,21). Table 3 summarizes the morphologies of the tonoplasts in Figs. 5 and 6, the ESR data in Figs. 1 and 2 and the fatty acid enrichment in Table 1. Table 3, and Figs. 5 and 6 show that, in contrast to the plasmalemma, the most important parameter in determining vacuole membrane morphology is the fatty acid supplement rather than growth temperature. However, the degree to which particles are aggregated does increase slightly above the phase transition, and about 10% of the tonoplast fracture faces from stearolate-enriched cells grown at 30° are unique in that they exhibit large flattened areas with particles confined to the ridges defining the flattened areas. In some, the particles were arranged in hexagonal arrays.

When the stearolate enrichment was poor, due to revertants,(Table 1B, □) and a large amount of other unsaturates were present, the particles were dispersed as in Fig. 6. If enrichment was poor in non-revertants and little or no unsaturates were present (Table 1B,○), the particles aggregated as in Fig. 5.

TABLE 2

Data summary, plasmalemma

Parameter	Growth temperature 18°	30°
Stearolate enrichment	76%	53%
Phase transition	25°	28°
Growth temperature	Below transition	Above transition
Particles	Random only	In hexagonal arrays and random
Invaginations	Rare	Frequent, narrow
Linolenate enrichment	73%	72%
Phase transition	9°	10°
Growth temperature	Below transition	Above transition
Particles	Random only	In hexagonal arrays and random
Invaginations	Rare	Frequent, broad

TABLE 3

Data summary, tonoplasts

Parameter	Growth temperature 18°	30°
Stearolate enrichment	76%	53%
Phase transition	25°	28°
Growth temperature	Below transition	Above transition
Relief	Irregular	Irregular, many with large flattened areas
Particles	Aggregated	Very aggregated
Linolenate enrichment	73%	72%
Phase transition	9°	10°
Growth temperature	Above transition	Above transition
Relief	Irregular	Irregular
Particles	Random	Random

4. DISCUSSION

Using fatty acid desaturase mutants, we have grown yeast cells whose membranes are enriched with either stearolate or linolenate. The fatty acid enrichment of these membranes is reflected in their ESR spectra, their phase transitions and in their structural features; fatty acid composition, rather than the position of the phase transition relative to growth temperature, appears to be the more important variable in determining membrane morphology.

The use of a spin label motion parameter offers a convenient and simple method for observing phase transitions in samples of molecular dimensions. We have defined a phase transition as a non-linearity of a spin label motion parameter displayed on an Arrhenius plot and treat this as a phenomenon, without further quantitative description.* It was previously observed that Arrhenius plot non-linearities of sweet potato mitochondria and rat-liver mitochondria occur at temperatures coincident with discontinuities in oxygen uptake and chilling sensitivity of the organism from which the mitochondria were extracted (24). Characteristic Arrhenius discontinuities depending on the average chain length of yeast mutant fatty acids (2), and coincidence between Arrhenius plot discontinuities and optical melting point of fatty acids have also been observed. These coincidental occurrences indicate that some structural change (or changes) occurs at the temperature where the activation energy of spin label motion changes. For present purposes it is sufficient to consider the discontinuities in Arrhenius plots as an event which correlates with other physical and biological parameters.

In our experiments lipid supplements but not the nature of the basal growth medium had reproducible effects on the fatty acid composition (Table 1), ESR determined phase transition (Table 1, Figs. 1 and 2) and freeze-fracture faces of yeast *ole-1* cultures. Under these same conditions, growth temperature has no detectable effect on fatty acid composition (Table 1) or ESR determined phase transition (Table 1, Figs. 1 and 2). However, growth temperature is reflected in the freeze-fracture faces of yeast plasmalemmas (Figs. 3 and 4, Table 2). The plasmalemma fracture face shown for stearolate supplemented cultures grown at 30° is identical to that reported by Moor and Muhlethaler (18) and others (19,20) for wild type yeast grown at unspecified temperature. These authors reported that young cells show no invaginations while older cells show an average of 15 per square micron. Our data and the general observations of other workers would indicate that the effect of temperature on plasmalemma morphology is not the result of a direct physical effect of temperature on structure but probably an indirect reflection of the greater growth and metabolic rate at the higher temperature.

It was previously shown that *ole-1* yeast mutants could be enriched with a particular unsaturated fatty acid by supplementing the growth medium with that same fatty acid (1). Later results with the mutants (25) indicated that linolenate- and stearolate-rich cells could be attained at 18° as well as at 30°. Our results confirm this and also show that changes in fatty acid composition alter membrane lipid mobility and tonoplast morphology. Yeast cells with a high enrichment in stearolate give a characteristic Arrhenius plot as

* The line shapes which result from the methyl ester of 12NS are sufficiently broad that the origin of the Arrhenius non-linearity cannot presently be ascribed to viscosity changes. Spin labels having aliphatic chains such as 12NS may exist in equilibrium between a bent conformer where the n-oxyl group is localized in polar zones and an extended conformer where the n-oxyl group is localized in hydrocarbon areas (22). This mixed condition causes line broadening resulting from heterogeneity of the n-oxyl group's local environments. Consequently, a change in partitioning between the bent and extended conformers may be the cause of, or a contributor to, the Arrhenius discontinuity. This will be treated in detail elswhere (22,23).

do those with a high enrichment of linolenate. At a given temperature the Arrhenius plot characteristic of stearolate showed reduced spin label motion compared to that of the plot characteristic of linolenate. Also the average point of discontinuity in the stearolate plots is 25° while that in the linolenate plot is 10°. These findings were substantiated by examining cultures showning poor stearolate or linolenate enrichment where the Arrhenius plots were not of the characteristic type for the corresponding fatty acid supplement. These results are consistent with other studies (2,6-9) which have found that fatty acid composition affects the temperature of phase transition.

Fatty acid composition also affects the morphological features of the plasmalemma and vacuole. In the case of the plasmalemma, the effects appear to be quite minor, a slight change in the structure of the invaginations as seen in Figs. 3 and 4 and noted in Table 2. In the case of the vacuole there are dramatically aggregated particles in stearolate enriched cells (Fig. 5). Tourtellotte *et al.* (6) and James and Branton (11) observed aggregated particles in freeze-fractured Mycoplasma membranes enriched in stearate, while membranes enriched in oleate or linoleate showed evenly distributed particles. Tourtellotte *et al.* (6) reported ESR data showing that growth at 37° was above the lipid phase transition for oleate enriched cells but below the lipid phase transition for stearate enriched cells. In our yeast cells the most important factor influencing particle distribution is fatty acid composition (Table 3). However, as indicated in Table 3, a significant number of stearolate enriched cells growing above the phase transition showed a further modification over those growing below the phase transition. The particles were more aggregated and the membranes were flattened. These modifications may represent a conformational transition of the membrane components into a "preferred" state at temperatures above the phase transition. A more complete series of the various vacuolar profiles observed is available (26).

Revertant cultures with high concentrations of unsaturates have tonoplast fracture faces similar to those for linolenate enriched cells (Fig. 6). Those which are not revertants but are low in unsaturates and high in saturates show particle aggregation similar to that for stearolate enrichment. These freeze-fracture observations on cultures with poor enrichment suggest that the fatty acid composition of the whole cells is indicative of that for the vacuoles.

Detectable differences in freeze-fracture faces were observed only in the plasmalemma and tonoplast. There may be corresponding changes in other membranes, but their scarcity under these experimental conditions or, in the case of mitochondria, difficulty in characterizing even the wild type membranes by freeze-fracture led to inconclusive data.

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