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The ability of cholestanol to support growth of yeast sterol auxotrophs has been examined. Growth on this stanol was precluded unless minute quantities of ergosterol were available, a phenomenon designated sparking. The low levels of ergosterol were insufficient to support growth alone. The data indicate that cholestanol is fulfilling a bulk membrane requirement(s) while ergosterol satisfies a high specificity sparking function(s).

A variety of sterols and stanols were analyzed for their ability to satisfy these two sterol functions in yeast sterol auxotrophs. While many sterols and stanols satisfied bulk membrane requirements, only those possessing a C-5,6 unsaturation or capable of being desaturated at C-5 in vivo fulfilled the sparking function. Addition of a bromo or iodo moiety to the sterol side

chain had no effect on the ability of cholesterol to satisfy either sterol function. However, the presence of a keto or hydroxy group on the sterol side chain completely abolished the ability of cholesterol to satisfy either sterol requirement. Unsaturation of the A-ring or β-saturation of a C-5,6 unsaturation rendered both sterol and stanol unsuitable for either function.

The C-28 methyl group of ergosterol, while found not to be required for growth, allowed for greater ease of in vivo desaturation at C-5. As a result some sterols and stanols lacking the C-28 methyl were incapable of satisfying the sparking requirement while identical compounds possessing the C-28 methyl were able to fulfill the sparking function(s). These data are extended to hypothesize a role for the C-28 methyl group as well as other functional moieties of ergosterol in yeast. Physiological studies are also undertaken in an attempt to define the sparking phenomenon better.

Defining Roles for Sterols in *Saccharomyces cerevisiae*: Characterization of Bulk Membrane and High Specificity Sparking Functions

by

Russell John Rodriguez

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Redacted for privacy

Chairman of the Department of Microbiology

Redacted for privacy

Dean of Graduate School

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I have come to realize that a persons experience in graduate school is heavily influenced by three factors. These include the specific research problem, the major professor, and the environment provided by fellow graduate students and friends. My research problem has been one of continual challenge and interest, not to exclude frustration, and has evolved to a more sophisticated problem than originally expected. I cannot express enough gratitude for Dr. Leo Parks who has not only been responsible for my training as a microbial physiologist but has also been a constant source of friendship, support, and encouragement. The environment which I have experienced in graduate school has been superb and I wish to express sincere thanks and extend a loving hug to all of my friends. If gratitude could be expressed in numbers, infinity would be the only way to convey thanks to Gael Kurath. Gael was always there for support and encouragement but most importantly for sharing the beauty of life with me. Special thanks are also due to Gael for editing my manuscripts not only for

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INTRODUCTION

The importance of sterol molecules in the maintenance of biological membranes has been the subject of much investigation (1,2,3). It is believed that the major role of sterols is as bulk membrane components which regulate fluidity. However, it has been suggested that sterols may also have a more specific function(s) (4). Evidence for this comes from work with *Dermestes vulpinus* (5) and *Mycoplasma capricolum* (4,6). In these studies a synergistic effect was observed between cholesterol and sitosterol, or cholesterol and lanosterol, a phenomenon referred to as a sparing effect. It is important to note that in each case cholesterol was not acting to initiate growth but rather to supplement or modify growth.

The yeast *Saccharomyces cerevisiae* has been used extensively to study the function(s) of sterols. Research has involved studying wild type cells grown anaerobically in the presence of sterols (7), mutant strains blocked in the late stages of ergosterol biosynthesis (8,9), and sterol auxotrophs (10,11,12). To the best of my knowledge no data have been published demonstrating specific roles for sterols in yeast.

The importance of specific functional moieties of sterols has been alluded to by a number of researchers (7,13,14). Anaerobic

studies with wild type yeast indicate that the C-28 methyl and C-3 hydroxyl of ergosterol are critical for growth (7). In contrast, experiments done with a sterol auxotroph (13) suggest that a C-3 hydroxyl is not required and that a methyl ether at C-3 will supplement growth equally as well as free sterol. Studies with sterol auxotrophs have also suggested that the C-28 methyl of ergosterol is not required for growth (13,4,15). In most such studies nuclear unsaturations in the B-ring were not considered important for growth.

Data presented in this thesis reveals that highly purified cholestanol does not support growth of the sterol auxotrophs FY3 and GL7, strains independently derived from Saccharomyces cerevisiae. These yeast can, however, be induced to grow on cholestanol by the addition of very small quantities of ergosterol. We have designated this phenomenon the sparking of growth. Furthermore the sterol requirement satisfied by ergosterol and not cholestanol has been termed the sparking function. The sterol requirement satisfied by cholestanol is referred to as the bulk membrane function.

A number of different sterols and stanols have been studied for their ability to satisfy the bulk membrane and sparking functions. The sparking requirement is only satisfied by sterols possessing a C-5,6 unsaturation or capable of being desaturated at C-5 in vivo. A number of sterols and stanols lacking a C-5,6 unsaturation are shown to be capable of satisfying bulk membrane

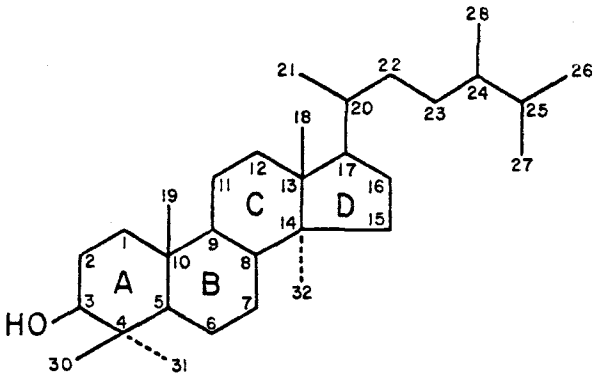
functions but not the sparking function. Data are also reported which indicate that a functional Δ^5-desaturase enzyme is required for cell growth and that *in vivo* specificity of the Δ^5-desaturation reaction is enhanced by the presence of a C-28 methyl on the sterol substrate.

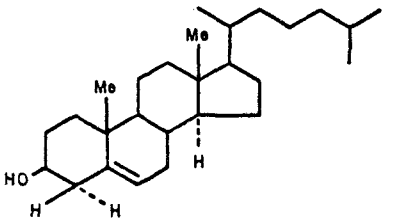
Experiments were also performed in an attempt to define the sparking phenomenon physiologically. These as well as genetic mapping data for the Δ^5-desaturase gene are presented.

LITERATURE REVIEW

Sterols are complex tetracyclic alcohols possessing a hydroxyl group at C-3 of ring A and a branched aliphatic side chain at C-17 of ring D (figure 1). These lipids are derived from isoprene subunits and are derivatives of perhydrocyclopentanophenanthrene. Sterols were first identified in the 19th century although at least one species, cholesterol, was isolated early in the 18th century (16). Since that time a wealth of knowledge, as well as more than 19 Nobel prizes, have been obtained concerning these lipids, and yet no specific physiological role(s) has been assigned.

Sterols have been found in all major groups of organisms from viruses to humans, but not in all species. Every known eukaryotic organism requires sterols during part or all of its life cycle (16). Only a few groups of eukaryotes are incapable of sterol biosynthesis. These include two groups of fungi, Pythia and Phytophthera, and the insects (16). In the absence of exogenous sterols Pythia and Phytophthera species are able to grow vegetatively but are unable to undergo sexual reproduction (16). Insects have an absolute growth requirement for sterol, and since they are unable to synthesize these lipids they must obtain them elsewhere. The insects do this by consuming other sterol producing eukaryotic organisms and incorporating dietary sterols into their own membranes. It has recently been discovered that a number of bacteria, both gram positive and gram negative, possess sterols or related compounds called terpenes (16). Many photosynthetic

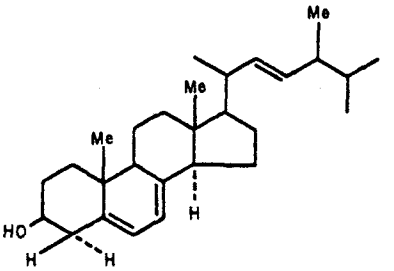




CHOLESTA-5-EN-3β-OL

Δ^5 CHOLESTENE-3β-OL

CHOLESTEROL



ERGOSTA-5,7,22-TRIEN-3β-OL

$\Delta^{5,7,22}$ ERGOSTATRIEN-3β-OL

ERGOSTEROL

Figure 1. Structure, carbon numbering and ring designation for C-32 sterols. Structure for ergosterol and cholesterol are also shown.

bacteria also contain sterols (16). In addition, sterols have been discovered in the protein coats of certain budding viruses, although the significance is unknown (17). The ubiquity with which sterols occur in nature suggests that eukaryotic organisms have evolved from the same or very similar ancestry (3). Further support for a common eukaryotic ancestry comes from the fact that while not all eukaryotes synthesize sterols, those that don't still possess some of the same sterol biosynthetic enzymes found in other eukaryotes and are capable of modifying dietary sterols (16).

While all eukaryotes require sterols for their existence, there is great diversity in the number of different sterols. One major group of organisms, the vertebrate animals, show very little diversity with regard to sterols. In the majority of all animals cholesterol is the predominant sterol. This is not the case for plants and fungi. In plants three sterols predominate, campesterol, stigmasterol, and sitosterol, the ratios of which fluctuate from species to species. Fungi as a general rule synthesize ergosterol although great variation from this is recorded (16). The group of eukaryotes with the most diverse sterol pattern is the marine invertebrates where more than 50 different sterols have been identified (18,19).

Despite the great diversity of sterol species in nature, almost all sterols which predominate in eukaryotes have five common characteristics: 1) a C-5,6 unsaturation, 2) no substituents at C-14 and C-4 of the D and A ring respectively, 3) a methyl group at

the juncture of ring A and B, 4) an aliphatic side chain at C-17 of ring D, and 5) an H atom or C_1 or C_2 moiety at C-24 of the side chain. If one considers minor sterols the number of different sterols increases around 10 to 20 fold. The significance of minor sterols, however, is unknown.

The biosynthetic pathways for the different sterols vary considerably but all eukaryotes have in common the early portion of the sterol biosynthetic sequence (figure 2a). This begins with acetyl-CoA which through a series of enzymatic reactions forms mevalonic acid. Mevalonic acid is then converted to a C-5 phosphorylated isoprene intermediate (isopentenylpyrophosphate) which is the building block of sterols (20). The C-5 units are polymerized to yield a variety of compounds, one of which is squalene. Squalene is then epoxidized to form the last intermediate of the early pathway, squalene-epoxide. The sterol pathway branches at this point such that squalene-epoxide is either cyclyzed to lanosterol in non-photosynthetic eukaryotes or to cycloartenol in photosynthetic eukaryotes. It is important to note that while all eukaryotes share this early pathway from mevalonic acid to squalene-epoxide, this portion of the pathway is not exclusive for sterol biosynthesis. An intermediate of this portion of the sterol pathway, farnesyl pyrophosphate, is also an intermediate of dolichol (21), ubiquinone (16), and carotenoid (16) biosynthesis. In addition it is from these early reactions in sterol biosynthesis that the isopentenyl groups of serine and

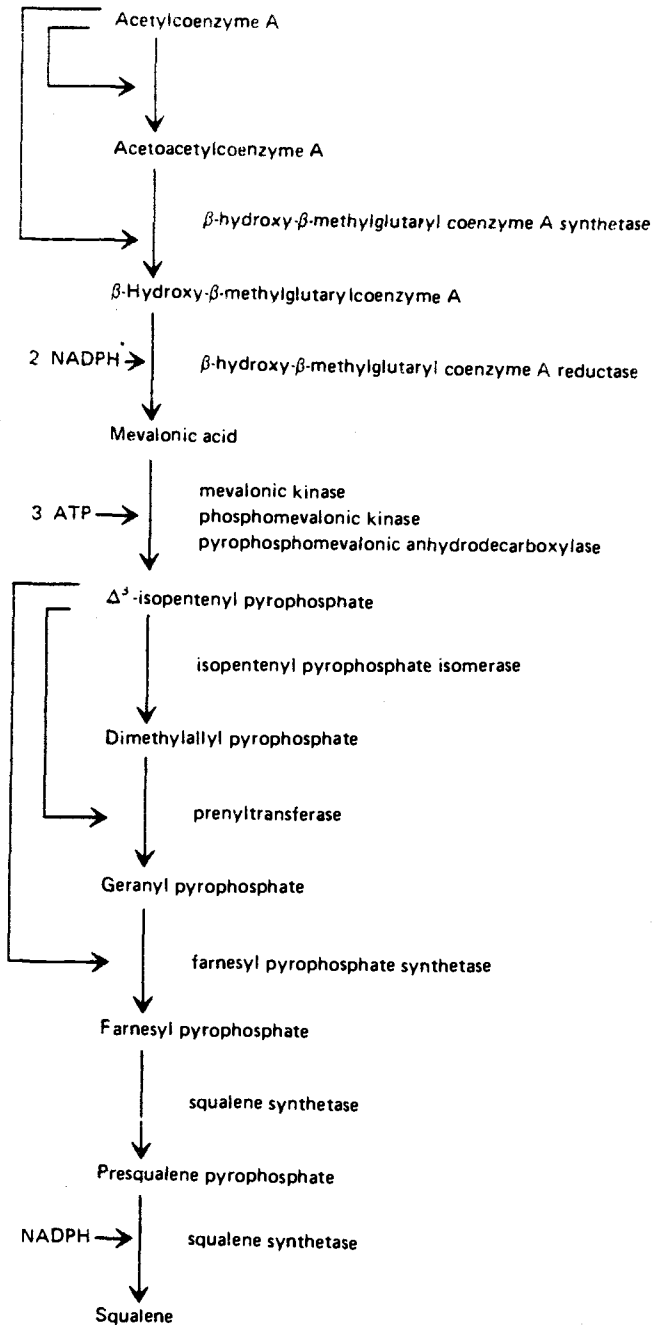


Figure 2a. Sequence of reactions in the early portion of sterol biosynthesis.

tyrosine tRNA's are derived (22). It is most likely that these other cellular components ensure uniformity in this early portion of sterol biosynthesis.

After formation of either lanosterol or cycloartenol there is great variation in the pathway between eukaryotic species. Regardless of the organism or specific pathway involved, sterol biosynthesis is one of the most complex and energetically expensive pathways in the cell. One reaction in particular, cyclization of squalene epoxide, has been viewed as possibly the most complex enzymatic reaction (23) the cell undertakes. In Saccharomyces cerevisiae there are at least 25 reactions in ergosterol biosynthesis, which together cost the cell 10 ATP and 16 NADPH molecules per molecule of ergosterol formed. This does not include the estimated 12 ATP equivalents (24) necessary for the trans-methylation reaction (25) at C-24 of the side chain.

The late portion of the ergosterol biosynthetic pathway is shown in figure 2b. Although the pathway from acetyl-CoA to lanosterol is quite specific, progression from this intermediate is not (1,26,27,28). In wild type cells, however, there is one major route from lanosterol to ergosterol (28) indicated by the heavy line in figure 2b. Two steps in this pathway, squalene epoxidation (I → II) and lanosterol demethylation (III → VII) require oxygen (29,30,31,32), and as a result yeast can grow anaerobically only when sterol is supplied (33). In addition yeast also require unsaturated fatty acid (UFA) and methionine under anaerobic conditions (34).

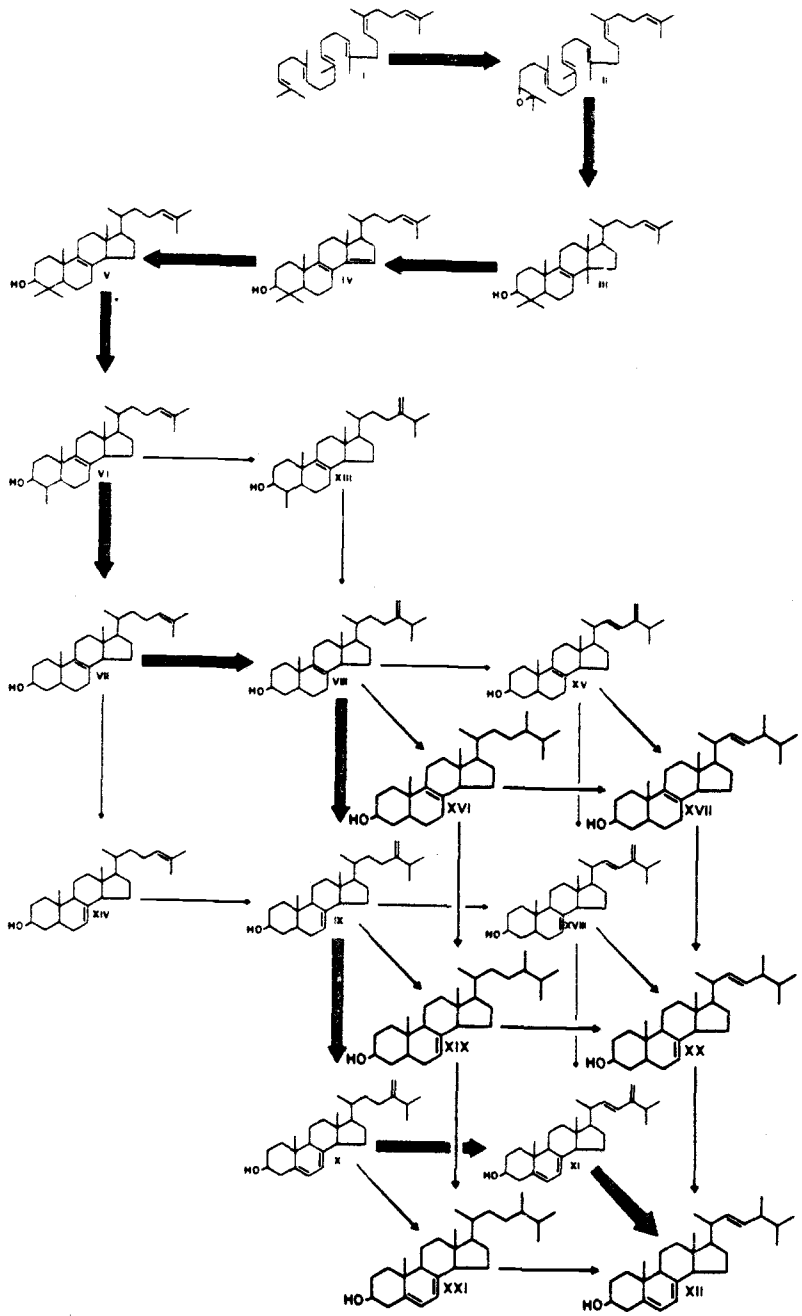


Figure 2b. Schematic sequence of reactions in the late portion of the ergosterol biosynthetic pathway.

Since cells must coordinate cellular reactions with growth, it is imperative that they regulate high energy demand pathways such as sterol biosynthesis. Regulation of ergosterol biosynthesis is only beginning to be understood and there are many reactions which are likely candidates for regulation. Many cellular pathways have been shown to be regulated at the beginning as well as throughout the pathway, particularly at branch points (35). It is apparent that the irreversible conversion of HMG-CoA to MVA is a regulatory point in sterol biosynthesis. This enzyme has been studied extensively and found to be involved in regulating cholesterol biosynthesis in animals (36-39) as well as ergosterol biosynthesis in yeast (40-43). Little is known about regulation of the remainder of the ergosterol pathway. From mevalonic acid to squalene there are branch points leading to other required compounds such as dolichols and ubiquinones which are also likely regulatory sites. Although there are no branch sites after squalene (to other cellular components), there are two reactions which demand energy. The first is demethylation of lanosterol to zymosterol which consumes 4 NADH. The second reaction, which is considerably more expensive for the cell, is transmethylation at C-24 to convert zymosterol into fecosterol (VII → VIII). While much attention has been devoted to regulation of C-24 transmethylation in yeast (44), it appears that this enzyme is not a major control point of sterol biosynthesis (1).

Although sterols were among the first complex microbial products analyzed extensively, very little is known about specific physiological roles. Sterols have been found to occur in only two forms in S. cerevisiae: as free alcohols or esterified to long chain fatty acids (1). Sterols can account for 2 to 10% of cell dry weight in these yeast (45) depending on growth conditions (1). During log phase most of the sterol in yeast exists as free alcohol in membranes. However, as the culture grows to late log phase steryl esters begin to accumulate and can represent 90% of the total sterol pool in stationary phase cells (46). Most of the fatty acids which are esterified to sterols are C:16 and C:18 species (47). It has been established that steryl esters are not utilized as energy reserve (49). However, it has been shown that when stationary phase cells are inoculated into fresh medium the accumulated steryl ester is rapidly hydrolyzed and is incorporated as free sterol when cells enter log phase again (49). It has been found that steryl esters as well as triglycerides accumulate in lipid droplets and are not associated with cell membranes (50). This may allow the cell to esterify and immobilize sterol intermediates such that they are unable to be membrane inserted (1).

Almost 100 years after its identification, cholesterol was recognized to affect membrane order (51). It was determined that cholesterol condensed or decreased fluidity of a lecithin bi-layer membrane. The degree to which cholesterol condensed the bi-layer

was dependent on the fatty acid substituents on the lecithin and on the molar ratio of cholesterol to lecithin. This was the first evidence that sterols serve as bulk membrane components which affect fluidity. Since that time a wealth of knowledge has been obtained on the physical effects of sterols on model and natural membranes. By use of physical techniques such as electron spin resonance (ESR), fluorescence polarization, nuclear magnetic resonance (NMR), and differential scanning calorimetry (DSC), investigators have been able to study sterol-membrane interactions (52).

It is well established that variation in membrane lipid composition causes changes in the Arrhenius kinetics of intrinsic membrane enzymes (53-56). A fatty acid deficient mutant revealed that cytochrome C oxidase is affected by the lipid environment of the inner mitochondrial membrane (57). It was later found that this enzyme also responded to changes in membrane sterol composition (58,9). Cobon and Haslam (59) found that altering the sterol composition of wild type yeast cells by growing them anaerobically caused changes in the Arrehnius kinetics of the mitochondrial ATPase. Sterols have also been shown to affect mitochondrial L-kynurenine-3-hydroxylase and the chitin synthetase enzyme but not the plasma membrane ATPase (8,60). Osmotolerance of mitochondria and spheroplasts (61), transmembrane potentials (62), and permeability to nickel (63) and cations (64,65) are also affected by membrane sterol composition.

As a result of physical analyses performed with sterols, both on model membrane and natural systems, a general consensus has been arrived at explaining the physiological role of sterols. The basic structure of a membrane regardless of its components is a bi-layer. The components of membranes are quite mobile (66) and as such require surroundings with which to move. Phospholipid bi-layers are normally quite rigid structures and over a temperature range will undergo phase transition from a crystalline state (low temperature) to a liquid crystalline state (high temperature) (2). The temperature at which the bi-layer undergoes transition is dependent on the fatty acid substituents (52). If bi-layers are formed in the presence of increasing concentrations of sterol, the enthalpy or magnitude of the bi-layer phase transition will diminish to the point of elimination when the bi-layer contains approximately 30 mole % sterol. Yeast plasma membranes contain about 30 mole % ergosterol and when analyzed by DSC and fluorescent polarization are not found to undergo a lipid phase transition (67). From these observations and data on the effect of sterols on membrane enzymes mentioned earlier, it has been concluded that sterols serve as bulk membrane components which act to modulate membrane fluidity (2,3,7). As a result of sterols membrane fluidity of an organism is a relatively constant value over a normal range of environmental conditions.

It has been suggested that while sterols may only serve as bulk membrane components, there may be sterol rich and sterol poor

domains in natural membranes. This has been examined primarily in mammalian cells with certain viral receptor sites (68), cationic pumps (69), and inner and outer membrane leaflets of certain cell types (66,14). Recently (60) it was observed that there is variation with regard to distribution of sterol in yeast plasma membranes. In yeast the plasma membrane ATPase is not sensitive to the polyene antibiotic nystatin, which selectively binds sterol, but chitin synthetase is. This antibiotic has a particularly high affinity for ergosterol (70). By using a nystatin resistant yeast (a strain which does not synthesize ergosterol) Bottema and Parks (60) were able to show that in wild type cells nystatin inhibits chitin synthetase activity but that in the nystatin resistant mutant nystatin had no effect on chitin synthetase activity. Plasma membrane ATPase activity was not affected by nystatin in either system. This can be interpreted to mean that the chitin synthetase is in a sterol rich domain and the plasma membrane ATPase is not.

Recently it has been suggested that sterols may be involved in more specific cellular roles (4). Evidence for this comes from work with Dermestes vulpinus (5), and Mycoplasma capricolum (6). In these studies synergistic effects were observed between cholesterol and other sterols in regard to growth and/or development. In Dermestes vulpinus it was discovered that plant sterols as well as many other sterols were not adequate for the development of larvae to pupae. However, addition of small amounts

of cholesterol (insufficient to show an effect alone) allowed for this development. This phenomenon is not true for all insects since Heliothis zea was able to pupate in the presence of plant sterols (71). In Mycoplasma capricolum (6) it was observed that low levels of cholesterol will increase growth rates and yields for cells cultured with lanosterol, a phenomenon termed sparing. Concomitant with addition of cholesterol to lanosterol grown mycoplasma (4) was a decrease in the Km of uptake for unsaturated fatty acids. It is important to note that the addition of elevated levels of UFA eliminated the need for cholesterol in the lanosterol grown mycoplasma cultures. It was concluded (72) that cholesterol was required for UFA uptake and that dual functionality of sterols existed in mycoplasma. More recently Dahl et al. (73) concluded that cholesterol also regulated RNA and protein synthesis in Mycoplasma capricolum. The fact that sparing cholesterol can be replaced by increased levels of fatty acid implies that the cholesterol is not involved in a specific interaction. It may be that cholesterol is being localized in the mycoplasma membrane and while not affecting overall membrane fluidity (73), might affect local regions. If this is the case then a similar phenomenon may explain why fatty acids alleviate the need for cholesterol. Another explanation for the mycoplasma data is that cholesterol and UFA are taken up at specific sites of the membrane which may be associated with expansion or growth of the membrane. This growth site(s) may require a less fluid environment than lanosterol can

accommodate and therefore function better with cholesterol and/or fatty acid.

In this thesis a new system is described which has led to the discovery that sterols are required for two separate classes of functions in S. cerevisiae. Structural specificities necessary for sterols to satisfy these requirements are also investigated, and their significance to cell physiology is discussed.

MATERIALS AND METHODS

Strains of *Saccharomyces cerevisiae* and growth media:

The sterol auxotroph RD5 (α, *hem1*, *erg3*, *erg7*) was derived from a cross between a sterol auxotroph FY3 (10), and a sterol mutant JR1 (74). RD5-R is a revertant form of RD5 and is described in the text. GL7 was obtained from Dr. T. Buttke (Mississippi Medical Center at Jackson). FY1 is a heme mutant and sterol auxotroph, and is able to synthesize ergosterol in the presence of δ-aminolevulinic acid (10). 3701b-n3 was derived by EMS mutagenesis of 3701b, a sterol wild type (9). The sterol mutants JR1, JR3 and JR4 were isolated from S288C, a wild type (74). LS-60 and LS-61 are sterol mutants that were independently isolated from S288C (Salerno, L. F. and Parks, L. W. unpublished results). These sterol mutants are further characterized in table 3. The genetic mapping strains X4037-14C (a, *gal1*, *leu2*, *arg9*, *ile3*, *met14*, *lys7*, *pet17*, *trp1*) and K396-27B (a, *ura3*, *ade1*, *his1*, *leu2*, *lys7*, *met3*, *trp5*) were obtained from the Berkeley culture collection and Dr. J. Profitt (Oregon State University) respectively. Sterol mutants were cultured on rich (YPD) medium containing 1% yeast extract, 1% peptone, and 2% dextrose. Sterol auxotrophs were cultured in growth medium buffered with 50 mM potassium succinate to pH 5.5 and containing the following: 0.67% yeast nitrogen base (Difco), 2% dextrose; 0.002% of isoleucine, glutamate, serine, glutamine, histidine, tyrosine, and uracil;

0.003% of proline, glycine, alanine, and tryptophane; 0.004% of lysine, methionine, leucine, and adenine; 0.005% of valine and phenylalanine; 0.001% of aspartate and asparagine; and 0.015% of threonine and cysteine. For solid medium 1.5% agar was added. A mixture of palmitoleic acid-oleic acid (1:4, v/v) in tyloxapol-ethanol (1:1, v/v) was added to a final concentration of 100 µg/ml as a source of unsaturated fatty acid. Sterols were added in a mixture of tyloxapol-ethanol (1:1, v/v).

Preparation of inocula for growth studies:

To determine whether various sterols and stanols satisfied bulk membrane requirements, inocula were derived from auxotrophic strains grown in medium containing 5 µg/ml of ergosterol. Stationary phase cells were harvested, and washed two times with medium lacking sterol. Pellets were resuspended in the same volume of sterol-less medium, and 0.02 ml transferred to 5 ml fresh medium containing 5 µg/ml of the sterol or stanol being tested for satisfying bulk function. To assay lipids for their ability to satisfy sparking requirements, inocula cells were depleted of endogenous sterol as follows: the sterol auxotrophs were cultured on ergosterol to stationary phase; 0.02 ml of cells were then transferred to fresh medium containing cholestanol. The cholestanol culture was grown to stationary phase, harvested by centrifugation (500 x g for 2 minutes), washed and resuspended to the same volume with medium devoid of sterol. Then, 0.02 ml aliquots were added to 5 ml fresh media containing 5 µg/ml of the

sterol or stanol being tested for its ability to satisfy "sparking" function. In all experiments growth was monitored using a Klett-Summerson photoelectric colorimeter equipped with a green filter. Cultures were aerated in a Scientific Industries rocking gradient incubator operated isothermally at 28°C.

Preparation of radioactive sterols:

[4-^{3}H]Cholestanol was prepared from cholesterol by the method of Nace (75). ^{3}H-Ergosterol was prepared by growing FY1 in the presence of aminolevulinic acid (50 μM) and 100 μCi/ml of ^{3}H-acetate. Cells were grown to stationary phase and extracted for sterols as will be described.

Purification of sterols and stanols:

All sterols and stanols used in these studies were HPLC purified with the exception of cholestanol. Cholestanol was purified by argentation chromatography on silica gel thin layer plates (76). HPLC analysis (77) was performed on a Altex model 332 gradient liquid chromatograph coupled to a Hitachi model 155-40 spectrophotometer. Sterols were purified on a 10 mm I.D. x 250 mm ODS-Ultrasphere column (5 μ particle size). A solvent system of ethanol:methanol:H_2O (10:86:4, v/v/v) was pumped through the column at a rate of 6 ml/minute. Sterols were monitored for ultraviolet absorption at 210 nm. After purification the lipids were evaporated to dryness under N_2 and quantitated by gas-liquid chromatography (GLC) (78). In all cases sterols and stanols were

dissolved in isopropanol for HPLC purification and chloroform for GLC analysis.

Sterol extraction and analysis:

Cultures (10 ml) were grown to stationary phase and harvested by centrifugation (500 x g for 2 minutes). Cells were acid labilized (79) and alkaline saponified with methanolic KOH (46). Saponified samples were extracted three times with hexane and the solvent evaporated under N_2 gas. Samples were resuspended in either isopropanol for HPLC analysis, chloroform for GLC analysis (78), or hexane for UV absorbance from 310 nm to 210 nm.

HPLC analysis of cellular sterol content was performed on a 0.4 mm I.D. x 250 mm ODS-Ultrasphere column (5 μ particle size). A solvent system of ethanol:methanol:H_2O (10:86:4, v/v/v) was pumped through the column at 1.5 ml/min. Sterols were detected by ultraviolet absorption at 210 nm.

Screening sterol mutants for the presence of $\Delta^{5,7}$-sterol:

Sterol mutants were grown in one liter of YPD to stationary phase. Cells were harvested by centrifugation (500 x g for 2 minutes) and washed twice with distilled H_2O. Sterols were extracted as described above. Saponified extracts were resuspended in 0.5 ml of isopropyl alcohol and 250 μl were injected onto a preparative HPLC column (ODS-Ultrasphere, 10 mm I.D. x 250 mm). Sterols were monitored at 282 nm for detection of a $\Delta^{5,7}$-diene. Peaks appearing at this wavelength were isolated, evaporated under

N_2, resuspended in 0.5 ml hexane, and analyzed for ultraviolet absorbancy from 310 nm to 210 nm on a Beckman DU-8 spectrophotometer.

Conversion of sterols in vivo:

Sterol auxotrophs were analyzed for their ability to modify supplemented sterols. The organisms were cultured in medium with 5 µg/ml cholesterol to stationary phase and inoculated into 10 ml of medium containing 5 µg/ml of a sterol or stanol of interest. These cells were grown to stationary phase and the sterols analyzed and quantitated by GLC, HPLC, and UV as described above.

Mutagenesis of FY3 and selection of Δ^5-desaturase mutants:

The procedure of Brusick (80) was used for acridine orange mutagenesis. After a 15 minute exposure to acridine orange, cells were plated on defined medium containing ergosterol. These cells were transferred and replica plated onto medium containing cholestanol. After colony formation the cells were replica plated onto medium containing either cholestanol, ergostanol, Δ^7-cholestenol, or cholesterol. Clones were selected which were unable to grow on cholestanol, ergostanol, and Δ^7-cholestenol and able to grow on cholesterol.

Genetic studies:

Genetic mapping of the Δ^5-desaturase gene was done according to Sherman et al. (81). Asci were dissected following digestion

for 15 minutes with a solution of glusulase:water (1:1, v/v). Micromanipulations were performed on a W. M. Brower dissection microscope.

Nystatin resistance was determined by stamping yeast segregants from a YPD master plate onto medium containing yeast nitrogen base (0.67%), glucose (2%), all required amino acid supplements, and various concentrations of nystatin (2 to 7 units/ml). Growth was determined at 24 and 48 hrs. Nystatin stock solutions were prepared fresh in dimethylformamide and added to the media just prior to being poured into plates.

Uptake of ergosterol and cholestanol:

Analyses were performed with RD5-R under sparking conditions. Cholestanol cycled cells were inoculated into two 250 ml Klett flasks with 30 ml of defined medium containing 10 ng/ml of ergosterol and 5 µg/ml of cholestanol. In one flask the ergosterol added was uniformly labelled with ^{3}H-acetate while the cholestanol was not radiolabeled. In the second flask the ergosterol was not radioactive but the cholestanol was labelled at the C-2 of ring A with ^{3}H. At designated time points duplicate samples (0.5 ml) of culture were taken and filtered through a 0.45 µ Millipore filter and washed two times with 5 ml of a succinate buffer (1% tergitol, 50 mM succinate, pH 5.5) and once with 5 ml of H_2O. The filters were air dried and counted in POPOP-PPO (0.1 g/l of 1,4-bis [2(5-phenyloxazolyl)] benzene and 3.0 g/l 2,5-diphenyloxazole in toluene) scintillation cocktail for 5 minutes in a Beckman model LS 8000 scintillation counter.

Materials:

Ergosterol, cholesterol, lanosterol, amino acids, nucleotide bases, fatty acids, and detergents were purchased from Sigma. Growth media supplies were from Difco. Cholestanol was purchased from Applied Science, Sigma, and Cal-Biochem. Δ^4-cholestenol and $\Delta^{3,5}$-cholestadienol were obtained from Research Plus. Stigmasterol was from Calbiochem, campesterol from Supelco, and sitosterol from Applied Science. The halogenated as well as the oxygenated sterols were supplied by Dr. Eliahu Caspi. Highly purified cholestanol as well as the remaining sterols and stanols used in these studies were generously supplied by Dr. Henry Kircher. [4-^{3}H]cholesterol and [^{3}H]acetate were from ICN. Nystatin was from Sigma and glusulase from Endo Laboratories. All solvents were either from Mallinckrodt or Amachem and were glass distilled prior to use. GLC equipment was from Supelco and HPLC equipment from Beckman.

RESULTS

Cholestanol supplementation:

The sterol auxotrophs FY3 and GL7 have been used in a number of studies to determine the effect of different sterols on growth and cellular metabolism (10,11,12). In one of these studies (12) cholestanol was shown to support growth of GL7 to the same degree as cholesterol. We have found that, when highly purified, cholestanol is unable to support the growth of FY3 or GL7. Results presented here indicate that the contradiction between these two studies is due to the purity of the cholestanol and to the sterol content of inoculum cells.

Cholestanol obtained from commercial sources was capable of supporting growth of FY3 and GL7 to the same degree as ergosterol and cholesterol, even after purification by TLC and recrystallization. However, when cholestanol was purified by HPLC or argentation chromatography it was unable to satisfy the sterol requirement of FY3 and GL7. Analysis of TLC purified cholestanol by GLC showed a single peak which co-eluted with cholesterol. HPLC analysis revealed that each sample of cholestanol, with the exception of that supplied by Dr. Henry W. Kircher, possessed a compound which absorbed ultraviolet light at 210 nm and eluted in a volume similar to cholesterol. Cholestanol does not absorb ultraviolet light at 210 nm and elutes in a much greater volume than cholesterol (elution volume determined with [^{3}H]cholestanol) (77). Both components of the cholestanol were isolated to purity

by preparative HPLC, quantitated by GLC, and assayed for their ability to support growth of FY3. While FY3 was capable of growth on the compound which absorbed ultraviolet light, it was unable to grow on the purified cholestanol. HPLC or TLC (argentation) purified cholestanol was used for the remainder of this study.

In addition, we observed that FY3 or GL7 cells grown to stationary phase on ergosterol or cholesterol were able to grow when subcultured into medium containing cholestanol, regardless of whether or not the inoculum was washed free of residual sterol (figure 3). When these cholestanol-grown cells were subinoculated into fresh medium containing cholestanol no growth was observed (figure 3). It is important to note that cells transferred from a culture grown on ergosterol or cholesterol to medium devoid of sterol or stanol were unable to grow. One explanation for these observations is that when these auxotrophs are grown on ergosterol or cholesterol they accumulate excess sterol which is able to support growth in the presence of cholestanol but not in its absence. Since the excess sterol in the cell is a finite quantity it can only support a certain number of cell doublings. Therefore if the inoculum from a sterol grown culture is limited to a small number of cells, the number of doublings made possible by the residual sterol would be insufficient to increase culture turbidity. This was found to be the case when the inoculum consisted of 10^3 cells rather than 10^5 cells.

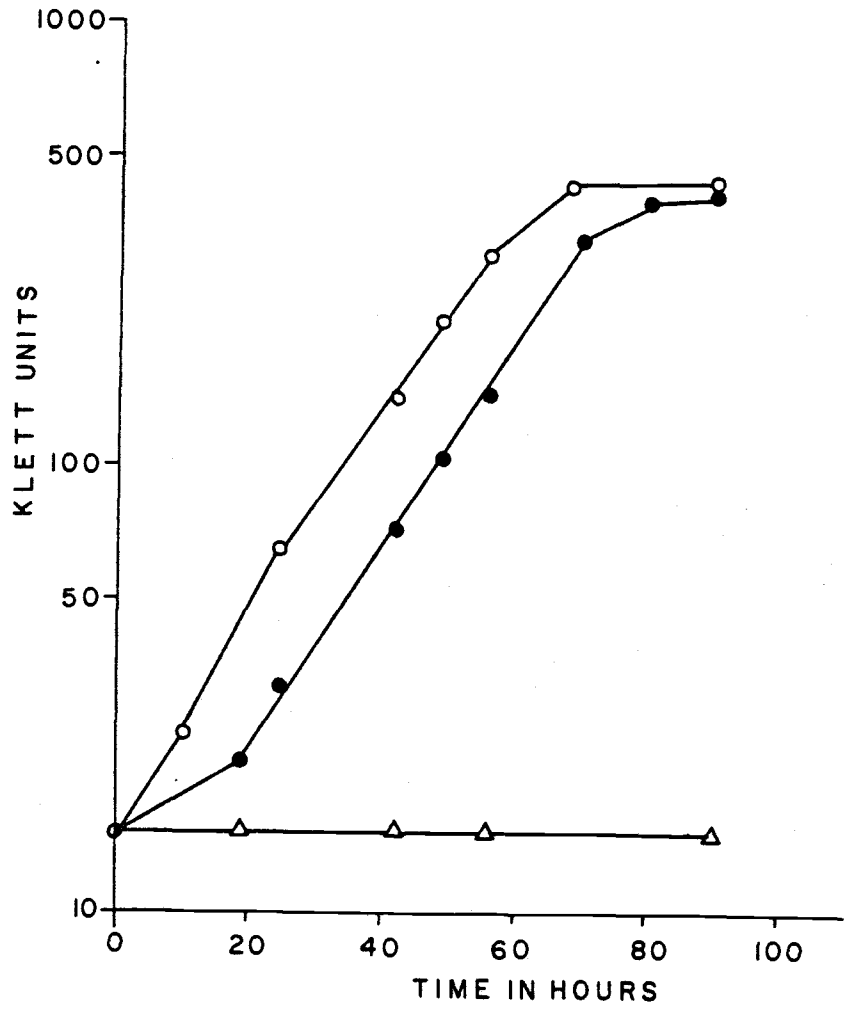


Figure 3. Growth of FY3 on ergosterol, 5 µg/ml, (0), subsequent transfer of ergosterol grown cells to medium containing cholestanol (●), and subinoculation of cholestanol-grown cells into medium containing cholestanol (Δ).

Sparking of growth on cholestanol by addition of ergosterol:

The data suggesting that accumulated excess sterols may be responsible for growth of FY3 and GL7 on a stanol which by itself was non-supportive of growth led us to investigate a phenomenon we refer to as sparking of growth. An example of sparking is the ability of an auxotroph to grow on purified cholestanol when minute quantities of ergosterol are added. The low levels of ergosterol in themselves did not support growth. Figure 4 represents the determination of the minimal concentration of ergosterol capable of supporting growth of FY3. At ergosterol concentrations greater than 5 µg/ml, growth rates and cell yields were similar. However, at lower concentrations both parameters of growth diminished and at 10 ng/ml no increase in culture turbidity was detected. When cells were inoculated into media containing 100, 50 or 10 ng/ml of ergosterol and 5 µg/ml of cholestanol, growth rates and cell yields were equivalent to those of cultures grown with 5 µg/ml of ergosterol (figure 5). The only difference between cultures grown on 5 µg/ml of ergosterol and those sparked to grow on cholestanol by ergosterol were increased lag times prior to the onset of growth. These lag times, which increased as the ergosterol concentration decreased, may be due to an increase in the amount of cholestanol relative to ergosterol, causing a decrease in ergosterol availability. Alternatively, lag times may be explained by specific deposition or "processing" of ergosterol. The sterol requirement satisfied by ergosterol is designated the high

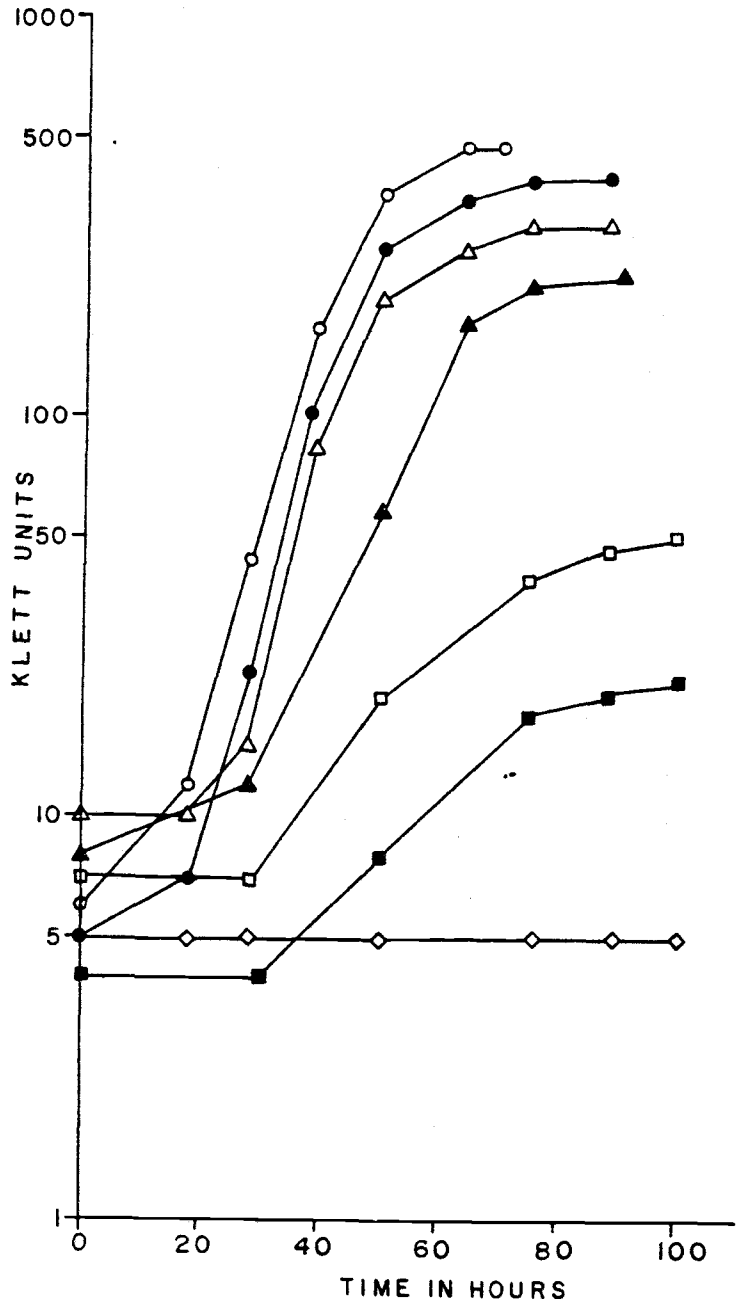


Figure 4. Growth of FY3 in medium containing the following concentrations of ergosterol: 5 and 10 µg/ml (O), 1 µg/ml (●), 0.5 µg/ml (Δ), 0.25 µg/ml (▲), 0.1 µg/ml (□), 0.05 µg/ml (■), and 0.01 µg/ml (◇).

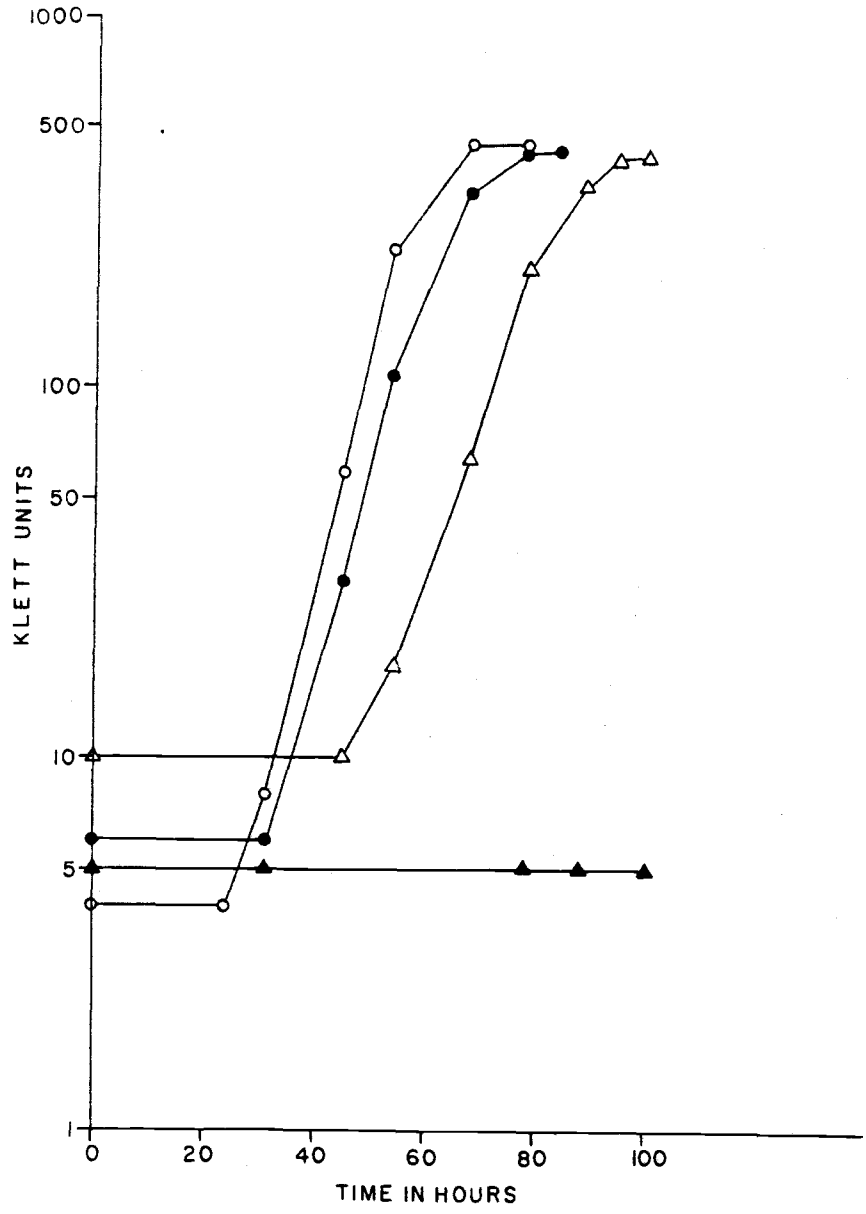


Figure 5. Sparking the growth of FY3 on cholestanol by 0.1 µg/ml (○), 0.05 µg/ml (●), and 0.01 µg/ml (Δ) of ergosterol. The ability of cholestanol to support growth is also shown (▲).

specificity sparking function while that fulfilled by cholestanol is the bulk membrane function.

Derivation of Δ^5-desaturase mutants:

A sterol analysis of FY3 grown under sparking conditions revealed that it desaturated cholestanol to form cholesterol (table 1) and began to grow on this stanol after very long lag periods (100-150 hrs). In order to study the phenomenon of sparking in greater detail we attempted to isolate a strain which completely lacked Δ^5-desaturating capacity. This was attempted by two methods: 1) transfer of genetic material between FY3 and either JR1 or 3701b-n3 (nystatin resistant strains defective in Δ^5 desaturase); and 2) acridine orange mutagenesis of FY3 and screening for a Δ^5-desaturase-less strain.

Matings of FY3 with JR1 and FY3 with 3701b-n3 yielded four classes of mutants with regard to Δ^5-desaturase: 1) wild type, 2) sterol producing without Δ^5-desaturase activity, 3) sterol auxotrophic possessing Δ^5-desaturase activity, and 4) sterol auxotrophic lacking Δ^5-desaturase activity. Isolates from this last class of mutants were designated RD5. Although growth studies were possible with RD5, it was very unstable and reverted to Δ^5-desaturase competent within 7 days. The revertant form of RD5 was designated RD5-R. RD5, RD5-R, and FY3 appeared to differ only in their ability to desaturate sterols and stanols at C-5.

The second approach was taken to isolate a more stable line of mutants using acridine orange as a mutagen. This compound has been

Table 1. Conversion of sterols and stanols by RD5, RD5-R, and FY3.[a]

Lipid	% Major Product	% Minor Product
$\Delta^{8,14}$-ergostadienol	$\Delta^{5,7}$-ergostadienol (100)	-
Δ^{7}-ergostenol	$\Delta^{5,7}$-ergostadienol (67)	Δ^{7}-ergostenol (33)
Δ^{8}-ergostenol	Unidentified sterol (60)	$\Delta^{5,7}$-ergostadienol (40)
$\Delta^{7,22}$-ergostadienol	Ergosterol (78)	$\Delta^{7,22}$-ergostadienol (12)
Δ^{0}-ergostanol[b]	Δ^{0}-ergostanol (95)	Δ^{5}-ergostenol (5)
Δ^{7}-cholestenol	$\Delta^{5,7}$-cholestadienol (80)	Δ^{7}-cholestenol (20)
Δ^{8}-cholestenol[c]	Unidentified sterol (60)	$\Delta^{5,7}$-cholestadienol (40)
Lanosterol	C-14 methyl fecosterol (100)	-
lophenol	$\Delta^{5,7}$-cholestadienol (73)	Δ^{7}-cholestenol (17)
Δ^{0}-cholestanol[c]	cholestanol (95)	cholesterol (5)
Δ^{7}-campestenol	$\Delta^{5,7}$-campestenol (80)	Δ^{7}-campestenol (20)

[a]Cells were transferred from ergosterol grown culture to media containing 5 µg/ml of the above lipids. Cells were harvested in stationary phase and analyzed for sterol content (Materials and Methods). Except where indicated numerical values were similar for all three auxotrophs.

[b]Stanol converted by RD5-R and FY3 only.

[c]Stanol converted by FY3 only.

reported to cause deletion mutations in yeast (80,82,83). Five hundred colonies from a mutagenized culture were transferred by replica plating onto media containing cholestanol, ergostanol, Δ^7-cholestenol, or cholesterol. Fifty clones were picked which grew only on cholesterol and were screened further for Δ^5-desaturase activity by monitoring conversion of the stanols in vivo. Forty of these strains appeared to be of the RD5 type. All of them were unstable and reverted to a form identical to RD5-R with respect to Δ^5-desaturase. This phenomenon was observed in 3 successive attempts at mutagenesis.

Δ^5-desaturase activity in sterol mutants:

Since no stable sterol auxotrophs were obtained which lacked Δ^5-desaturase enzyme activity it was of interest to determine if any sterol mutants reported to accumulate non-Δ^5-sterols were devoid of this enzyme activity. Six sterol mutants (table 2) were screened for the presence of Δ^5-sterols. These strains comprised three sterol mutant phenotypes; 1) defective in Δ^5-desaturase, 2) defective in $\Delta^{8\rightarrow7}$ isomerase, and 3) double mutants defective in Δ^5-desaturase and C-14 demethylase. In addition to their respective major sterols (table 2), all of these mutants were found to contain minute quantities of a sterol which eluted in the same volume as ergosterol when analyzed by HPLC, and possessed a $\Delta^{5,7}$ unsaturated nucleus as determined by ultraviolet scanning (i.e. peaks at 294, 282, 272, 264 nm with a 282 nm maximum). These mutants were observed to synthesize 1.5-3.5 mg of their respective major sterol

Table 2. Major sterol accumulation by yeast mutants with altered sterol biosynthetic pathways.

Mutant[a]	Enzymic Defect	Major Sterol
JR1	Δ^5-desaturase	Ergosta-7,22-dienol
JR3	$\Delta^{8\rightarrow7}$-isomerase	Ergost-8-enol
JR4	Δ^5-desaturase : C-14 demethylase	14-methyl-ergosta-8,24(28)-dienol
3701b-n3	Δ^5-desaturase	Ergosta-7,22-dienol
L5-60	Δ^5-desaturase : C-14 demethylase	14-methyl-ergosta-8,24(28)-dienol
L5-61	Δ^5-desaturase : C-14 demethylase	14-methyl-ergosta-8,24(28)-dienol

[a]References to these strains are listed in Materials and Methods.

per liter of stationary phase cells (approximately 10 grams wet weight). In contrast, the same quantity of cells synthesized 12-25 µg of $\Delta^{5,7}$ sterol (ergosterol). This represents an approximate 135-fold difference between the $\Delta^{5,7}$-sterol and the major sterol produced by these mutants. It is interesting to note that this concentration of ergosterol in 1 liter (average 18.5 ng/ml) is very close to the lower limit of levels of ergosterol required for sparking auxotrophic growth on cholestanol.

Mapping the gene encoding Δ^5-desaturase:

The inability to derive an auxotrophic mutant completely lacking Δ^5-desaturase activity, and the fact that a number of reported sterol mutants lacking this function possessed some activity, suggested that this enzyme was critical for cell growth. It was therefore of interest to map the location of the gene encoding the Δ^5-desaturase. This was accomplished by mating a sterol mutant, JR1, defective in the Δ^5-desaturase, and the mapping strains X4037-14C and K396-27B.

Tetrads from these crosses were analyzed by classical genetic techniques (81). Linkage between two genes is established by segregation patterns of two markers in pairwise fashion. In a multifactorial cross (GH x gh) if both G/g and H/h show 2:2 segregation three types of asci are found: parental ditype (PD), GH GH gh gh; non-parental ditype (NPD) Gh Gh gH gH; and tetratype (T), GH Gh gH gh. Gene linkage is observed when PD >> NPD and the distance between two genes is determined by recombination

frequencies. A single recombination event between two genes on the same chromosome will result in a T ascus, more than that will yield NPD. Using the equation $x = \frac{100}{2}\left[\frac{T + GNPD}{PD + NPD + T}\right]$ the gene encoding Δ^5-desaturase was shown to be linked to ilv3 and met3 on chromosome 10 (table 3). Since the desaturase gene was approximately 31 centiMorgans (cM) from the ilv3 gene and 28 cM from the met3 gene it meant the Δ^5-desaturase gene was located approximately 25 cM from the centromere on the left side of the chromosome.

Effect of the sterol nucleus on the sparking and bulk membrane requirements of sterol auxotrophs:

Although it was not possible to derive a stable Δ^5-desaturase minus strain, we were able to perform experiments which better defined the sparking phenomenon. Growth experiments under sparking and non-sparking conditions were performed on the yeast sterol auxotrophs RD5, RD5-R, and FY3 (table 4). A variety of sterols and stanols were used to elucidate specific structural moieties which are required to fulfill the sparking and/or bulk membrane functions.

In order to test the ability of sterols and stanols to satisfy the sparking function(s), the auxotrophs were cycled on cholestanol after growth on ergosterol (Materials and Methods). In this way intracellular levels of ergosterol were diluted to a concentration insufficient for sparking growth. This was demonstrated by failure of these cycled cells to grow in medium containing 5 μg/ml of cholestanol. The cholestanol cycled cells were then inoculated

Table 3. Genetic mapping of the erg3 gene.

Cross No.	Strains	Marker Segregation with erg3	Spore Types PD	NPD	T	Total	Map Distance
1	JR1 x K396-27B(812)	ura3	5	3	15	23	
		ade1	6	2	15	23	
		his1	2	3	18	23	
		leu2	6	5	12	23	
		lys7	2	3	18	23	
		met3	10	0	13	23	28.2
		trp5	4	2	17	23	
2	JR1 x X4037-14C	gal1	2	2	7	11	
		leu2	2	1	8	11	
		arg9	2	1	6	9	
		ile3	4	0	7	11	31.8
		met14	2	2	7	11	
		lys7	0	2	7	9	
		pet17	2	3	6	11	
		trp1	2	1	8	11	

Table 4. Ability of sterols and stanols to satisfy bulk membrane and sparking requirements of yeast sterol auxotrophs.

IUPAC - IUB designation	Common designation	Bulk membrane function[a]			Sparking function[b]		
		RD5	RD5-R	FY3	RD5	RD5-R	FY3
5α-cholestan-3βol	cholestanol		+		-	-	+[c]
5α-cholestan-3αol	epicholestanol		+		-	-	+[c]
5β-cholestan-3βol	coprostanol		-		-	-	-
5α-cholest-4-en-3βol	Δ^4-cholestenol		-		-	-	-
cholest-5-en-3βol	cholesterol		+		+	+	+
5α-cholest-7-en-3βol	Δ^7-cholestenol		+		+[c]	+[c]	+[c]
5α-cholest-8-en-3βol	Δ^8-cholestenol		+		-	-	+[c]
5α-cholesta-8,24-dien-3βol	zymosterol		+		+[c]	+[c]	+[c]
cholesta-3,5-dien-3βol	$\Delta^{3,5}$-cholestadienol		-		-	-	-
4α-methyl-5α-cholest-7-en-3βol	lophenol		+		+[c]	+[c]	+[c]
4,4,14-trimethyl-5α-cholesta-8,24-dien-3βol	lanosterol		+		-	-	-
5α-ergostan-3βol	ergostanol		+		-	+[c]	+[c]
ergosta-5,7,22-trien-3βol	ergosterol		+		+	+	+
ergost-5-en-3βol	Δ^5-ergostenol		+		+	+	+
5α-ergost-7-en-3βol	Δ^7-ergostenol		+		+[c]	+[c]	+[c]
5α-ergost-8-en-3βol	Δ^8-ergostenol		+		+[c]	+[c]	+[c]
ergosta-5,22-dien-3βol	$\Delta^{5,22}$-ergostadienol		+		+	+	+
5α-ergosta-7,22-dien-3βol	$\Delta^{7,22}$-ergostadienol		+		+[c]	+[c]	+[c]
24α-methyl-cholest-5-en-3βol	campesterol		+		+	+	+
24α-ethyl-cholest-5-en-3βol	sitosterol		+		+	+	+
24α-ethyl-cholest-5,22-dien-3βol	stigmasterol		+		+	+	+

[a]Determined by the growth response of cells derived from ergosterol grown culture (to allow for intracellular sparking levels of ergosterol) inoculated into media containing the above lipids at 5 μg/ml. Results were identical for all three auxotrophs.

[b]Determined by the growth response of cells cycled on cholestanol (to dilute residual sterol) and inoculated into media containing the lipid (5 μg/ml) indicated.

[c]Supplemented sterol or stanol listed was desaturated at C-5 in vivo by the indicated auxotroph.

into medium containing 5 μg/ml of the sterol or stanol of interest. In order for the auxotrophs to grow under these conditions, the available lipid had to satisfy both bulk membrane and sparking functions. Ten cholesta-derivatives were analyzed and only four (cholesterol, Δ^7-cholestenol, zymosterol, and lophenol) were growth supportive of RD5 and RD5-R (table 4). While both mutants grew on Δ^7-cholestenol, RD5 did so with a much longer lag period (figure 6). Although the lag periods differed, growth rates on Δ^7-cholestenol and cholesterol were equivalent. When these experiments were repeated with FY3, Δ^8-cholestenol in addition to those above also supported growth. After a lag period of 100-150 hours, cholestanol also supported FY3 growth (table 1). All ergosta- derivatives (table 4 and figure 7) with the exception of ergostanol supported growth of RD5 albeit with extended lag times for Δ^7 and Δ^8-ergostenols. RD5-R and FY3 had growth patterns identical to RD5 on the ergosta-derivatives with the exception of ergostanol (table 4). Stigmasterol, sitosterol, and campesterol supported growth of all three auxotrophs while lanosterol was incapable of fulfilling the sparking requirement.

In order to test various sterols and stanols for their ability to satisfy bulk membrane function alone it was necessary to provide cells with sparking amounts of ergosterol. This was accomplished in two different ways. In the first method, ergosterol grown auxotrophs were cycled on cholestanol to remove residual ergosterol in the cells. The cells were then placed in media containing 10

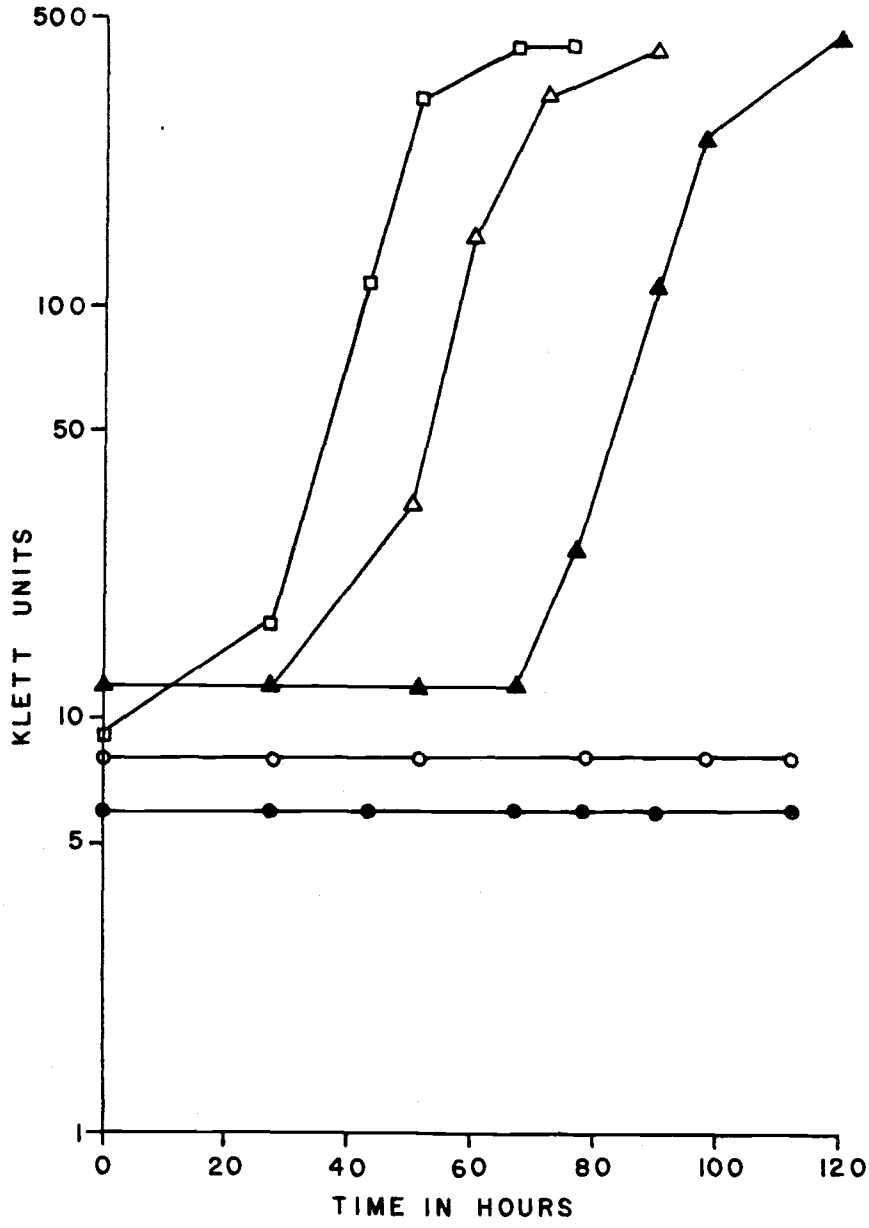


Figure 6. Growth of RD5 and RD5-R on cholesta-derivatives after cells were cycled on cholestanol. Both auxotrophs on cholestanol, 0; Δ^4-cholestanol and Δ^8-cholestenol, ●; cholesterol, □. Growth of RD5-R (Δ) and RD5 (▲) on Δ^7-cholestenol.

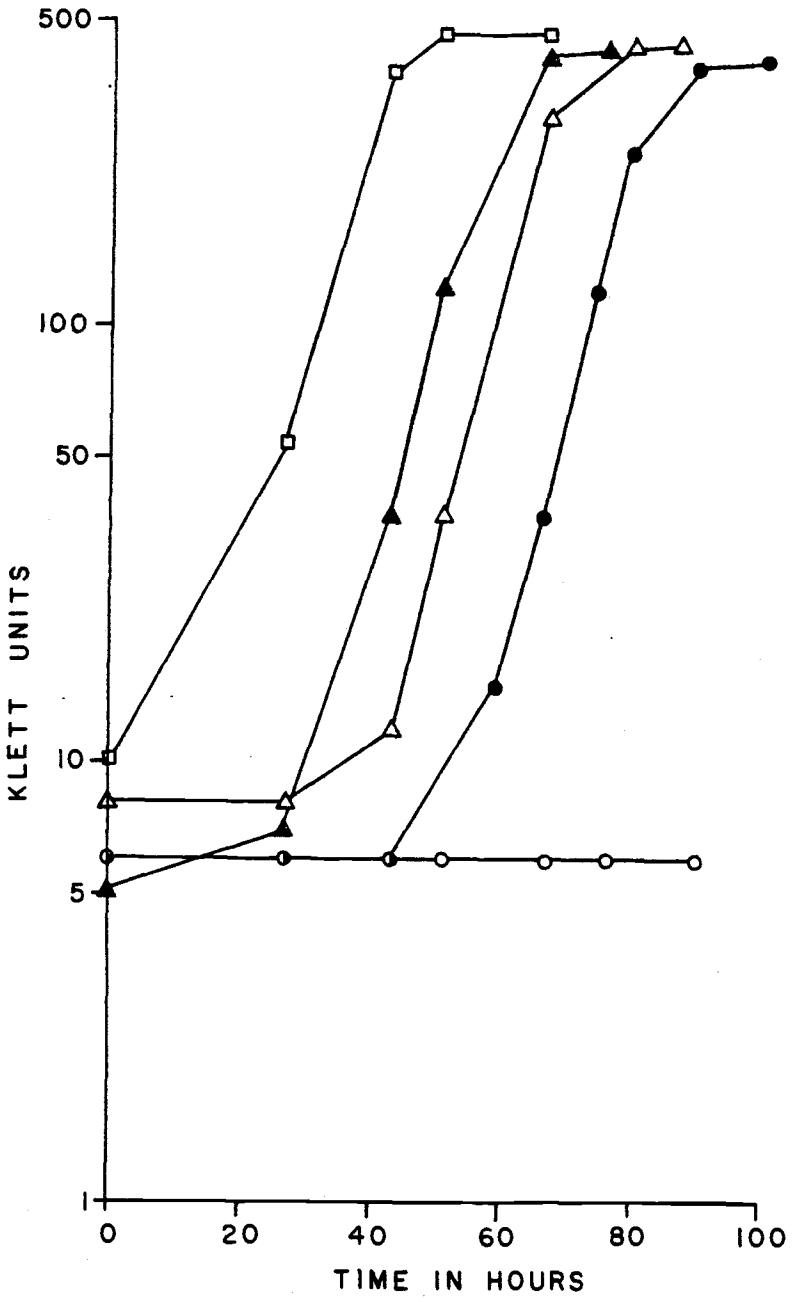


Figure 7. Growth of RD5 and RD5-R on ergosta-derivatives after cells were cycled on cholestanol. Both auxotrophs on Δ^8-ergostenol (Δ), Δ^7-ergostenol (▲), and ergosterol (□). Growth of RD5 (0) and RD5-R (●) on ergostanol.

ng/ml of ergosterol to satisfy the sparking requirement(s), and 5 µg/ml of a lipid to be analyzed for its ability to fulfill bulk membrane function(s). An alternative method was to use ergosterol grown cells as inocula for these experiments. As shown earlier these cells contain sufficient levels of ergosterol to satisfy the sparking function(s), but not enough for the bulk membrane function(s). This was supported by the fact that ergosterol grown cells will grow in medium containing 5 µg/ml of cholestanol but not in medium devoid of sterol. The results of growth experiments with cells prepared either way were the same. All three auxotrophs showed identical responses (with respect to bulk membrane function) to the various lipids tested. As shown in table 4 and figure 8, all lipids analyzed supported growth, hence satisfied bulk membrane function, with the exception of Δ^4-cholestanol, $\Delta^{3,5}$-cholestadienol, and coprostanol. To demonstrate further that growth on these lipids (figure 8) was due to sparking levels of ergosterol in the inocula cells, stationary phase cells were subsequently transferred to fresh medium containing 5 µg/ml of the same lipid supplement in which they were grown after transfer from ergosterol culture. These cells no longer contain enough residual ergosterol to satisfy the sparking function as explained by cholestanol cycling. As figure 8 depicts, none of the lipids supported growth under these conditions.

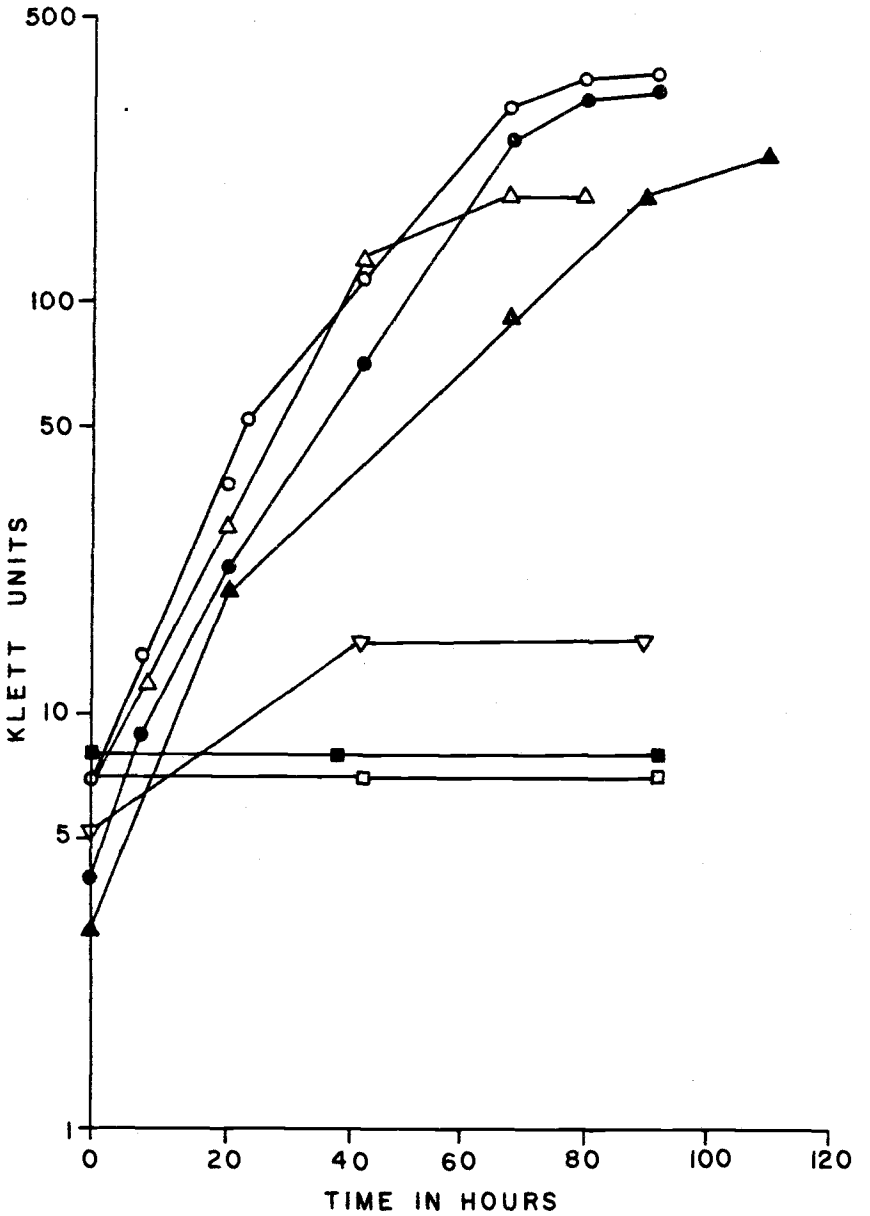


Figure 8. Growth of RD5 and RD5-R on various sterols and stanols. Transfer of cells grown on ergosterol to medium containing cholestanol (O), Δ^8-cholestenol (●), epicholestanol (Δ), lanosterol (▲), Δ^4-cholestenol (□), and no sterol (▽). After reaching stationary phase on cholestanol, cells were inoculated into media containing each of the above sterols or stanols (■).

Modification of sterols and stanols *in vivo*:

It appeared from the above experiments that the sparking requirements of the auxotrophs could be satisfied by many different sterols and stanols (table 4). However, detailed analyses revealed that all three auxotrophs could only be sparked for growth by sterols possessing C-5,6 unsaturations or by sterols and stanols which could be desaturated at C-5 *in vivo* (table 4). It is important to note that while Δ^5-sterols appear necessary for the sparking function(s), this feature is not imperative for bulk membrane function (table 4). Conversion *in vivo* of a number of non-Δ^5-sterols and stanols by the sterol auxotrophs is presented in table 2; RD5 was unable to desaturate cholestanol, ergostanol, or Δ^8-cholestenol. These three lipids were able to fulfill bulk membrane requirements but were unable to satisfy sparking requirements of RD5 (table 4). Results for RD5-R were similar to RD5 with the exception of ergostanol which was desaturated at C-5 and sparked growth. FY3 was able to desaturate all three lipids at C-5 and all three fulfilled both bulk and sparking requirements.

The cholesta- and ergosta-side chains are unable to be trans-methylated by Saccharomyces (84). This made it possible to determine the importance of a C-28 methyl to the auxotrophs. We found that this methyl had little or no effect on the ability of a sterol or stanol to satisfy bulk membrane function. It did, however, greatly influence the ability of sterols or stanols to fulfill sparking requirements. This is shown by the observation

that RD5 was sparked for growth by Δ^8-ergostenol but not by Δ^8-cholestenol, both of which satisfied bulk membrane function equally well (table 4). An identical phenomenon was seen with RD5-R on ergostanol versus cholestanol. The influence of the C-28 methyl is also apparent in the variation in lag times for growth of RD5 and RD5-R on Δ^7-ergostenol versus Δ^7-cholestenol (figures 6 and 7).

Effect of the sterol side chain on the sparking and bulk functions:

To determine how side chain modifications affected the ability of a sterol to satisfy the bulk membrane function alone, it was necessary to provide the cells with nanogram amounts of a sterol which would satisfy the sparking requirement. This was accomplished as described above. As shown in table 5 all of the sterols tested with the exception of the oxygenated sterols supported growth and therefore satisfied the bulk membrane requirement.

When these sterols (table 5, figure 9) were analyzed for their ability to fulfill the sparking requirement as well as the bulk membrane function, the same results were obtained. Only the oxygenated sterols were unsuitable for either sterol function. To ensure that these results were not due to *in vivo* modification of sterols, stationary phase cells were analyzed by HPLC and GLC for sterol content. Although we showed earlier that cholesterol was not modified *in vivo* it was possible that functional moieties on the side chain modified sterols were being removed. In every case, the respective sterols were found unaltered by the cells.

Table 5. Growth of RD5-R on sterols with different side chain modifications.

	Sterol	Ability to Satisfy	
		Bulk Requirements	Sparking Requirements
1)	26-nor-25(RS)-25-bromocholesterol	+	+
2)	26-bromocholesterol	+	+
3)	26-nor-25-hydroxy-cholesteryl-3-acetate	-	-
4)	26-nor-25-ketocholesteryl-3-acetate	-	-
5)	26-nor-25(RS)-hydroxy-cholesterol	-	-
6)	26-nor-25-ketocholesterol	-	-
7)	cholesta-5,25-diene-3β-ol	+	+
8)	26-iodo-cholesterol	+	+
9)	cholesterol	+	+

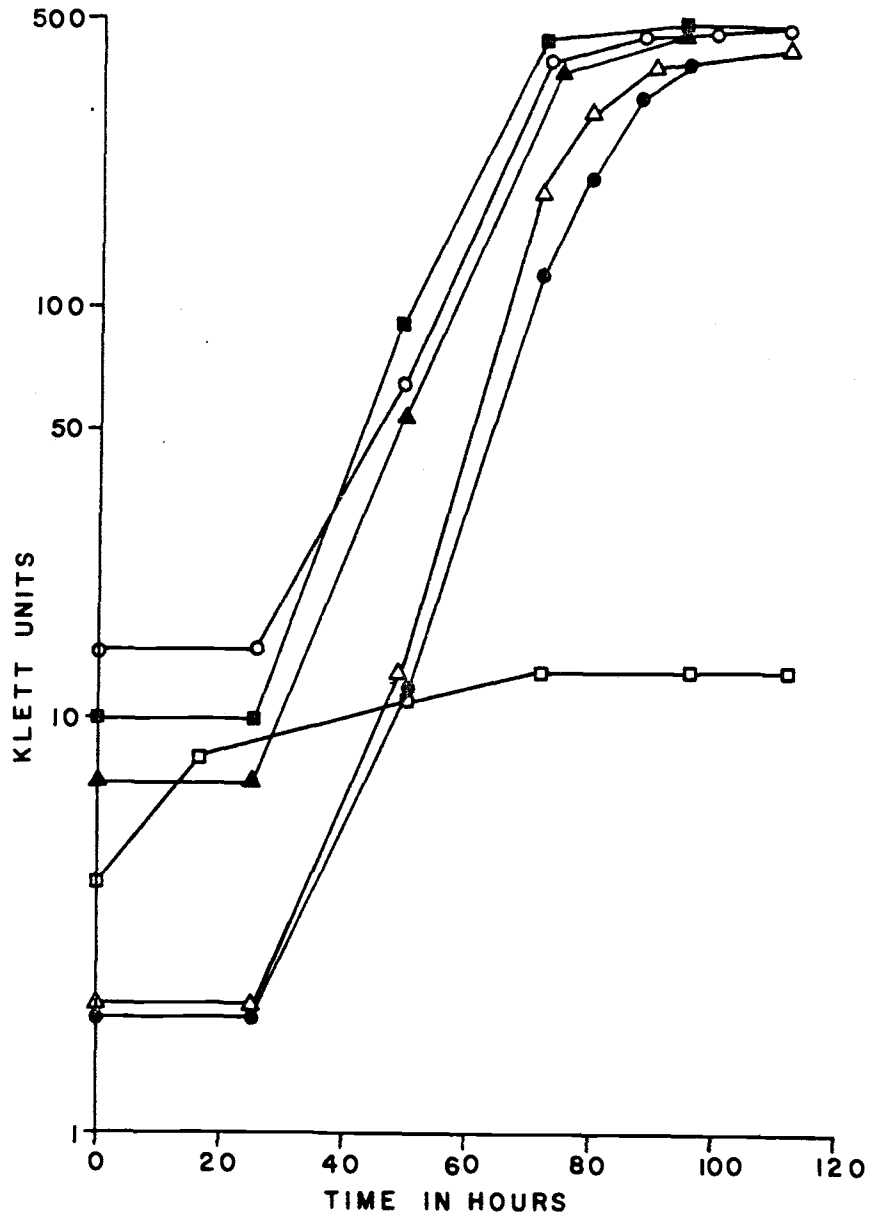


Figure 9. Growth of RD5-R on cholesterol (▲); 26-bromocholesterol (Δ); 25-bromocholesterol (●); $\Delta^{5,25}$-cholestadienol (■); iodocholesterol (0); and 25-hydroxycholesterol or 25-ketocholesterol (□). The curve for 25-ketocholesterol was virtually identical to that of 25-hydroxycholesterol, differing at some points by only 4 Klett units. Inocula for these growth studies were derived from cells which had been cycled on cholestanol.

It was of interest to determine if 25-keto and 25-hydroxy cholesterols were either growth inhibitory or were not transported into the cells. Cells grown in medium containing cholesterol (1 µg/ml) with and without 25-keto or 25-hydroxycholesterol (5 µg/ml) were analyzed for growth rate and final cell yield in order to determine if the oxygenated sterols were growth inhibitory. As shown in table 6, growth rates of RD5-R in the presence of either oxygenated sterol were approximately 15% lower than with cholesterol alone. However, final cell yields were the same in all three cultures (table 6). In order to assess transport of the oxygenated sterols, stationary phase cells from the same cultures were analyzed by HPLC and GLC for sterol content. Cholesterol was detected in all three cultures to about the same extent. However, in both cultures supplemented with an oxygenated sterol the respective oxygenated sterol was found to be incorporated to levels greater than cholesterol indicating functional transport.

Uptake of sterol and stanol by RD5-R:

It was of interest to determine how sparking amounts of ergosterol as well as bulk amounts of cholestanol were incorporated by the cells. Since such a small amount of ergosterol (10 ng) was used as compared to 5 µg/ml of cholestanol dual label experiments were not attempted. Two cultures were grown under sparking conditions with one containing ^{3}H-ergosterol and the other ^{3}H-cholestanol. At designated time points samples were taken and analyzed for cell density, cell number, and incorporated

Table 6. Growth of RD5-R in the presence and absence of oxygenated sterols.

Sterol Supplement	Growth Rate (doublings/hr)	Final Cell Yield (Klett units)
Cholesterol	0.20	450[a]
Cholesterol + 25-ketocholesterol	0.17	450[b]
Cholesterol + 25-hydroxycholesterol	0.17	450[b]

[a]Culture grew to this density in 51 hours.

[b]Culture grew to this density in 60 hours.

radioactivity. The results are shown in figure 10. In both cultures the first time point revealed that the cells either took up sterol and stanol immediately upon addition or that there was non-specific binding. After the first time point, however, the amount of label in the cells decreased until or just prior to the onset of growth. From that time there was a slow increase in the amount of cholestanol taken up and an even slower increase for ergosterol. At mid log phase, the cells stopped incorporating ergosterol but continued accumulating cholestanol at the same rate as in early log phase.

Throughout the entire culture cycle there was a predominance of cholestanol compared to ergosterol. On a molecular basis, at the start of growth there were approximately 1.6×10^{12} molecules of cholestanol per cell and 4×10^{8} molecules of ergosterol per cell constituting a 4×10^{3} fold difference. In stationary phase the values decreased to 2.5×10^{9} and 1.4×10^{5} respectively, which was a 1.78×10^{4} fold difference.

Physiological analyses on the sparking phenomenon:

Since it was apparent from uptake studies that more ergosterol was associated with sparked cells immediately after inoculation, it was of interest to determine if this sterol was being transported. In order to determine this, cholestanol cycled RD5-R cells were inoculated into medium containing only sparking (10 ng/ml) levels of ergosterol. At various times after inoculation aliquots of culture were washed two times with medium devoid of

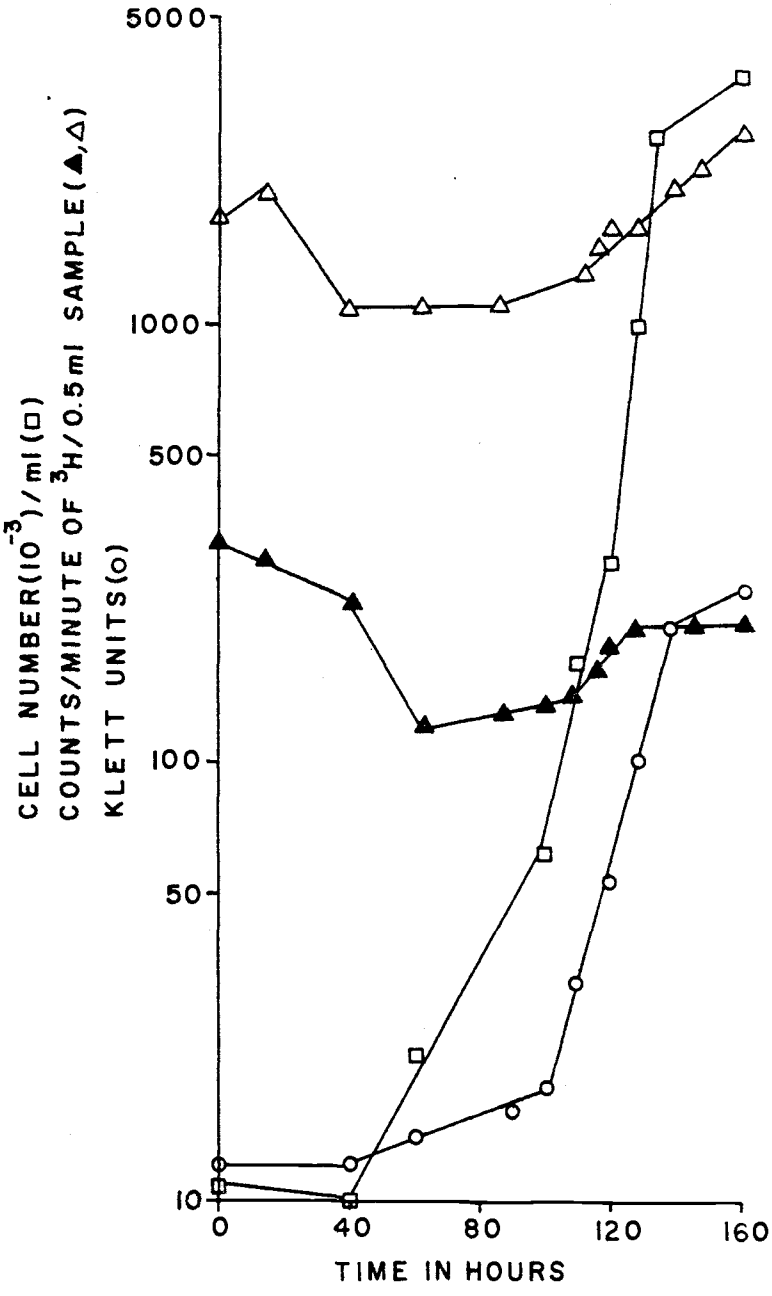


Figure 10. Uptake of ^{3}H-cholestanol (△) and ^{3}H-ergosterol (▲), and growth of RD5-R (○) under sparking conditions. Change in cell number throughout growth is also shown (□).

sterol and transferred to medium containing only cholestanol (5 µg/ml). The rationale was that cells would grow when transferred to cholestanol medium only if they had taken up sparking amounts of ergosterol. As shown in figure 11 transferred cells did not grow unless they were pre-incubated in ergosterol medium for at least 24 hrs. Even at that there was a 34 hour lag period before the onset of growth. This lag period occurred for all transferred cultures that grew regardless of the preincubation period.

When this experiment is carried out in a different manner, similar results are obtained. If cholestanol cycled cells are inoculated into medium containing only sparking ergosterol and bulk cholestanol is added at different times after inoculation there is a constant lag period up to 40 hours. As shown in figure 12 cholestanol (5 µg/ml) was added to cultures containing 10 ng/ml ergosterol at time = 0, 6.5, 15, 24, and 40 hours after inoculation. In all cases growth commenced soon after 40 hrs with all sparked cultures obtaining similar growth yields. It is interesting to note that the same lag period was observed in the presence of 5 µg/ml of cholesterol. To ensure that the lag times were not due to conditioning of the medium, a control was inoculated at time zero into medium containing no sterol. After 66 hours 10 ng/ml of ergosterol and 5 µg/ml of cholestanol were added. Again growth did not commence until after 110 hrs, approximately a 40 hour lag period. This control also demonstrated that there was not a "starvation" adaptation by cells occurring at 40 hrs.

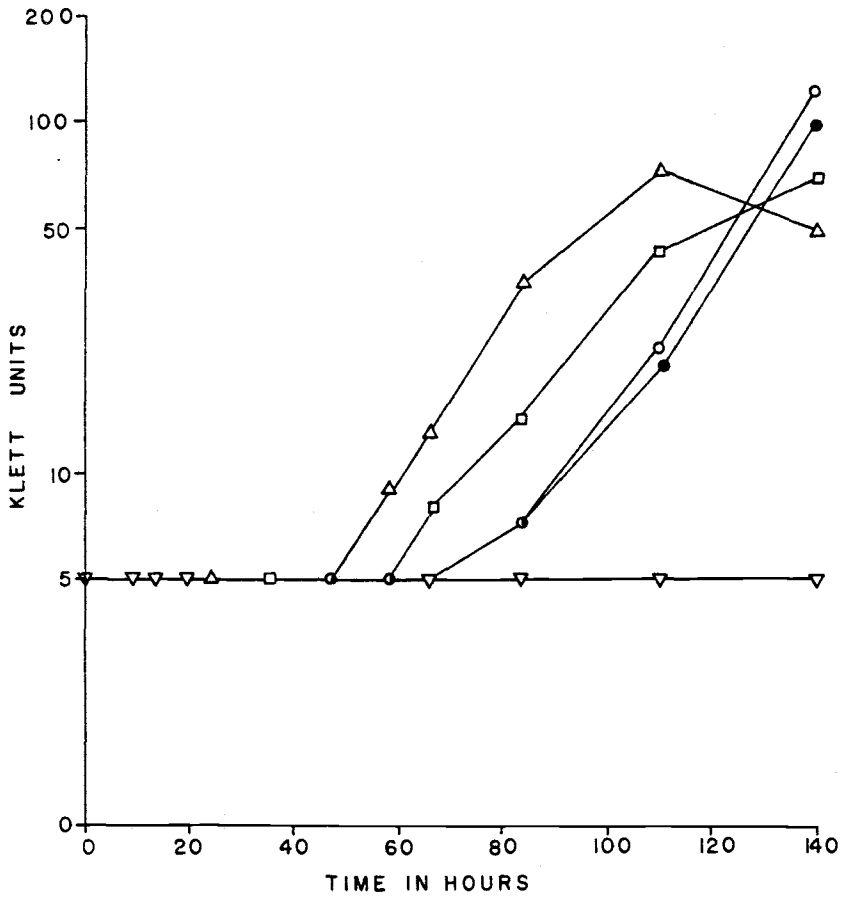


Figure 11. Transfer of cholestanol cycled RD5-R cells, after incubation in medium containing 10 ng/ml ergosterol, to medium containing 5 µg/ml cholestanol. Transfer after 0, 9, 13, and 19 hours (▽), 24 hours (△), 35 hours (□), 47 hours (○), and 58 hours (●).

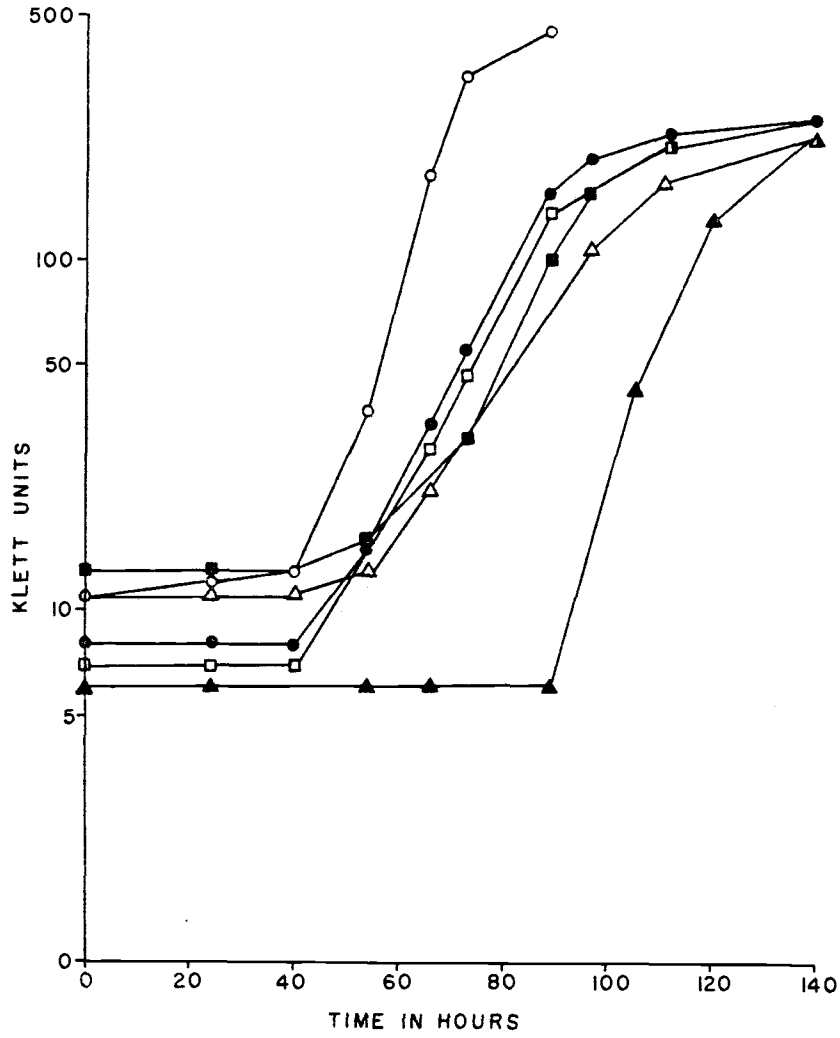


Figure 12. Growth of cholestanol cycled RD5-R cells upon addition of 5 µg/ml cholestanol to the same medium in which cells were pre-incubated with 10 ng/ml ergosterol. Cholestanol was added at 0 (△), 7 (△), 15 (■), 28 (□), and 40 (●) hours. Also shown is growth on 5 µg/ml cholestanol [added at time zero (○)] and growth on ergosterol (10 ng/ml) and cholestanol (5 µg/ml) after 66 hours of pre-incubation in the absence of these compounds (▲).

To demonstrate that the lag periods were resulting from an ergosterol dependent phenomenon, the experiment was done in reverse with regard to cholestanol and ergosterol. Cholestanol cycled cells were inoculated at time zero into medium containing only cholestanol 5µg/ml as sterol supplement. At designated times after inoculation 10 ng/ml of ergosterol was added to the cultures. In every case there was a 25 to 45 hour lag period (figure 13). It is interesting that the lag periods decreased from 45 to 25 hours with increasing length of incubation in medium containing only cholestanol as sterol supplement.

Reassessing the level of ergosterol required for sparking:

From uptake and sterol/stanol preincubation studies it was apparent that sterols need not be added simultaneously to a culture and that addition of cholestanol after ergosterol did not lengthen lag periods. It was of interest to determine how much ergosterol had to be added to spark growth if cholestanol was added much later. As shown in figure 14 the level of ergosterol was able to be decreased 10 fold to 1 ng/ml. This constituted a 5000 fold difference in the ratio of cholestanol/ergosterol present in the medium.

Effect of glucose on sparking:

It was of interest to determine if the sparking growth lag period required a carbon and energy source. Cells were inoculated into medium containing only sparking (10 ng/ml) level of ergosterol

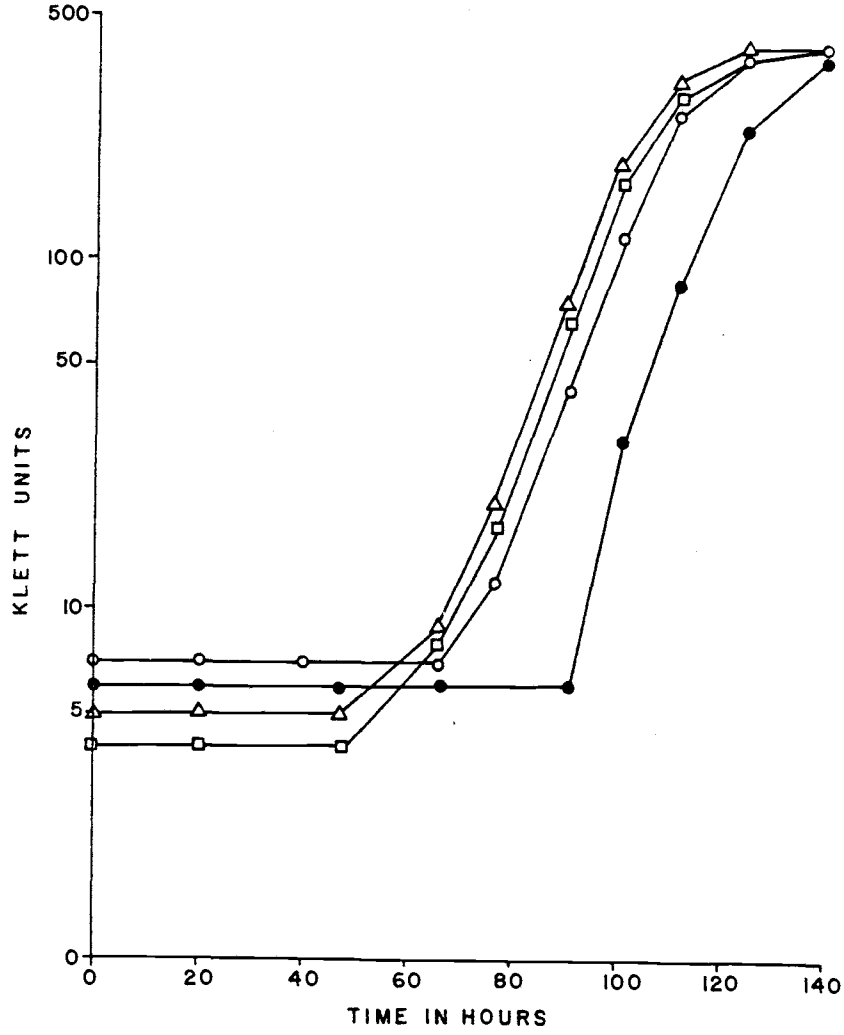


Figure 13. Growth of cholestanol cycled RD5-R cells upon addition of 10 ng/ml ergosterol to the same medium in which cells were pre-incubated with 5 µg/ml cholestanol. Ergosterol was added at 17 (△), 24 (□), 39 (○), and 66 (●) hours.

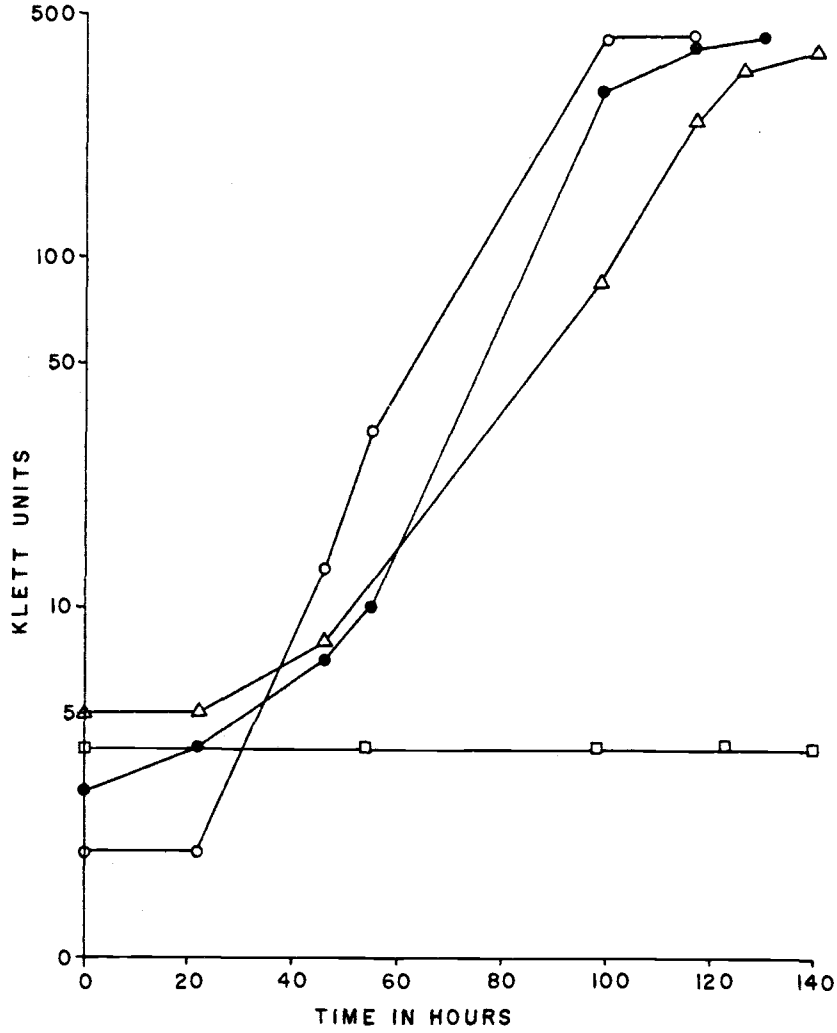


Figure 14. Growth of cholestanol cycled RD5-R cells after addition of 5 µg/ml cholestanol to cultures pre-incubated for 40 hours in media containing decreasing amounts of ergosterol: 10 ng/ml (○), 5 ng/ml (●), 1 ng/ml (△), 0.5 ng/ml (□). The figure depicts growth after addition of cholestanol.

with decreasing concentrations (2 to 0%) of glucose. After 28 hours, 5 µg/ml of cholestanol and glucose (to 2%) was added to each culture. It was observed that not only was glucose not required for the sparking lag period, cells grew sooner and better without glucose (figure 15). As the concentration of glucose in pre-incubated sparking culture decreased, so did the lag period before growth commenced after cholestanol addition. In addition subsequent growth rates (doublings/hour) increased for those cultures preincubated with decreasing amounts of glucose.

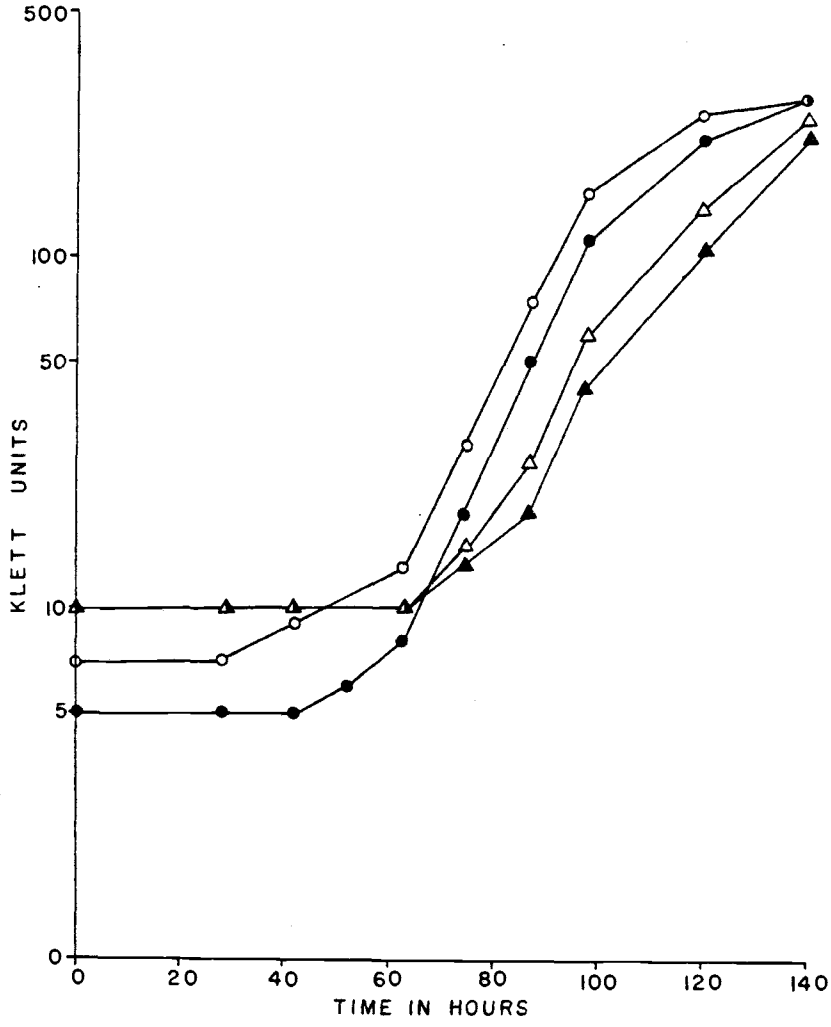


Figure 15. Growth of cholestanol cycled RD5-R cells after addition of cholestanol (5 µg/ml) and glucose (to 2%) to cultures pre-incubated for 40 hours in the presence of ergosterol (10 ng/ml) and decreasing amounts of glucose: 2% (▲), 1% (△), 0.1% (●), 0.01% (○), 0% (○).

DISCUSSION

Data presented in this thesis deals with the roles of sterols in Saccharomyces cerevisiae. Results demonstrate that this yeast requires sterols for two separate classes of functions. This was elucidated by the fact that cholestanol supports growth of FY3 only in the presence of minute amounts of Δ^5-sterol. Therefore, while cholestanol is unable to support growth by itself, it is capable of satifying, in part, the sterol requirement. Also, since there is a low but definite level of Δ^5-sterol needed for growth, it suggests that these supplements are satisfying different requirements. It is important to note that higher concentrations of ergosterol are capable of satisfying both classes of functions since cells can grow on it alone. It is conceivable to divide the "function" of sterols in yeast cells into two major classes, one which can be satisfied by cholestanol or ergosterol and another which cannot be satisfied by cholestanol. The function(s) satisfied by relatively high concentrations (5 µg/ml) of cholestanol or ergosterol are considered to involve bulk membrane properties such as fluidity. We suggest that the function(s) satisfied by minute quantities (10 ng/ml) of ergosterol and not cholestanol are much more specific but have not yet been identified. We refer to this phenomenon as the "sparking" of growth because it differs from previously reported observations (4). In our experiments ergosterol acts to initiate and maintain growth rather than modify growth. The cellular need for small amounts of ergosterol is designated the sparking require-

ment and the need for cholestanol, is the bulk membrane function. This is the first report of multiple functions in yeast. This is also the first detailed demonstration of such a highly specific requirement for sterol in eukaryotic organisms.

Mutants described in this thesis (RD5, RD5-R, and FY3) appear to differ only in their ability to utilize certain sterols and stanols for bulk membrane and/or sparking functions. Supplementation of the sparking function by a sterol or stanol was dependent on the presence of a C-5,6 unsaturation on the sterol or the ability of the strain to desaturate a non-Δ^5-sterol (or stanol) at this position *in vivo*. Clearly, the lack of the C-28 methyl group as well as the Δ^7 and Δ^{22} unsaturations of ergosterol have no effect on the ability of a Δ^5-sterol to fulfill the sparking function. Mutagenesis and screening of cells was carried out in an attempt to isolate a stable mutation in the gene encoding the Δ^5-desaturate enzyme. In every case strains were isolated with the impaired Δ^5-desaturase activity of RD5, but no strain was obtained which completely lacked this enzyme. All mutagenized strains reverted quickly to obtain a greater Δ^5-desaturase capacity (equivalent to RD5-R). It is interesting that we did not observe reversion of RD5-R to FY3 with regard to Δ^5-desaturase. We therefore hypothesize that this enzyme is required by the cells, at least at a low level, and that complete loss of this function constitutes a lethal event.

Analysis of sterol mutants reported to lack Δ^5-desaturase (74,9) provided support for this hypothesis. All of the presumed sterol mutants defective in this enzyme were found by HPLC analysis to contain small amounts of $\Delta^{5,7}$-sterol (ergosterol), indicating some Δ^5-desaturase activity. The $\Delta^{5,7}$-sterol in each sterol mutant was present in concentrations equal to or greater than that necessary for sparking growth of sterol auxotrophs on cholestanol. This was true even for C-14 demethylase : Δ^5-desaturase double mutants. Sterol mutants accumulating non-Δ^5-sterols as primary sterols may only be viable because of leakiness in the gene encoding Δ^5-desaturase such that sparking levels of Δ^5-sterol are available. Further support for this hypothesis comes from the fact that the major sterol of the sterol mutant JR4 (14-methylfecosterol) is unable to satisfy the sparking requirement(s) of RD5-R (85).

Growth of the sterol auxotrophs on many sterols and stanols was dependent on modification only at the C-5 position. The efficiency of Δ^5 desaturation was dependent on two structural features: the C-28 methyl group and another B-ring unsaturation (table 4). The influence of the C-28 methyl is best illustrated by the inability of Δ^8-cholestenol to fulfill the sparking requirement of RD5 but Δ^8-ergostenol does (figures 6 and 7). Although these strains were able to grow on Δ^8-ergostenol, the sterol was converted in part to $\Delta^{5,7}$ ergostadienol while Δ^8-cholestenol was not converted. This same phenomenon is seen with RD5-R on

ergostanol versus cholestanol. The effect of a B-ring unsaturation can be observed with either RD5 or RD5-R in the presence of Δ^7-cholestenol compared with cholestanol (figure 6). Δ^7-cholestenol is desaturated at C-5,6 and satisfies the sparking requirement of either strain but neither is true with cholestanol. This information can be interpreted to mean that although the C-28 methyl group and other B-ring unsaturations are not required for either sterol function (bulk or sparking), their major role may be to enhance enzyme specificity of Δ^5-desaturase.

The bulk membrane requirement of these auxotrophs appears to be satisfied by many different sterols and stanols. Of the compounds tested, only coprostanol, Δ^4-cholestenol, $\Delta^{3,5}$-cholestadienol, 25-ketocholesterol and 25-hydroxycholesterol were incapable of satisfying this requirement. We have determined that these lipids are not growth inhibitory and that the inability of the oxygenated sterols to satisfy bulk function was not due to an inability to be incorporated by these cells. It is apparent from these studies that while Δ^5-sterols are required to satisfy sparking requirements, they are not necessary for bulk membrane function. In addition, the presence of C-5,6 unsaturation does not ensure that a sterol will satisfy either requirement as shown with the above sterols. It is interesting that addition of a bromo or iodo, group to the sterol side chain had no effect on the ability of cholesterol to satisfy the sterol requirements but an oxygen atom did. This phenomenon is obviously not due to steric

consideration since an iodide moiety has a greater atomic diameter than oxygen. However, this may result from detrimental hydrogen bonding with oxygen.

Uptake of sterol and stanol under sparking conditions follow different kinetics. It appears that little or no lipid is incorporated until just prior to or as growth commences. Cholestanol is incorporated at a continously increasing amount per sample, however, the total amount of cholestanol per cell decreased throughout growth. Incorporation of ergosterol per sample occurred until mid-log phase and remained at a constant value for the remainder of growth. The rate of ergosterol incorporation seemed, in part, to coincide with growth rate. The total amount of ergosterol per cell also decreased throughout growth. The final ratio of cholestanol/ergosterol in sparked cells was 1.78×10^4. While these cells contain approximately 1.4×10^5 molecules of ergosterol/cell, there were 2.5×10^9 molecules of cholestanol/cell. From growth studies it is apparent that in order for sparked cells to grow to stationary phase they must have ergosterol available until mid-log phase. However the cells will begin to grow if ergosterol is present for at least the normal lag period for sparked cells. It appears that the cell is able to regulate ergosterol uptake and that the amount of this sterol required for sparking is not taken up all at once, rather it is incorporated during early to mid-log phase.

The fact that such a repeatable lag phase occurred prior to sparked growth suggested the cells were undergoing some physiological adjustment. It is apparent that the "adjustment" is ergosterol dependent since pre-incubation of medium and cells in the absence of this sterol does not decrease the lag period. During the lag period the cells must take up a low level of ergosterol which sparks the cells to grow on cholestanol. This may result when ergosterol reaches a critical concentration somewhere in the cell or is processed and deposited at a specific location(s). To determine if the adjustment or lag period was energy dependent cells were starved for carbon and energy sources (glucose). As the glucose level decreased to 0% during ergosterol pre-incubation, the lag period decreased and the cells grew faster on cholestanol. This indicated that not only was the adjustment not energy dependent (at least glucose derived energy), it was accomplished sooner in the absence of glucose. Growth in the glucose deprivation experiments was very slow to start and then quickly accelerated until slowing once again prior to stationary phase. However with increasing amounts of glucose, the fast growth portion of the cycle decreased. When log phase cells are starved for glucose (86) they have been shown to stop growing upon entering G_1 phase. It is possible that a similar phenomenon is occurring in cells pre-incubated with ergosterol, but starved for glucose. It has recently been reported that in mammalian cells cholesterol is required during G_1 phase of growth (87). A similar phenomenon may be occurring in yeast sterol auxotrophs.

It is interesting that different sterols and stanols show varying lag periods prior to growth of these auxotrophs (figures 6 and 7). It is possible that minor differences in solubilities of the lipids in the medium result in differences in availability to the cells. In addition, uptake Km's for the different lipids may vary considerably. However, these do not appear to be major factors since sparking quantities of ergosterol reduce lag times significantly (eg. FY3 grown on cholestanol). Reduction in lag periods by sparking levels of ergosterol (Δ^5 sterol) may result from alterations in membrane properties such that uptake of other lipids would occur with greater ease. However, in light of anisotropy data reported with mycoplasma grown with lanosterol and cholesterol (6) it seems unlikely that sparking amounts of Δ^5 sterol would significantly alter membrane fluidity.

A more probable explanation for lag period variations is that a Δ^5-sterol or a converted form of it is required for a very specific function(s). If this is the case then the auxotrophic cells must first take up a sterol or stanol and, if the lipid does not contain a C-5,6 unsaturation, desaturate at C-5, and deposit the sterol in a specific required location(s). From this model the lag times for growth on different lipids may result from variation in: 1) transport from the plasma membrane to the site of C-5 desaturation, 2) specificity of the Δ^5-desaturase enzyme, 3) transport of the sterol from the site of desaturation to a required location(s), and/or 4) further modification of the Δ^5-sterol and

transport to and from this site. Intracellular sterol transport mechanisms undoubtedly function in the deposition of sterol and maintenance of cellular membranes. These transport processes may only function efficiently with Δ^5-sterols. These data may be further extended to suggest that the Δ^5-desaturase enzyme has more than one cellular function. In light of recent evidence suggesting a possible hormone system in yeast (88), it is conceivable that the sterol Δ^5-desaturase enzyme may be involved in such a system.

Sterol auxotrophs of Saccharomyces cerevisiae exhibit selectivity with regard to sterols capable of satisfying the sterol requirements. This appears to be a relatively specific process in which either nuclear changes (β versus α saturation of a C-5,6 unsaturation) or side chain alterations (addition of a keto or hydroxy moiety at C-24) significantly alter the ability of a sterol or stanol to fulfill bulk membrane and/or sparking requirements.

Subsequent to our original report of diverse sterol functions in yeast (89), a similar observation has been described in this organism (90).

It is important to discriminate between the sparking phenomenon (89), sterol synergism (90), and sparing (5,6,72,73). The phenomenon of sparking is illustrated by the observation that cholestanol cycled RD5-R will not grow on cholestanol (5 μg/ml) unless minute quantities of a Δ^5-sterol (10 ng/ml) are present. It is important to note that under sparking conditions the Δ^5-sterol acts to initiate rather than modify growth. A Δ^5-sterol appears to be an absolute growth requirement for Saccharomyces cerevisiae.

Sparing is demonstrated by the ability of cholesterol (0.5 µg/ml) to increase growth of Mycoplasma capricolum on lanosterol (10 µg/ml). Under sparing conditions there is a decrease in the Km for oleic acid transport (5). Fluctuations in RNA and protein biosynthesis have also been reported to occur when this organism is grown on different sterols (73). As a result of studies with Mycoplasma it has been concluded that cholesterol coordinately regulates RNA, protein, and phospholipid biosyntheses (73). However, there are two major criticisms of this conclusion: 1) Under sparing conditions the cholesterol can be replaced either by doubling the supplemented fatty acid concentration (5) or by addition of egg phosphatidylcholine (73). This indicates that there is not a specific role for sparing cholesterol but rather a generalized membrane effect which is compensated by other lipids. 2) Decreases in RNA, protein, and phospholipid biosyntheses occur when Mycoplasma capricolum is supplemented with sterols other than cholesterol. However, the results may be ascribed to variations in growth rates which also occur and coincide with the respective biosynthetic decreases.

Sterol synergism was described with the yeast sterol auxotroph, GL7; it grows better in the presence of 0.3 µg/ml cholesterol plus 0.1 µg/ml ergosterol than on either sterol, at these same concentrations, alone (90). Analyses with the above pair of sterols as well as two other pairs of sterol have revealed that, depending on the sterol supplements, there are differences in

phospholipid biosynthesis. This study has led to the conclusion that sterol regulates phospholipid biosynthesis and that it is the side chain of ergosterol that makes this sterol optimal for yeast. However, these interpretations may be unjustified for the following reasons: 1) Analysis of growth data for GL7 grown on different sterol combinations (used to distinguish the importance of the ergosterol side chain) shows little if any variation in log phase growth rates. This indicates that the C-28 methyl and C-22(23) unsaturation of ergosterol are not as critical as suggested. In addition slight differences which may occur under these conditions may arise due to impurities in the sterols used. In those studies sterols were purified by recrystallization, a method which we have found insufficient for removing inhibitory breakdown products from sterols. Sterol impurities can be removed by HPLC or TLC purification. 2) The incorporation of oleate into phospholipid is proposed to be regulated by sterol. However, these data can be interpreted two ways. One interpretation which has been proposed is that at synergistic levels, ergosterol is taken up by the cells and specifically regulates oleate uptake and hence phospholipid biosynthesis (90). An alternative interpretation is that the ergosterol is taken up and alters the physical state of the cell membranes. Since synergistic ergosterol constitutes one-fourth of the sterol supplement this is a strong possibility. Alterations in membrane fluidity may then be compensated by mechanisms which are not specifically related to ergosterol, such as modulation in phospholipids.

Clearly the sparking phenomenon differs from sparing or sterol synergism. The need for a Δ^5-sterol appears to be an absolute growth requirement in yeast sterol auxotrophs. Since a Δ^5-sterol is not required by Mycoplasma capricolum (i.e. it grows on lanosterol alone) direct comparison with the yeast system could be suspect. The sparking phenomenon seems to involve a triggering or switching on of cell growth. While this suggests very specific roles for Δ^5-sterols none have been demonstrated. Since such small amounts of Δ^5-sterol (1 ng/ml) are necessary and since they have such a significant effect on cell growth, it is conceivable that the role of sparking Δ^5-sterol is not membrane related. Irregardless of the specific role, data presented in this thesis suggest that sterols may play a much more important role in yeast cellular physiology than previously thought.

LITERATURE CITED

1. Parks, L. W. 1978. Metabolism of sterols in yeast. CRC Crit. Rev. Microbiol. 6, 301-341.

2. Demel, R. A., and B. DeKruyff. 1976. The function of sterols in membranes. Biochim. Biophys. Acta 457, 109-132.

3. Nes, W. R. 1974. Role of sterols in membranes. Lipids 9, 596-615.

4. Dahl, J. S., C. E. Dahl, and K. Bloch. 1981. Effect of cholesterol on macromolecular synthesis and fatty acid uptake by Mycoplasma capricolum. J. Biol. Chem. 256, 87-91.

5. Clark, A. J., nd K. Bloch. 1959. Function of sterols in Dermestes vulpinus. J. Biol. Chem. 234, 2583-2588.

6. Dahl, J. S., C. E. Dahl, and K. Bloch. 1980. Sterols in membranes: growth characteristics and membrane properties of Mycoplasma capricolum cultured on cholesterol and lanosterol. Biochem. 19, 1467-1472.

7. Nes, W. R., B. C. Sekula, W. D. Nes, and J. H. Adler. 1978. The functional importance of structural features of ergosterol in yeast. J. Biol. Chem. 253, 6218-6225.

8. McLean-Bowen, C. A., and L. W. Parks. 1981. Corresponding changes in kynurenine hydroxylase activity, membrane fluidity, and sterol composition in Saccharomyces cerevisiae mitochondria. J. Bacteriol. 143, 1325-1333.

9. Thompson, E. D., and L. W. Parks. 1974. The effect of altered sterol composition on cytochrome oxidase and S-

adenosylmethionine: Δ24 sterol methyltransferase enzymes of yeast mitochondria. Biochem. Biophys. Res. Commun. 57, 1207-1213.

10. Taylor, F. R., and L. W. Parks. 1980. Adaptation of Saccharomyces cerevisiae to growth on cholesterol: selection of mutants defective in the formation of lanosterol. Biochem. Biophys. Res. Commun. 95, 1437-1445.

11. Taylor, F. R., and L. W. Parks. 1981. An assessment of the specificity of sterol uptake and esterification in Saccharomyces cerevisiae. J. Biol. Chem. 256, 13048-13054.

12. Buttke, T. M., and K. Bloch. 1981. Utilization and metabolism of methyl-sterol derivatives in the yeast mutant GL7. Biochem. 20, 3267-3272.

13. Lala, A. K., T. M. Buttke, and K. Bloch. 1979. On the role of the sterol hydroxyl group in membranes. J. Biol. Chem. 254, 10582-10585.

14. Clejan, S., R. Bittman, and S. Rottem. 1981. Effects of sterol structure and exogenous lipids on transbilayer distribution of sterols in the membrane of Mycoplasma capricolum. Biochem. 20, 2204-2207.

15. Buttke, T. M., S. D. Jones, and K. Bloch. 1980. Effect of sterol side chains on growth and membrane fatty acid composition of Saccharomyces cerevisiae. J. Bacteriol. 144, 124-130.

16. Nes, W. R., and M. L. McKean. 1977. Biochemistry of steroids and other isopentenoids. University Park Press, Baltimore.

17. Pal, R., W. A. Petri, and R. R. Wagner. 1980. Alteration of the membrane lipid composition and infectivity of vesicular stomatitis virus by growth in a Chinese hamster ovary cell sterol mutant and in lipid-supplemented baby hamster kidney clone 21 cells. J. Biol. Chem. 255, 7688-7693.

18. Minale, L., and G. Sodano. 1977. Marine natural product chemistry. Plenum Press, New York, 87-109.

19. Bohlin, L., H. P. Gehrken, P. J. Scheuer, and C. Djerassi. 1980. Minor and trace sterols in marine invertebrates XVl. 3ξ-hydroxymethyl-A-nor-5α-gorgostane, a novel sponge sterol. Steroids 35, 295-302.

20. Weete, J. D. 1980. Lipid biochemistry. Plenum Press, New York, 261-298.

21. Babezinski, P., and W. Janner. 1973. Involvement of dolicholmonophosphate in the formation of specific mannosyl-linkages in yeast glycoproteins. Biochem. Biophys. Res. Commun. 54, 1119-1124.

22. Karst, F., and F. Lacroute. 1977. Ergosterol biosynthesis in Saccharomyces cerevisiae. Mutants deficient in the early steps in the pathway. Molec. Gen. Genet. 154, 269-277.

23. Maugh II, T. H. 1982. Completing the puzzle of steroid synthesis. Science 218, 1297.

24. Atkinson, D. E. 1977. Cellular energy metabolism and its regulation. Academic Press, New York, 72-75.

25. Parks, L. W. 1958. S-adenosylmethionine and ergosterol synthesis. J. Am. Chem. Soc. 80, 2023.

26. Fryberg, M., L. Avruch, A. C. Oehlschlager, and A. M. Unrau. 1975. Nuclear demethylation and C-24 alkylation during ergosterol biosynthesis in Saccharomyces cerevisiae. Canad. J. Biochem. 53, 881-889.

27. Pierce, A. M., R. B. Mueller, A. M. Unrau, and A. C. Oehlschlager. 1978. Metabolism of Δ^{24}-sterols by yeast mutants blocked in removal of the C-14 methyl group. Canad. J. Biochem. 56, 794-800.

28. Pierce, A. M., A. M. Unrau, and A. C. Oehlschlager. 1979. Azasterol inhibitors in yeast. Inhibition of the Δ^{24}-sterol methyltransferase and the 24-methylene sterol $\Delta^{24(28)}$-reductase in sterol mutants of Saccharomyces cerevisiae. Canad. J. Biochem. 57, 201-208.

29. Aries, V., and B. H. Kirsop. 1978. Sterol biosynthesis by strains of Saccharomyces cerevisiae in the presence and absence of dissolved oxygen. J. Inst. Brew. 84, 118-122.

30. Reddy, V. V. R., D. Kupfer, and E. Caspi. 1977. Mechanism of C-5 double bond introduction in the biosynthesis of cholesterol by rat liver microsomes. Evidence for the participation of microsomal cytochrome b_5. J. Biol. Chem. 252, 2797-2801.

31. Hata, S., T. Nishino, M. Komori, and H. Katsuki. 1981. Involvement of cytochrome P450 in Δ^{22}-desaturation in ergosterol biosynthesis in yeast. Biochem. Biophys. Res. Commun. 103, 272-277.

32. Osumi, T., S. Taketan, H. Katsuki, T. Khara, and I. Matsumoto. 1978. Ergosterol biosynthesis in yeast. Pathways in the late stages and their variation under various conditions. J. Biochem. 83, 681-691.

33. Andreasen, A. A., and T. J. B. Stier. 1953. Anaerobic nutrition of Saccharomyces cerevisiae. I. Ergosterol requirement for growth in a defined medium. J. Cell. Comp. Physiol. 41, 23-36.

34. Andreasen, A. A., and T. J. B. Stier. 1954. Anaerobic nutrition of Saccharomyces cerevisiae. II. Unsaturated fatty acid requirement for growth in a defined medium. J. Cell. Comp. Physiol. 43, 271-281.

35. Lehninger, A. L. 1977. Biochemistry. Second edition. Worth Publishers, Inc., New York, 623-749.

36. Shah, S. N. 1981. Modulation in vitro of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase in brain microsomes: Evidence for the phosphorylation and dephosphorylation associated with inactivation and activation of the enzyme. Arch. Bioch. Biophys. 211, 439-446.

37. Volpe, J., and K. A. Obert. 1981. Coordinate regulation of cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme

A synthetase but not 3-hydroxy-3-methylglutaryl coenzyme A reductase in C-6 Glia. Arch. Biochem. Biophys. 212, 88-97.

38. Schroepfer Jr., G. J. 1981. Sterol biosynthesis. Ann. Rev. Biochem. 50, 585-621.

39. Brown, M. S., and J. L. Goldstein. 1980. Multivalent feedback regulation of HMG CoA reductase, a control mechanism coordinating isoprenoid synthesis and cell growth. J. Lipid Res. 21, 505-517.

40. Bard, M., and J. F. Downing. 1981. Genetic and biochemical aspects of yeast sterol regulation involving 3-hydroxy-3-methylglutaryl coenzyme A reductase. J. Gen. Microbiol. 125, 415-420.

41. Berndt, J., M. Boll, M. Lowel, and R. Gaumert. 1973. Regulation of sterol biosynthesis in yeast: induction of 3-hydroxy-3-methylglutaryl-CoA reductase by glucose. Biochem. Biophys. Res. Commun. 51, 843-848.

42. Quain, D. E., and J. M. Haslam. 1979. The effects of catabolite derepression on the accumulation of steryl esters and the activity of β-hydroxymethylglutaryl-CoA reductase in Saccharomyces cerevisiae. J. Gen. Microbiol. 111, 343-351.

43. Trocha, P. J., and D. B. Sprinson. 1976. Location and regulation of early enzymes of sterol biosynthesis in yeast. Arch. Biochem. Biophys. 174, 45-51.

44. Parks, L. W., C. McLean-Bowen, M. McCammon, and P. R. Hays. 1979. S-adenosylmethionine: Δ^{24}-sterol methyltransferase:

studies on its regulation and physiological role. In: Transmethylation, eds. E. Usdin, R. T. Borchardt, C. R. Creveling, Elsevier/North Holland, New York. 319-327.

45. Dulaney, E. L., E. O. Staply, and K. Simpf. 1954. Ergosterol production by yeasts. Appl. Microbiol. 2, 371-379.

46. Bailey, R. B., and L. W. Parks. 1975. Yeast sterol esters and their relationship to the growth of yeast. J. Bacteriol. 124, 606-612.

47. Madyastha, P. B., and L. W. Parks. 1969. The effect of cultural conditions on the ergosterol ester components of yeast. Biochim. Biophys. Acta 176, 858-862.

48. Bartlett, K., and E. I. Mercer. 1974. Variation in the levels and composition of the sterols and sterol esters of Phycomyces blakesleanus with age of culture. Phytochem. 13, 1115-1121.

49. Taylor, F. R., and L. W. Parks. 1978. Metabolic interconversion of free sterols and steryl esters in Saccharomyces cerevisiae. J. Bacteriol. 136, 531-537.

50. Clausen, M. K., K. Christiansen, P. K. Jensen, and O. Behnke. 1974. Isolation of lipid particles from bakers yeast. FEBS Lett. 43, 176-179.

51. Leathes, J. B. 1925. Role of fats in vital phenomena. Lancet 208, 853-856.

52. Chapman, D. 1968. Recent physical studies of phospholipids and natural membranes. In: Biological membranes. Physical

fact and function, ed. D. Chapman. Academic Press Inc., London. 125-199.

53. Raison, J. K. 1973. The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane associated enzyme systems. Bioenerg. 4, 285-309.

54. Sizer, I. W. 1943. Effects of temperature on enzyme kinetics. Adv. Enzymol. 3, 35-63.

55. Eletr, S., M. A. Williams, T. Watkins, and A. D. Keith. 1974. Perturbations of the dynamics and lipid alkyl chains in membrane systems: Effect on the activity of membrane bound enzymes. Biochim. Biophys. Acta 339, 190-201.

56. Warren, G. B., P. A. Toon, N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe. 1974. Reversible lipid titrations of the activity of pure adenosine triphosphate-lipid complexes. Biochem. 13, 5501-5507.

57. Ainsworth, P. J., E. R. Tustanoff, and A. J. S. Ball. 1972. Membrane phase transitions as a diagnostic tool for studying mitochondriogenesis. Biochem. Biophys. Res. Commun. 52, 320-326.

58. Kimelberg, H., and D. Papahadjopoulus. 1974. Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on (Na^+ - K^+)-stimulated adenosine triphosphatase. J. Biol. Chem. 249, 1071-1080.

59. Cobon, G. S., and J. M. Haslam. 1973. The effect of altered membrane sterol composition on the temperature dependence of

yeast mitochondrial ATPase. Biochem. Biophys. Res. Commun. 52, 320-326.

60. Bottema, C. D. K., C. A. McLean-Bowen, and L. W. Parks. 1983. Role of sterol structure in the thermotropic behavior of plasma membranes of Saccharomyces cerevisiae. Submitted to Biochim. Biophys. Acta.

61. McLean-Bowen, C. A., and L. W. Parks. 1982. Effect of altered sterol composition on the osmotic behavior of sphaeroplasts and mitochondria of Saccharomyces cerevisiae. Lipids 17, 662-665.

62. McLean-Bowen, C. A., and L. W. Parks. 1981. The effect of sterol on the energy producing capacity of yeast mitochondria. Chem. Phys. Lipids 29, 137-145.

63. Kleinhans, F. W., N. D. Lees, M. Bard, R. A. Hock, and R. A. Woods. 1979. ESR determination of membrane permeability in yeast sterol mutants. Chem. Phys. Lipids 23, 145-154.

64. Bard, M., N. D. Lees, L. S Burrows, and F. W. Kleinhans. 1978. Differences in crystal violet uptake and action-induced death among yeast sterol mutants. J. Bacteriol. 135, 1146-1148.

65. LeGrimellec, C., and G. Leblanc. 1978. Effect of membrane cholesterol on potassium transport in Mycoplasma mycoides var. capri (PG3). Biochim. Biophys. Acta 514, 152-163.

66. Green, C. 1977. Sterols in cell membranes and model membrane systems. In: Biochemistry of lipids 11, eds. T. W. Goodwin,

H. L. Kornberg, and D. C. Phillips. University Park Press, Baltimore. 101-154.

67. Parks, L. W., C. A. McLean-Bowen, C. K. Bottema, F. R. Taylor, R. A. Gonzales, B. W. Jensen, and J. R. Ramp. 1982. Aspects of sterol metabolism in the yeast Saccharomyces cerevisiae and in Phytophthora. Lipids 17, 187-196.

68. Hennache, B., G. Torpier, and P. Boulanger. 1982. Adenovirus adsorption and sterol redistribution in KB cell plasma membrane. Exp. Cell. Res. 137, 459-463.

69. Giraud, F., M. Claret, K. R. Bruckdorfer, and B. Chailley. 1981. The effects of membrane lipid order and cholesterol on the internal and external cationic sites of the $Na^+ - K^+$ pump in erythrocytes. Biochim. Biophys. Acta 647, 249-258.

70. Hamilton-Miller, J. M. T. 1973. Chemistry and biology of the polyene macrolide antibiotics. Bacteriol. Rev. 37, 166-196.

71. Ritter, K. S., and W. R. Nes. 1981. The effects of the structure of sterols on the development of Heliothis zea. J. Insect Physiol. 27, 419-424.

72. Dahl, J. S., C. E. Dahl, and K. Bloch. 1982. Role of membrane sterols in Mycoplasma capricolum. Rev. Infect. Diseases 4, 593-596.

73. Dahl, J. S., and C. E. Dahl. 1983. Coordinate regulation of unsaturated phospholipid, RNA, and protein synthesis in Mycoplasma capricolum by cholesterol. Proc. Natl. Acad. Sci. 80, 692-696.

74. Ramp, J. R. 1981. Nystatin resistant mutants in yeast. Honors Thesis, Oregon State University, Corvallis, Oregon.

75. Nace, H. R. 1951. An improved hydrogenation of cholesterol to cholestanol. J. Am. Chem. Soc. 73, 2379.

76. Kircher, H. W. 1974. Improved synthesis of 7-dehydrositosterol. Lipids 9, 623-624.

77. Rodriguez, R. J., and L. W. Parks. 1982. Application of high-performance liquid chromatographic separation of free sterols to the screening of yeast sterol mutants. Anal. Biochem. 119, 200-204.

78. Neal, W. D., and L. W. Parks. 1977. Sterol 24(28)methylene reductase in Saccharomyces cerevisiae. J. Bacteriol. 129, 1375-1378.

79. Gonzales, R. A., and L. W. Parks. 1977. Acid-labilization of sterols for extraction from yeast. Biochim. Biophys. Acta 489, 507-509.

80. Brusick, D. J. 1970. The mutagenic activity of ICR-I70 in Saccharomyces cerevisiae. Mutation Res. 10, 11-19.

81. Sherman, F., G. R. Fink, and J. B. Hicks. 1981. Methods in yeast genetics. Cold Spring Harbor Press, Cold Spring Harbor.

82. Magni, G. E., R. C. Von Borstel, and S. Sora. 1965. Mutagenic action during meiosis and antimutagenic action during mitosis by 5-amino acridine in yeast. Mutation Res. 1, 227-230.

83. Culbertson, M. R., L. Charnas, M. Tina Johnson, and G. Fink. 1977. Frameshifts and frameshift suppressors in Saccharomyces cerevisiae. Genet. 86, 745-764.

84. Moore, Jr., J. T., and J. L. Gaylor. 1969. Isolation and purification of an S-adenosylmethionine: Δ^{24}-sterol methyltransferase from yeast. J. Biol. Chem. 244, 6334-6340.

85. Taylor, F. R., R. J. Rodriguez, and L. W. Parks. 1983. A requirement for a second sterol biosynthetic mutation to allow for viability of a sterol C-14 demethylation defect in Saccharomyces cerevisiae. J. Bacteriol. in press.

86. Pringle, J. R., and L. H. Hartwell. 1982. The Saccharomyces cell cycle. In: The Molecular Biology of the Yeast Saccharomyces. Life cycle and inheritance. eds, J. N. Strathern, E. W. Jones, and J. R. Broach. Cold Spring Harbor Press, Cold Spring Harbor. 97-142.

87. Quesney-Huneeus, V., H. A. Galick, M. D. Siperstein, S. K. Erickson, T. A. Spencer, and J. A. Nelson. 1983. The dual role of mevalonate in the cell cycle. J. Biol. Chem. 258, 378-385.

88. Feildman, D., Y. Do, A. Burshell, P. Stathis, and D. S. Loose. 1982. An estrogen-binding protein and endogenous ligand in Saccharomyces cerevisiae: Possible hormone receptor system. Science 218, 297-298.

89. Rodriguez, R. J., F. R. Taylor, and L. W. Parks. 1982. A requirement for ergosterol to permit growth of yeast sterol

auxotrophs on cholestanol. Biochem. Biophys. Res. Commun. 106, 435-441.

90. Ramgopal, M., and K. Bloch. 1983. Sterol synergism in yeast. Proc. Natl. Acad. Sci. 80, 712-715.