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Title: Coordinated Regulation Of Plasma Membrane Lipids In Saccharomyces cerevisiae

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Plasma membranes isolated from a yeast sterol auxotroph (RD5-R) grown on 1,5, and 15 μg ml^{-1} exogenous concentrations of sterol showed no discontinuity in plots of steady-state fluorescence anisotropy. Liposomes constructed from phospholipid and sterol extracted from RD5-R grown on different sterols indicated exogenously supplied sterol modulated cellular phospholipids such that lipid phase transitions were avoided. Liposomes derived from sterol and phospholipid extracted from the same culture exhibited no lipid phase transitions. However, when phospholipid extracted from a culture grown on a specific sterol was mixed with sterol extracted from a heterologous culture grown on a different sterol to form liposomes, discontinuities were detected in the anisotropy measurements of the liposomes produced. Quantitative analyses revealed that the exogenously supplied sterol regulated specific phospholipid species, fatty acid composition, and sterol to phospholipid ratios in yeast auxotrophs.

Analyses of the free sterol, steryl ester, and fatty acids from yeast secretion mutants indicated that the sterol content (free and esterified) remained relatively constant over a growth range of 24 to 34°C. The saturated fatty acid components (C16:0 and C18:0) increased while the unsaturated fatty acids (C16:1 and C18:1) decreased as a function of growth temperature.

Coordinated Regulation Of Plasma Membrane Lipids In *Saccharomyces cerevisiae*

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COORDINATED REGULATION OF PLASMA MEMBRANE LIPIDS IN SACCHAROMYCES CEREVISIAE

INTRODUCTION

An enormous variety of sterol structural types exist in nature (1,2,3,4). Almost without exception, the principal sterols of an organism appear to have a 3-β-OH, an alkyl side chain of about five carbons in length, and an unsaturation at C5-6. While the modifications to this basic structure that are seen in various organisms appear to be generally unobtrusive, it is reasonable to assume that those changes impart some selective advantage to that particular organism. Some digressions from the consensus structure described above are energetically expensive for the cell. This is particularly true for the transalkylation reactions at C24 found in many plants and animals.

While it is interesting to speculate on the reasons for different sterols in different organisms, such exercises are of little heuristic value, since most ensuing speculations are not directly testable in controlled experiments. Because of two recent developments in yeast physiology and genetics it is possible to address two issues related to this subject. First, sterol auxotrophs have been obtained that have an absolute requirement for sterols. These cells have the added advantage that they are able to accumulate sterols from the growth medium by a passive accumulation mechanism (5,6) that is generally nonspecific for

the sterol being taken up by the cell. This permits feeding to the cell a large variety of sterol types (7,8). The question can then be asked: is there a difference in the physiology of the cell, if "unusual" sterols are used to replace the normal sterol? Second, secretion mutants have been obtained that are conditionally restrictable in the maturation of the yeast plasma membrane. Since the principal functional role of sterol appears to be in the plasma membrane, it is now possible to ask: are there differences in sterol metabolism in these mutants which can be attributed to the defect in membrane formation?

This dissertation is the result of a study of these two questions. It has been found that the sterol being provided to an organism mandates modifications in lipid metabolism to accommodate the structural features of that sterol. While a major modification in sterol metabolism was observed in one mutant that is defective in membrane synthesis, the altered sterol metabolism was not the cause of defective membrane growth at the restrictive temperature.

LITERATURE REVIEW

Yeast Sterols

The ascosporus yeast Saccharomyces cerevisiae is a typical eukaryotic organism with respect to membranous organelles, component phospholipids, fatty acids, and proteins. S. cerevisiae like other eukaryotes incorporates sterol into its membranes (1,4). In this yeast the natural sterol is ergosterol rather than cholesterol which is found in many other eukaryotic cells (1,4). Ergosterol, isolated over 90 years ago, has been structurally and chemically defined (1).

Ergosterol (ergosta-5,7,22-trien-3β-ol) (Figure 1) has a perhydrocyclopentanophenanthrene nucleus (9) which consists of three six-membered rings (A, B, C) and a five-membered ring (D). There are double bonds between carbons 5-6, 7-8 and 22-23. The molecule is termed a lipid alcohol due to the hydroxyl at the C3 position (1,4,10). The side chain (C20 to C27) attached at C17 is modified relative to the cholesterol-like structures, in that ergosterol has an extra carbon at C24 (1,4,11).

While the sterol occurs principally as the free alcohol, another in vivo form of ergosterol is ergosteryl ester, in which the C3 β-hydroxyl is linked to an unsaturated fatty acid, principally C16 or C18 (12). Sterol esterification activity was shown to increase markedly as the culture entered the stationary phase of growth even though the rate of ergosterol biosynthesis decreased (12). Additionally, sterol intermediates in ergosterol biosynthesis, of which only trace amounts

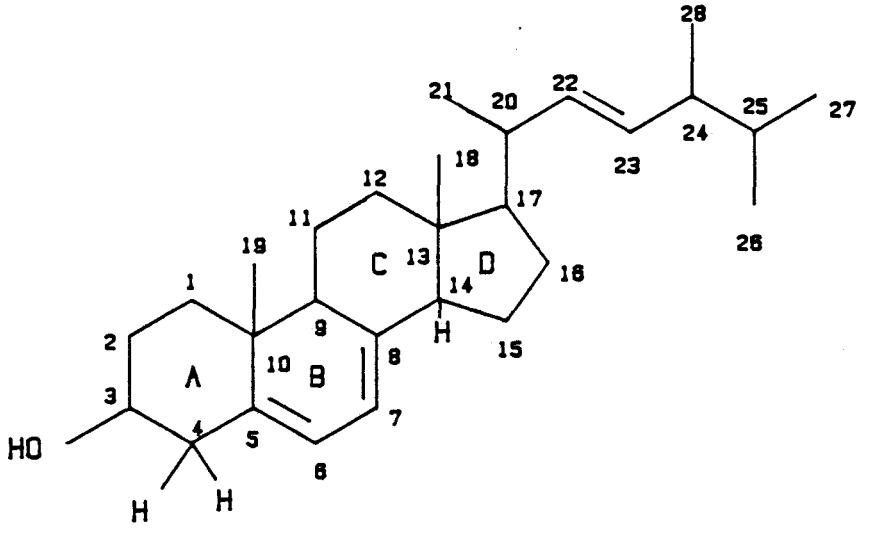

Figure 1. Structure of ergosterol. The endogenous S. cerevisiae sterol is ergosterol. Shown are the ring designations and carbon numbering system of ergosterol (1,4).

of free sterol (<0.1%) are found during log phase growth are esterified and can account for approximately 16% of the steryl ester pool (12). In sterol uptake experiments using yeast auxotrophs, it was shown that there is an apparent hierarchy of esterification in which, the percentage of a sterol in the ester pool increased as the similarity to ergosterol decreased.

Sterol Biosynthesis

Ottke et al. (13) showed that most of the carbon in ergosterol was supplied via acetyl-CoA, the precursor of mevalonic acid. The condensation reaction of acetoacetyl-CoA and acetyl-CoA (Figure 2) is the committed step in sterol biosynthesis (11). After mevalonate formation, a series of rearrangements leads to the formation of isopentyl pyrophosphate (10). After isopentyl pyrophosphate formation, in a second series of condensation reactions, three molecules of isopentyl pyrophosphate form farnesyl pyrophosphate. Two molecules of farnesyl pyrophosphate condense, forming the C30 hydrocarbon, squalene. The late reactions of sterol biosynthesis in yeast (Figure 2) involve cyclization of squalene into lanosterol which requires molecular oxygen for activity of the yeast oxidosqualene cyclase. The C8=9 double bond is also isomerized to the C7-8 position; the demethylation at C14 occurs prior to the two C4 demethylation steps (1). Further along the pathway are three reactions not found in animal cell cholesterol synthesis. These reactions modify the side chain attached at C17. There is a transalkylation (45) at C24, a desaturation producing the C22=23 double bond and a reduction of the C24(28) unsaturation (1,14).

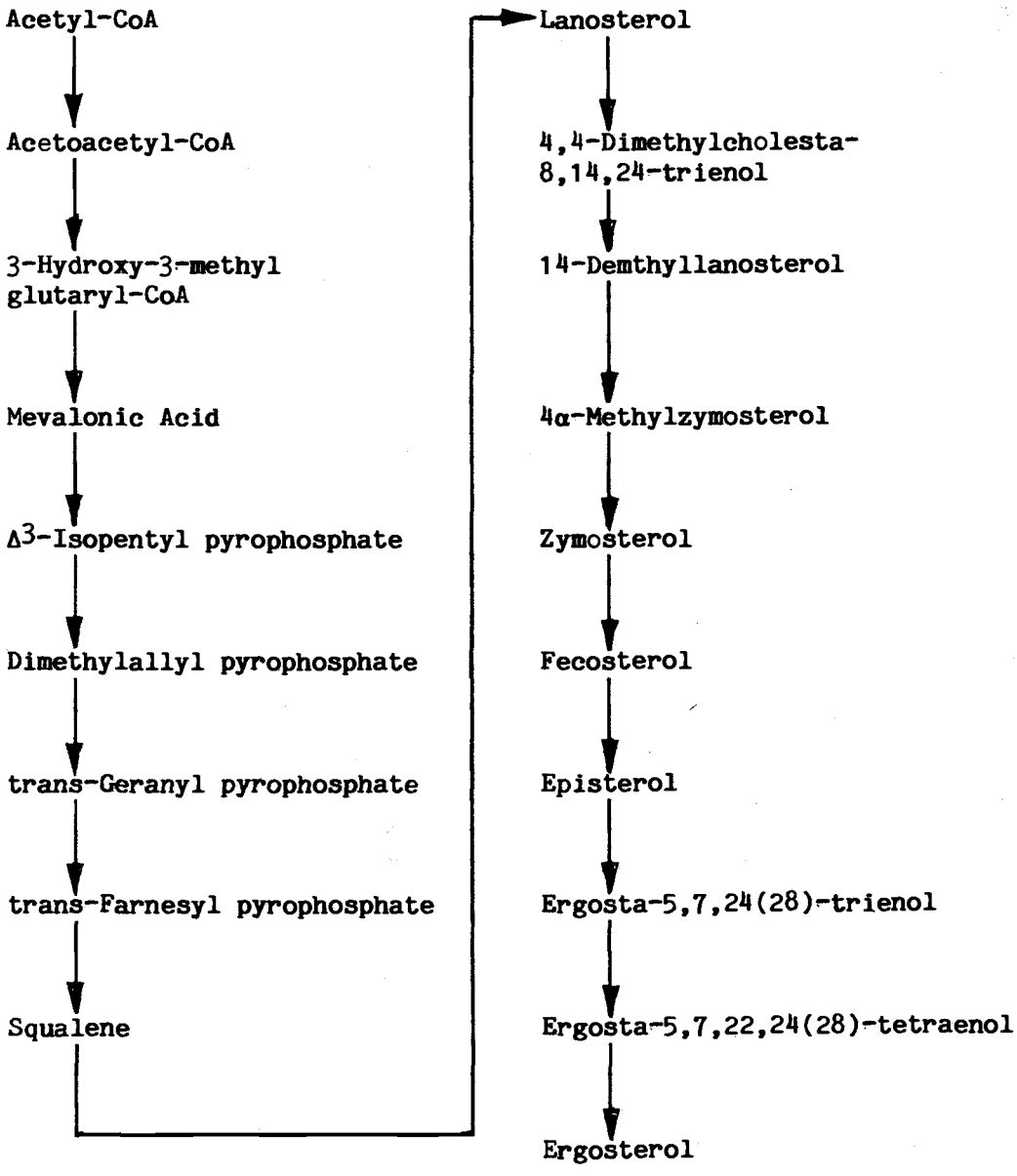

Figure 2. Yeast sterol biosynthetic pathway. The pathway is customarily divided into early and late stages with the demarcation of early and late being the conversion of squalene to lanosterol (1, 4).

Sterol Energetics

Ergosterol biosynthesis is perhaps one of the most metabolically demanding processes in the yeast cell. A conservative estimate of the energy requirements for the pathway from acetate to ergosterol is 16 NADPH, at least 10 ATP, and 12 ATP equivalents for the C24 side chain transmethylation (1,8,15).

Sterol Functional Roles

Generally, the primary role of sterol has been viewed as a modulator of membrane fluidity to prevent dramatic changes under fluctuating environmental conditions (2). Analyses using a yeast auxotroph RD5-R indicated there are at least four different levels of sterol function, operationally defined as a function of the exogenous C5-6 unsaturated sterol concentration. These levels are designated as 1) sparking (1-10 ng ml^{-1}), 2) critical domain (100 ng ml^{-1}), 3) domain (0.5-1.0 µg ml^{-1}) and 4) bulk ($\geq$ 15 µg ml^{-1}) (7,8,16,17,18).

Sparking and bulk functions were defined by the observation that a sterol auxotroph was unable to grow unless a low exogenous concentration (1-10 ng ml^{-1}) of C5-6 unsaturated sterol was present in the growth medium even though exogenous cholestanol (a fully saturated form of cholesterol) was present in adequate amounts (5 µg ml^{-1}). Under these conditions, the C5-6 unsaturated sterol fulfilled the sparking microrequirement and cholestanol fulfilled the bulk role in the membrane (8). The critical domain was defined by the ability of the auxotroph to grow on lanosterol (5 µg ml^{-1}) only when 100 ng ml^{-1} of C5-6 unsaturated sterol was added to the growth medium as well. The

fourth function, domain level, was observed when the auxotroph was grown in medium supplemented with less than 5 μg ml^{-1} of C5-6 unsaturated sterol. The domain level is perhaps best characterized by a measurable difference in the final growth yield when the auxotroph is cultured on a range of sterol concentrations (0.5-5 μg ml^{-1}). Here, the growth rate is unchanged over the concentration range used (8).

Yeast Phospholipids

The yeast Saccharomyces cerevisiae contains a mixture of phospholipids. These are phosphatidic acid (PA), cytidine diphosphate diglyceride (CDP-DG), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL) (4). Of these, the five major phospholipids are PC (31%), PI (28%), PE (18%), PS (8%) and CL (6%). The values here are the average amount of these phospholipid classes found in mutants and wildtype cells cultured under various environmental conditions (4).

When the temperature profile of phospholipids from wildtype cells grown to stationary phase was examined, it showed a shift to more C18 (50% increase between 10-34°C) and less C16 unsaturated fatty acid (43% decrease between 10-34°C) (19).

In a comparative study of sterol mutants and wildtype cells, there were differences of 1-3% between the different phospholipid headgroup types. However, the mutants had more PI and PC but less PE than the wildtype (14). These results are based on analyses of sterol mutants

which produced as an end product of sterol biosynthesis, a non-ergosterol sterol.

When the inositol auxotroph MC13 was starved for inositol, it stopped growing and phosphatidylinositol synthesis ceased. Furthermore, when MC13 cells were supplemented with inositol, [^{14}C]acetate labeled acylglyceride activity was shown to decrease and accumulated as [^{14}C]phospholipid. In cultures starved for inositol, the [^{14}C]phospholipid fraction decrease was mirrored by an increase in [^{14}C]triacylglycerol (20).

In an _in vitro_ system, using vesicles prepared from total yeast extract, Daum and Paltauf showed that a yeast cytosolic protein catalyzed the transfer of essentially any phospholipid from donor (yeast lipids) to acceptor membranes (yeast mitochondria) (21). The phospholipids transferred in order of decreasing transfer activity are PE, PC, and PI, the major yeast phospholipids. The temperature at which the transferase enzyme is 50% inactivated was 48°C, a temperature higher than that for optimal growth of yeasts. The PC and PI transfer activity was also lost simultaneously. Inhibitor experiments with iodoacetamide, p-chloromercuribenzoic acid, dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide, p-tosyl-L-lysinechloromethylketone and o-phenanthroline did affect transfer activity (21).

Using ^{32}P label, Ramirez _et al_. (22) were able to to show that in the tested yeast secretion mutants (23), phospholipid synthesis continued at the non-permissive temperature even though the overall phospholipid variation did not appear very different from X2180-1A, the wildtype strain. It was further shown that after temperature up-shift,

spheroplasts made from the secretion mutant strains had an increased osmotic sensitivity which indicated a failure of membrane growth (22). In the secretion mutants (23,22), phospholipid was shown to increase at the restrictive temperature, however, in striking contrast, another temperature sensitive mutant (24) stopped ^{32}P incorporation and was coordinate for all the major classes of yeast phospholipid. With respect to phospholipid content, Fernandez et al., demonstrated that the amount of phosphatidylinositol in an inositol auxotroph was twice that of the wildtype while the phosphatidylserine content was only one-half the amount found in the wildtype (25).

Phospholipid biosynthetic reactions have been reported to occur in endoplasmic reticulum and mitochondria of yeasts. Kuchler et al. have shown that phosphatidate cytidylyltransferase is present in inner and outer mitochondrial membrane and in microsomes. Microsomes are small particles of 50-150 nm diameter, mostly endoplasmic reticulum fragments but in part derived from plasma membrane of homogenized cells and may still have attached ribosomes on the outer surface (26). Glycerophosphate acyltransferase, phosphatidylserine synthase, phosphatidylinositol synthase and choline phosphotransferase are located in microsomes and the outer mitochondrial membrane. The inner mitochondrial membrane also had enzymatic activity for phosphatidylserine decarboxylase and phosphatidylglycerophosphate synthase while the microsomal fraction had PE-methyl transferase activity (27). Activity of the CDP-DG synthase, as well as other membrane associated phospholipid N-methyl transferases which convert PE to PC, was repressed six to tenfold by addition of choline to medium

containing inositol. Addition of choline to inositol-less medium had no repressive effect on enzyme activity (28) and demonstrated the presence of a coordinated response to choline and inositol (29).

Fatty Acids

In S. cerevisiae, the typical saturated and unsaturated fatty acids are 14, 16, and 18 carbons long with a majority of unsaturated fatty acids having double bonds at position C9-10. Furthermore, Saccharomyces cerevisiae does not produce di or polyenoic unsaturated fatty acids although they will utilize these di and polyunsaturated fatty acids for growth (4).

Fatty acids which support anaerobic growth of yeasts appear to require a site of unsaturation (cis or trans) in the chain. In feeding experiments designed to elucidate anaerobic fatty acid requirements, C9-10 unsaturated fatty acids were most efficient at promoting growth. Saturated fatty acids with a chain length of 12 or more carbons were ineffective in promoting anaerobic growth of yeasts (30).

Yeast Plasma Membrane And Membrane Probes

The fluid mosaic model of Singer and Nicholson, describes the membrane as a two dimensional fluid matrix of oriented proteins and lipids. A major feature is the phospholipid and glycolipid molecules which form the bilayer geometry. The dual role of solvent for membrane protein and permeability barrier is also attributed to the lipids. Only a small portion of the membrane lipids interact specifically with proteins which are relatively free to diffuse laterally. These

proteins are hindered from flipping from one surface of the membrane to the other (10).

The plasma membrane is the site of specific attachment of macromolecules, glycolipids, and/or glycoproteins. These recognitive events at the membrane surface (internal and/or external) can be key regulatory mechanisms in morphogenesis, tissue formation, growth control, mytotube formation and differentiation (31). As such, the membrane components and composition have been optimized for activity in a lipid bilayer (31). Here, it has been proposed that sterol, phospholipid, and fatty acid are coordinately regulated.

Presumptive data supporting coordinated regulation were observed in Neurospora crassa (32), human lung macrophages (33), and yeast (34,35,20). It was also reported that lipid changes were differentially responsive to specific sterol structures in a yeast sterol mutant (36). Previous methods using S. cerevisiae to ascertain the function(s) of sterols have included: a) the use of anaerobic conditions to induce sterol auxotrophy (37), b) sterol mutants blocked at specific steps in the ergosterol biosynthetic pathway such that non-ergosterol intermediates are accumulated (38), c) mutants auxotrophic for sterol under aerobic conditions (38), and d) use of polyene antibiotics (1).

The foremost physiological role of phospholipid is the formation of bilayer membranes. In synthetic bilayers, phospholipids are known to form discrete lattices (L_{α}, considered to be an infinite array of planar sheets, a stacked bilayer; H_{II}, an infinite array of hexagonally stacked tubes; and C_{II}, a cubic close packed array of inverted spherical micelles) (39). These synthetic bilayers represent a model

membrane system with a controlled level of complexity (31) and are models for plasma membrane as well as other intracellular organelles.

In any of the lattice forms, the stable phase has the lowest free energy. Depending on the physical and chemical environment, membrane lipids may undergo a transition to a different lipid state. Since the cubic phase C_{II} is not a predicted stable state (39), the phase transition can be defined as a crossover point where the free energy of the L_{α} phase increases above that of the H_{II} phase and conversion of lamellar to hexagonal arrays minimizes the free energy in response to the altered environment (39). Below the transition temperature, the average molecular cross-sectional area is uniform while above the transition temperature, the cross section becomes tapered (altered head to tail geometry). More quantitatively, the shape would be determined as a function of the free energy (40). At the microscopic lipid-water interface are three distinct regions, 1) the water region which can be perturbed by polar head groups, 2) the polar group region which interacts chemically, electrostatically, and sterically with water and itself, and 3) the hydrocarbon chains which preserve the hydrophobic region. The chain packing in interstitial spaces and electrostatic interactions of charged lipids also affect total free energy (39).

Thermodynamically, there is a tendency for lipid monolayers to curl which reduces the energy of intrinsic curvature. This model proposes that protein mediates the introduction of hydrophobic molecules which locally disrupt the bilayer. Biological membranes appear as a mixture of bilayer (large intrinsic equilibrium radius of curvature) and non-bilayer (small radius of curvature) lipids. This

mixture has an equilibrium curvature radius of intermediate value such that the membrane is in a metastable state. By tipping the balance with a small triggering event (protein mediated introduction or modification of hydrophobic molecules into the bilayer or their removal) a reversible transition event (L_α to H_{II} or H_{II} to L_α) using one or a combination of hydrophobic, hydrophilic, or electrostatic processes, can occur (40).

To detect these changes the fluorescence of a chromophore probe, which is much more sensitive to the surrounding environment than absorption, is often used. If during the lifetime of the excited state, the chromophore tumbles fast enough to randomize its orientation (non-rigid system), then by the time light emission occurs, the intensities of parallel and perpendicularly polarized light are equal and the calculated polarization and anisotropy are minimized (41).

Since there is a significant order-disorder transition in membranes, differential scanning calorimetry and X-ray diffraction have also been used to show that at the transition temperature, there can be as much as a 20% change in the bilayer surface area with 6% reversibility in a concentrated multilamellar system (42). This is correlated with fluorescence polarization measurements used to determine the steady state anisotropy. In triple chain ammonium amphiphiles, Kunitake and Higashi (43), were able to show the presence of a thermotropic transition using DPH as a probe and, correlated the transition inflection points by differential scanning calorimetry.

The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene, appears to have similar orientational properties in phosphatidylcholine systems

(soybean, synthetic, and egg). There appeared to be a marked decrease in reorientational dynamics when unsaturation in the lipid chains was present (44). DPH, it is assumed, behaves as a stiff rod and the long axis distribution appears axially symmetric around the normal to the membrane surface (45). The lower limiting anisotropy has usually been interpreted in terms of a larger cone angle (wobble) and in a decreased lipid order. However, it has been reported that one orientation of DPH is parallel to the plane of the membrane and that the gel to liquid crystalline transition of the membrane/vesicle system may be interpreted as a change in the dynamic equilibrium between orientations of the DPH molecule (44,45). Examination of the structural effects of free fatty acids upon cell membranes and pure lipid bilayers using DPH revealed that the cis-unsaturated fatty acids decreased the DPH polarization while trans-unsaturated and saturated fatty acids did not affect DPH polarization (46). Fluorescent decay lifetime measurements indicated that the lipid structure in plasma membrane may exist as discrete domains into which DPH could partition (47).

In another study, rat liver plasma membranes (examined using DPH) showed a measurable lipid thermotropic transition between 18 to 30°C. The discontinuities were indicative of a phase transition and were confirmed by differential scanning calorimetry (48). Similar discontinuities (thermotropic transitions) were observed in rat intestinal plasma membranes (49) and hepatocytes (47). Temperature induced reversible transitions of gel to liquid crystal are not the only detected transitions. Barkai et al. were able to show that the fluorescent probe DPH was useful in detecting the effect of hydrostatic

pressure on rat lung surfactant. At a temperature above the lipid critical temperature (temperature above which the predominant lipid phase is liquid crystalline), a few hundred atmospheres were required to convert melted lipid back to a solid phase (50).

Fluorescent probes, localized at different depths in the lipid bilayer, were used by Zavoico et al., to demonstrate that alkanols, i. e. n-alcohols (pentanol, decanol and tetradecanol) perturb the bilayer at different positions (51). Here, one perturbing compound was shown to have pleiotropic effects on membrane order at different depths in the bilayer. This was detected at the site of perturbation and distant from it (using different fluorescent probes). These results indicated the interior of the bilayer (occupied by acyl chains) was more susceptible to perturbation by n-alcohols than the region near the phospholipid headgroups (51).

In another model system using dipalmitoylphosphatidylcholine and a cholesterol analogue (cholesta-4,6-dien-3-one) to form multilamellar liposomes, the transition temperature as determined by fluorescence depolarization (using the probe DPH) and differential scanning calorimetry were within 0.1°C (52). In liposomes prepared from phosphatidylcholine, both sterol analogue and probe molecule were randomly distributed in the bilayer (53). In this case, polarization of DPH was found to increase with increasing sterol content for cholesterol and other sterols differing in ring structure or in the C17 side chain.

Cholesterol has a dual effect on fluidity of phospholipid bilayers. The ordered array of lipid acyl chains in the gel phase is

fluidized while the liquid crystalline phase fluidity was reduced by addition of cholesterol (54). In S. cerevisiae, with nyr5 (nystatin resistance variant), electron paramagnetic resonance studies showed that stationary phase cultures had a more rigid membrane than exponential cultures due to increased packing of sterol into the membrane (55). In studies conducted on the disordering effects of ethanol in S. cerevisiae plasma membrane, it was shown that the discontinuities in fluorescence steady state anisotropy plots were made less abrupt by the addition of increasing amounts of sterol and eventually eliminated by the addition of even more sterol into the plasma membrane (56).

Presumptive evidence that sterol is incorporated into the bilayer among the acyl chains is based on the lack of phospholipid headgroup displacement with and without sterol (57). The intercalation then, of sterol among the acyl chains, affects acyl chain ordering by decreasing the available interstitial chain space. The plasma membrane is enriched with free sterol and spheroplasts containing a sterol with a C17 unsaturated side chain were shown to be more resistant to osmotic stretching (11,58).

A general criticism of the fluorescence probe technique is the degree of perturbation of the lipid structure by the probe. Several experiments using cetylmethylammonium bromide have shown that as long as the concentration of probe is kept below a limiting value, micelle parameters including size, internal viscosity, critical micellar concentration (CMC) remain unchanged (59).

Environmental Stress

Maintenance of membrane integrity and stability under adverse environmental stress has been attributed to the presence of sterols in the plasma membrane. However, for cellular growth and division, plasma membranes must undergo at least two basic kinds of instability: 1) rupture (pore formation and/or membrane fragmentation) and 2) buckling (bending or folding of the membranes) (60). Plasma membrane stability in interactions with other membranes or surfaces is important to understanding the mechanisms of membrane adhesion, fusion, cell division, and detachment (60).

At least two different factors control the membrane physical state: 1) the lipid components and 2) the environment surrounding the membrane. Cells often respond to environmental stress that causes a deranged physical state of the membrane by altering the phospholipid composition of the membrane (61). A typical phospholipid has a saturated or monounsaturated fatty acid at the sn1 position and an unsaturated fatty acid at the sn2 position. The distribution of the double bond and its location in the acyl chain affect the physical properties of the membrane (61). Specific changes therein may allow the cell to survive in an otherwise adverse environment.

Poikilotherms are known to alter their membrane lipid composition in response to environmental temperature. This appears to occur primarily in the fatty acyl chains of the membrane phospholipids as membrane fluidity is more effectively modulated by changing the fatty acid components of the membrane (61,62). Generally, the model states that as the growth temperature decreases, there is a shift from

saturated fatty acids to monounsaturated fatty acids, the average chain length decreases, and the amount of branched fatty acids increases with alteration in the kind of branches. All of this produces a lower gel to liquid crystalline phase transition temperature. Implicit in this model is the fact that an increased growth temperature produces the opposite effects. This phenomenon of keeping the membrane fluidity relatively constant has been termed homeoviscous adaptation. Further, the rates of change are reported to occur within one to two generations of a growth temperature alteration. The processes of modification of preexisting fatty acids and de novo synthesis of new fatty acids custom made for the new growth temperature also occur with a specificity for modification at the sn1 position over the sn2 position. Here, as the temperature decreases, the sn1 position will have a higher percentage of the longer chains than the sn2 position. This particular modification/de novo synthesis combination also produces a lower gel to liquid crystalline transition than if the opposite occurred (sn1 shorter chain and sn2 longer chain). Again, the reverse is implicit in a growth temperature increase (61,62).

In the eukaryotic poikilotherm Nerium oleander, grown at 20/15°C or 45/32°C day/night temperatures, Raison et al. demonstrated that discontinuities in plots of spin label motion, fluorescence intensity, and polarization ratio as a function of temperature occurred at different temperatures in leaf liposome preparations. Analyses of the unsaturated fatty acids indicated there was an approximate fourfold increase in oleic acid (C18:1) for plants cultured at the higher temperature (63).

Another aspect of the model must include optimization of other membrane lipid components. It was shown by Starr and Parks (64) that the sterol biosynthetic rate appeared to be optimized for the temperature at which growth of yeast was also optimal (30°C). Above this temperature, sterol biosynthesis was severely inhibited.

Yeast Secretion Mutants

The secretory-vesicle-membrane assembly model hypothesizes that new plasma membrane, membrane proteins and perhaps other lipids will first appear at the bud region, a site of eventual division of mother and daughter cells (4). Membrane bound organelles known to be involved in the secretory process include the nuclear membrane, endoplasmic reticulum, Golgi, vacuoles, secretory vesicles and plasma membrane (4,23,65,66,67,68).

The temperature sensitive secretion mutants (23) comprise at least 23 known complementation groups. Of these, one class was found to accumulate thermoreversibly, small vesicles (0.05-0.07 μm in diameter) in the bud region of a dividing yeast cell (66). Other classes of secretory mutants were found which accumulate endoplasmic reticulum, Golgi stacks, Berkeley bodies, or no organelle at all (4,23,65,66,67,68). Genetic manipulation of these mutants (double mutants for secretory organelle accumulation) has been used to determine that the order of major events in the yeast secretory pathway (4,67) is as indicated in Figure 3. Analysis of the secretory mutants suggested a single linear secretion path but, the results (23,65,66,67,68,69) are also consistent with parallel secretion

pathways and the separation of secretion and plasma membrane assembly (70). If cell surface assembly (membrane biogenesis) is mediated by a separate class of vesicles, they should fuse with the bud region of the plasma membrane (70) since polarized surface growth is the norm in S. cerevisiae.

While transport to the vacuole and plasma membrane is intracellular, the yeast plasma membrane has been shown to be active in endocytotic processes. The dye Lucifer Yellow, which is membrane impermeable and fluorescent, is taken up by an active energy requiring process that is not saturable (70,71) and all vesicle accumulating strains are defective in dye uptake at the restrictive temperature (70).

Analyses of phospholipid synthesis (22) indicated that the rate of phospholipid synthesis increased as a function of the growth temperature. When shifted to the restrictive growth temperature, phospholipid synthesis first increased then decreased. Spheroplasts made from secretion mutant strains showed increased osmotic fragility at the restrictive temperature and has been interpreted as restricted membrane growth. Furthermore, substantial increases in PC and PE which are known to increase membrane fragility were not observed (22).

Another temperature sensitive strain (not isolated from the HMSF secretion mutants) exhibited a defect in lipid biosynthesis in the early part of the pathway. In this mutant, free fatty acids and steryl ester increased over a four hour temperature up-shift, phospholipid decreased, and the triglyceride level remained relatively constant. Fatty acid analysis by chain length showed an approximate 3% increase

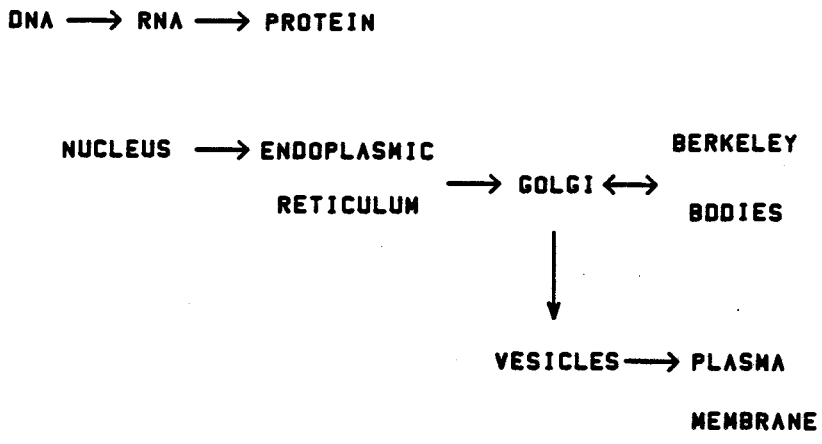

Figure 3. Secretion pathway. The typical eukaryotic secretion pathway (4) found in Saccharomyces cerevisiae is depicted.

for 16:0 and 1% increase for 18:0 saturated fatty acid at the restrictive temperature (24).

MATERIALS AND METHODS

Yeast Strains

The strains of Saccharomyces cerevisiae used in this study are listed in Table 1 with the pertinent auxotrophic markers. The wildtype strains for the temperature sensitive secretion mutants are X2180-1A and 1B. To minimize the effects of different strains, the HMSF yeast secretion mutants were used. All were isolated from the X2180-1A or X2180-1B after ethyl methane sulfonate mutagenesis (66).

The sterol auxotroph, RD5-R was derived from a cross between the sterol auxotroph, FY3 (72), and the sterol mutant JR1 (73), and has been described in detail (7). RD5-R is defective in δ-aminolevulinic acid (alv) synthase due to the pleiotropic hem1 mutation. This results in heme, sterol, unsaturated fatty acid, and methionine auxotrophy (72).

Media

Rich growth medium consisted of yeast extract (.5%, w/v), tryptone (2%, w/v) dextrose (2%, w/v) (YETD) and was used for growth of haploid strains from stock slants. A second medium, (a minimal defined growth medium) consisted of yeast nitrogen base without amino acids (.67%, w/v) and dextrose (2%, w/v) (YNBD). The minimal medium was used for growth of strains not requiring dietary supplements.

The sterol auxotrophs were cultured on yeast nitrogen base without amino acids (.67%, w/v), casamino acids (1%, w/v), methionine (0.005%,

Table 1

STRAIN LIST

Strains	Genotype and Organelle Accumulated[a]
X2180-1A	a, CUP1, SUC2, mal, gal2, secretion wildtype, temperature sensitive wildtype and parental for HMSF strains
X2180-1B	α, CUP1, SUC2, mal, gal2, secretion wildtype, temperature sensitive wildtype and parental for HMSF strains
HMSF1[b]	sec1-1 vesicles[c]
HMSF134	sec5-24 vesicles
HMSF136	sec6-4 vesicles
HMSF143	sec9-4 vesicles and Berkeley bodies
HMSF154	sec11-7 none
HMSF169	sec14-3 Berkeley Bodies and vesicles (11), Golgi (8)
HMSF171	sec15-1 vesicles
HMSF175	sec17-1 endoplasmic reticulum and small vesicles[d]
HMSF178	sec19-1 endoplasmic reticulum, Berkeley bodies, vesicles and small vesicles
HMSF179	sec20-1 endoplasmic reticulum
HMSF180	sec21-1 endoplasmic reticulum
HMSF183	sec22-3 endoplasmic reticulum and small vesicles
HMSF190	sec23-1 endoplasmic reticulum
RD5-R	α, hem1, erg3, erg7

[a] intracellular membrane bound organelle (23,65,66,67,68).
[b] genotype of all HMSF strains except for secretory mutation is: a, CUP1, SUC2, mal, gal2 (36,37,78).
[c] 80-100 nm diameter vesicles.
[d] 40-60 nm diameter vesicles.

w/v), adenine (0.005%, w/v), uracil (0.002%, w/v), and dextrose (2%, w/v) (YNB/CA/D). Each supplemented sterol (cholesterol, ergosterol, stigmasterol, and sitosterol) was dissolved in a mixture of tyloxapol:ethanol (1:1, w/v) and added to a final concentration of 1, 5, 10, and 15 μg ml^{-1} in the growth medium. Auxotrophic cells used in liposome preparation were grown on this medium at 15 μg ml^{-1} final sterol concentration. This concentration of sterol was used so that these cells had enough exogenous sterol to satisfy bulk membrane requirements (8). Unsaturated fatty acid requirements for the auxotrophs were fulfilled by addition of 80 μM alv or by supplementing the medium with a mixture of oleic acid/palmitoleic acid (4:1, v/v, dissolved in tyloxapol:ethanol, 1:1, v/v) to a final concentration of 100 μg ml^{-1}.

Growth Conditions

Secretion mutants (HMSF strains) were grown at the permissive temperature (24°C) to the mid-exponential and stationary phase of the culture cycle and harvested by centrifugation. Another growth regime consisted of growth to mid-log (experimentally defined as 100 Klett units) followed by a temperature up-shift to the non-permissive temperature (38-39°C) for two hours after which the cells were harvested by centrifugation. Initially these strains were tested for differences in type and quantity of free and esterified sterol under the growth conditions just described. Growth was followed turbidimetrically using a Klett-Summerson photoelectric colorimeter equipped with a green filter. The free and esterified sterol extracts

from the secretion mutants were compared by thin-layer chromatography. Based on these results, a representative set of the secretion mutants was selected on the basis of the accumulated organelle at the non-permissive temperature from Table 1. The strains X2180-1A (wildtype), HMSF134 (vesicle accumulation), HMSF154 (no organelle accumulated), HMSF169 (Berkeley Bodies, vesicles, and Golgi), and HMSF190 (Endoplasmic Reticulum, ER) were used. In repeating the temperature up-shift experiments for quantitative analyses, the cultures were grown in duplicate to mid-log and either harvested or temperature up-shifted. Here, either one liter of culture (analyses based on mg dry weight of cells) or a given number of cells (analyses based on $1X10^8$ cells) was harvested. For sterol analyses, the cells were either lyophilized and steamed in the presence of dimethyl sulfoxide (DMSO) or alkaline saponified (38). For fatty acid and phospholipid analyses, the lyophilized and DMSO treated cells were extracted and quantitated as described (38). For temperature profile experiments, these strains were also cultured in duplicate on YNBD at 26-40°C in 2°C steps on a temperature gradient incubator to stationary phase and harvested. The cell pellets were then lyophilized, steamed in the presence of DMSO, and total cellular lipids extracted and quantitated.

For liposome preparations, the sterol auxotroph RD5-R, was cultured on YNB/CA/D supplemented with 80 µM alv and one of the following HPLC purified sterols: cholesterol, ergosterol, sitosterol, and stigmasterol. The final exogenous sterol concentration was 15 µg ml^{-1}. In cultures used for liposome reconstitution studies, growth was to late log phase and the cells were harvested by centrifugation,

washed, frozen, lyophilized, steamed in dimethyl sulfoxide (DMSO) and the lipids extracted.

Radiolabeled Extracts

In experiments using ^{14}[C]acetate, the cultures were split at mid-log and labeled acetate added just prior to temperature up-shift. Half of each culture was temperature up-shifted for two hours and the other half was incubated at 24°C for two hours. At 30 minute intervals, yeast were harvested from both non-temperature shifted and temperature shifted cultures, washed with glass distilled water and frozen. The cell samples were then lyophilized, DMSO treated and steamed prior to extraction of phospholipid, sterol, and steryl ester (38). This was followed by TLC using the method of Skipski and Barclay (74). The phospholipid, free sterol, and steryl ester bands, visualized with iodine, were scraped and counted in a scintillation counter (Packard TriCarb model 3320).

Lipid Extracts

Lipids were extracted by either alkaline saponification or dimethyl sulfoxide (DMSO)-hexane extraction from lyophilized cells. In alkaline saponification, the cells are acid pretreated by steaming in 20 volumes of 0.1 N HCl for 20 min. After steaming, the cells were washed with distilled water and resuspended in 2 ml 0.5% pyrogallol (w/v), 2 ml 60% aqueous potassium hydroxide (w/v), and 3 ml methanol. Boiling chips were added and the mixture refluxed for 1-2 hours at 60-80°C. After cooling, the mixture was extracted by addition of 5 ml

hexane. The hexane top layer was removed, saved, and the mixture extracted two more times with hexane. Emulsions, when present, were removed by addition of 0.5 ml of methanol (38).

For DMSO-hexane extraction, enough DMSO was added to just saturate the cell pellet, and then steamed for one hour. After cooling, 5 ml of methanol-water (1:1, v/v) and 5 ml of hexane were added, vortexed, and centrifuged (500 x g, 5 min.). The hexane layer was removed, saved, and the mixture extracted two more times with hexane. Emulsions were clarified by addition of 0.5 ml methanol (38).

With both lipid extraction methods, the extract was dried under nitrogen with gentle heating. For quantitation, 100 µl of a 1 mg ml^{-1} (w/v) of cholesterol in chloroform was added prior to the hexane extraction step in the secretion mutant strains. The added cholesterol was used as a means of determining the extraction efficiency. In RD5-R samples, the same amount and concentration of extraction standard was used but it was not the same sterol as the one used for growth of the cells (e. g. for cells cultured on cholesterol, sitosterol (1 mg ml^{-1}) was used as an extraction standard).

Thin-Layer Chromatography

Sterol extracts (alkaline saponified and those steamed in DMSO) were purified from other lipids by thin-layer chromatography (TLC). The dried lipid extracts were solvated in chloroform and applied to silica gel plates (EM Laboratories Silica Gel 60 F-254, 20 cm^2). The plates were chromatographed in solvent-I (isopropyl ether:glacial acetic acid, 96:4, v/v) until the solvent front reached 12 cm above the

baseline. The plates were dried and rechromatographed in the same direction in solvent-II (hexane:diethyl ether:glacial acetic acid, 90:10:1, v/v/v) to within 1 cm of the top of the plate as described by Skipski and Barclay (74). Sterols were visualized by short wavelength UV (sterols with a conjugated double bond system) or by iodine staining (sterols without a conjugated double bond system).

Free sterols were recovered by scraping the silica gel containing sterol off of the plate and transferring the silica gel to Pasteur pipettes into which a cotton plug and powdered sodium sulfate had been previously inserted. The sterols were eluted from the column using three 2 ml volumes of chloroform:methanol (4:1, v/v) and dried under nitrogen with gentle heat.

Steryl esters were recovered by scraping the silica gel containing the ester into a screw cap test tube and saponifying for 2-3 hours at 60-80°C after addition of 6% methanolic KOH (w/v). Hydrolyzed ester (free sterol) was then recovered by extraction with hexane and dried under nitrogen with gentle heating.

The phospholipid band, which remained at the baseline, was also scraped and extracted from the silica gel using chloroform:methanol (1:1, v/v) and dried with N_2 and heat. Phospholipids were further separated by two other TLC systems, a two-dimensional system (75) using solvent-I (chloroform:methanol:acetic acid, 65:25:10, v/v/v) in the first dimension and solvent-II (tetrahydrofuran:methanol, 3:1, v/v) in the second dimension, or a one-dimensional HPLC system (76) modified for use with TLC (isopropyl alcohol:hexane:water, 53:40:7, v/v/v).

Liposome Preparations

RD5-R, cultured on YNB/CA/D supplemented with sterol (15 μg ml^{-1}) and 80 μM δ-aminolevulinic acid, was harvested at late log phase and extracted using the DMSO-hexane method described (38). The thin-layer chromatography system of Skipski and Barclay (74) was used to separate the phospholipid and sterol fractions. The sterols and phospholipids were quantitated as described in the sterol and phospholipid analyses sections.

The combinations for phospholipid and sterol used in liposome preparations are as shown in Table 2 and the molar ratios at the time of mixing are as indicated in Table 3. Components for liposomes (extracted phospholipids and sterols, Table 3) were prepared by solubilization in 5 ml of chloroform and then 1 ml volumes of the appropriate phospholipid and sterol were mixed. These mixtures were dried under N_2, and then, resuspended in buffer (10 mM TRIS, 1 mM EDTA, pH 6.8) containing fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (77,78) by vortex mixing and water bath sonication.

Plasma Membrane Isolation

RD5-R cells were converted to spheroplasts using a dialyzed extracellular preparation of lyticase enzyme from Oerskovia xanthineolytica. Spheroplasts were osmotically lysed and the plasma membrane vesicles were separated on a 10-40% sucrose step gradient using a modified procedure of Bottema et al. (77). For secretion mutant strains, a different lytic enzyme was used, NovoZym234^{tm} (Novo Industrie A|S Bagsvaerd, Denmark) (79). In both cases, only the lytic enzyme and its

Table 2

LIPOSOME RECONSTITUTION

Phospholipid from Cells Grown On	Sterol Used In Liposome		
	Cholesterol	Ergosterol	Sitosterol
Cholesterol	+	-	-
Ergosterol	-	+	-
Sitosterol	-	-	+

Liposomes were made by mixing the appropriate phospholipid extract and sterol from RD5-R with the probe DPH as described (77). Plots (+) of fluorescence anisotropy versus 1/K have no change in slope, while a (-) denotes a plot with a slope discontinuity.

Table 3

LIPOSOME COMPONENTS

Exogenous Sterol in Medium	Intracellular Sterol Whole Cell Total (nmol mg^{-1} dry wt.)		Phospholipid as Phosphate Whole Cell Total (nmol mg^{-1} dry wt.)		Homologous Sterol/PL (nmol $nmol^{-1}$)
	$\bar{X}$	SD	$\bar{X}$	SD	
Cholesterol	4.6	0.2	7.9	1.0	0.6
Ergosterol	2.5	0.1	5.9	0.2	0.4
Sitosterol	3.5	0.3	6.4	0.7	0.6
Stigmasterol	2.8	0.2	6.7	1.0	0.4

Free sterol and phospholipid quantitation for sterols and phospholipids used in liposome reconstitution. Sterols and lipids from RD5-R were extracted and chromatographed as described in Materials and Methods. In each case, the sterols and phospholipids were from cells cultured on the indicated sterol, 80 μM δ-aminolevulinic, acid and represent the mean molar ratios at the time of mixing. The mean and standard deviation are shown for sterol and phospholipid.

concentration differed. Briefly, the procedure consisted of harvesting appropriately treated cells (temperature up-shifted and no-temperature shift at mid-log for secretion mutants and, no temperature shift for RD5-R cultures); washing with glass distilled water and resuspending the cells in 0.5 M β-mercaptoethanol buffer containing 0.1 M TRIS, pH 9.3. After incubation for 15 minutes (30°C), these cells were washed two times in lyticase buffer (1.1 M sorbitol, 10 mM phosphate, 1 mM EDTA, pH 7.5), resuspended in either lyticase (in lyticase buffer) or in 3 mg ml^{-1} NovoZym234^{tm} (in lyticase buffer) and incubated for 30-45 min. The spheroplasts were washed twice with 1.2 M sorbitol in 10 mM TRIS, 1 mM EDTA, pH 7.4, and osmotically lysed by vortexing and water bath sonication after resuspension in lysis buffer (10 mM TRIS, 1 mM EDTA, pH 7.4).

The cell lysate was then loaded on 10-20-30-40% discontinuous sucrose gradients (2:2:2:2, v/v/v/v) containing 10 mM TRIS and 1 mM EDTA (pH 7.5). The gradients were centrifuged (4°C) at 16500 x g for 20 min using a Sorvall RC5-C equipped with an HB-4 swinging bucket rotor. The gradient was then fractionated by removal of the plasma membrane band located just below the interface of the 10% sucrose layer and cell lysate.

Phospholipid Analyses

For phospholipid analyses, total phospholipid as phosphate and individual phospholipid species were analyzed by the method of Ames (80). The reagents consisted of 10% (w/v) $Mg(NO_3)_2 \cdot 6H_2O$ in 95% ethanol, 0.5 N HCl, and a 1:6 (v/v) mixture of 10% ascorbic acid (w/v) : 0.42%

(w/v) $(NH_4)_2Mo_2O_4 \cdot 4H_2O$ in 1N H_2SO_4 (28.6 ml concentrated H_2SO_4 and 4.29 $(NH_4)_2Mo_2O_4 \cdot 4H_2O$ to a final volume of one liter). Absorbance at 820 nm was measured and compared against a serially diluted phosphate standard solution (KH_2PO_4, 2 mM).

Fatty Acid Analyses

Total fatty acids from phospholipid extracts were analyzed by gas liquid chromatography (GLC) after conversion to their methyl ester equivalent form (81,82). The procedure was a modification of the method described by Metcalfe et al. (81,82) using the following reagents: 0.5 N NaOH (in methanol), 10% $BaCl_3$ (in methanol), and an aqueous saturated salt solution (NaCl, 0.4 g ml^{-1}). The samples were steamed for two minutes after addition of 0.4 ml of methanolic NaOH, then, 0.5 ml of $BaCl_3$ was added and the samples steamed for an additional 2 minutes. Hexane was used to extract the fatty acid methyl esters (FAMEs) after addition of 1 ml of the saturated salt solution. Gas liquid chromatography for fatty acid methyl esters (FAMEs) was done using either a Varian 2740 or an HP5890A gas chromatograph with a 10% DEGS (Supelco) packed column (175°C, He carrier with a 20 ml min^{-1}, detectors and injectors at 190°C). Data acquisition and integration of peak areas was done using an IBM CS-9000 computer.

Fluorescence Anisotropy

For these studies, a facile, highly reproducible presumptive test to assess differences between the lipid component of various membranes and liposomal preparations was needed. For these analyses, the steady-

state fluorescence anisotropy (r_s) of the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (78) was used. While such measurements have been used to measure lipid layer microviscosity, inaccuracies with precise determination have been reported (37,83,84). Since the intent was to use the probe as an indicator of differences when comparing various liposome and membrane preparations, the data are reported simply as r_s values regardless of the specific physical state of the probe defined regions.

Steady-state fluorescence anisotropy changes were measured with a computerized anisotropy spectrofluorophotometer (77), using the probe DPH (78). Anisotropy was determined from the equation:

$$r_s = (I_{11}-I_1)/(I_{11}+2I_1)$$

where I_{11} and I_1 are the intensities of parallel and perpendicular polarized light respectively. At each temperature point, more than 1,000 individual anisotropy measurements were taken. The standard deviation of each anisotropy value plotted is 0.001. Here, temperature was determined within ±0.5°C. Absorbance (460 nm) was less than 0.40 for all samples, which contained DPH at a concentration of 1 μM in 10 mM TRIS, 1mM EDTA buffer, pH 6.8.

Using a second spectrofluorimeter (SLM Instruments Inc. series 8000) equipped with a water-jacketed cuvette holder, fluorescence anisotropy analyses of plasma membrane preparations from the secretion mutants were carried out from 12-42°C.

The steady-state polarization, P, was calculated from the equation $P=(R-1)/(R+1)$ and by substitution of $R=I_{11}/I_1$ into the equation $r_s = (I_{11}-I_1)/(I_{11}+2I_1)$, the steady-state fluorescence anisotropy was

determined. At each temperature point, triplicate measurements of the total photon counts were taken (>2X10^5 photons counted per data point measurement for statistical relevance).

Materials

Cholesterol, ergosterol, sitosterol, stigmasterol, DPH, bovine serum albumin, tyloxapol, TRIS and EDTA were purchased from Sigma. Sterols were purified by HPLC to remove trace impurities and other contaminating sterols (85). Lipid standards (myristic, (C14:0); palmitic, (C16:0); palmitoleic (C16:1); stearic (C18:0); and palmitoleic (C18:1) acids), NHI-D mixture, and phospholipid standards (CL, PC, PE, PI, and PS) were from Supelco as well. Solvents, (Mallinckrodt and Fisher) were redistilled prior to use. The HPLC (Altex model 332 liquid chromatograph) was purchased from Beckman.

RESULTS

Growth Of Yeast Strains

The growth characteristics of RD5-R and the representative set of secretion mutants are shown (Figures 4 and 5). The wildtype (X2180-1A typically had the fastest growth rate with RD5-R and the secretion mutants having a marginally slower growth rate (Figure 4) at 24°C. However, when the growth rates for these strains were examined after the temperature cycling (T=24°C to T=37-39°C for 2 hours followed by T=37-39°C to T=24°C) even the wildtype strain displayed a decreased growth rate (Figure 5) from which they did not recover (at least for the duration of the batch culture). During the temperature up-shift, there was an initial increase in turbidity (measured in Klett units) followed by a plateau (change in Klett units <50 for 3 hours). While these cells eventually reached stationary phase, the growth period was extented two to threefold compared to cells cultured at 24°C.

Effect Of Sterol On Plasma Membrane Properties

When the sterol auxotroph RD5-R was grown in medium supplemented with ergosterol, these exogenous concentrations of sterol (1, 5, and 15 $\mu g\ ml^{-1}$) were not the same as the intracellular concentration of free sterol in whole cells (Table 4). However, there is a corresponding increase in sterol content in both whole cell and plasma membrane samples as a function of the exogenous sterol concentration. It is of interest to note that, the sterol to phospholipid ratio at each

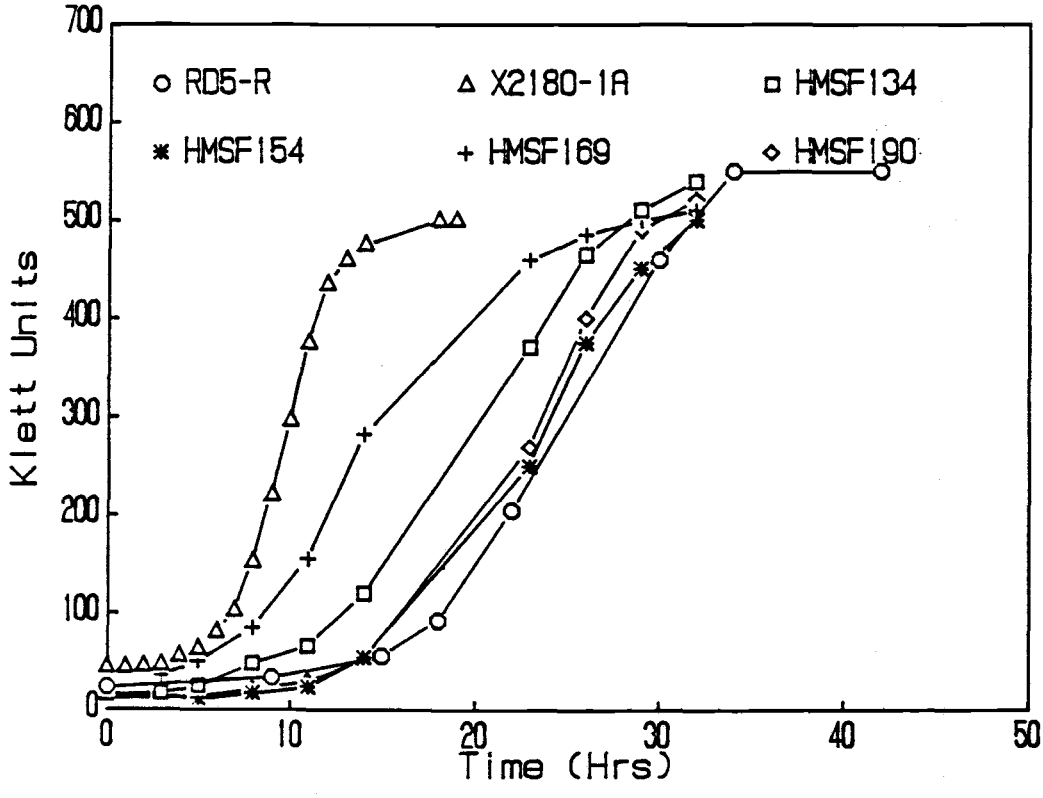

Figure 4. Growth of yeast strains. These strains were cultured on yeast nitrogen base dextrose (HMSF134, HMSF154, HMSF169, HMSF190, and X2180-1A) or on YNB/CA/D with unsaturated fatty acid and sterol supplements (RD5-R). In all cases, the growth temperature was 24°C.

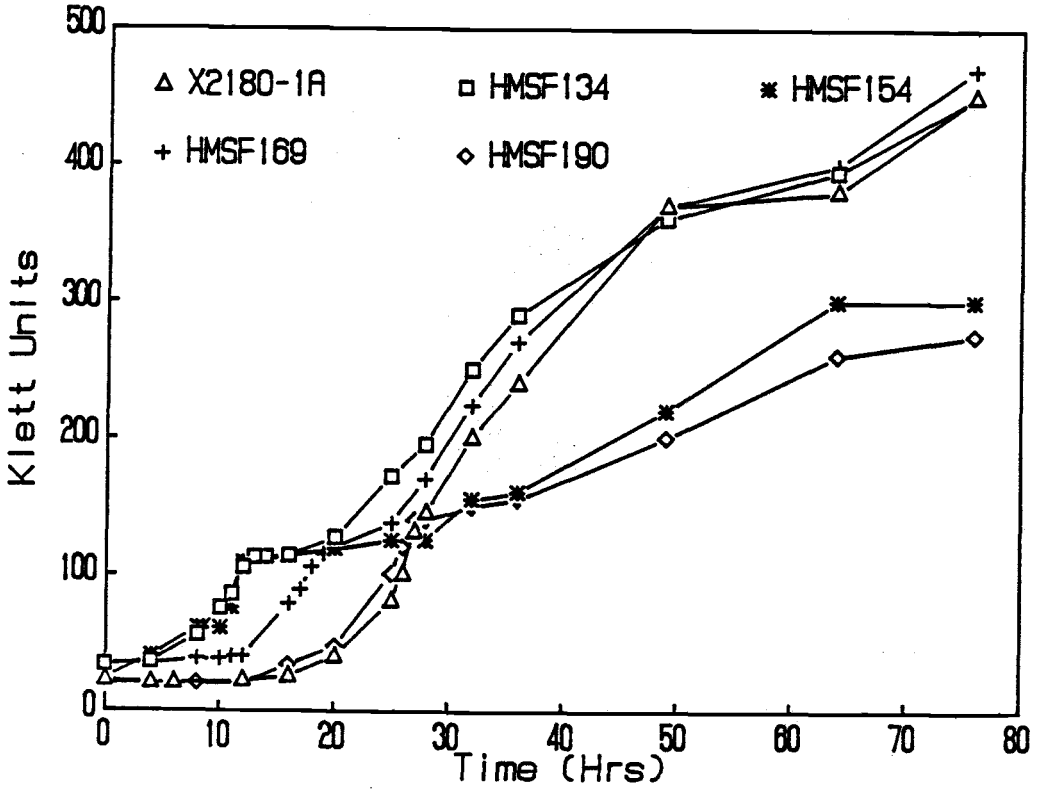

Figure 5. Dual temperature shift growth of secretion mutants. Cells were cultured on yeast nitrogen base with dextrose to 100 Klett units at 24°C then shifted to 37-39°C for 2 hours. At the end of 2 hours, the cultures were shifted back to growth at 24°C. The time period of the temperature upshift for each strain starts at 100 Klett units.

TABLE 4

COMPARATIVE ANALYSES OF RD5-R STEROL AND STEROL TO PHOSPHOLIPID RATIOS

Exogenous Ergosterol $\mu g\ ml^{-1}$	Endogenous Ergosterol		Sterol/Phospholipid Ratio	
	Whole[a,b] Cell $\mu mol\ mg^{-1}$ dry wt.	Plasma[c] Membrane $\mu mol\ ml^{-1}$	Whole[a,b] Cell $\mu mol\ \mu mol^{-1}$	Plasma Membrane $\mu mol\ \mu mol^{-1}$
1.0	0.0008	<.1	0.1	0.3
5.0	0.0028	0.1	0.4	0.3
10.0	0.0053	0.2	0.8	0.8
15.0	0.0063	0.3	0.7	0.7

[a]Data shown here are the result of triplicate samples and have an average error of less than five percent. These cells were cultured on different concentrations of ergosterol, and lipids were quantitated as described in Materials and Methods.

[b]Whole cell analyses (8).

[c]All plasma membrane preparations (1 liter cultures) were normalized to a given volume and absorbance at 420 nm.

exogenous sterol concentration is approximately equal for both whole cell and plasma membrane preparations indicating that the values obtained from whole cell analyses are a reasonable approximation of the sterol to phospholipid ratio of the plasma membrane.

When RD5-R was grown in medium supplemented with either cholesterol or ergosterol, the mean values were from 4.6 to 8.8 μg sterol mg^{-1} dry weight for ergosterol and cholesterol respectively. Fluorescence anisotropy measurements for plasma membrane prepared from these cultures were made over the temperature range of 15 to 39°C. The fluorescence anisotropy decreased with increasing temperature indicating an increase in probe mobility with increasing temperature. The plots (Figures 6 and 7) of fluorescence anisotropy revealed no discontinuities (i. e. a change of slope in the plot). Such a discontinuity is interpreted as a change in fluid state in at least the lipid microenvironment surrounding the probe molecule (8) (Table 2).

Liposomes

Reconstitution experiments were performed using phospholipids and sterols extracted from RD5-R cells grown on ergosterol, cholesterol, or sitosterol. Liposomes were constructed with phospholipid from cells grown on cholesterol by recombining them with sterols extracted from cells grown on ergosterol, cholesterol, or sitosterol (Table 2) (e. g. the amount of ergosterol added to phospholipid extracted from cells grown on cholesterol would be the same as the amount of ergosterol added to phospholipid extracted from cells grown on ergosterol). This same procedure was used in making liposomes with phospholipids from

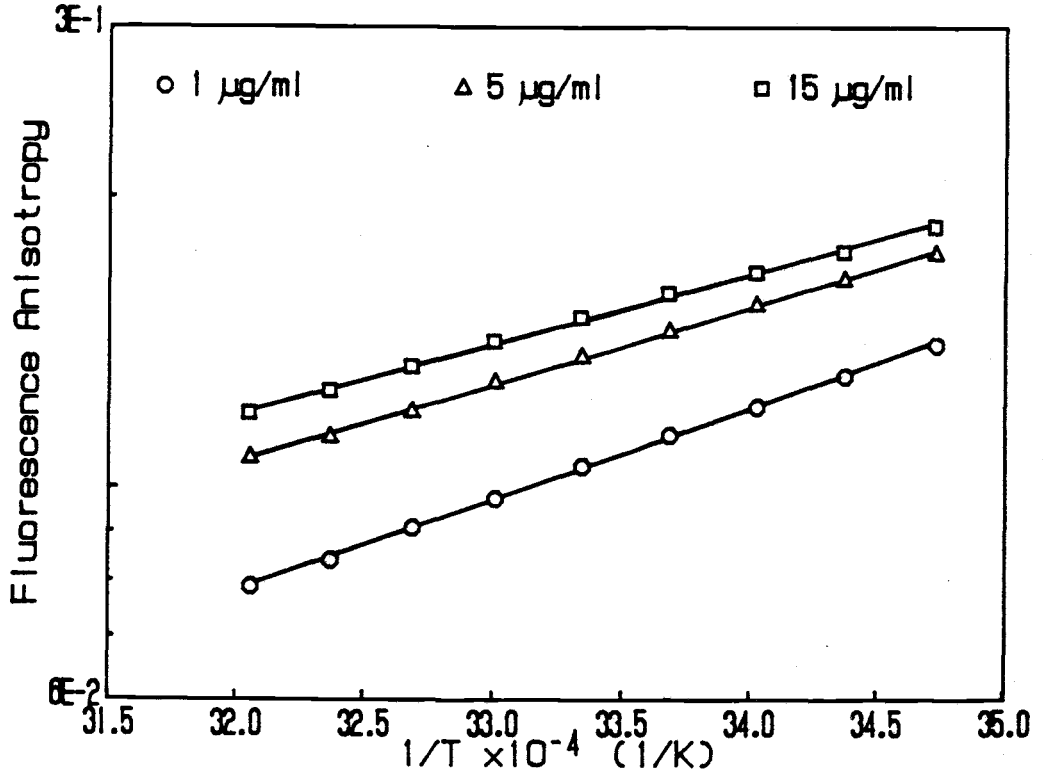

Figure 6. Fluorescence anisotropy of cholesterol supplemented RD5-R plasma membrane. Fluorescence anisotropy (r_s) of plasma membrane from RD5-R cultured in medium supplemented with 1, 5, or 15 μg ml^{-1} cholesterol and 80 μM δ-aminolevulinic acid.

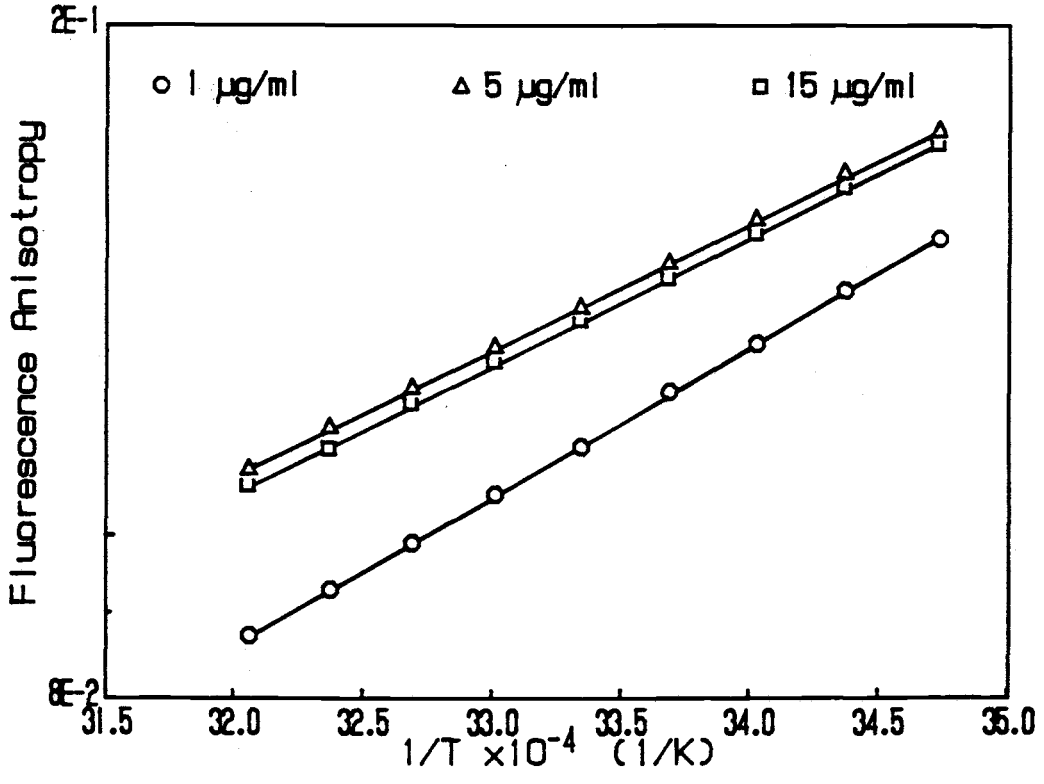

Figure 7. Fluorescence anisotropy of ergosterol supplemented RD5-R plasma membrane. Fluorescence anisotropy (r_s) of plasma membrane from RD5-R cultured in medium supplemented with 1, 5, or 15 µg ml^{-1} ergosterol and 80 µM δ-aminolevulinic acid.

cells grown on either sitosterol or ergosterol and the sterols from cells grown on ergosterol, cholesterol, or sitosterol. In all cases, the sterol to phospholipid mean molar ratio was 0.5 (SD of 0.14) (calculated from Table 3). Plasma membranes from cells grown on cholesterol, ergosterol, or sitosterol were also prepared and analyzed along with the liposomes.

In all cases, when the phospholipid fraction from cells grown on ergosterol was mixed with cholesterol (extracted from cells grown on cholesterol) or sitosterol (extracted from cells grown on sitosterol) a discontinuity in the r_s plot was observed (Figure 8, plots B, C). This phenomenon was also observed with phospholipid extracted from cells grown on cholesterol or sitosterol when recombined with either ergosterol or sitosterol for cholesterol grown cells (Figure 9, plots A, C) or with cholesterol or ergosterol for sitosterol grown cells (Figure 10, plots A, B). However, plots of steady state fluorescence anisotropy (Figure 9, plot B) for liposomes containing phospholipid and sterol from cells grown on cholesterol showed no discontinuity. Identical effects were found for liposomes containing phospholipid and sterol from ergosterol grown cells or phospholipid and sterol from sitosterol grown cells, Figures 8 (plot A), and 7 (plot C). These results, summarized in Table 2, compare favorably with fluorescence anisotropy plots (Figures 6 and 7) of RD5-R plasma membrane.

Sterol, Phospholipid, And Fatty Acid Analysis Of RD5-R

The data from Table 3 indicated an approximate 1.8 fold increase in extracted free cholesterol for RD5-R grown in medium containing

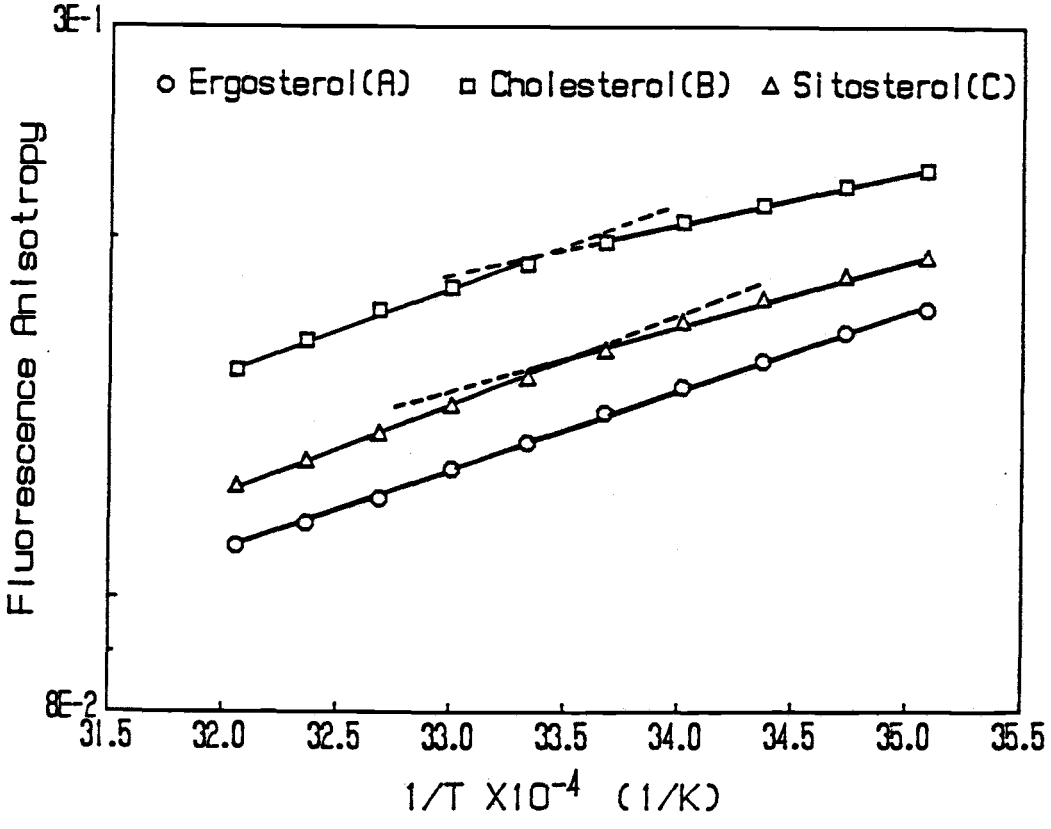

Figure 8. Fluorescence anisotropy of RD5-R liposomes (ergosterol). Fluorescence anisotropy (r_s) of liposomes reconstituted from phospholipid extracted from cells cultured on 15 µg ml^{-1} ergosterol and 80 µM alv supplemented medium. The phospholipid was mixed with sterol extracted from cells cultured on 15 µg ml^{-1} ergosterol (A), cholesterol (B), and sitosterol (C).

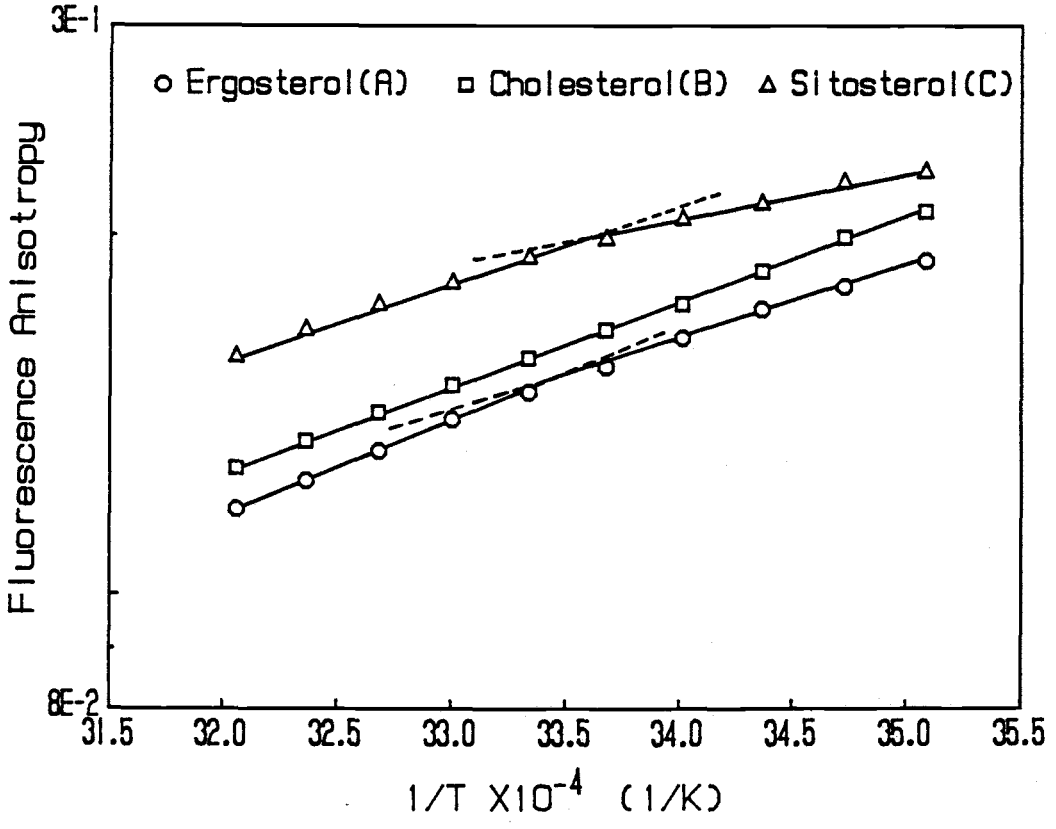

Figure 9. Fluorescence anisotropy of RD5-R liposomes (cholesterol). Fluorescence anisotropy (r_s) of liposomes reconstituted from phospholipid extracted from cells cultured on 15 µg ml^{-1} cholesterol and 80 µM alv supplemented medium. The phospholipid was mixed with sterol extracted from cells cultured on 15 µg ml^{-1} ergosterol (A), cholesterol (B), and sitosterol (C).

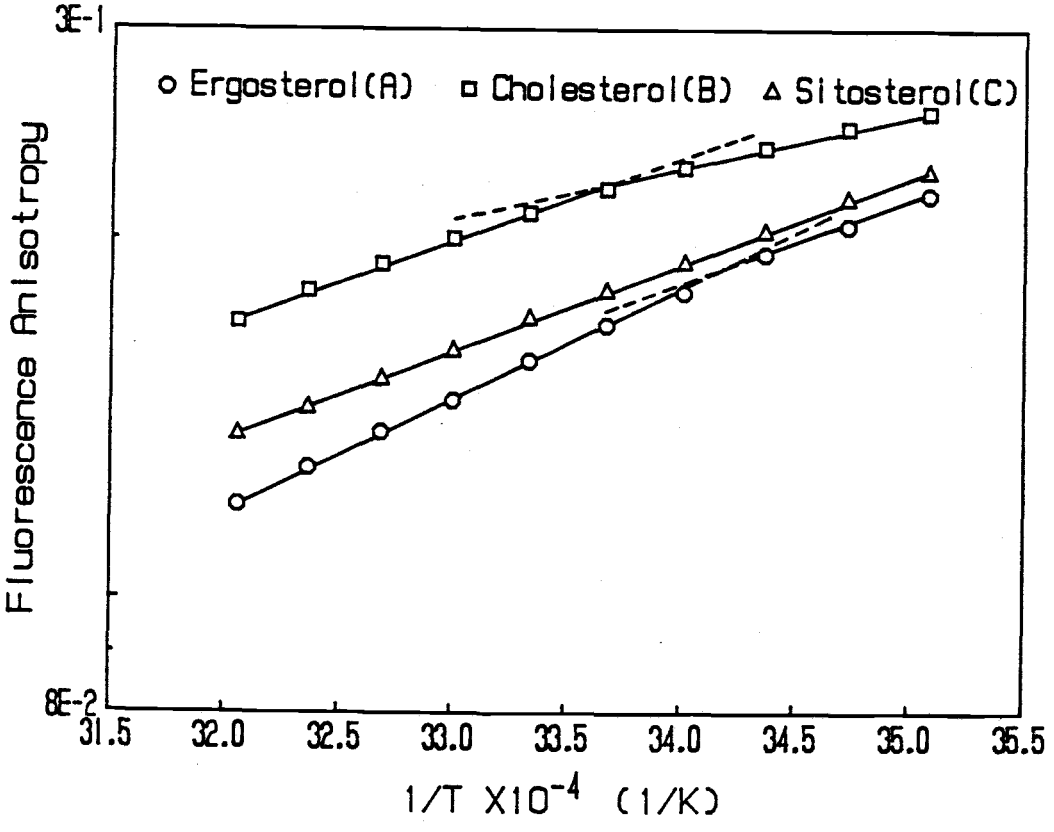

Figure 10. Fluorescence anisotropy of RD5-R liposomes (sitosterol). Fluorescence anisotropy (r_s) of liposomes reconstituted from phospholipid extracted from cells cultured on 15 µg ml^{-1} sitosterol and 80 µM alv supplemented medium. The phospholipid was mixed with sterol extracted from cells cultured on 15 µg ml^{-1} ergosterol (A), cholesterol (B), and sitosterol(C).

cholesterol compared to growth of RD5-R on ergosterol. The amount of phospholipid, however, remained relatively constant (total phospholipid as nmol mg^{-1} dry weight) as did the mean sterol to phospholipid ratio. Despite the constancy of the sterol to phospholipid ratio, analysis of the different phospholipids by head group (Table 5) revealed an approximate 1.7 fold increase in phosphatidylinositol (PI) for cholesterol grown cells compared to cells grown on ergosterol, sitosterol or stigmasterol. Phosphatidylethanolamine (PE) in cholesterol grown cells is approximately one-half of that found in ergosterol, sitosterol, or stigmasterol grown cells. The percentages of phosphatidylcholine (PC), phosphatidylserine (PS), and cardiolipin (CL) were not significantly different in cholesterol cultured cells compared to those from ergosterol and stigmasterol grown cells. In CL, the only change noted was for sitosterol grown cells, where, CL was only 1.2 percent of the total phospholipid compared to 6 to 10 percent in cholesterol, ergosterol, and stigmasterol grown cells.

Analysis of total fatty acids (Table 6) revealed a twofold increase in C16:1 and C18:1 fatty acid species and an approximate two-fold decrease in C16:0 fatty acid for cells cultured on cholesterol compared to cells cultured on ergosterol, sitosterol, and stigmasterol. Cells grown on ergosterol, sitosterol, or stigmasterol had smaller changes in their _in vivo_ phospholipid pattern.

Analysis of the fatty acid composition of individual phospholipid species (Table 7) indicated an elevated level of C16:1 and C18:1 for PC and an elevated level of C18:1 for PI in cells cultured on cholesterol compared to cells grown on exogenous ergosterol, sitosterol, and/or

Table 5

PERCENT OF TOTAL PHOSPHOLIPID HEAD GROUPS

Sterol In Growth Medium	Percent Of Phospholipids By Head Group									
	PC		PS		PI		PE		CL	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Cholesterol	32.3	0.8	10.0	0.8	28.5	2.0	23.1	3.9	6.2	0.8
Ergosterol	29.5	3.4	6.5	0.8	16.5	3.4	40.6	3.4	6.9	1.4
Sitosterol	34.2	2.4	9.2	1.2	18.9	3.1	36.6	2.0	1.2	0.2
Stigmasterol	28.7	1.2	7.0	2.6	15.9	5.1	37.6	2.6	10.8	2.0

Phospholipids (Table 3) were rechromatographed using a two dimensional TLC system as described in Materials and Methods. Individual phospholipids extracted from RD5-R were visualized using iodine and quantitated by the method of Ames (80).

Table 6

PERCENT OF TOTAL FATTY ACIDS BY CHAIN LENGTH AND UNSATURATION IN A STEROL AUXOTROPH

Sterol In Growth Medium	Percent Fatty Acid									
	C14:0		C16:0		C16:1		C18:0		C18:1	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Cholesterol	3.2	0.2	28.8	0.1	45.9	0.1	5.0	0.6	16.3	0.1
Ergosterol	5.9	0.2	58.2	0.1	22.5	0.1	6.8	0.2	6.4	0.1
Sitosterol	4.4	0.1	51.9	0.1	28.8	0.2	5.9	0.1	8.7	0.1
Stigmasterol	7.9	0.5	59.6	0.3	19.1	0.1	7.0	0.8	5.3	0.5

Fatty acid species were determined using total phospholipid extract from RD5-R. Methyl esters were made as described in Materials and Methods.

Table 7

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS IN RD5-R

Phospholipid Extracted From Cells Grown On	Percent Fatty Acid									
	14:0		16:0		16:1		18:0		18:1	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
PC										
Cholesterol	1.8	0.1	54.0	2.7	15.3	0.1	17.5	0.9	11.5	1.9
Ergosterol	4.4	1.1	79.6	4.8	3.4	0.3	10.1	4.8	2.5	0.9
Sitosterol	0.9	0.1	63.7	0.2	4.9	0.2	22.9	0.2	7.6	0.1
Stigmasterol	2.0	0.1	44.8	1.7	1.6	0.1	44.3	0.9	7.2	2.7
PS										
Cholesterol	1.5	0.2	58.7	7.8	5.4	1.5	21.4	3.4	13.0	2.8
Ergosterol	3.5	0.1	63.8	1.8	3.3	0.3	14.2	1.5	15.3	0.1
Sitosterol	3.9	0.5	70.7	9.4	7.0	0.7	7.8	3.1	10.7	7.3
Stigmasterol	1.2	0.3	44.2	6.0	3.4	3.1	26.8	4.3	24.4	1.7
PI										
Cholesterol	1.6	0.1	51.0	1.8	4.8	0.8	28.2	1.0	14.5	3.7
Ergosterol	2.1	1.0	59.3	3.7	2.6	0.7	27.8	2.4	8.2	2.9
Sitosterol	1.1	0.1	40.9	2.3	3.0	0.1	45.7	2.0	9.3	4.2
Stigmasterol	4.1	0.3	70.1	7.1	6.4	0.5	10.8	3.0	8.6	3.9
PE										
Cholesterol	3.9	0.4	73.3	2.7	6.4	0.2	6.5	0.6	9.9	1.5
Ergosterol	33.6	0.2	78.9	3.3	5.8	0.4	6.2	1.2	5.6	1.9
Sitosterol	11.8	0.1	76.5	0.5	6.7	0.2	7.2	0.1	7.8	0.5
Stigmasterol	22.8	0.7	43.7	1.1	5.1	0.6	36.3	0.1	12.1	1.2
CL										
Cholesterol	1.3	0.5	32.7	0.7	9.6	0.5	27.2	1.0	29.2	0.9
Ergosterol	2.7	0.1	74.6	0.4	1.7	1.5	10.5	0.7	10.6	0.4
Sitosterol	18.4	1.7	21.2	1.9	6.0	0.5	21.6	0.4	32.7	3.7
Stigmasterol	6.6	0.8	73.7	3.7	2.3	0.2	8.6	1.7	8.8	2.6

The fatty acid composition of the individual phospholipids were determined after two dimensional TLC. Methyl esters of the fatty acids from RD5-R were made as described in Materials and Methods.

stigmasterol. A two to tenfold depression of the level of C14:0 fatty acid in PE occurs in cholesterol cultured cells. However, there appears to be a generalized increase in the percentage of longer chain fatty acids (C14:0 and C16:0 compared to C16:1, C18:0, and C18:1) for cardiolipin. The remaining data revealed that other modifications made by these cells to the in vivo fatty acid composition of the individual phospholipid species were small.

Screening Of Secretion Mutants For Altered Sterol Metabolism

Preliminary growth experiments (Figure 5) showed that temperature sensitivity was detectable in these mutants after two hours at 37°C. Initial screening for altered sterol metabolism in the secretion mutants listed in Table 1 consisted of growing the strains to 100 Klett units, splitting the cultures in half and culturing them at either 24 or 37°C for an additional two hours before harvesting the cells. The cell extracts were then evaluated for differences in free and esterified sterol by TLC. As seen in Table 8, strain HMSF134 did not synthesize TLC-detectable amounts of steryl ester when incubated at the non-permissive temperature. The remaining secretion mutant strains tested (Table 8), however, are like the wildtype and begin to synthesize ester upon altering the growth temperature to 37°C. In the cultures incubated at 24°C, the level of steryl ester remained low since these cells are still in mid-exponential phase of growth and they do not normally begin ester synthesis in this stage of the culture cycle (in wildtype cells, ester synthesis begins upon entry of the cells into stationary phase (86,87).

Table 8

THIN-LAYER CHROMATOGRAPHY OF SECRETION MUTANT STERYL ESTERS

Strains		Free Sterol	Steryl Ester
X2180-1A	wildtype	+	+
HMSF1	sec1-1	+	+
HMSF134	sec5-24	+	-
HMSF136	sec6-4	+	+
HMSF143	sec9-4	+	+
HMSF154	sec11-7	+	+
HMSF169	sec14-3	+	+
HMSF171	sec15-1	+	+
HMSF175	sec17-1	+	+
HMSF178	sec19-1	+	+
HMSF179	sec20-1	+	+
HMSF180	sec21-1	+	+
HMSF183	sec22-3	+	+
HMSF190	sec23-1	+	+

These cells were cultured in YNBD at 24°C to 100 Klett units and then split into two equal volumes. One-half of the split culture was reincubated at 24°C and the other at 37°C for two hours. Cellular extracts were made from cells that were DMSO treated as described (38) and chromatographed using the solvent system of Skipski and Barclay (74).

These temperature up-shift split culture experiments were repeated and total sterols were quantitated on a per cell basis (Table 9). It was found that there were no large interstrain differences in total sterol for cells cultured under the same temperature regimen. For cells grown to stationary phase, the range of total sterol was 0.4 to 0.6 μg $1X10^8$ $cells^{-1}$. There is an approximate three to fourfold increase in total sterol content in cells cultured to mid-exponential phase and harvested but the amount of sterol was relatively constant between secretion mutant phenotypic groups. For cells subjected to temperature up-shift, the total sterol content ranged from 0.4 to 0.9 μg $1X10^8$ $cells^{-1}$, an amount not substantially different from cells grown to stationary phase.

However, when sterols were extracted from these cells using the DMSO extraction method of Parks et al. (38) to preserve the ester component, a very different sterol pattern was observed (Table 10). In one of the strains (HMSF134) it was noted that free sterol and steryl ester biosynthesis appeared normal when this strain was grown to stationary phase, but steryl ester synthesis (detected by GLC analysis of the steryl ester fraction after saponification in 6% methanolic KOH) did not become activated under conditions of a temperature up-shift as did the other representative strains. Further examination of the steryl ester synthase in HMSF134 showed that the enzyme preparation from cells subjected to temperature up-shift was at least as active as that extracted from other secretion mutant strains that had been subjected to similar thermal cycling (T. Lewis, personal communication).

Table 9

TOTAL STEROL IN SECRETION MUTANTS AND THEIR PARENT

	TOTAL STEROL (μg sterol $1X10^8$ cells ml^{-1})				
	wildtype X2180-1A	vesicles[a] HMSF134	none[a] HMSF154	Golgi[a] HMSF169	ER[a] HMSF190
sta nts[b]	0.4	0.4	0.6	0.5	0.5
ml nts[c]	1.6	1.3	2.0	2.0	1.1
ml ts[d]	0.5	0.9	0.6	0.4	0.5

Sterols from different classes of secretion mutants (μg sterol $1X10^8$ $cells^{-1}$) obtained by alkaline pyrogallol saponification (38).

[a]accumulated structures on temperature up-shift.
[b]sta nts - stationary phase, no temperature shift.
[c]ml nts - mid-exponential growth phase, no temperature shift.
[d]ml ts - mid-exponential growth phase, temperature shift to 37°C.

Table 10

SECRETION MUTANTS FREE AND ESTERIFIED STEROL PER CELL

	wildtype X2180-1A	vesicles[a] HMSF134	none[a] HMSF154	Golgi[a] HMSF169	ER[a] HMSF190
FREE STEROL (μg sterol $1X10^8$ $cells^{-1}$)					
sta nts[b]	0.4	0.4	0.2	0.4	0.3
ml nts[c]	1.4	1.2	1.4	1.4	1.0
ml ts[d]	0.2	0.9	0.4	0.3	0.3
STERYL ESTER (μg sterol $1X10^8$ $cells^{-1}$)					
sta nts	0.3	0.2	0.1	0.2	0.2
ml nts	0.1	0.1	0.4	0.5	0.3
ml ts	0.3	0.1	0.5	0.1	0.2
RATIO FREE STEROL (FS) TO STERYL ESTER (SE) (FS/SE)					
sta nts	1.3	2.0	2.0	2.0	1.5
ml nts	14.0	12.0	3.5	2.8	3.3
ml ts	0.7	9.0	0.8	3.0	0.7

These cells were lyophilized and steamed in 100% DMSO.
[a]accumulated structures on temperature up-shift.
[b]sta nts - stationary phase, no temperature shift.
[c]ml nts - mid-exponential growth phase, no temperature shift.
[d]ml ts - mid-exponential growth phase, temperature shift to 37°C.

Whether these strains were compared on a per cell basis or by dry weight, the data (Tables 10 and 11) indicated that the steryl ester synthase had not been activated in the same manner as the wildtype or the other secretion mutant strains tested. The data for HMSF134, Table 10, showed a 9 and 12-fold free sterol to total steryl ester ratio in mid-log temperature up-shifted and non-temperature shifted cultures respectively, when the cells are compared on a per cell basis. A comparison of similar data from cells quantitated by dry weight showed a 10 and 12.5-fold free sterol to total steryl ester ratio when cultured under the same conditions. Of the other HMSF strains tested, there was a range of free sterol to total steryl ester of 0.6:1 to 5:1 when both quantitative methods are considered.

Effect Of Temperature On Secretion Mutant Plasma Membranes

Analysis of the steady-state fluorescence anisotropy for plasma membrane preparations showed that in each strain, X2180-1A, HMSF134, HMSF154, HMSF169 and HMSF190, there was a discontinuity in the plots for strains not subjected to a temperature up-shift (Figure 11). However, under conditions of temperature up-shift, strain HMSF134 plasma membrane preparations displayed no discontinuity while the plots for X2180-1A, HMSF154, HMSF169, and HMSF190 displayed a discontinuity similar to that for non-temperature up-shifted cells (Figure 12). In each case, except for HMSF134, the temperature at which the discontinuity appears is shifted to a higher temperature by 3-6°C. The temperature at which these discontinuities appear (33-36°C in non-temperature shifted cells, and 36-42°C in temperature up-shifted cells)

Table 11

SECRETION MUTANTS FREE AND ESTERIFIED STEROL BY DRY WEIGHT

	wildtype X2180-1A	vesicles[a] HMSF134	none[a] HMSF154	Golgi[a] HMSF169	ER[a] HMSF190
FREE STEROL (µg sterol mg^{-1} dry wt.)					
sta nts[b]	2.0	2.0	0.8	2.1	2.1
ml nts[c]	2.5	2.5	2.5	2.3	2.3
ml ts[d]	3.8	2.0	1.7	2.2	1.9
ERGOSTERYL ESTER (µg sterol mg^{-1} dry wt.)					
sta nts	0.2	0.2	0.2	0.5	0.4
ml nts	0.4	0.1	0.8	0.3	0.2
ml ts	0.3	0.1	0.5	0.7	0.5
TOTAL STERYL ESTER (µg sterol mg^{-1} dry wt.)					
sta nts	1.4	1.2	1.1	1.9	2.0
ml nts	0.5	0.2	1.8	1.4	0.6
ml ts	1.4	0.2	2.7	2.4	2.0
RATIO FREE STEROL (FS) TO STERYL ESTER (SE) (FS/SE)					
sta nts	0.7	1.7	0.7	1.1	1.1
ml nts	5.0	12.5	1.4	1.6	3.8
ml ts	2.7	10.0	0.6	0.9	1.0

These cells were lyophilized and steamed in 100% DMSO.
[a]structures accumulated on temperature up-shift.
[b]sta nts - stationary phase, no temperature shift.
[c]ml nts - mid-exponential growth phase, no temperature shift.
[d]ml ts - mid-exponential growth phase, temperature shift to 37°C.

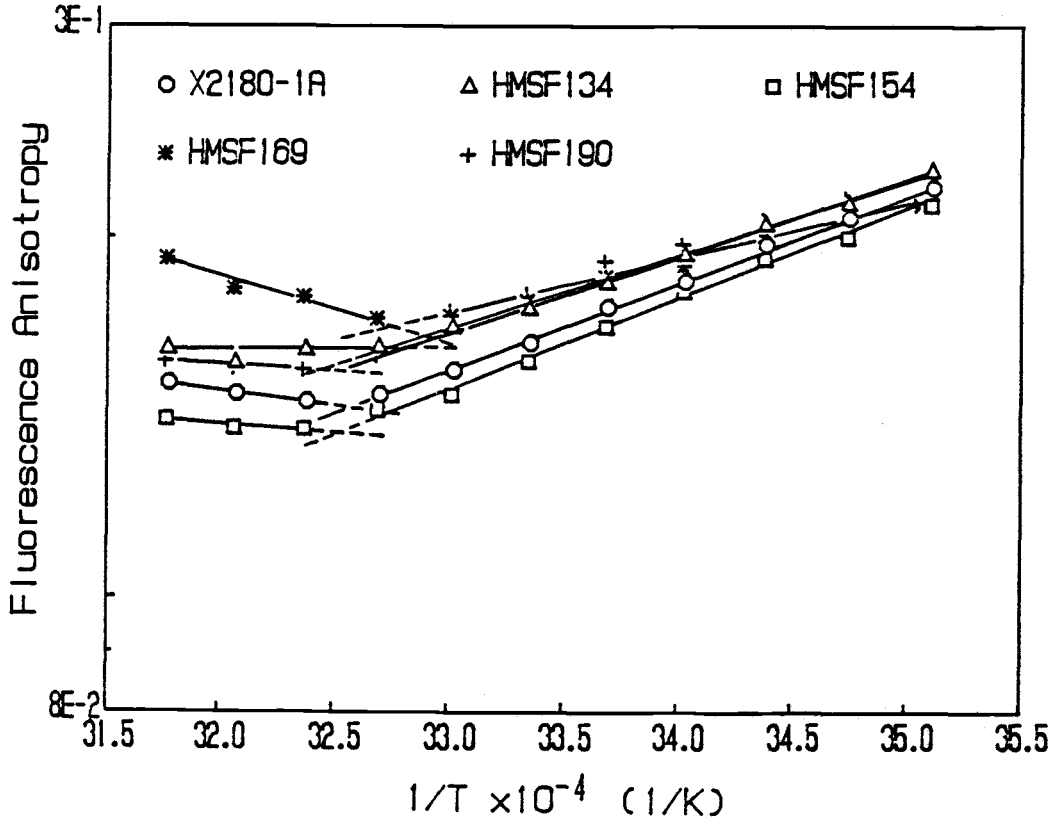

Figure 11. Fluorescence anisotropy of plasma membrane from secretion mutants (24°C). Plasma membranes were isolated from secretion mutants cultured at 24°C to mid-log phase. The wildtype (X2180-1A) is shown for comparison over the range of 12 to 42°C.

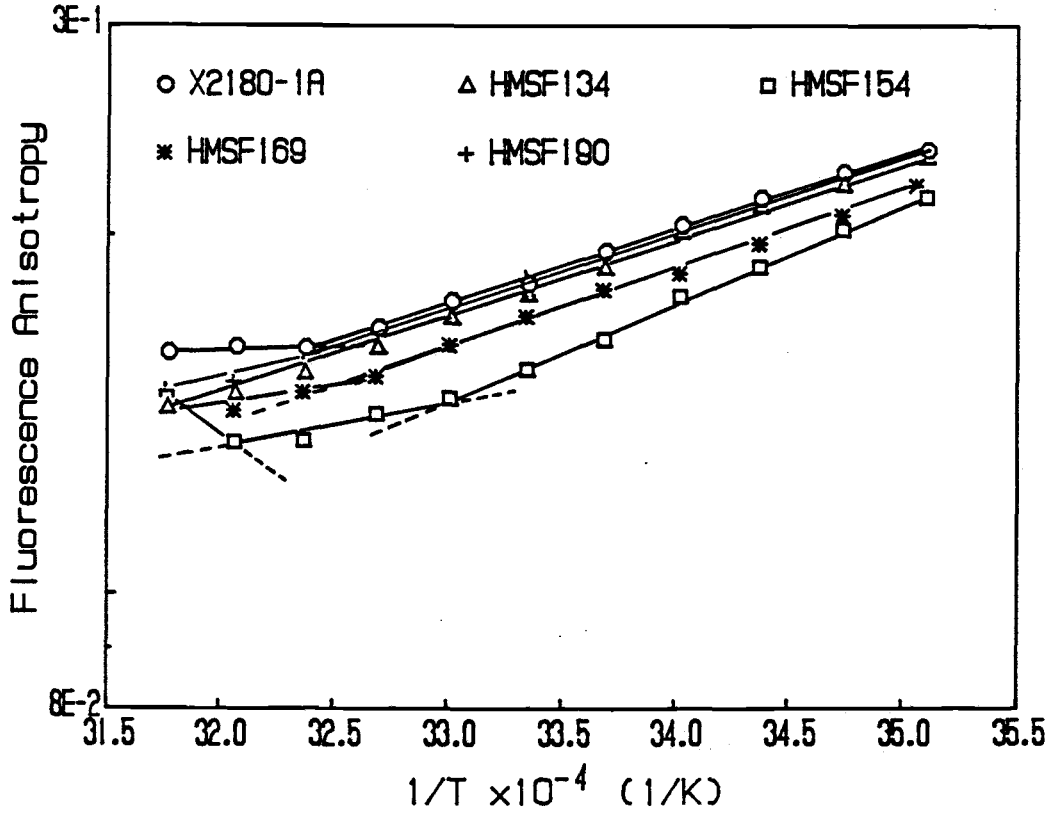

Figure 12. Fluorescence anisotropy of plasma membrane from secretion mutants (37-39°C). Plasma membranes were isolated from secretion mutants cultured at 24°C to mid-log phase then temperature up-shifted to 37-39°C for 2 hours before harvesting. The wildtype (X2180-1A) is shown for comparison over the range of 12 to 42°C.

is above the optimal growth temperature range of 28-32°C for S. cerevisiae.

Fatty Acid Analyses Of Secretion Mutants

While analyses of fatty acids (Table 12) from cells cultured to stationary (no temperature shift), mid-log (no temperature shift), and mid-log (with subsequent temperature up-shift) did not reveal any large differences, comparative analyses of the fatty acids from cultures grown to stationary phase at different temperatures did. As seen in Figures 13, 14, 15, and 16, as the temperature increased, there was a general increase in the content of C16:0 and C18:0 until a growth temperature just less than the restrictive temperature was reached. When the culture reached the restrictive temperature, there appeared to be a 2 to 3-fold increase in the amount of C16:0 in the secretion mutant and wildtype (X2180-1A) strains which grew at 40°C (Figure 13). For the C18:0 component (Figure 15), the increase was not as pronounced except for HMSF134. The C16:1 and C18:1 components decreased as the growth temperature increased and like the saturated component, when a growth temperature just below the restrictive temperature was reached, there was a large decrease in C16:1 and C18:1 (Figures 14 and 16).

Radiolabel Analyses

Using the wildtype (X2180-1A), HMSF134, and HMSF169 strains it was found that HMSF134, under the conditions of the temperature shift, did not esterify sterol to the same extent as that found in the wildtype and HMSF169. A further observation was that even under conditions of

Table 12

PERCENTAGE OF TOTAL FATTY ACIDS BY CHAIN LENGTH AND UNSATURATION IN SECRETION MUTANTS

Strain	Percentage Fatty Acid Type									
	C14:0		C16:0		C16:1		C18:0		C18:1	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
X2180-1A										
sta-nts[a]	3.2	0.1	39.0	3.6	17.5	3.2	37.7	7.7	4.2	1.1
ml-nts[b]	3.4	0.8	44.1	7.0	20.0	3.6	29.4	3.0	3.3	0.9
ml-ts[c]	2.4	1.5	37.5	6.8	23.6	3.8	32.3	3.9	4.3	0.7
HMSF134										
sta-nts	1.9	0.5	35.2	3.7	16.9	4.7	44.2	10.6	3.6	0.7
ml-nts	5.0	2.8	49.8	5.1	18.3	1.8	26.1	2.7	2.7	0.6
ml-ts	<1.0	0.1	35.1	7.1	20.8	3.7	36.1	5.7	10.2	2.2
HMSF154										
sta-nts	<1.0	0.1	34.6	5.8	14.6	1.1	44.6	6.2	6.2	1.1
ml-nts	2.9	2.5	50.1	1.4	20.9	0.3	23.6	2.0	2.5	0.1
ml-ts	3.3	0.2	33.8	7.6	23.7	3.9	34.6	10.9	6.3	2.5
HMSF169										
sta-nts	1.9	0.4	42.1	0.3	17.3	0.2	35.5	0.6	3.0	0.5
ml-nts	2.5	1.0	52.2	1.4	13.9	2.6	31.1	4.3	2.0	0.1
ml-ts	<1.0	0.1	31.1	3.9	17.7	2.8	43.9	5.6	7.0	1.3
HMSF190										
sta-nts	0.9	0.3	36.9	2.7	15.0	3.5	43.0	4.9	4.7	1.4
ml-nts	2.5	0.1	45.2	11.8	12.4	1.6	36.3	10.0	4.9	4.9
ml-ts	1.6	0.2	46.2	5.2	13.7	4.0	34.7	3.2	4.2	2.7

Lipid extracts obtained after steaming in the presence of DMSO as described (38). Analyses were conducted by GLC as described in Materials and Methods.

[a]sta nts - stationary phase, no temperature shift.
[b]ml nts - mid-exponential growth phase, no temperature shift.
[c]ml ts - mid-exponential growth phase, temperature shift to 37°C.

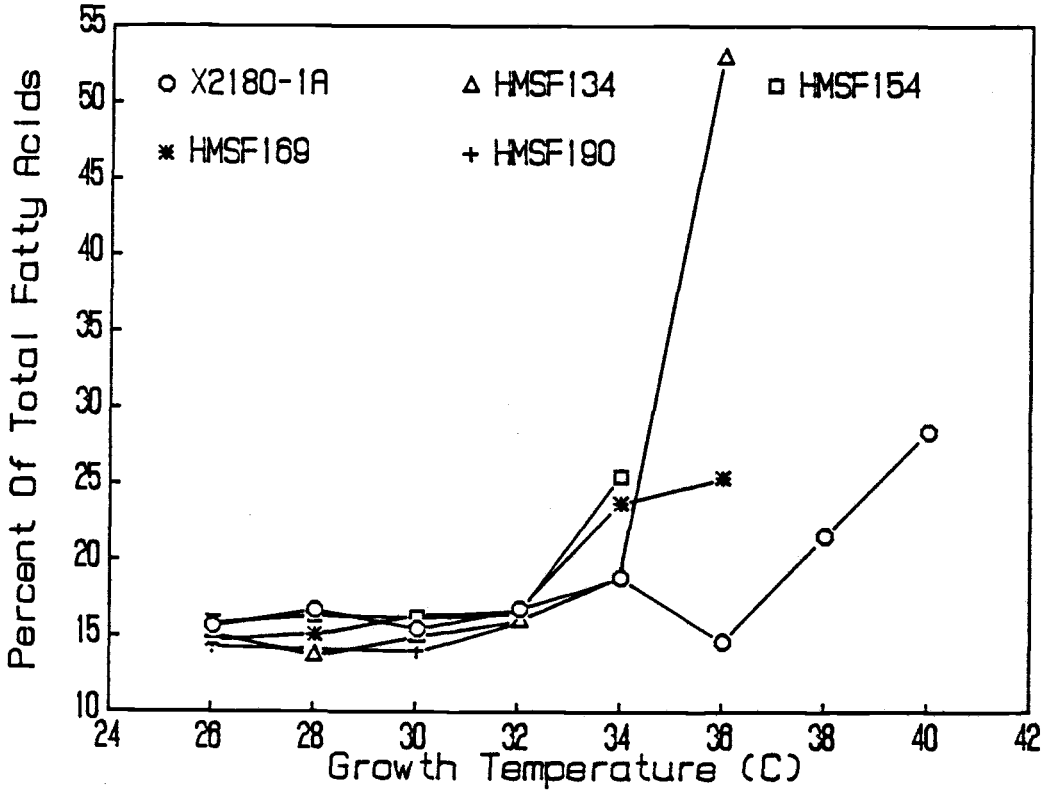

Figure 13. Percent C16:0 in secretion mutants. These cultures were grown to stationary on YNBD over a temperature range of 26 to 40°C using a temperature gradient incubator.

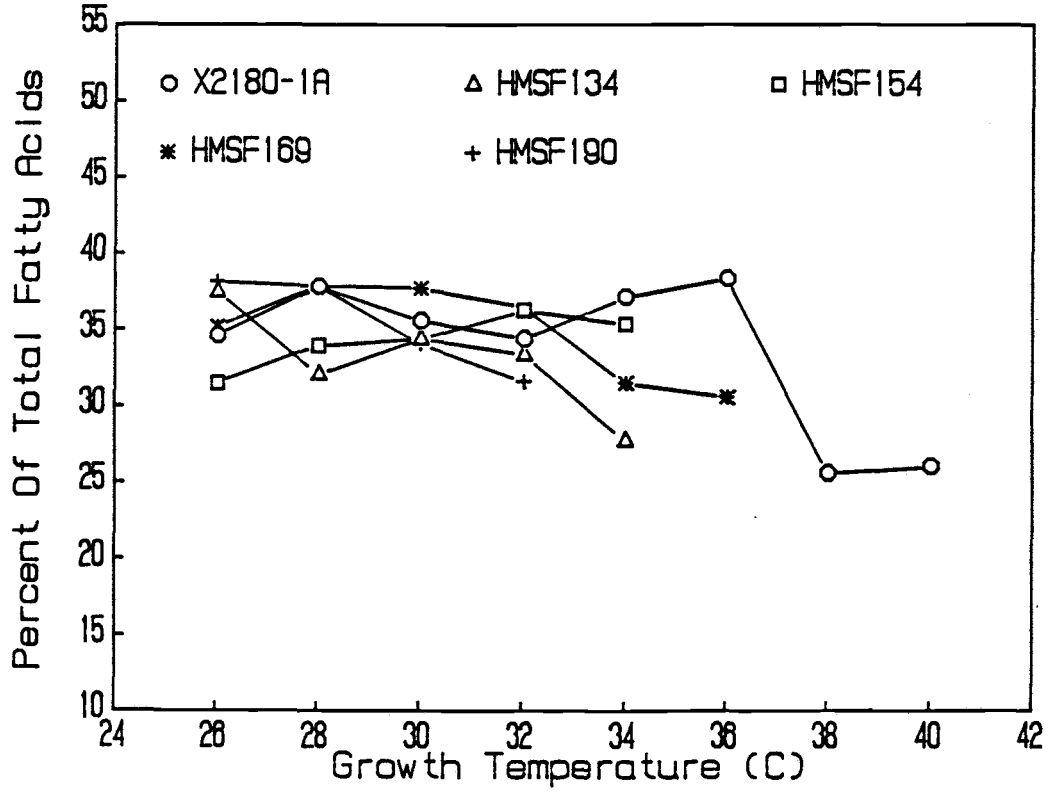

Figure 14. Percent C16:1 in secretion mutants. These cultures were grown to stationary on YNBD over a temperature range of 26 to 40°C using a temperature gradient incubator.

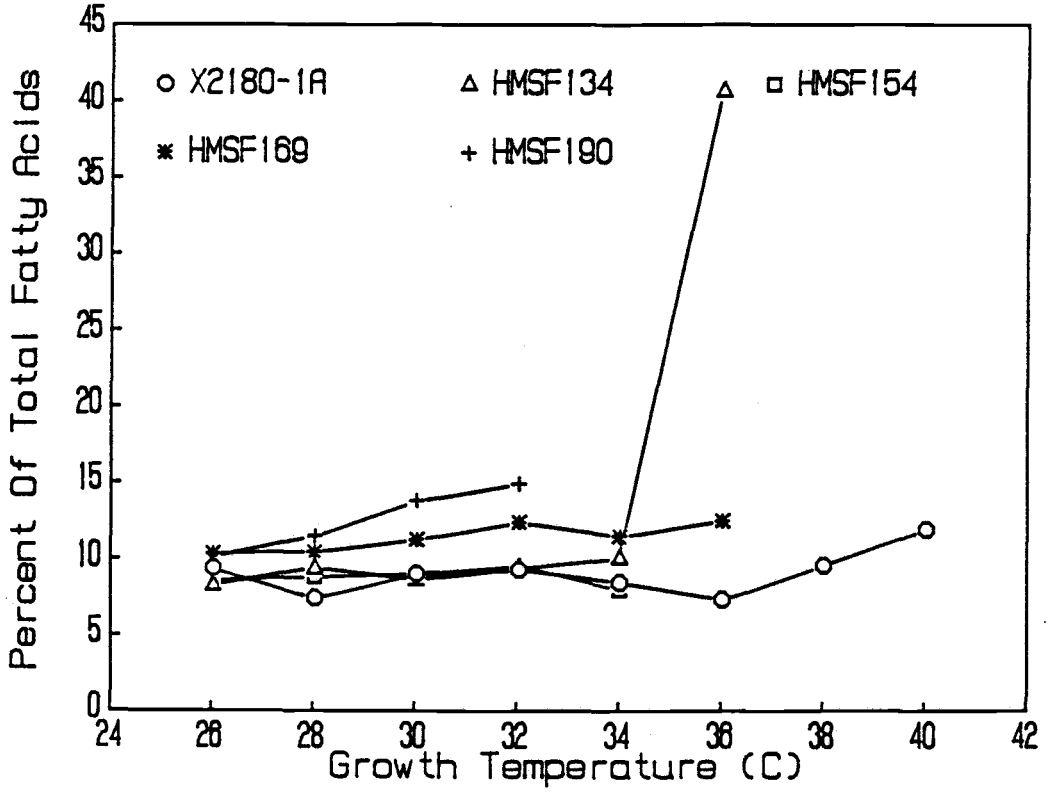

Figure 15. Percent C18:0 in secretion mutants. These cultures were grown to stationary on YNBD over a temperature range of 26 to 40°C using a temperature gradient incubator.

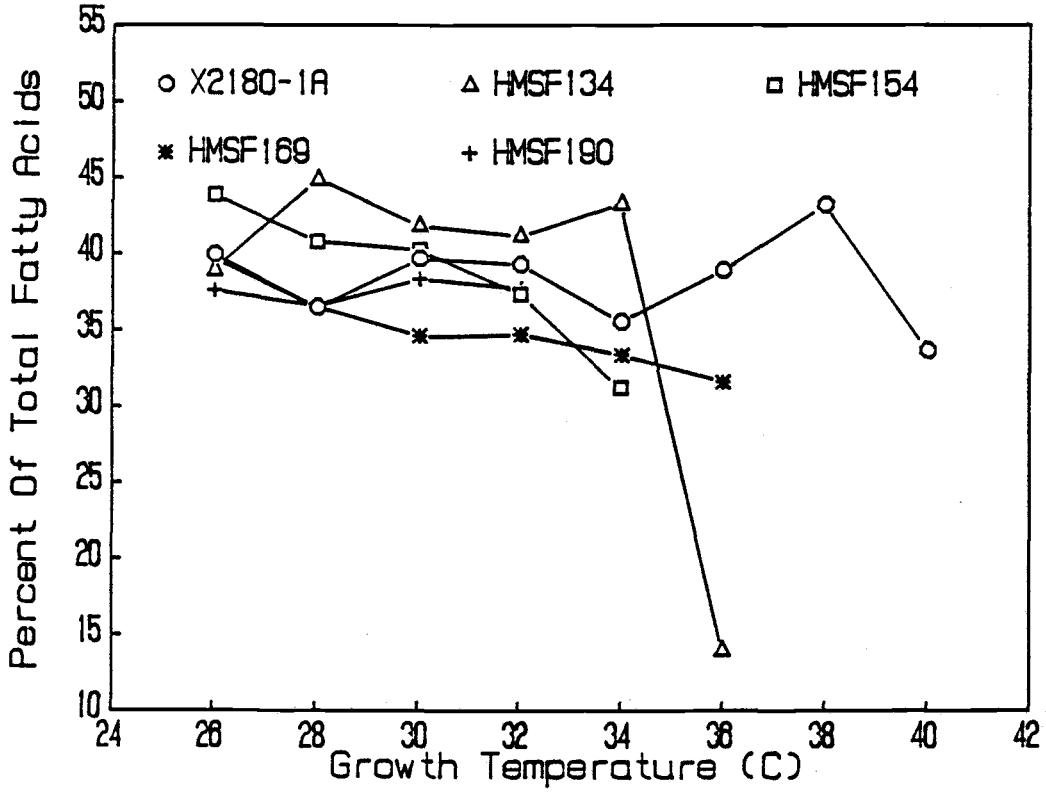

Figure 16. Percent C18:1 in secretion mutants. These cultures were grown to stationary on YNBD over a temperature range of 26 to 40°C using a temperature gradient incubator.

temperature up-shift, sterol biosynthesis did not stop (measurable incorporation of ^{14}[C]acetate into the free sterol fraction continually increased over the duration of the temperature shift) (Figures 17, 18, and 19).

Temperature Profile Studies

When X2180-1A, HMSF134, HMSF154, HMSF169, and HMSF190 were cultured using a temperature gradient incubator (T=24°C to 40°C in 2°C increments), all strains grew at temperatures below the restrictive temperature. An analysis of the free sterol, steryl ester, and fatty acid patterns by TLC revealed that as long as these strains grew ($T_{growth}<T_{restrictive}$) the cells had detectable levels of steryl ester and free sterol (Table 13). However, examination of the fatty acids by chain length showed that for C16:0 saturated fatty acids, there was a general increase as the growth temperature increased (T>32°C). This trend also appeared in the C18:0 lipid class but was not as pronounced at 32°C. Examination of the unsaturated fatty acids C16:1 and C18:1 revealed a general decrease with a marked decrease starting at 32°C in the secretion mutants and wildtype cells (Figures 13, 14, 15 and 16).

Examination of the sterol levels in secretion mutant cells cultured to stationary at different temperatures revealed that there was a 30 to 50% decrease in the amount of detectable free sterol at 30-32°C as there was at 32-34°C. The amount of steryl ester detected, however, appeared to be strain dependent (Table 13).

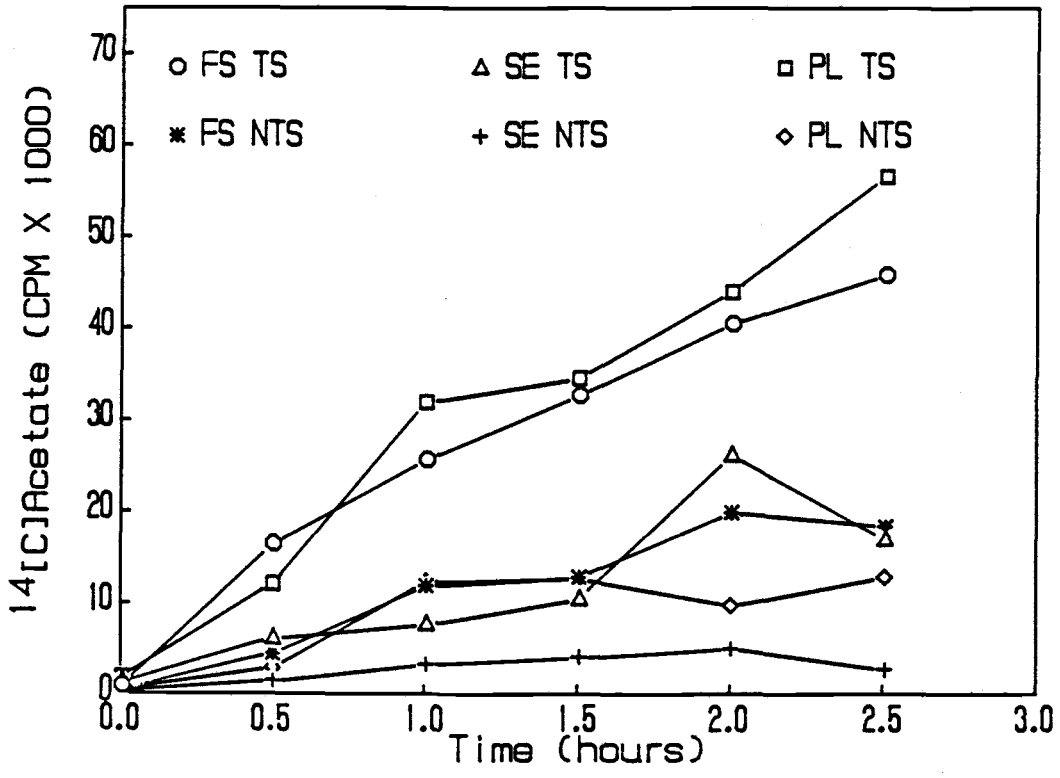

Figure 17. Incorporation of ^{14}[C]acetate as free sterol, steryl ester, and phospholipid in X2180-1A. The cultures were grown to mid-log at 24°C and split. One set was reincubated at 24°C (NTS) and the other set was incubated at 37-39°C (TS). Labeled acetate, 75 µl of 0.1 µCi µl^{-1} (56 mCi mmol^{-1}), was added to each set of cultures and 25 ml volumes harvested at 30 min. intervals. The samples were steamed in the presence of DMSO and hexane extracted prior to TLC separation for phospholipid (PL), free sterol (FS), and steryl ester (SE). TLC lipid bands were visualized with iodine vapor, scraped and counted as described in Materials and Methods.

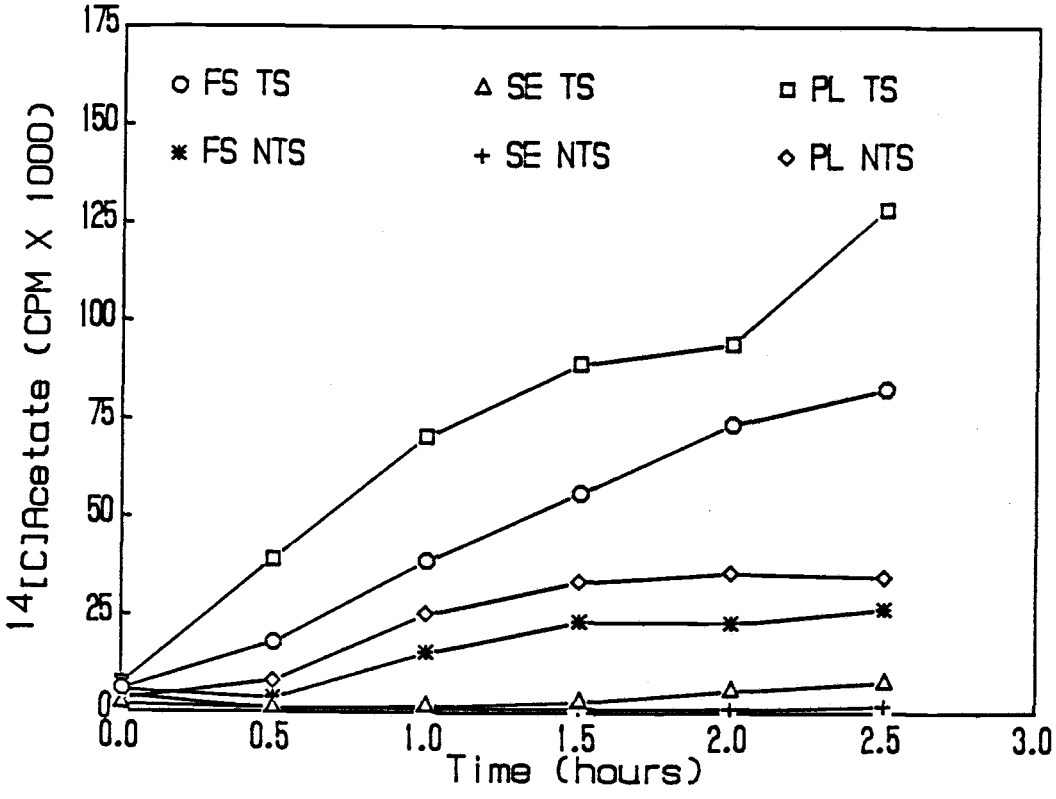

Figure 18. Incorporation of ^{14}[C]acetate as free sterol, steryl ester, and phospholipid in HMSF134. The cultures were grown to mid-log at 24°C and split. One set was reincubated at 24°C (NTS) and the other set was incubated at 37-39°C (TS). Labeled acetate, 75 µl of 0.1 µCi µl^{-1} (56 mCi mmol^{-1}), was added to each set of cultures and 25 ml volumes harvested at 30 min. intervals. The samples were steamed in the presence of DMSO and hexane extracted prior to TLC separation for phospholipid (PL), free sterol (FS), and steryl ester (SE). TLC lipid bands were visualized with iodine vapor, scraped and counted as described in Materials and Methods.

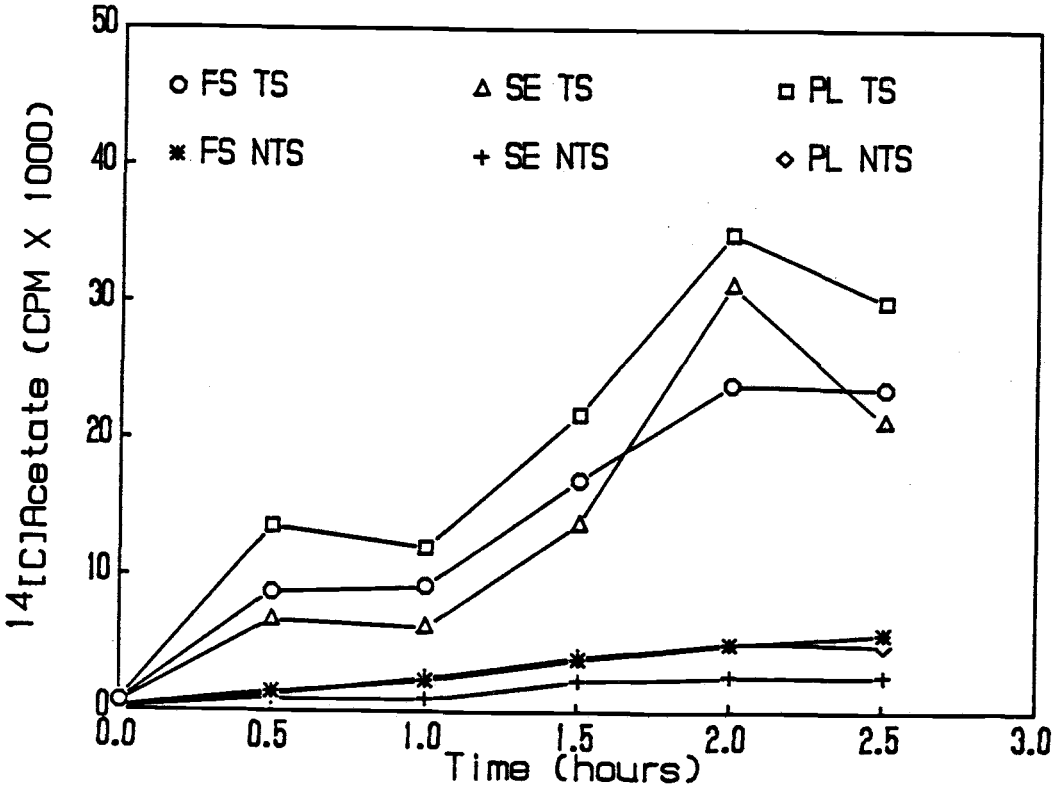

Figure 19. Incorporation of ^{14}[C]acetate as free sterol, steryl ester, and phospholipid in HMSF169. The cultures were grown to mid-log at 24°C and split. One set was reincubated at 24°C (NTS) and the other set was incubated at 37-39°C (TS). Labeled acetate, 75 µl of 0.1 µCi µl^{-1} (56 mCi mmol^{-1}), was added to each set of cultures and 25 ml volumes harvested at 30 min. intervals. The samples were steamed in the presence of DMSO and hexane extracted prior to TLC separation for phospholipid (PL), free sterol (FS), and steryl ester (SE). TLC lipid bands were visualized with iodine vapor, scraped and counted as described in Materials and Methods.

Table 13

STEROL TEMPERATURE PROFILE FOR SECRETION MUTANTS

	Temperature (°C)							
	26	28	30	32	34	36	38	40
Strain	Free Sterol (μg sterol mg^{-1} dry weight)							
X2180-1A	1.7	2.3	2.8	3.2	2.3	1.4	1.0	0.5
HMSF134	0.6	0.6	0.6	0.7	1.0	nd	nd	nd
HMSF154	0.8	0.8	0.9	0.9	nd	nd	nd	nd
HMSF169	0.8	0.9	1.1	0.8	0.3	0.3	nd	nd
HMSF190	0.7	0.7	0.8	0.4	nd	nd	nd	nd
	Steryl Ester (μg sterol mg^{-1} dry weight)							
X2180-1A	0.4	0.5	0.5	0.3	0.2	nd	nd	nd
HMSF134	0.6	0.9	0.8	1.3	1.1	nd	nd	nd
HMSF154	0.7	0.7	1.0	1.2	nd	nd	nd	nd
HMSF169	0.6	1.4	1.3	1.1	0.4	nd	nd	nd
HMSF190	2.0	2.3	2.1	1.3	nd	nd	nd	nd

[a]nd - not determined.

DISCUSSION

The data presented in this thesis demonstrates the adaptability of the yeast cell to numerous environmental and physiological insults. In the yeast sterol auxotroph, replacement (in feeding experiments) of the evolutionarily advantageous sterol, ergosterol, indicated that lipid metabolic and compositional adaptation was required for maintenance of cell viability and growth. Specifically, these changes resulted in an altered phospholipid and fatty acid composition that could now accommodate the structural differences in the dietary sterol. These changes generally leave the yeast cell cultured on one sterol, morphologically indistinct from a yeast cell grown in the presence of a different sterol, yet, they are physiologically distinct. Because of this uniqueness, the yeast secretion mutants which are conditionally dysfunctional at elevated temperatures appeared to be an ideal vehicle for examining the function of sterol in plasma membrane and membrane formation.

Effects Of Sterol On Plasma Membrane

The fluorescence anisotropy measurements and lipid analyses for RD5-R collectively indicate a functional mechanism for modulating the fluid state of the plasma membrane which compensates for changes brought about by specific sterols. The r_s plots of plasma membranes for the different concentrations of sterols (Figures 6 and 7), also indicate that RD5-R can compensate for differences of sterol type and

concentration when the cells are capable of synthesizing unsaturated fatty acid. These cells can synthesize unsaturated fatty acid when fed the heme precursor, δ-aminolevulinic acid.

Experiments using artificial membranes indicated that one function of sterol in a biological membrane is stabilization of membrane phospholipid structure (58). Gallay et al. have shown by differential scanning calorimetry that the lipid transition enthalpy (gel to liquid crystalline phase transition) of dipalmitoylphosphatidylcholine vesicles was eliminated by addition of cholesterol (88). Bottema et al. have shown this lipid transition enthalpy is not present in yeast plasma membranes containing sterol(s) (77). These results have been interpreted to mean that the presence of sterol modifies the membrane bilayer structure such that no gel to liquid crystalline transition occurs (figures 6 and 7).

Quantitation of the lipids extracted from cells grown on different sterols showed no major alterations in the total amount of phospholipid present although there were significant changes in PI and PE (Table 5) in cells grown on cholesterol compared to cells grown on ergosterol, sitosterol, or stigmasterol. The increase in PI and decrease in PE overall, increase the type of phospholipids with bulky headgroups and the acyl chain spacing. These differences in PI and PE species can be interpreted as a physiological response to growth on cholesterol (i. e. a modification of the membrane lipid components) which results in a plasma membrane having fluid state properties similar to those cells grown on ergosterol, sitosterol, or stigmasterol. Analyses of total fatty acids (Table 6) indicate a twofold decrease in C16:0, a doubling

of C16:1, and a 2.5-fold increase in C18:1 for cells adjusted to growth on cholesterol compared to cells cultured on ergosterol, sitosterol, or stigmasterol. These changes increase the fluid nature of the membrane which offsets the condensing effect of cholesterol.

In liposomes (Figures 8, 9, and 10) fluorescence anisotropy data showed that compensatory changes to the non-sterol lipid components as a function of the sterol in the growth medium appeared to be specific for the exogenously supplied sterol. Fluorescence anisotropy plots of reconstituted liposomes showed that the composition of extracted phospholipid was such that only a specific sterol would eliminate the change in slope. These changes to plasma membrane phospholipid and fatty acids in response to exogenous sterol, formed different and mutually exclusive membrane systems dependent upon the sterol supplied in the growth medium.

Synthesis of membranes requires regulation of availability of phospholipid unless it is present in excess (4). In wildtype yeast cells, it has been shown that triacylglycerol and phospholipid pools can interchange during growth and that fatty acid species in membrane phospholipid may be controlled by selective incorporation (19). These results provide evidence for a model in which there is coordinated regulation of sterol, phospholipid and fatty acid. In essence, when RD5-R is grown on cholesterol compared to growth on ergosterol, it was found that the phospholipid composition was altered such that there was an increase in phospholipid having a large uncharged head group (PI) with a concomitant decrease in phospholipid species having a smaller charged head group (PE). There is also a shift toward increased

unsaturated fatty acid in cells grown on sterol without a C24 alkyl group (cholesterol). The C24 alkyl group of ergosterol is β-oriented while sitosterol and stigmasterol are both α-oriented confirming that under these growth conditions the presence or absence of the C24 alkyl group is important rather than its configuration (36). While the C24 alkyl group absence appears to be responsible for changes in the phospholipid species, structural differences in ergosterol, sitosterol, and stigmasterol may account for the subtle differences seen in total fatty acid and fatty acid chain length specificity for phospholipid head group.

Although RD5-R can physiologically adjust its phospholipid and fatty acid composition when fed different dietary sterols and δ-aminolevulinic acid, it can also grow when the growth medium contains sterol and unsaturated fatty acids (oleate:palmitate 4:1 v/v) (7). It has been observed that there is an increased tendency of this strain to clump when the chain length of the unsaturated fatty acid supplement is decreased. The ability to grow using unsaturated fatty acids of chain length less than 16 carbons indicated there is no strict chain length requirement for fatty acid in membrane phospholipid.

Previous studies have used similar (30,35,36) or identical (34) yeast systems but did not address in vivo adjustment of phospholipid and fatty acid. Although these reports deal with physiological changes to phospholipid and fatty acid, their results are based on providing the cells with an exogenous supply of unsaturated fatty acid. The results reported here have addressed the problem of in vivo modulation of cellular phospholipid, saturated, and unsaturated fatty acid by

allowing the cells to biosynthesize their own saturated and unsaturated fatty acids. Here, only RD5-R has been utilized so that the manifestation of strain heterogeneity is eliminated when addressing the alterations in lipid composition when RD5-R is grown on different sterols.

The yeast system as observed here, appears to be a paradigm membrane system for eukaryotic cells. The lipid changes that were observed when the dietary sterol structure was altered have predictable changes in lipid classes. Evidence for this is found in altered fatty acid patterns and in steady state fluorescence anisotropy plots.

Effects Of Temperature On Plasma Membrane

The secretion mutants provide a unique model for studying membrane biogenesis. A temperature sensitive mutation for plasma membrane formation has been used to assess differences in the membrane lipid component as a function of growth temperature. The results from analyses of total cellular fatty acids are supportive of the model for poikilotherms presented in the literature review. In addition to modulating the fatty acid and phospholipid composition, S. cerevisiae has the lipid alcohol ergosterol. Ergosterol like cholesterol, a membrane fluidizing agent, alters the thermotropic state of the native yeast membrane such that no discontinuities are observed in steady state fluorescence anisotropy measurements.

In cells wildtype for ergosterol and sterol biosynthesis (secretion mutants) there does not appear to be any large increase or decrease in free sterol content for cells cultured to stationary phase

between 26-40°C. There was, however, a slight increase in free sterol mg^{-1} dry wt. as the growth temperature increased (Table 13). Instead, the cells appear to optimize the physical state of the plasma membrane by modifying the fatty acids attached to the sn1 and sn2 positions of the membrane phospholipids. The detected shift in the discontinuity for fluorescence anisotropy plots (Figures 11 and 12) show that a cellular readjustment to the plasma membrane has occurred.

When the non-sterol lipids from the secretion mutants were examined, the predominant changes appeared to be the fatty acids (Figures 13, 14, 15, and 16). The saturated fatty acids (C16:0 and C18:0) increased while the unsaturated fatty acids (C16:1 and C18:1) decreased. Larger fluctuations appeared at a temperature just below the restrictive temperature in each different class of secretion mutant as well as the wildtype (X2180-1A). Based on the data presented here, modifying the fatty acid chain length and unsaturation appears to be the preferred method of maintaining plasma membrane integrity for growth at different temperatures when the sterol structure is fixed (i. e. yeasts wildtype for sterol synthesize only ergosterol as a normal end product). Comparing the fatty acid data with the sterol analyses (Table 13) indicates that the secretion mutants tested do not have an altered sterol metabolism which produces the membrane synthesis defect. In one strain, HMSF134, which has an altered steryl ester synthesis metabolism under the conditions of the temperature up-shift, there does not appear to be a functional correlation of altered sterol metabolism and membrane formation.

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