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PERTURBATIONS OF THE DYNAMICS OF LIPID ALKYL CHAINS IN MEMBRANE SYSTEMS: EFFECT ON THE ACTIVITY OF MEMBRANE-BOUND ENZYMES

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SUMMARY

Cellular and membrane systems that are well defined with respect to lipid compositions were obtained from cultures of unsaturated fatty acid auxotrophs of *Saccharomyces cerevisiae* supplemented with specific 18-carbon unsaturated fatty acids.

Lipophilic spin labels were used to probe the physical state of the lipids in the resulting membrane systems. Arrhenius plots for the O_2 uptake of cells grown on lactate, and for the motion of a spin label in the lipid moieties of these cells display discontinuities at the same temperature. The characteristic temperature which defines the change in slopes in these plots is dependent upon the physical characteristics of the unsaturated fatty acid that supplemented a given culture. It is inferred that the changes in slopes originate from temperature-dependent changes in the physical state of lipid moieties.

Adamantane, a hydrophobic quasi-spherical molecule, was used as a lipophilic perturber to cooperatively hinder the axial ordering of phospholipid alkyl chains. Experimental evidence indicates that adamantane lowers the transition temperatures detected from the temperature dependence of the motion of spin labels and from the Arrhenius plots of O_2 uptake.

Additional experiments were carried out to determine the effect of other perturbing molecules and of mixtures of unsaturated fatty acids on the temperature dependence of spin-label motion.

The approximate coincidence of transition temperatures in both O_2 uptake and spin-label experiments infers that membrane-dependent physiological functions are dependent upon the physical state of membrane lipids.

INTRODUCTION

Structures resembling the lamellar phase characteristic of phospholipid disper-

sions in excess water have been detected in a variety of membrane systems. For example, X-ray diffraction studies of membranes of *Mycoplasma laidlawii* [1] and *Escherichia coli* [2] have demonstrated the presence of layered structures of lipids characterized by close packing of the fatty alkyl chains. Similarly, spin-label studies of erythrocytes, oriented with respect to the laboratory magnetic field by hydrodynamic shear [3], and of sarcoplasmic reticulum vesicles, oriented by prolonged centrifugation [4], have demonstrated that a majority of lipid molecules are oriented in arrays perpendicular to the membrane surface.

The lamellar phase of lipids dispersed in excess water undergoes temperature-induced transitions [5, 6] which preserve the lamellar character but affect the close order spacing of alkyl chains. Similar transitions have been detected in many biological membrane systems [1, 2, 5, 7–12] as well as in aqueous dispersions of lipids extracted from these membranes. The temperatures of these transitions have been correlated with the temperature dependence of the activities of several membrane-bound enzymes in mitochondria [8], in hepatic microsomes [11], in *E. coli* [12], and in sarcoplasmic reticulum [13]. These transition temperatures, which probably reflect changes in the energy barriers between ordered and disordered states of lipid methylene groups, are indicative that lipid ordering is an important factor in determining the functional properties of membrane-bound enzymes. Transition temperatures are also a convenient parameter with which to characterize the apolar interactions in the membranes. In this study, as previously [9], we use unsaturated fatty acid auxotrophs of yeast with relatively controlled fatty acid composition. These enriched cells show characteristic temperature dependences of spin-label motion and O_2 uptake on the nature of the unsaturated fatty acid supplementing the growth medium.

As a convenient model system we used sonicated vesicles of asolectin and micelles of sodium dodecylsulfate.

MATERIALS AND METHODS

Growth of yeast cultures

Two mutants of *Saccharomyces cerevisiae* were used in this study. They have been described genetically and biochemically previously [14]. KD115 is a respiratory competent mutant and KD46 is a nuclear petite. They are both deficient in fatty acid Δ^9 desaturase activity, and the only unsaturated fatty acids found in their membrane systems are those added to the growth media. KD115 was grown to late logarithmic phase in 25-ml cultures in 125-ml flasks at 30 °C, either in a reciprocating water bath shaker or in a gyrorotatory incubator shaker. The media contained 1% yeast extract (Difco), 2% bactopeptone (Difco), 1% DL-sodium lactate, 1% Tergitol NP-40 (Union Carbide) made to pH 6.0 with K_2HPO_4. The fatty acids were added at a level of 10^{-4} M. Stearolic acid ($18{:}1\Delta^{9\equiv}$) was dissolved in 70% ethanol and added in a volume not greater than 0.5% of the total volume of the medium. KD46 was cultured in the same way, except 2% dextrose was used instead on 1% DL-sodium lactate. In experiments where mixtures of fatty acids were added, the secondary fatty acid was added in 5 moles%. For example, the data presented in Fig. 5 is from KD46 grown on $1 \cdot 10^{-4}$ M $18{:}1\Delta^{9\equiv}$ plus $5 \cdot 10^{-6}$ M of either $18{:}1\Delta^{6}cis$ or $18{:}1\Delta^{11}cis$ (therefore 5 moles% of the secondary fatty acid). The presence of adamantane did not affect the growth rate or cell yield.

Stearolic acid was obtained from the Lachat Co. and was assayed by thin-layer chromatography as better than 99%. All other fatty acids were obtained from the Hormel Institute with purity claimed as better than 99%. These were not further analyzed. Adamantane and butylated hydroxytoluene were obtained from the Aldrich Chemical Co. Adamantane was dissolved in 70% ethanol and was added at a concentration of about $5 \cdot 10^{-4}$ M in a volume of ethanol which was also approx. 0.5% of the total volume of media. A saturated solution (approx. 10^{-1} M) of adamantane was made with hot 70% ethanol.

The yeast inoculum was added by suspending the cells in autoclaved distilled water and by adding an aliquot to the cultures. This produced an initial concentration of about 10^5 cells/ml of media. Growth of the culture was monitored turbidimetrically with a Klett–Summerson photometer. When the cells reached late logarithmic phase (about 375 Klett units, or 0.75 *A*), they were harvested by centrifugation, washed once with 1% Tergitol NP-40 solution, then twice with phosphate buffer at pH 4.5. Before washing, an aliquot was taken from the culture to test for the presence of revertants by serial dilution followed by replica plating. The washed cells were either used directly in the O_2 uptake measurements on KD115 or they were shaken overnight in phosphate buffer to deplete them of endogenous substrates.

Preparation of cells for oxygen uptake

Temperature dependence of the O_2 uptake was measured with a Clark electrode (Yellow Spring Instruments), refrigeration unit (Varian, Walnut Creek, Calif.) and a constant temperature water circulator (Haake). The harvested cells were pelleted, washed once with 1% Tergitol and twice with a 0.1 M phosphate buffer at pH 4.5. O_2 uptake ($8 \cdot 10^7$ cells/ml) was measured in a 3.0-ml vessel with 0.3 mM DL-sodium lactate as the carbon source. The O_2 electrode was calibrated with air-saturated water at various temperatures. The concentration of O_2 in the air-saturated water was calculated from published tables with the assumption of a constant atmospheric pressure of 754 mm Hg.

Preparation of yeast fatty acids for gas–liquid chromatography

The washed cells from each 25-ml culture were saponified in 5 ml 2 M KOH, with an appropriate amount of heptadecanoic acid in methanol added as an internal standard. The samples were refluxed for 1 h, then 10 ml redistilled light petroleum was added, and the non-saponifiable material extracted (Vortex mixer) into the light petroleum phase, which was discarded. The remaining aqueous layer was acidified with 1 ml 5 M H_2SO_4, and 10 ml light petroleum added. The fatty acids were extracted into the light petroleum layer and then methylated by adding 0.2 ml of concentrated H_2SO_4 in 5 ml methanol and heating to boiling for about 1 h (0.5 ml final volume) After cooling, 1 ml water and 2 ml light petroleum were added. The methyl esters were extracted into light petroleum, the light petroleum evaporated under N_2, and the methyl esters dissolved in iso-octane before gas–liquid chromatography.

The mole% determinations were carried out on a Perkin–Elmer model 900 gas chromatograph equipped with a stainless steel 3 mm inside diameter by 200 cm long column packed with Chromosarb W 60/80 mesh, acid washed, coated with 15% diethyleneglycol succinate. Column temperature was maintained at 180 °C. Peak areas were determined by triangulation and by cutting and weighing the appropriate

part of the chart paper. By employing a quantitative fatty acid standard (Hormel Institute, Austin, Minn., GLC 15) it was determined that major peaks were within 5% of the claimed percentages and minor peaks were within 12%.

Preparation of phospholipid vesicles

Aqueous dispersions of phospholipids (50–100 mg/ml) in a Tris buffer at pH 7 were sonicated for 90 min in an ice-bath. The sonic disrupter (Sonifier W185D, Heat Systems Ultrasonics) was operated at 60 W. Thioglycerol was added as an antioxidant. The preparation was then centrifuged at 105 000 $\times g$ for 30 min in a Beckman L2-65B centrifuge. The supernatant was divided into 10-ml aliquots and perturbing molecules, dissolved in 95% ethanol, were added to these aliquots to give the desired molecular ratios. These were sonicated for 7 min at 30 W to effect mixing and were centrifuged again for 30 min at 105 000 $\times g$ to separate aggregates that may have formed from the suspensions.

Spin-label studies

The synthesis of the lipophilic molecular probe methyl-12-nitroxide stearate has been described already [15]. The probe molecules were added to all preparations at an appropriate concentration of 1 per 200 alkyl chains or 1 per 100 phospholipid molecules. They were dissolved first in ethanol at a concentration of $3 \cdot 10^{-3}$ M, then mixed with the membrane preparations, either directly or after evaporation of the ethanol. The measured spectra were the same with either method.

The spin-labeled probe methyl-12-nitroxide stearate is largely insoluble in aqueous solution. It is soluble in membrane systems, however, and its paramagnetic spectra exhibit the same solvent and polarity effects as do spectra obtained in solvents such as octadecane and other aliphatic hydrocarbons [16]. From this, and from studies in aqueous lipid dispersions, it is inferred that methyl-12-nitroxide stearate is incorporated into the hydrophobic regions of lipid moieties. The probe is far from spherical; however, the measured spectra are approximately the same as those measured in the case of isotropic motion of a quasi-spherical nitroxide whose rotational correlation time may be derived from spectral paremeters using the formula

$$\tau_c = K(W_{-1} - W_0) \tag{1}$$

K is obtained from the spin-label crystal parameters, W denotes a first derivative line width and the subscripts -1 and 0 refer to the high and midfield lines, respectively. This formula is relatively valid as long as τ_c is $< 10^{-9}$ s and the probe concentration is low. Since in this case the motion is not strictly isotropic the local probe concentration is not very low and since the derived τ_c values are of the order of $2 \cdot 10^{-9}$ s, the analysis does not apply strictly. It is possible still to use Eqn 1 to define an empirical motion parameter R_i with which to describe this quasi-isotropic motion:

$$R_i = W_{-1} - W_0 \tag{2}$$

where we have set the constant K of Eqn 1 to 1. With the assumption of Lorentzian line shapes, the equation for the empirical motion parameter becomes

$$R_i = W_0 \left[(h_0/h_{-1})^{\frac{1}{2}} - 1\right] \tag{3}$$

where h refers to first derivative line heights. While this parameter describes the motion

of methyl-12-nitroxide stearate empirically, it also describes, indirectly, the physical characteristics of the solvent matrix with which the probe interacts. This and similar probes have been used in conjunction with this empirical method of analysis of the EPR spectra to derive information on the physical state of membrane hydrophobic regions [8–11].

The electron paramagnetic resonance spectra were recorded on a modified JEOL ME-1X spectrometer equipped with a variable temperature unit. The sample temperature was estimated with an accuracy of $\pm$ 0.5 °C.

RESULTS AND DISCUSSION

Perturbation of phospholipid vesicles: Turbidity studies

The object of these measurements was to investigate whether the solubilization of perturbing molecules in the hydrophobic regions of phospholipid vesicles would result in gross perturbations of vesicular geometry and size. Adamantane was chosen as the perturbing molecule because it is a saturated hydrocarbon of quasi-spherical shape. It is not water soluble and, if present in lipid alkyl regions where it is soluble, it would be expected to disrupt the hexagonal packing of methylene groups which characterizes the bilayer phase of phospholipids.

Fig. 1 summarizes the results of turbidity measurements on suspensions of asolectin vesicles in which various concentrations of adamantane were solubilized. Since turbidity stayed constant on adamantane no gross perturbations of the size or the geometry of the vesicles were detected.

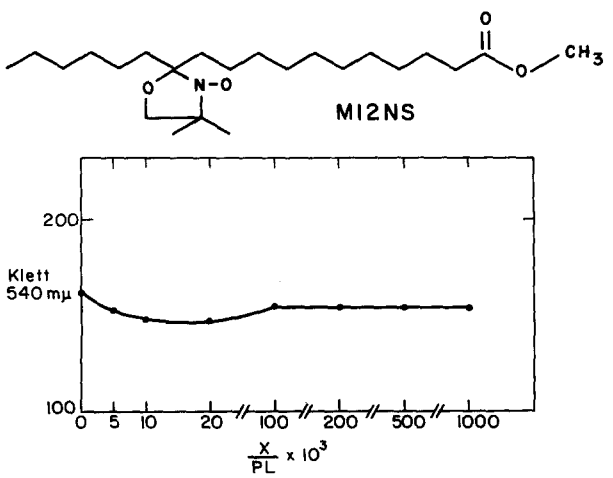


Fig. 1. Light scattering from aqueous suspensions of sonicated phospholipid vesicles containing hydrophobic molecules solubilized in alkyl regions at various concentrations.

To verify that the interactions between adamantane and alkyl chains do result in disruption of the ordering of methylene groups, we repeated these measurements with sodium dodecylsulfate micelles instead of lipid vesicles. The geometry of micelles is expected to be much more sensitive to the packing of the methylene groups than

that of bilayer (or multibilayer) structures. Fig. 2 shows the results of turbidity measurements on suspensions of sodium dodecylsulfate micelles in which various concentrations of perturbing molecules were solubilized. It is clear from the optical absorption data that the perturbers used, adamantane and butylated hydroxytoluene, perturb the geometry of the micelles. Apparently, hexane does not affect the geometry as it is able to orient along the axes of the molecules forming the micelles. In that case the character of the packing of methylene groups is not expected to differ. Note that according to these turbidity measurements, micelle size increases with the addition of an effective perturber until each micelle contains an average of one perturber molecule, i.e. about one perturber per 65 matrix molecules.

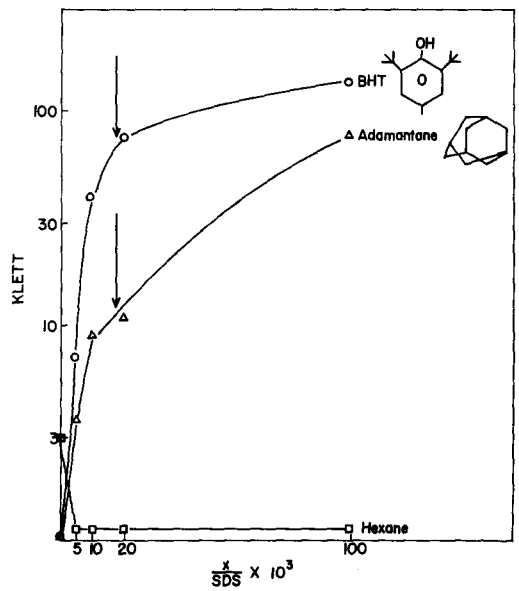


Fig. 2. Light scattering from aqueous suspensions of sonicated sodium dodecylsulfate micelles. Various concentrations of perturbing molecules were solubilized in the micelles as indicated. Butylated hydroxytoluene (BHT) (○–○); adamantane (△–△), and hexane (□–□).

Perturbation of phospholipid vesicles: Spin-label studies

The transition temperature associated with the reordering of lipid alkyl chains depends on the position and nature of unsaturated sites [9–12]. It was concluded that these unsaturated sites interfere with the packing and ordering of alkyl chains. Thus it was reasonable to assume that other means of interfering with the ordering of alkyl chains may be equally effective in perturbing alkyl interactions. The solubilization of a molecule such as adamantane in lipid hydrophobic regions is expected to perturb the axial symmetry of packed alkyl chains and, therefore, the transition temperature associated with the reordering of these chains.

The spin probe methyl-12-nitroxide stearate was used to study the temperature dependence of the fluidity of hydrocarbon regions in pure asolectin vesicles and in vesicles containing adamantane. Fig. 3 shows the temperature dependences of the motion parameter R_i of methyl-12-nitroxide stearate in asolectin vesicles and in aso-

lectin vesicles containing adamantane. The concentration of adamantane added is 1 per 20 lipid molecules. Both curves exhibit abrupt changes in slopes on either side of a transition temperature. Similar transition temperatures detected in the same manner in other membrane systems were interpreted in terms of order–disorder transitions in the lipid alkyl chains [9–13], and correlated with the results of calorimetric and other measurements [5, 11, 13].

It is clear from the data shown in Fig. 3 that the presence of one molecule of adamantane per 20 phospholipids is sufficient to perturb the ordering state of lipid alkyl chains and to lower the transition to a lower temperature. This is consistent with our hypothesis that adamantane would disrupt the axial symmetry of alkyl chains and hinder the orderly hexagonal packing of methylene groups that this axial symmetry implies. It is of interest that we have observed the lowering of the transition temperature at even lower concentrations of adamantane. Hexane, in contrast, does not lower the transition temperature when present at these concentrations.

In the case of the vesicles containing adamantane, the slope above the transition temperature is nearly the same as that of the vesicles without adamantane. This indicates that adamantane interferes little with the disordered state (melted state) and that it acts mainly as a symmetry disrupter of the solid state. We have also used butylated hydroxytoluene to disrupt asolectin vesicles. This molecule, however, has a hindered hydroxyl which may to a limited extent participate in amphiphilic interactions. The resulting disruption reflected by the data shown on the Arrhenius plot is not as simple as that shown in the case of adamantane, presumably due to the dual nature of the hydrophobic and amphiphilic character, although at the same concentration butylated hydroxytoluene lowers the transition temperature about 10 °C.

Perturbation of alkyl chain interactions in biological membranes

Fig. 4 shows plots of R_i for methyl-12-nitroxide stearate in KD115 yeast cells grown on lactate and enriched with oleic acid, with and without adamantane. The transition temperature was lowered in the cells grown with adamantane in the growth medium. We assume that adamantane molecules are solubilized in membrane hydrophobic regions during uptake by the organism of the oleic acid supplementing the growth medium. It is not known in what concentration the perturbing molecules are present in the membranes. The fatty acid compositions of the organisms, obtained from growth media with and without adamantane, respectively, were determined by gas–liquid chromatography. No appreciable differences were noted. Enrichment in the supplemented unsaturate was in all cases between 75 and 79% of total fatty acid composition.

Unsaturated sites as effective perturbers

In membrane systems homogeneous in position and nature of unsaturated sites in the alkyl chains, the distance between these sites and polar regions helps to determine the transition temperature [9]; consequently this leads to the question of whether a small fraction of other unsaturated sites, at a different position from that of the primary ones, will perturb the ordering of lipid chains. Fig. 5 shows data obtained from the motion of methyl-12-nitroxide stearate in yeast cells (KD46) enriched with $18{:}1\Delta^{9\equiv}$ plus 5 moles% of either $\Delta^{6}cis$ or $\Delta^{11}cis$ octadecenoic acids. Fig. 6 shows the results of similar experiments where oleic acid ($18{:}1\Delta^{9}cis$) is used instead

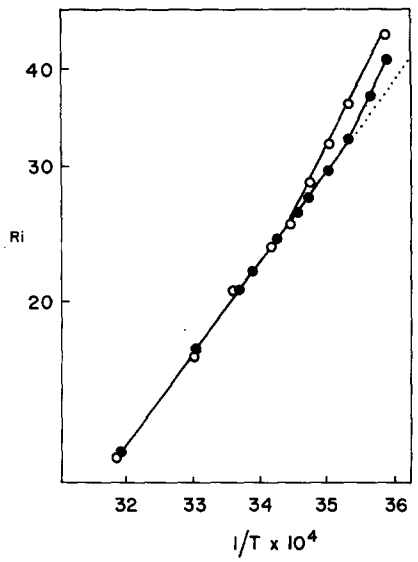
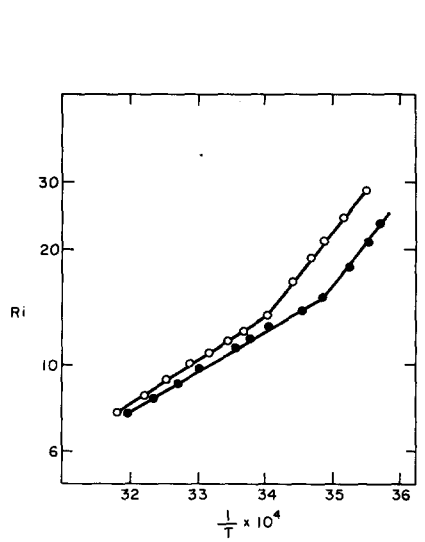


Fig. 3. Temperature dependence of the motion parameter (R_1) of the spin-labeled probe in sonicated aqueous dispersions of asolectin vesicles (○) and asolectin vesicles with adamantane (●). The molar ratio of adamantane to asolectin is 1:20.



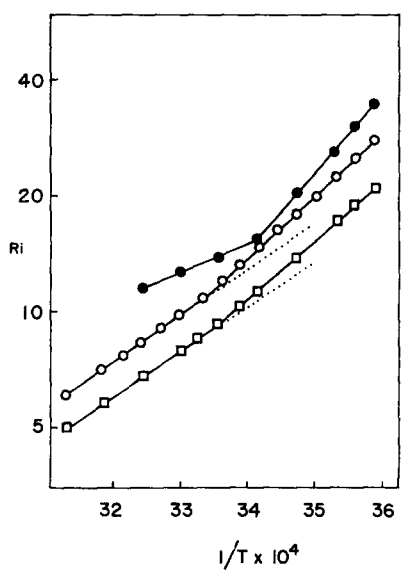
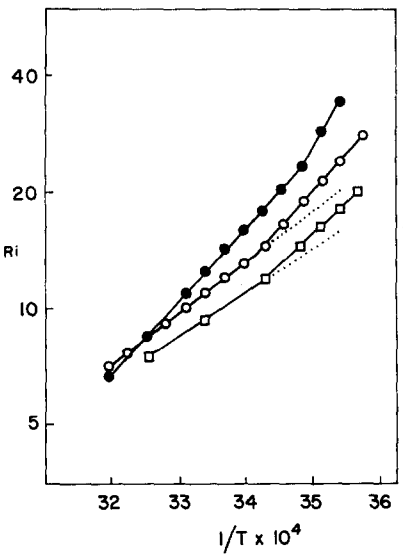


Fig. 4. Temperature dependence of the motion parameter (R_1) of the spin-labeled probe in yeast cells (KD115) grown on lactate and enriched with oleic acid with (●) and without (○) adamantane in the growth medium.

Fig. 5. Temperature dependence of the motion parameter (R_1) of methyl-12-nitroxide stearate in yeast cells (KD46) enriched with 18:1$\Delta^{9}\equiv$ (○), 18:1$\Delta^{9}\equiv$plus 5 moles% of 18:1Δ^{6}*cis* (●), and 18:1$\Delta^{9}\equiv$ plus 5 moles% of 18:1Δ^{11}*cis* (□). Cells were grown on glucose.



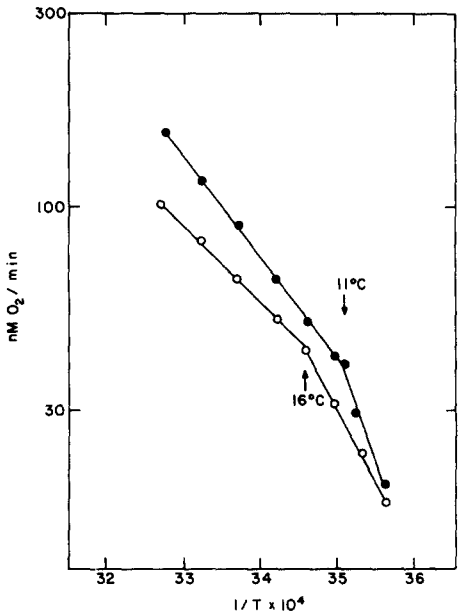


Fig. 6. Temperature dependence of the motion parameter (R_1) of the spin-labeled probe methyl-12-nitroxide stearate in yeast cells (KD46) enriched with 18:1Δ^9*cis* (○), with 18:1Δ^9*cis* plus 5 moles% of 18:1Δ^6*cis* (●), and with 18:1Δ^9*cis* plus 5 moles% of 18:1Δ^{11}*cis* (□). Cells were grown on glucose.

Fig. 7. Temperature dependence of the O_2 uptake by a yeast culture (KD115) grown on lactate and enriched with oleic acid (○) or oleic acid in the presence of adamantane in the growth medium (●).

of 18:1$\Delta^{9\equiv}$. In both cases the "perturbing" fatty acids were added to the growth medium at 5% the concentration of the major supplement, oleic or stearolic acid. The proportion of oleic or stearolic acid reached 40–50% of the total fatty acid composition and growth rates of the cultures having different fatty acid compositions were similar. Analysis by gas–liquid chromatography was possible on cultures supplemented with stearolate (18:1$\Delta^{9\equiv}$) plus a Δ^9*cis* alexinic fatty acid since the retention time for methyl stearolate is considerably longer than methyl oleate on polyester columns. These data showed that supplementation with 5 moles% of 18:1Δ^6*cis* resulted in an 18-carbon monoene distribution of 18:1$\Delta^{9\equiv}$ (80%) and 18:1Δ^6*cis* (20%). Supplementation with $1 \cdot 10^{-4}$ M 18:1$\Delta^{9\equiv}$ plus $5 \cdot 10^{-6}$ M 18:1Δ^{11}*cis* (5 moles%) resulted in an 18:1 monoene distribution of 18:1$\Delta^{9\equiv}$ (82%) and 18:1Δ^{11}*cis* (18%). Supplementation with 18:1$\Delta^{9\equiv}$ plus 18:1Δ^9*cis* resulted in higher enrichment in Δ^9*cis* (25%, 28%) than in either of the other *cis* monoenes. Where Δ^6*cis* and Δ^{11}*cis* were supplemented at 5 moles% their enrichment was enhanced about a factor of four. We estimate that simultaneous enrichment with 18:1Δ^9*cis* at 10^{-4} M plus 5 moles% of either the Δ^6*cis* or Δ^{11}*cis* results in about the same or less percent of the secondary monoene. The data of Fig. 4 reflect mitochondrial-dependent growth whereas Figs 5 and 6 are of a nuclear petite which has no capability of mitochondrial-dependent growth. Therefore, taken together, the data in Figs 4–6 indicate that the ESR-detectable transition is the same for a given fatty acid composition whether or not growth, is dependent on mitochondrial function.

The plots in Figs 5 and 6 indicate that a $\Delta^6 cis$-unsaturated site is effective in perturbing the ordering of alkyl chains in the host systems ($\Delta^9 cis$ or $\Delta^{9\equiv}$) and in lowering the respective transition temperatures. $\Delta^{11} cis$ -unsaturated sites appear to have no such effects. This is consistent with the results previously published [9] which indicated that, in homogeneous systems, the ordered region of alkyl chains is between the position of unsaturated sites and the amphiphilic interface. $\Delta^{11} cis$ does not perturb effectively the host systems $\Delta^9 cis$ or $\Delta^{9\equiv}$ as the region beyond carbon-9 presumably is relatively disordered in either case.

Perturbation of membrane activity

In order to understand membrane functionality it is important to determine whether the perturbation of lipid interactions in membrane systems, as detected by the motion of spin labels, results in a perturbation of the physiological activity of the host cell.

O_2 uptake is a metabolic parameter which may be limited by a number of different enzymatic steps, since it reflects the activities of one or more membrane-bound enzymes. As has been shown in previous studies [11–13] the activities of membrane-bound enzymes do reflect the physical state of membrane lipids. More specifically, the transition temperatures associated with lipid melts or order–disorder transitions have been shown to correlate with transitions in the Arrhenius plots of enzyme activities. Fig. 7 shows O_2 uptake plotted against temperature for KD115 yeast grown on lactate and enriched with oleic acid, with and without adamantane in the growth medium. Two activation energies are apparent on either side of transition temperatures. Without adamantane the transition temperature is at about 16 °C; the presence of adamantane noticeably lowers the break point. These values are in the same direction and are in general agreement with the transition temperatures determined from the temperature dependence of the motion of a lipophilic spin-labeled probe (Fig. 4). Thus the ordering of the lipid alkyl chains and perturbation of that ordering are of biological significance. Fig. 8 demonstrates similarly the perturbing effect of adamantane on the O_2 uptake of KD115 yeast cells enriched with stearolic acid. The differences in O_2 uptake between the two types of cultures in Figs 7 and 8 reflect differences in number of cells used. Similar O_2 uptakes occur with equal numbers of cells.

It is of interest that the transition temperatures reported by the labeled probes are in general agreement with the temperatures obtained from O_2 uptake experiments. First, this means that the probes do not perturb the vital functions of the cell appreciably. Second, this means that the bulk measurement of O_2 uptake by a cell population is influenced by a membrane phenomenon. While the spin label probes its local environment it is the information transmitted to the boundaries of this local environment that is important. Therefore, the boundaries of the spin-label domain are sensitive to influence mediated by cooperative interactions. Since the alkyl regions of membranes have generally been determined to exist in ordered hexagonal arrays any change of state or any introduced perturbation is expected to behave as a long-range influence, sensed by a spin label dissolved in the hydrocarbon interior. The location of methyl-12-nitroxide stearate is not precisely known; however, as determined here and previously [16] the hyperfine coupling constant reflects a hydrocarbon domain. The spin-label concentration was maintained at about 1 spin label/100 phospholipids; therefore,

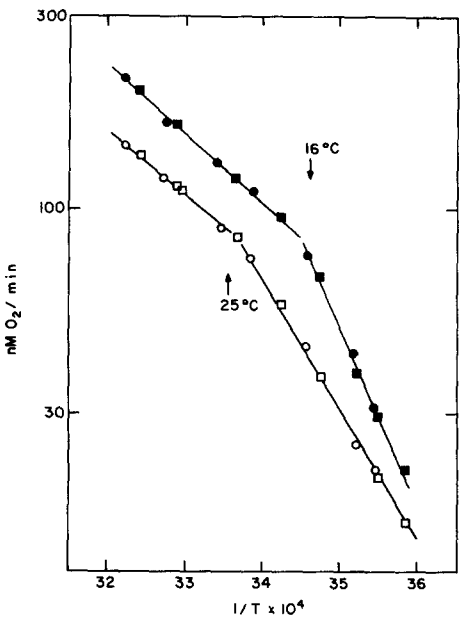


Fig. 8. Temperature dependence of the O_2 uptake by a yeast culture (KD115) grown on lactate and enriched with stearolic acid (○, □) or stearolic acid in the presence of adamantane in the growth medium (●, ■).

any significant aggregating of spin labels would have been apparent as an exchange narrowed contribution superimposed on the mid-line or any significant non-random distribution would have been apparent as electron–electron dipole broadening of all three lines. Neither of these effects was observed at the spin-label concentration used although they were observed when attempts were made with higher spin-label concentrations. Methyl-12-nitroxide stearate has been used in several studies to detect membrane lipid transitions and is apparently reliable as a phenomenological monitoring device. Since no organism synthesizes oxazolidines on fatty acids there is no way of adding such a spin label to a membrane without locally perturbing the environment of the nitroxide. The cooperative transmission of information to the local environment of the spin label is more important than the precise structure of the non-perturbing part of the spin-label molecule. No doubt many other spin labels and spin-label parameters can be used to obtain the same information as is obtained here.

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