AN ABSTRACT OF THE THESIS OF

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Leo W. Parks

Sterols of the yeast Saccharomyces cerevisiae are found in membranes with a free C-3,β hydroxyl group or stored in lipid droplets as esters of long-chain fatty acids. We have examined the distribution of cholesterol into free sterol and steryl ester pools of a sterol auxotrophic strain of Saccharomyces cerevisiae. Varying the amount of cholesterol available to cells has shown that steryl esters are accumulated as a small proportion of total sterol until a free sterol content of about 1 μg per milligram dry weight cells is attained. At cellular concentrations of free sterol above this threshold amount, the steryl ester pool receives a greater proportion of the total sterol incorporation, and the free sterol receives less. Since free sterol and steryl esters are known to be interconvertible within the cell, the occurence of a threshold of esterifiable sterol indicates that the cell attempts to maintain an optimal membrane sterol concentration through the regulation of components involved in this function.

We have also conducted a study into a possible mechanism of

sterol uptake control. Wild type yeast do not accumulate exogenous sterols under aerobic conditions, and a mutant allele conferring sterol auxotrophy (erg7), could only be isolated in strains with a heme deficiency. δ-Aminolevulinic acid (ALA), fed to a hem1 (ALA synthetase$^-$) erg7 (2,3-oxidosqualene cyclase$^-$), sterol auxotrophic yeast strain inhibits sterol uptake, and growth is negatively effected when intracellular sterol is depleted. The inhibition of sterol uptake (and growth of sterol auxotrophs) by ALA is dependent on the ability to synthesize heme from ALA. A procedure was developed which allows selection of strains which will take up exogenous sterols, but have no apparent defect in heme or ergosterol biosynthesis. One of these sterol uptake control mutants possesses an allele which allows phenotypic expression of sterol auxotrophy in a heme competent background.

Sterol Assimilation by *Saccharomyces cerevisiae*

by

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CONTRIBUTIONS OF AUTHORS

Russell Rodriguez performed the experiment of Fig. 1.2.

Fred Taylor made the original observation of the inhibitory effect of ALA upon strain FY3, and suggested the possibility of heme biosynthesis having a negative effect upon sterol uptake.

ACKNOWLEDGEMENTS

I would like to acknowledge the people whose help has made it possible for me to pursue this degree. My parents, Robert and Phyllis Lewis, have been a great source of encouragement and love. I hope that whatever I may achieve in my life can measure up as a fitting tribute to those wonderful people. My sisters, Mary and Elaine, have also provided alot of greatly appreciated moral support and companionship throughout the years. I have also been able to count on my "brothers", Mark Severson, Larry Duhamell, and Mike Weisser to provide some good times or at least some form of excitement during the past fifteen or so years.

The friends and colleagues I have met at Oregon State University will enrich my life a great deal more than any degree could. Among those people, Rusty Rodriguez and Cindy Bottema took the time to look after me in my early years in the lab. Chris Low and R. P. Griffiths have done so more recently with their generous donations of technical expertise that have saved me alot of mental anguish. Craig Banner, Kevin Marchitto, Debbie Schons, Paul Bellatty, Bruce Bolding, Bob Gilmore, Greg Pierce, and Ralph Tripp, among others, have provided company in my extracurricular activities which provided relief from the long hours in the lab.

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INTRODUCTION

Sterols are lipids found in significant amounts in the membranes of most eukaryotes. In the yeast, Saccharomyces cerevisiae, sterols are found in free hydroxyl and fatty acyl ester forms. This organism has many advantages for genetic and physiological studies, such as a well characterized genome with many mutant types available, a heterothallic mating system allowing meiotic recombination and segregation of alleles during sporulation, isolation of mutants from a haploid genome, and simple culture techniques. Being a eukaryotic organism, it posesses many of the basic characteristics of higher organisms and could provide useful information applicable to systems which lack the ease of study found in yeast.

A sterol requirement has been demonstrated for the growth of S. cerevisiae under anaerobic conditions (1)[1] and by strains possessing certain genetic defects (in heme biosynthesis or certain steps of the sterol biosynthetic pathway). Using a sterol auxotrophic strain, at least two functions for sterols have been defined in S. cerevisiae. These have been differentiated by structurally specific requirements for the initiation of growth and a less specific requirement for sterols fulfilling a "bulk" function (8)[2].

Sterol metabolism by S. cerevisiae has been studied using wild type as well as sterol auxotrophic strains. Sterol esterification

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and the hydrolysis of steryl esters display specificity for particular sterol structures (14)[1]. This specificity leads to the prevalence of ergosterol, the natural end product of sterol biosynthesis in yeast, in the free (hydroxyl) fraction. Sterol is readily interconverted between free and ester forms with the greatest accumulations of esters occurring under circumstances of reduced growth rate. Wild type S. cerevisiae has been found to accumulate little or no exogenous sterol when provided in an aerobic culture medium (17)[1] while sterol auxotrophic strains take up many sterols with different apparent K_m's of uptake (13)[2]. Together with the finding of relatively constant cellular free sterol contents (14)[1], these observations allow one to realize that the yeast cell precisely regulates its membrane sterol composition both quantitatively and qualitatively.

The text which follows describes experiments intended to show the relationship between free sterol content and sterol esterification for the purpose of defining functional quantities of sterol, and to examine the priority with which sterol pools are utilized. Data are also presented which lend insight to the means by which sterol uptake is controlled by S. cerevisiae.

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[2] literature cited, page 22

CHAPTER 1

THE FREE CHOLESTEROL-DEPENDENCE OF NET STERYL ESTER SYNTHESIS BY STEROL AUXOTROPHIC *SACCHAROMYCES CEREVISIAE*

Thomas A. Lewis, Russell J. Rodriguez, and Leo W. Parks

Abstract: Sterols of the yeast Saccharomyces cerevisiae are found in membranes with a free C-3,β hydroxyl group or stored in lipid droplets as esters of long-chain fatty acids. We have examined the distribution of cholesterol into free sterol and steryl ester pools of a sterol auxotrophic strain of Saccharomyces cerevisiae. Varying the amount of cholesterol available to cells has shown that steryl esters are accumulated as a small proportion of total sterol until a free sterol content of about 1 μg per milligram dry weight cells is attained. At cellular concentrations of free sterol above this threshold amount, the steryl ester pool receives a greater proportion of the total sterol incorporation, and the free sterol receives less. Since free sterol and steryl esters are known to be interconvertible within the cell, the occurence of a threshold of esterifiable sterol suggests that the cell attempts to maintain an optimal membrane sterol concentration through the regulation of components involved in sterol esterification and hydrolysis.

INTRODUCTION

Sterols are required for the normal growth of nearly all eukaryotic organisms. Alhough they exist intracellularly in the free hydroxyl form and as esters of long-chain fatty acids, their most important functions in the yeast, Saccharomyces cerevisiae seem to be served by the free form, which is primarily found in cellular membranes. The relative importance of free sterol in S. cerevisiae is evidenced by observations of steryl ester hydrolysis in response to sterol limitation (1,6,14), the occurrence of steryl esters in lipid droplets (11), and by cultures of yeast which grow normally with little or no steryl esters (5,13). These observations have indicated that steryl esters represent a storage pool of sterol which may be hydrolysed when cellular demand for free sterol is highest, and augmented when free sterol demand is reduced. In this manner, the yeast cell's system of sterol esterification and steryl ester hydrolysis appears to function in maintaining membrane sterol content within an optimal concentration range for growth. Mammalian cells also show an apparent free sterol maintainence. This is illustrated by the hydrolysis of steryl esters during periods of net free sterol excretion, and esterification of free cholesterol during periods of excess cholesterol availability (4,9). From this, it seems plausible that cells are able to respond to free sterol levels with appropriate sterol metabolic activities. Control of membrane lipid composition in response to sterol has been suggested in other work with S. cerevisiae (3,7).

If the major role of steryl esters is to serve as a reserve

source of free sterol, it would not be physiologically sound for the cell to allow net esterification to occur when free sterol is present in less than optimal amounts. This implies that there is a minimal amount of cellular free sterol which the cell requires for physiological function and which the esterification system does not act upon. Yeast cells with no restrictions upon sterol biosynthesis maintain free sterol concentrations within a narrow range (14). Because of this characteristic free sterol homeostasis, wild type, aerobically-grown yeast are not amenable to studies designed to produce an insufficient membrane sterol content. To reveal if there is a free sterol concentration range which is not readily esterified and a range which is readily accessible for esterification due to adequate sterol content such manipulations were desired.

The existence of a concentration threshold would be of particular interest from the aspect of membrane sterol regulation. Since biochemical parameters often represent important physiological boundaries, this membrane sterol concentration threshold may define the concentration of sterol which fulfills a particular functional role in membranes. The concentration of sterol found in cells whose growth has stopped due to a limiting supply of sterol would define the lower limit of this functional quantity, this amount being insufficient to allow further increase in cell mass. Here we describe experiments meant to define these functional quantities of sterol in a sterol auxotrophic strain of S. cerevisiae observe the process of quantitative membrane lipid regulation.

The natural sterol of yeast is ergosterol, which differs from

cholesterol by its degree of unsaturation and the occurrence of a methyl group on side-chain carbon number 24. Cholesterol can satisfy the growth requirements of yeast sterol auxotrophs and is readily esterified (13). We have chosen to use cholesterol to examine sterol incorporation into sterol auxotrophic strain RD5-R for the additional reasons that it is available in radioisotope form, in high purity, and is much more refractory than ergosterol to oxidative degradation during the growth of the organisms. By manipulating cellular sterol concentrations through changes in the exogenous cholesterol supply, we were able to observe the distribution of sterol into free and steryl ester pools. The range of cellular sterol contents could be defined physiologically as growth-limiting, minimally satisfying, or excess on the basis of cell yields.

MATERIALS AND METHODS

Yeast strains and growth conditions. S. cerevisiae strain RD5-R (hem1 erg3 erg7) was used throughout this work and has been described previously (8). It is defective in heme biosynthesis due to a mutation in the gene for δ-aminolevulinic acid synthase, and in sterol biosynthesis due to mutations in genes for 2,3-oxidosqualene cyclase and C5,6 desaturase.

The medium used was 0.67% yeast nitrogen base (Difco), 2% dextrose, 1% casamino acids, 0.01% methionine, 0.01% uracil, 0.002%adenine sulfate, 50mM potassium succinate, pH 5.5. Sterol was added from a 5 μg/ml stock solution in tyloxapol:95% ethanol, 1:1, (v/v). Unsaturated fatty acids (oleic and palmitoleic, 4:1, v/v) were added from a 10% (v/v) solution in tyloxapol:95% ethanol to a

final concentration of 0.005% (v/v).

All cultures were incubated at 30°C with aeration. In experiments using stationary phase cells, cultures were grown in 250ml side-arm flasks. For sterol titration experiments, pre-growth in limiting quantities of sterol was accomplished in a 1l flask until no growth was observed over an 8 hour interval. Sterol was either added to this 500ml culture and 50ml samples removed at time intervals, or 50ml aliquots were transferred to sterile 250ml side-arm flasks before receiving additional sterol. In experiments in which sterol was quantitated throughout growth, cells were grown in a 1500 ml fermentor (MultiGen, New Brunswick), and samples yielding approximately 50 mg dry weight of cells were removed during growth. Aliquots of culture medium were removed into tubes compatible with the spectrophotometer, and growth monitored with a Klett-Summerson colorimeter equipped with a green filter. RD5-R cell density was found to be proportional to Klett units according to the formula:

log cells per milliliter = (log Klett + 1.86)/0.6486, as determined using a Coulter Counter.

Sterol quantitation. [^{14}C]cholesterol, specific activity approximately 5μCi/mg, was prepared by adding [^{14}C]cholesterol (60 mCi/mmol) to a benzene solution containing a weighed amount of unlabelled cholesterol in a volumetric flask and diluting to volume with benzene. Aliquots of this solution were used for precise specific activity determination before the solution was dried under N_2 and redissolved in Tyloxapol-95% ethanol, 1:1 (vol/vol).

Sterol was extracted from cells after washing twice with a solution of 0.5% Tergitol NP-40, and once with distilled water. These washed cells were then lyophilized and extracted with dimethyl sulfoxide by steaming for 1hr. The DMSO/cell suspension was extracted twice with 5ml of n-hexane after dilution into aqueous 0.9% NaCl:methanol, 4:1 (v/v). The hexane extracts were then dried under N_2 and applied to silica gel thin layer plates. Lipid classes were separated using the solvent system of Skipski and Barclay (12), and visualized with I_2 vapor. Bands migrating with standards of cholesterol and cholesteryl oleate were scraped from plates, put into scintillation vials, and counted in a Beckman LS8000 liquid scintillation counter using toluene, PPO (2,5-diphenyloxazole), POPOP (7,4-bis-(5-phenyloxazolyl) benzene) scintillator. Extraction efficiency was calculated by counting radioactivity remaining in the aqueous phase after hexane extraction. Determinations were performed in triplicate and means reported. Standard error of the means was less than 4% for all reported values.

Gas chromatographic analysis was used to quantitate non-radioactive cholesterol using the same extraction procedure described above, and with ergosterol, and ergosteryl myristate as internal standards. Sterols and steryl esters were eluted from silica gel using chloroform:methanol, 4:1 (v/v). Eluted steryl esters were converted to the free form by alkaline hydrolysis with 6% KOH in methanol (75°C, 2 hrs.). Sterols were quantitated using a Varian aerograph series 2700 gas chromatograph equipped with a Hewlett-Packard 3390A integrator.

Chemicals. [4-^{14}C]cholesterol was from New England Nuclear Corp. Cholesterol, unsaturated fatty acids, Tyloxapol, Tergitol, PPO, POPOP, and nucleotide bases were from Sigma Chemical Corp. Ergosterol was also from Sigma and was recrystallized before use. Ergosteryl myristate was prepared from ergosterol and myristoyl chloride (Eastman Chemical Co.) as described previously (2). Solvents were from Mallinkrodt, Inc. and were redistilled before use.

RESULTS

Growth of RD5-R on different cholesterol concentrations. To demonstrate exogenous cholesterol concentrations which are growth limiting or in excess of cellular growth requirements, a culture of RD5-R was incubated with seven different concentrations of cholesterol covering a 50-fold range. The results are shown in Figure 1.1 and demonstrate that at concentrations of 2 µg/ml or less, the growth yield is less than maximal but the growth rate is not affected by these lower initial concentrations of cholesterol in the medium. This result prompted us to ask how cellular sterol was being distributed by the yeast cells and how much cellular sterol pools fluctuated throughout growth.

Sterol content and distribution in cells throughout growth under conditions of sterol-limitaton and sterol-excess. We sampled cells of RD5-R grown with 1 µg/ml, 5 µg/ml, and 15 µg/ml cholesterol throughout exponenential and stationary phases of their culture cycles and quantitated free and esterified cholesterol. The results

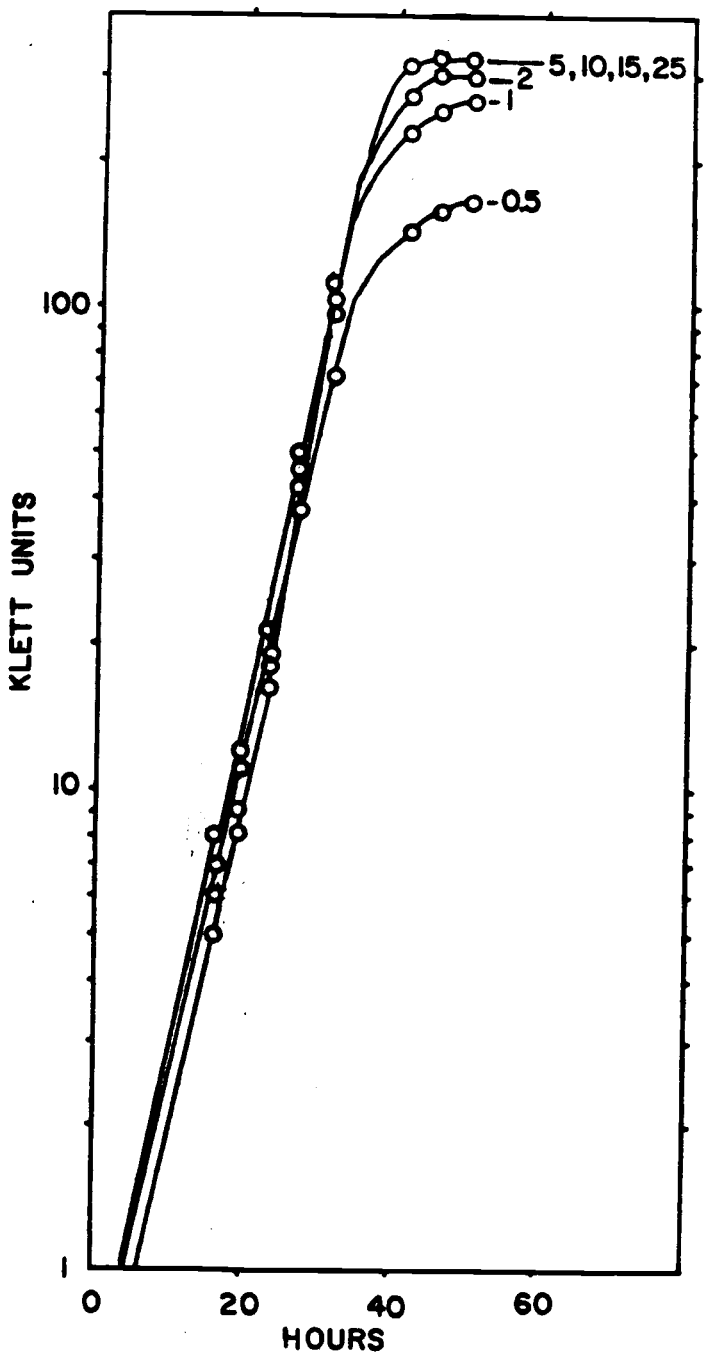

Figure 1.1. Growth of RD5-R on different cholesterol concentrations. Strain RD5-R was innoculated into identical media containing 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 5 µg/ml, 10 µg/ml, 15 µg/ml, and 25 µg/ml cholesterol, as indicated to the right of each curve.

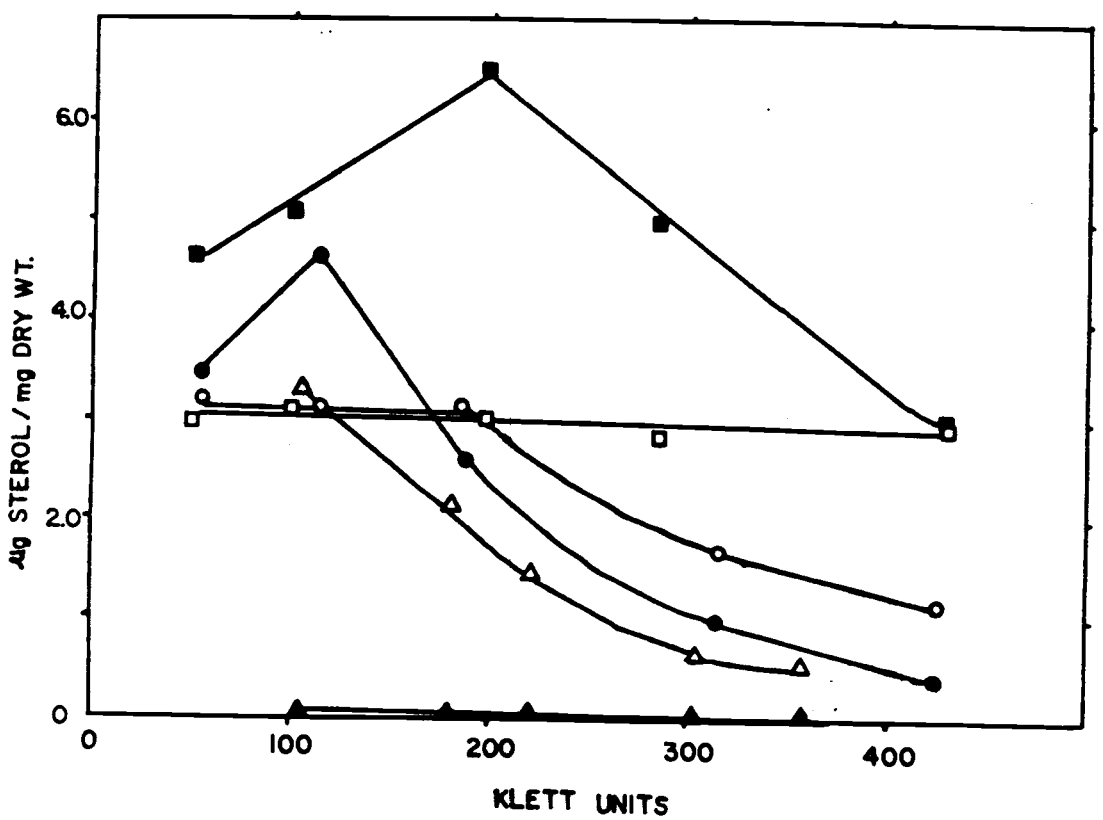

Figure 1.2. Free sterol and steryl ester content throughout growth of RD5-R on different concentrations of cholesterol. Open symbols represent free sterol content (µg/mg dry weight) and filled symbols represent steryl ester content (µg/mg dry weight) for RD5-R grown with △, 1 µg/ml; ○, 5 µg/ml; and □, 15 µg/ml cholesterol.

of these analyses are shown in Figure 1. 2 in which cellular sterol is plotted as a function of cell density. From these data some interesting points emerge. The greatest accumulation of sterol was reached relatively early in the culture cycle (for reference see Fig. 1.2), and the partitioning of the remaining sterol favored the free fraction since the free sterol content of the cells declined only after the steryl ester fraction had already shown a decrease or was very small.

Quantitations based on cell mass do not directly tell if steryl ester was hydrolysed or merely diluted by additional growth. By calculating the amount of steryl esters per ml of culture, net hydrolysis or esterification can be ascertained. The µg steryl ester per ml of the 15-µg/ml culture did not decrease over the period observed whereas the 5- and 1-µg/ml cultures did show net decreases (data not shown). These points seem to illustrate some characteristic of the sterol uptake function in strain RD5-R which causes the cells to accumulate excess sterol early in growth and merely maintain free sterol in the late stages, if enough exogenous sterol is available. The observations most relevant to our study of the control of sterol interconversion reveal that free sterol concentration is maintained where possible but steryl esters are diluted or hydrolysed by stationary phase. Sterol quantitations performed on stationary phase RD5-R cells grown with comparable amounts of exogenous sterol then must represent levels remaining after the net hydrolysis, or dilution of steryl esters.

Specific incorporation of sterol in stationary phase cells.

The amounts of sterol remaining in the cultures of Figure 1.2 seemed to bear out the premise that the free sterol content of the cell dictates whether or not steryl esters are synthesized or hydrolysed. Furthermore, it supports our model wherin a critical free sterol content exists which the cell attempts to maintain through the hydrolysis of steryl esters. One means by which we chose to observe this prioritization of sterol distribution was to quantitate free sterol and steryl esters in cells grown to stationary phase with limiting to excess cholesterol concentrations. By plotting these specific quantities as a function of the total sterol content we can determine if there is an observable change in the way sterol is incorporated over the range of cellular sterol contents. The results of this experiment are shown in Figure 1.3. The specific incorporations into free sterol and steryl esters both showed two slopes over the range tested. The inflection points of both plots occur at a total incorporation of about 1 µg per mg dry weight.

The data of Figure 1.2 made us aware that RD5-R grown with 15 µg/ml cholesterol or less undergoes a decrease in cellular sterol content as stationary phase is approached. The point at which the slopes of free sterol incorporation and sterol ester incorporation change as a function of total sterol content seems to demonstrate a change in the cell's ability to maintain its free sterol content.

The data of Figure 1.3 were obtained in order to observe the way sterol utilization changes throughout the course of sterol depletion. These were obtained from separate cultures having access to different amounts of cholesterol and should represent different

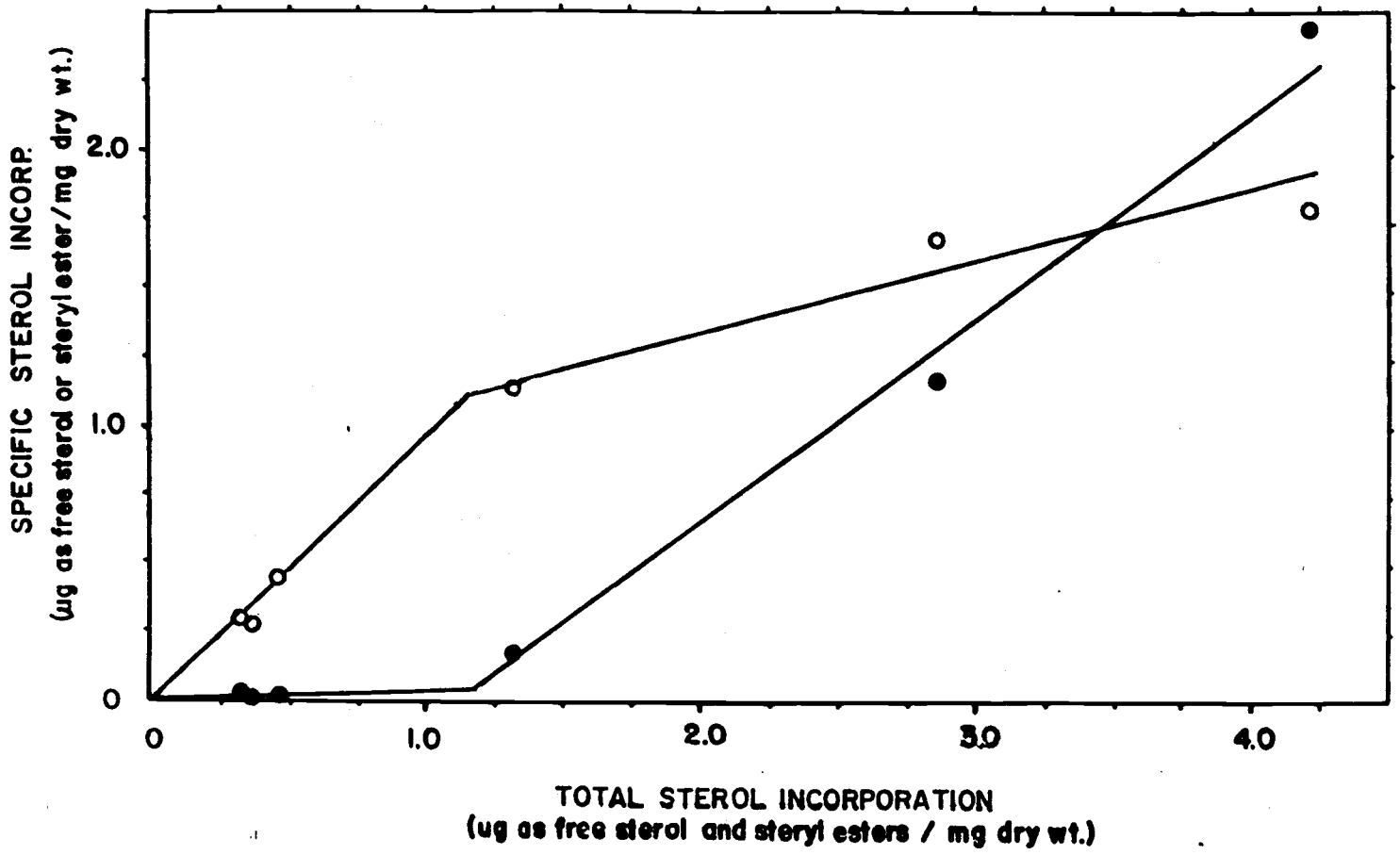

Figure 1.3. Specific incorporation of sterol in stationary phase RD5-R grown with different concentrations of cholesterol. Strain RD5-R was grown to stationary phase with various concentrations of ^{14}C-cholesterol (0.49, 0.96, 1.66, 4.46, 9.38, 14.09, and 22.4 µg/ml) and µg free cholesterol in free and ester fractions determined after extraction and separation, using liquid scintillation counting. Open circles represent free sterol contents, and filled circles represent steryl ester contents.

stages of sterol depletion interrupted by the cessation of growth. The data of figure 1. 2 were obtained by sampling cultures as they underwent decreases in cellular sterol content. When these are plotted in the manner of Figure 1.3, there is good agreement between the data with the exception that the 1-µg/ml culture never incorporated sterol into steryl esters even though accumulations exceeded 1 µg/mg dry weight. This discrepancy may indicate that the factors governing sterol distribution are subject to culture cycle variation.

Titration of cellular free sterol. Since the data of Figure 1.3 had apparently demonstrated the ability of the yeast cell to hydrolyse steryl esters to supply free sterol through the course of sterol depletion, we then examined whether the same changes in sterol specific incorporation would occur in cells undergoing the reverse process; that is, whether free sterol incorporation would have priority over steryl ester synthesis until a critical cholesterol content is attained by cells undergoing a net increase in sterol content. To observe if this were the case, cells which had been maximally depleted of sterol could be examined, quantitating free sterol and steryl esters after the addition of more sterol, and effectively titrating the critical free sterol level.

It has been observed that if cells have been grown continuously with an ample supply of sterol (20 µg per milliter) signifigant incorporations of exogenous sterol only occurred in cultures which were actively growing (10). It has subsequently been found that prior sterol depletion can allow sterol uptake in the

absence of growth[1]. This makes the titration experiment possible by merely adding sterol to sterol-depleted cells and either following incorporation over a time course, or quantitating sterol in several batches of these cells after equal exposure to different amounts of sterol. Both experiments were performed in which no growth of the cultures was observed. The results of both experiments are plotted in Figure 1.4 and show that the resulting patterns of sterol incorporation are identical. The fact that the data agree so well indicates that cells at equilibrium with external sterol do represent intervals in the normal progression of sterol distribution by cells undergoing net sterol increases. This figure also shows the slope changes evident in Figure 1.3, but a much more accurate determination of the inflection points is possible with the greater number of data points in this vicinity. Again, the inflection points coincided at a total sterol content near 0.95 µg per milligram dry weight. Before this critical sterol content is reached, sterol is incorporated almost entirely into the free fraction. Above this point, steryl ester accumulation exceeds free sterol accumulation. The agreement between data of Figures 1.3 and 1.4 show that the yeast cell's sterol interconversion system obeys the same equilibria whether the cell is experiencing a net decrease in sterol content or a net increase.

It is interesting to note that total incorporations below the inflection point represent 44% of the sterol in the medium, and total incorporations after this point represent 51-56%. This could reflect the effect of the increased amount of steryl esters upon cellular

[1] R. T. Lorenz, manuscript in preparation

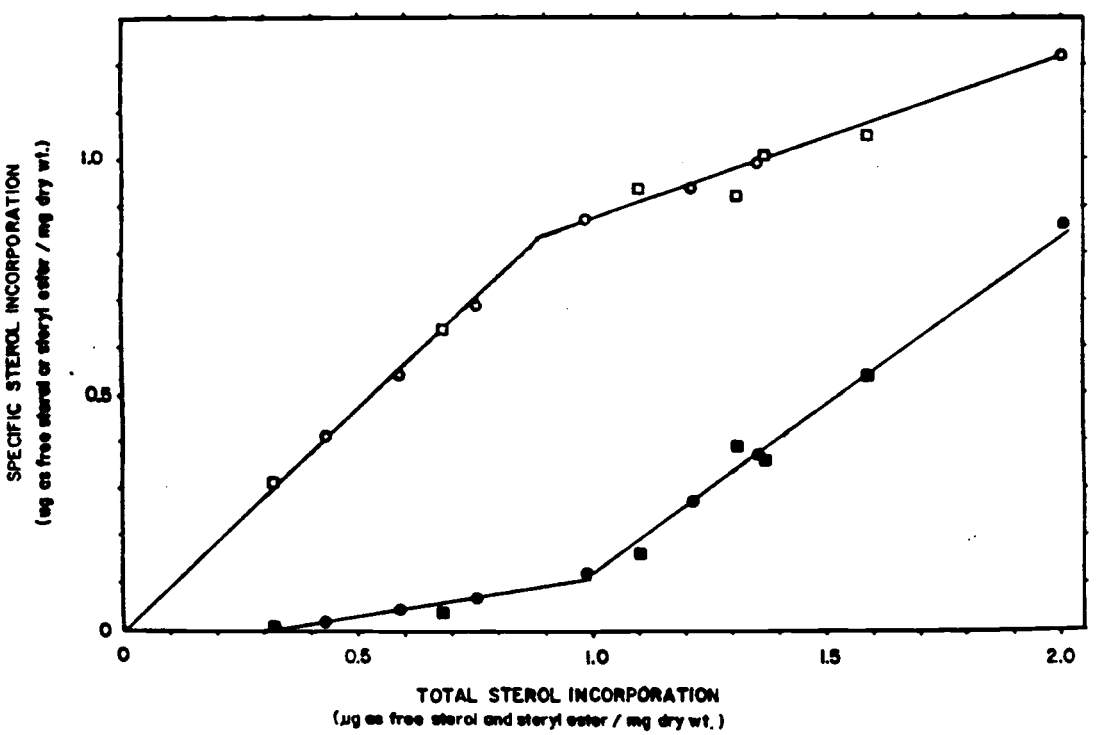

Figure 1.4. Titration of cellular free sterol by addition of sterol to sterol-depleted RD5-R. Strain RD5-R was grown to stationary phase on 1 µg/ml ^{14}C-cholesterol. These cells were then either washed and collected (those that gave the lowest points on graph) or received additional ^{14}C-cholesterol. The open symbols represent µg cholesterol in free sterol and filled symbols represent cholesterol in steryl ester. Circles were samples obtained from cells given different amounts of cholesterol (0.25, 0.5, 1, 1.5, 2, 3, and 6 µg/ml) and collected after an equivalent amount of time. Squares represent data obtained from cells collected after different lengths of time (0.75 to 18 hours) after addition of cholesterol (3.5 µg/ml).

sterol content which may otherwise be limited by an equilibrium between free sterol of the membrane and that in the medium.

DISCUSSION

The control over the sterol esterification/hydrolysis cycle in our system (sterol auxotrophic S. cerevisiae) must depend upon characteristics of cellular membranes, as well as the components of the steryl ester synthesizing and hydrolysing systems. The data given here show the net changes in cellular sterol pools over an easily obtainable range of total sterol contents. These data have aided in defining 3 functional levels of cellular free sterol content in S. cerevisiae. One constitutes the minimal amount of free sterol which can be obtained by outgrowth under circumstances of sterol limitation, and is associated with little or no steryl ester content. Another level of sterol defines a range of greater sterol concentration which is poorly esterified and may contribute important characteristics to the membranes. A final, but even higher concentration range is maximally esterified, indicating that its importance as a structural component of cellular membranes is not great.

One hypothesis to describe free sterol concentration functionalities is that sterol of the free fraction is relatively unavailable to esterification below a threshold level of the esterification system. This would regard all free sterol as being distributed homogeneously in the membrane. In this description, the minimal free sterol concentration represents a level of sterol which

cannot impart the necessary properties upon the membrane to allow further growth. An alternative view is that this concentration represents the number of distinct sites in the membrane which have high affinity for sterol, and perhaps a sterol requirement for physiological function. The affinity of these sites for sterol relative to the steryl ester synthesisizing apparatus would account for the existence of a fraction of the free sterol pool which is unavailable for ester synthesis. Perhaps what we have observed in sterol incorporation experiments is the titration of such sites. These models of functional quantities of free sterol can be tested in experiments which measure the exchangeability of sterol in the putative high affinity sites by labelling to distinguish it from superfluous amounts added as a chase.

The models described do not mention possible regulatory mechanisms for sterol metabolic enzymes such as induction/repression, post-translational modification and/or allosteric activation. The mechanism(s) which controls sterol metabolism and its responsiveness to sterol concentration is being investigated. The models and the experiments presented here were intended to explain how the yeast cell maintains its free sterol content within the narrow range found with wild type strains. Our results using a sterol-auxotrophic strain and an "animal sterol", do show a level of control over free sterol content but not as precise as found in the more natural system. Other factors which are probably also involved in the control of membrane sterol content but not studied here would be sterol synthetic control, physical properties of sterols, and culture

cycle phenomenon. Elucidation of the contributions of each of these factors should also provide a better understanding of the regulation of lipids in cellular membranes.

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CHAPTER 2

INVOLVEMENT OF HEME BIOSYNTHESIS IN THE CONTROL OF STEROL UPTAKE BY SACCHAROMYCES CEREVESIAE

Thomas A. Lewis, Fred R. Taylor, and Leo W. Parks

ABSTRACT: Wild type yeast do not accumulate exogenous sterols under aerobic conditions, and a mutant allele conferring sterol auxotrophy (erg7), could only be isolated in strains with a heme deficiency. δ-Aminolevulinic acid (ALA), fed to a hem1 (ALA synthetase$^-$) erg7 (2,3-oxidosqualene cyclase$^-$), sterol auxotrophic yeast strain inhibits sterol uptake, and growth is negatively effected when intracellular sterol is depleted. The inhibition of sterol uptake (and growth of sterol auxotrophs) by ALA is dependent on the ability to synthesize heme from ALA. A procedure was developed which allows selection of strains which will take up exogenous sterols, but have no apparent defect in heme or ergosterol biosynthesis. One of these sterol uptake control mutants possesses an allele which allows phenotypic expression of sterol auxotrophy in a heme competent background.

INTRODUCTION

Biosynthetic controls in microorganisms generally maximize the utilization of exogenously available nutrients over those endogenously synthesized. This spares the organism the metabolic expense of forming those compounds. An anomalous situation appears to exist in the yeast Saccharomyces cerevisiae with regard to the twenty-eight carbon membrane lipid, ergosterol. Under aerobic conditions, wild-type yeast strains synthesize large amounts of ergosterol and do not incorporate significant amounts of exogenous sterol (17). Under anaerobic conditions, sterol cannot be produced by the organism and exogenously supplied sterol is utilized by the cells (1). The physiological advantage of this system is not apparent, because it would seem more efficient to curtail endogenous synthesis of sterol when it is available in the medium rather than to prevent its transport and require synthesis to occur.

Anaerobic conditions have been used to induce a sterol requirement in order to study the structural features of sterols needed for growth of this organism (8). Because of the difficulty of manipulating the anaerobic system, attempts have been made to develop an aerobic alternative for probing structure-function relationships in sterols. Auxotrophs which are unable to synthesize sterols offer a means of restricting sterol synthesis; however, the isolation of mutants which are tightly and unconditionally blocked in sterol synthesis and which are otherwise prototrophic has not been reported previously. An apparent requirement for a concomittant genetic defect in heme synthesis along with sterol auxotrophy has been

observed by Gollub et. al. (4) and in our laboratory (unpublished data). Attempts to segregate recombinant, 2,3-oxidosqualene cyclase mutants from the hem (heme biosynthesis-defective) backgrounds in which they were originally isolated, have been unsuccessful. The hem mutations somehow allow for the viability of these sterol auxotrophic strains.

The first sterol auxotroph used in our laboratory, FY1, was derived from one of the first sterol-requiring mutants isolated, Ole3, shown to lack δ-aminolevulinic acid (ALA) synthetase activity (19); this locus is now referred to as hem1. The hem1 mutant requires unsaturated fatty acid, methionine, and sterol in the heme-deficient condition, but may also be supplemented by ALA alone to relieve these requirements. Strain FY1 posesses the hem1 allele and grows poorly on cholesterol as its sterol supplement. A rapid growing variant, FY3, was selected from this genetic background and was found to have acquired an additional mutation in ergosterol biosynthesis at the point of cyclization of 2,3-oxidosqualene (15).

We present here our studies on the control of sterol uptake by S. cerevisiae and the effects elicited by restoring the potential for heme synthesis by heme mutants. These studies were performed by using nutritional supplementation to manipulate the potential for heme synthesis by FY3 and other heme mutants. In addition, a genetic approach was taken by using a technique which allows the identification of strains that accumulate increased amounts of exogenous sterol.

MATERIALS AND METHODS

Yeast Strains. The yeast strains used in this study are listed in Table 1.

Media and Growth Conditions. The standard growth medium used was adjusted to pH 5.5 and consisted of 0.67% Yeast Nitrogen Base (Difco Laboratories), 0.05M succinic acid, 2% glucose, and the following at 0.02%: methionine, uracil, histidine, tyrosine, tryptophan, phenylalanine, leucine, lysine, and adenine sulfate. Sterol was added at a concentration of 20 µg/ml from a stock solution of 4 mg/ml in Tergitol Nonidet P-40-95% ethanol (1:1,vol/vol). Unsaturated fatty acid supplementation was either as 1% Tween 80 or as a mixture of oleic and palmitoleic acids (4:1,vol/vol) at 0.01% final concentration added from a 10% (vol/vol) solution in Tergitol-ethanol. ALA was supplemented to a final concentration of 0.005% (300µM), from a 1% stock solution in 95% ethanol. Protoporphyrin IX and hematin were added to a final concentration of 10 µg/ml from 1% (wt/vol) solutions in 0.01N KOH in 50% ethanol.

For depletion of sterol, cells of strain FY3 were grown to maximal cell density in medium containing cholestanol (5α-cholestan-3β-ol) in place of sterol supplement, as described by Rodriguez et. al.(12).

Cultures were grown aerobically with shaking at 30°C. Growth was measured using a Klett-Summerson photoelectric colorimeter equipped with a green filter. Klett units were found to be

TABLE 1. Descriptions of yeast strains used.

Strain	genotype/phenotype	source or reference
X2180-1A	a SUC2 *mal* *gal2* CUP1	Y.G.S.C.*
X2180-1B	α SUC2 *mal* *gal2* CUP1	Y.G.S.C.
FY1	α *hem1* *met* *ura*	16
FY3	α *hem1* *met* *ura* *erg7*	16
G204	α *hem1* *his4*	18
G207	α *hem10* *his4*	18
G210	α *hem11* *his4*	18
G214	α *hem15* *his4*	18
G216	α *hem13* *his4*	18
G121	a *hem12* *ura2-33*	18
GL7	a *hem3* *erg12*	T.M. Buttke and 4
TL-Upc27	a SUC2 *mal* *gal2* *CUP1* UpcI	this study
TY27-1	a *erg7* UpcI	this study

* Y.G.S.C., Yeast Genetic Stock Center, Berkeley, CA.

proportional to FY3 cell density according to the formula: log cells per milliliter = (log Klett + 1.86)/0.6486 in the range of 5 to 500 Klett, as verified by direct cell enumeration with a Coulter Counter.

Measurement of sterol accumulation throughout culture cycle. Cells were grown in the media described above with sterol and Tween 80. [4-^{14}C]cholesterol was present at an activity of 0.01 μCi/ml. This corresponds to a specific activity of approximately 0.18 mCi/mmol. Cell samples were collected from the growing cultures by removing 5- or 10-ml portions and centrifuging at 500g for 10 minutes. These cell pellets were washed twice with 10mM phosphate buffer (pH 7.0) containing 0.5% Tergitol Nonidet P-40 and again with buffer alone. A suspension of wild-type, un-labelled carrier cells (5ml) was added to samples of low cell densities. The washed cell pellets were frozen, lyophilized, and extracted with chloroform, after dimethylsulfoxide treatment, as described previously (16). Free sterol and steryl esters were resolved by thin layer chromatography (TLC) on silica gel plates using the solvent system of Skipski et al. (14). Bands were visualized with I_2 vapor, and those migrating with standards of cholesterol and cholesteryl palmitate were scraped into scintillation vials and counted in a Beckman LS 8000 scintillation counter using toluene/POP (2,5-diphenyloxazole)/POPOP (7,4-bis-(5-phenyloxazolyl)benzene) scintillator. The amount of sterol taken up per cell was measured as cpm/(milliliter of sample X Klett units).

Assay of Sterol Uptake. The ability of strains of yeast to take up sterol under aerobic conditions was measured by growing cells

to stationary phase in medium with added cholesterol. The cells were harvested and washed as described above, and the sterols extracted following acid labilization and saponification (5). Sterols were purified by TLC as described above, and bands scraped from plates and eluted with chloroform-methanol (4:1, vol/vol). This purified extract was then dried under N_2 and dissolved in diethyl ether before injection into a Varian 2700 gas chromatograph. Chromatograms were compared with those obtained from extracts of the same cells grown without added sterol, to ensure that endogenously produced sterols which may coelute with cholesterol did not give false-positive results.

Isolation of sterol uptake control mutants. A culture of wild type S. cerevisiae strain X2180-1A was mutagenized to approximately 10% survival with ethyl methanesulfonate by the procedure of Lindegren et al. (6). Dilutions of mutagen-exposed cells were spread onto the standard medium described above, without sterol, and solidified with 17g of agar per liter. Plates with 100-500 colonies were replica inoculated by adhering the colonies onto nitrocellulose membrane discs (Millipore Corp., type HA, 9-cm diameter) and placing the filters, with the colonies facing up, onto standard media with 0.02 μCi of [^{14}C]cholesterol (0.39 mCi/mmol) per ml. The original master plate was stored under refrigeration. After three days incubation at 30°C, the filters were removed from plates and alternately blotted three times on filter paper saturated with 0.5% Tergitol Nonidet P-40 solution, then dry filter paper, to remove nonincorporated radioactivity. After the filter was wetted again,

the colonies were transferred to paper filters (9.0cm, Whatman no. 1) by pressing the nitrocellulose and paper discs together. These were then placed in a 60°C oven until the colonies had dried thoroughly. The brittle nitrocellulose filter was removed, leaving the dried colonies adhered tothe paper. The filter discs were then autoradiographed by exposing them to Kodak XAR-5 X-ray film for three days at room temperature before they were developed. Colonies showing the greatest radioactivity were then picked from the original plate and screened for sterol uptake as described above, as well as for growth on minimal media and non-fermentable energy source (glycerol).

Determination of sterol intermediates accumulated. Strains were inoculated into standard media containing 0.2μCi of [1-^{14}C]acetate (specific activity, 55 mCi/mmol) per ml and 0.5% glucose plus the respective nutritional requirements and were grown to stationary phase. Non-saponifiable lipids were extracted and applied to silica gel thin-layer plates adjacent to pure standards of cholesterol, lanosterol, farnesol, and squalene. These samples were chromatographed using the solvent system cyclohexane-ethyl acetate (9:1, vol/vol). Bands were visualized by I_2 vapor. The distribution of radioactivity on plates was determined using a Packard model 7201 scanner.

Chemicals. Sterols, amino acids, hematin, ALA, and nucleotide bases were from Sigma Chemical Co. Protoporphyrin IX was from Calbiochem-Behring. Solvents were from Mallinkrodt, Inc. and were redistilled before use. Highly purified cholestanol was the gift of

Henry Kircher. [4-^{14}C]cholesterol (specific activity, 59.4 mCi/mmol) was from New England Nuclear Corp. [1-^{14}C]acetate (specific activity, 55 mCi/mmol) was from ICN Pharmaceuticals, Inc. Silica gel plates (F254, 0.25-mm thickness) were from E. Merck AG.

RESULTS

Growth response of hem1, and hem1 erg7 strains to ALA. The effect of ALA on the growth of strains FY1 (hem1) and FY3 (hem1 erg7) is shown in Fig. 2.1 and 2.2. The hem1 parent strain, FY1, showed growth similar to wild type yeast (data not shown) when ALA was added, whereas growth was poor with cholesterol plus Tween 80. No growth occurred without fatty acid or heme supplementation. The erg7 derivative, FY3, grows well on cholesterol plus tween 80 but gave a pattern of brief exponential growth followed by a period of transient growth inhibition when ALA was included in the same medium (Fig. 2.2). This type of growth pattern was also seen when ergosterol was used as sterol supplement (data not shown). When ALA was added to an exponentially growing culture of FY3, no inhibition of growth was seen (data not shown).

Three to five generations of growth with ALA supplementation were required to observe growth inhibition with strain FY3. Because strain FY1 did not require 3 generations with ALA supplementation to attain its optimal growth rate, it did not seem likely that three generations were required for ALA to be adequately metabolized by hem1 mutants. Instead, we reasoned that a cellular component

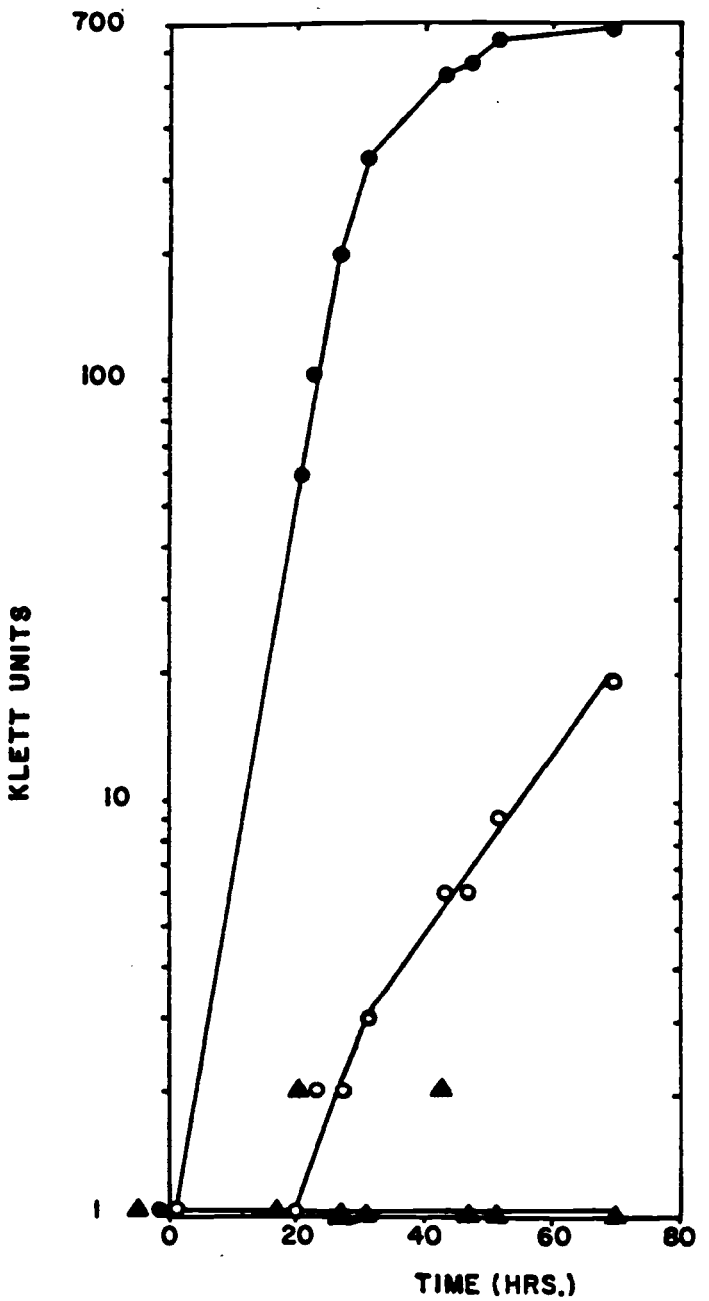

Figure 2.1. Growth of strain FY1. Symbols: ▲, medium containing 20µg/ml cholesterol and no unsaturated fatty acid or heme supplement; O, medium containing cholesterol plus 1% tween 80; ●, medium containing 300µM ALA.

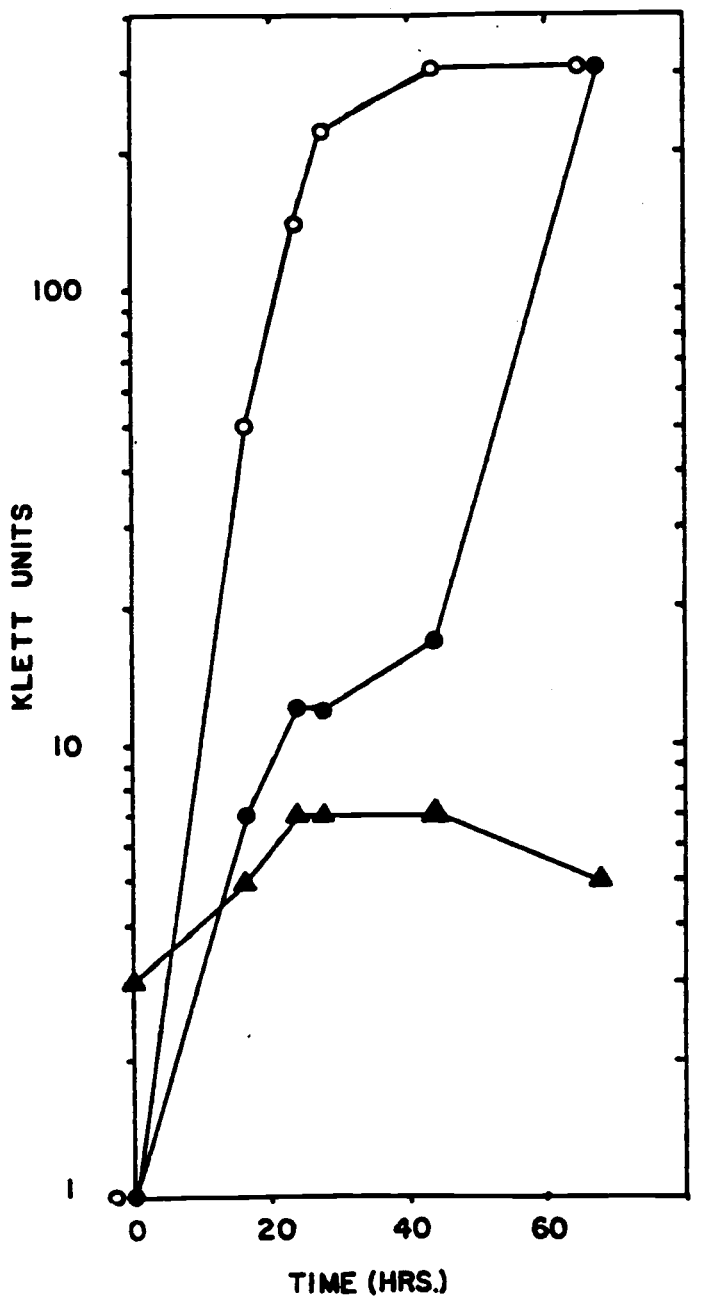

Figure 2.2. Growth of strain FY3. Symbols: ○ , medium with 20μg/ml cholesterol and 1% tween 80; ● , same medium plus 300μM ALA; ▲ , same as in the legend to Fig. 1.

essential for growth was being depleted in FY3 during the first three generations of growth with ALA. Since sterol is the only nutritional requirement of FY3 not shared by FY1 when these strains were grown with ALA, the possibility that sterol became growth limiting for FY3 during ALA supplementation was investigated.

Effect of ALA on sterol-depleted cells. The addition of ALA to FY3 during exponential phase had no apparent inhibitory effect, possibly because the sterol assimilated before ALA addition was enough to prevent dilution of sterol to levels below the limits of growth in the number of generations of the culture cycle which remained. By using cells which had already exhausted their supply of sterol, our explanation for the requisite growth prior to inhibition could be tested by observing if growth inhibition occurred earlier in these cells. If sterol depletion did affect the amount of growth which occurred before inhibition, other possible explanations for growth inhibition, such as another nutrient becoming growth limiting, or the accumulation of an inhibitory product after growth with ALA, would seem unlikely.

Depletion of endogenous sterol was accomplished by precycling FY3 cells on cholestanol, which cannot support the continued growth of sterol auxotrophs (12) but which allows growth to the point at which a minimum cellular sterol content is reached. Cholestanol-cycled FY3 showed no growth 100 hours after inoculation into ALA-containing medium (data not shown). Thus, depletion of sterol had the effect of preventing growth of FY3 in the presence of ALA, suggesting that ALA acts, as expected, by interfering with

sterol uptake by FY3.

Effect of ALA on sterol uptake by FY3. The ability of FY3 to take up exogenous sterol in the presence of ALA was examined more directly by growing cells in medium containing [^{14}C]cholesterol and monitoring accumulation of radioactivity into free sterol and steryl ester pools throughout growth. Both cellular sterol pools were examined because the steryl ester fraction has been found to fluctuate as a storage pool (2, 15) and could indicate the availability of sterol in the yeast cells. In the presence of ALA, growth retardation had an attendant decrease in free sterol per cell (Fig. 2.3). This figure also shows that the control culture completed the expected cycle of steryl ester hydrolysis and sterol esterification seen in wild-type cultures (15) but that the ALA-grown culture did not replenish the ester pool (Fig. 2.3C). It can also be seen that endogenous stores of sterol, (steryl esters), become maximally depleted at the time growth inhibition becomes apparent.

The effect of ALA on sterol uptake was also measured when ALA was added to exponential-phase cells (fig. 2.4). The growth of the culture receiving ALA paralleled the growth of the control in this experiment; however, the amount of free sterol per cell in ALA-exposed cells decreased continually from the point of ALA addition until growth stopped, while the amount of free sterol in control cells remained relatively constant. The amount of radioactive sterol appearing in steryl esters increased steadily as the stationary phase was approached by the control culture, but the ALA-supplemented culture again showed no increase in this fraction.

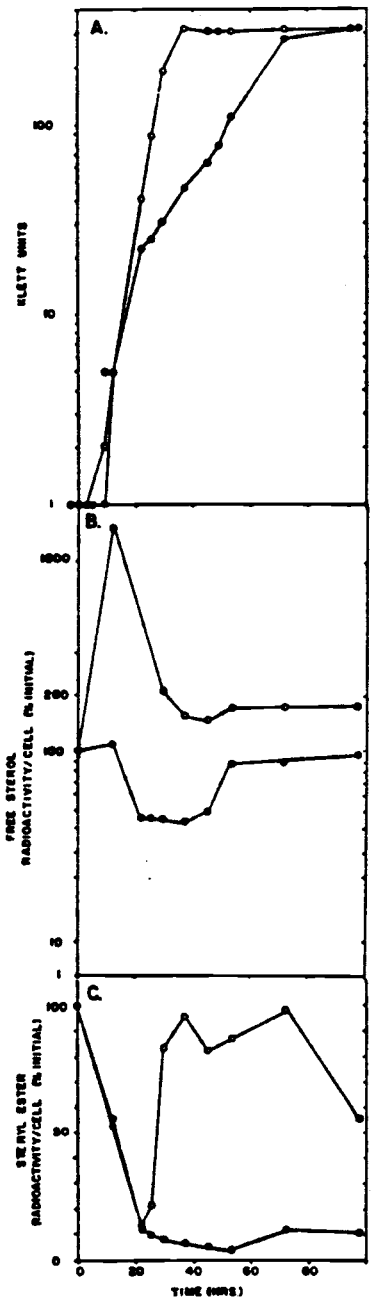

Figure 2.3. Effect of ALA supplementation on sterol accumulation by strain FY3. Inocula and initial samples were identical and from a culture grown to stationary phase on the same [^{14}C]cholesterol-containing medium used in this experiment. Cell samples were collected throughout growth, and lipids extracted, separated by TLC, and quantitated by liquid scintillation counting. Media and symbols are the same as those described in the legend to Fig. 2.2, except that sterol included radiolabel. (A.) Growth; (B.) relative amounts of free sterol; (C.) relative amounts of steryl esters, expressed as counts per minute/(milliliter of sample X Klett units).

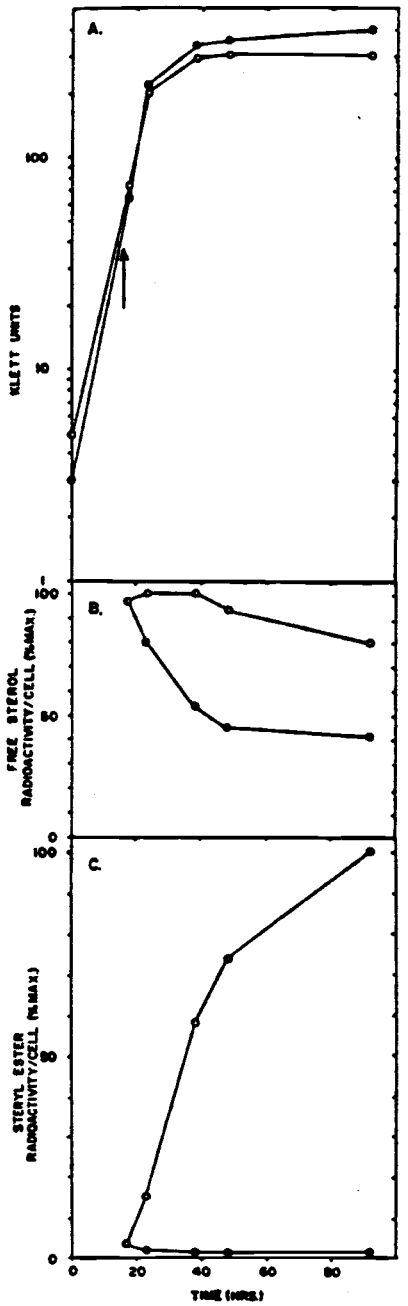

Figure 2.4. Effect of ALA addition during exponential growth on sterol accumulation by strain FY3. Growth conditions and symbols are the same as those described in the legend to Fig. 2.3, except that cells used as innocula in this experiment were not pre-labelled with [^{14}C]cholesterol. The arrow indicates the point of ALA addition. (A.) Growth; (B.) relative amounts of free sterol; (C.) relative amounts of steryl esters, expressed as counts per minute/(milliliter of sample X Klett units).
units).

Potential for hemoprotein synthesis and sterol uptake. The metabolic occurrence of ALA is unique to the heme biosynthetic pathway; therefore, it seemed most likely that heme or hemoprotein was responsible for the effects incurred during ALA supplementation. Before discarding the possibility that complete metabolism of ALA to heme was not necessary to manifest the effects of ALA upon sterol uptake, we decided to examine the effect of ALA on other heme mutants. Six mutants, each blocked in one of the eight steps of heme biosynthesis (Fig. 2.5), were grown with and without ALA supplementation. The uptake of cholesterol was measured under these circumstances in order to determine whether ALA itself or another subsequent precursor was responsible for the inhibition of sterol uptake. These mutants should either have their mutant phenotype suppressed (in the hem1 strain), or accumulate different porphyrin intermediates upon metabolism of ALA (18). It should be noted that only one of these strains (G121) required sterol, suggesting that the other mutations are leaky to some extent, but all were negative for catalase and growth on glycerol. The intracellular heme levels were also found to be below limits of detection (18). The results of examination of sterol uptake by nine heme mutants with various heme supplements are listed in Table 2. Cholesterol uptake was seen in all of these strains without ALA, and in all strains except the hem1 strain when ALA was present in the growth medium. ALA also had no effect on the uptake of sterol or growth of strain GL7 (hem3 [urogen I synthase$^-$] erg12 [oxidosqualene cyclase$^-$]), an independently isolated sterol auxotroph. Based on these data it seems unlikely

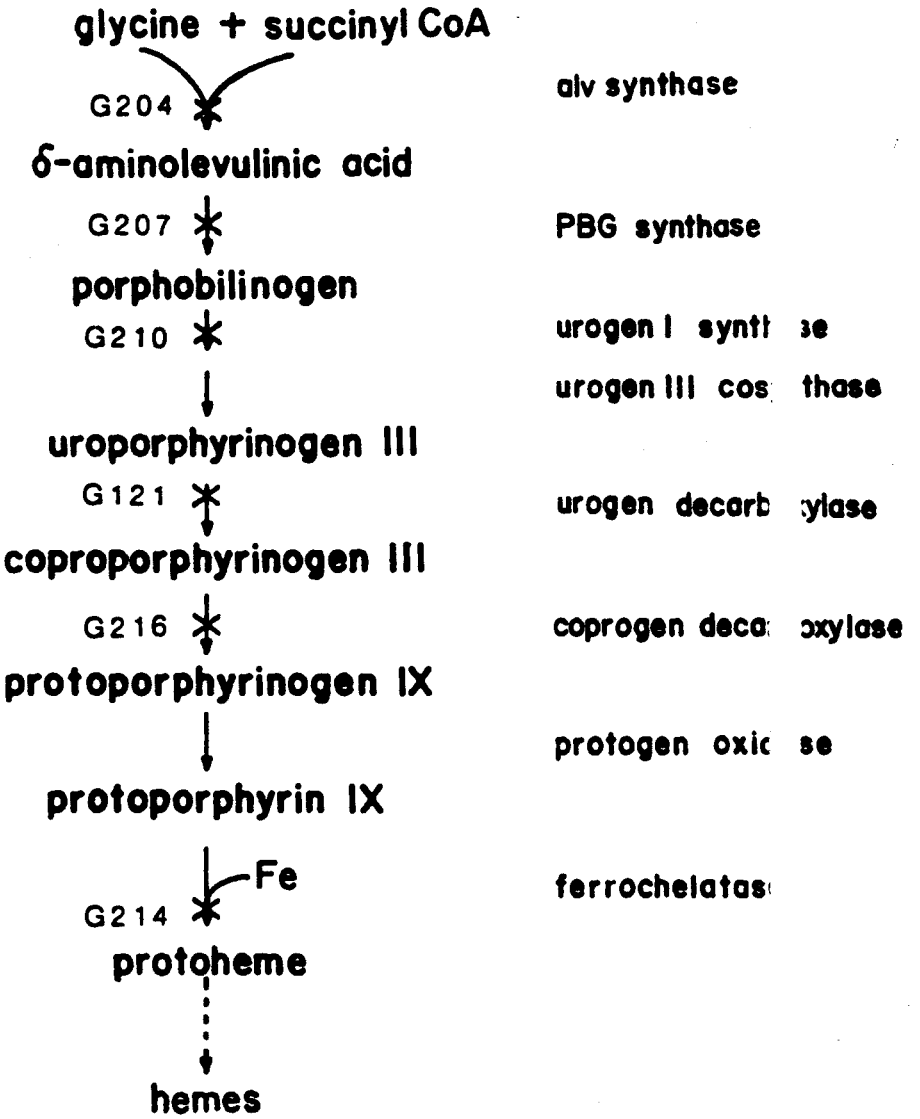

Figure 2.5. Heme biosynthetic pathway of S. c revisiae. Heme biosynthetic intermediates are listed on the left, n the order of the reaction sequence of heme biosynthesis indicatec by arrows. The enzymes catalyzing the reactions involved are liste on the right, next to their respective steps. The strain designat: ns, G204, G207, G210, G121, G216, and G214 are inserted in the igure at points corresponding to the biosynthetic defects in these st ains (indicated by crosses over the arrows of the reaction sequence).

Table 2. Effect of ALA and porphyrin heme supplements on heme mutants.

	Effect of supplements on:								
	Respiration[a]			Sterol Uptake[b]			Growth Inhibition[c]		
Strain	ALA	PPIX	hematin	ALA	PPIX	hematin	ALA	PPIX	hematin
G204	+	-	-	-	+	+	-	-	-
G207	-	-	-	+	+	+	-	-	-
G210	-	-	-	+	+	+	-	-	-
G121	-	-	-	+	+	+	-	-	-
G214	-	-	-	+	+	+	-	-	-
G216	-	-	-	+	+	+	-	-	-
FY1	+	-	-	-[d]	+[d]	+[d]	-	-	-
FY3	-	-	-	I[d]	N.D.	N.D.	+	-	-
GL7	-	-	-	+[d]	N.D.	N.D.	-	-	-

[a] Assayed by growth on glycerol as energy source. All strains listed are - with supplement.

[b] Assayed by gas chromatographic analysis using the wild type as negative control and cholesterol as exogenous sterol. All strains + with no supplement.

[c] Assayed turbidometrically. -, No noticeable difference in growth curves

[d] Assayed by [^{14}C]cholesterol incorporation. I, inhibited relative to control culture.

[e] ND, Not determined.

that ALA itself or another intermediate of the heme biosynthetic pathway is causing the inhibition of sterol uptake.

We also observed the effect of the porphyrins, PPIX and hematin, on sterol uptake by the heme mutants and sterol auxotrophs (Table 2). These porphyrins were less effective heme supplements than ALA because they did not restore respiratory competence (ability to grow on glycerol as energy source) or ergosterol synthesis to FY1 cells. PPIX was able to alleviate the unsaturated fatty acid requirements of FY1 and FY3 cells and allowed synthesis of sterols other than ergosterol, presumably precursors, by FY1 (data not shown). These porphyrins also did not prevent sterol uptake by the heme mutants G204, G207, G210, G121, G214, or G216. A limited effectiveness of porphyrin heme supplements, compared with ALA, was also observed previously by others (3). This evidence demonstrates that the efficiency with which a supplement affects cytochrome dependent metabolism (i.e. respiration or ergosterol biosynthesis) is related to its ability to inhibit sterol uptake.

Other evidence for the idea that cytochrome-restoring levels of heme synthesis must occur to prevent sterol uptake and the growth of sterol auxotrophs comes from the isolation of a strain, derived from FY3, which was not inhibited by ALA. This strain, PFY3A, was obtained from a culture of FY3 which had reached stationary phase in ALA-containing medium. PFY3A was identified by a greater accumulation of sterol in the presence of ALA, relative to other colonies of the population. This resistant strain had aquired the additional characteristic of an inability to grow on media devoid of

unsaturated fatty acid but supplemented with a low, sub-inhibitory concentration of ALA. FY3 will grow on this type of medium. PFY3A resembled FY3 in that it did not require unsaturated fatty acid when PPIX was included in the growth medium. Since unsaturated fatty acid is required by FY3 due to the lack of heme synthesis, and therefore cytochromes, this evidence indicated that an additional defect between the steps of ALA permeation and iron insertion into protoporphyrin IX had been aquired by PFY3A. The ability to incorporate ALA and a resulting accumulation of porphyrins by PFY3A was indicated by the pink coloration of PFY3A colonies grown with ALA and their fluorescence under long wavelength, UV illumination (Fig. 2.6). The phenotype of PFY3A provides an additional correlation between the ability to synthesize heme with the negative effect upon sterol uptake.

Genetic studies on sterol uptake control. The fact that sterol uptake occured when sterol was not required, (in the presence of a leaky heme mutation or under semi-anaerobic conditions [7]), and was inhibited when heme synthesis but not sterol synthesis could occur (FY3 with ALA) indicated that a system of sterol uptake control exists which responds to heme synthesis. On this basis, we assumed that it should be possible to obtain mutants which are uncoupled in this response and would take up sterol concurrent with heme and ergosterol synthesis. By using the autoradiographic technique described above, we screened for the uptake of sterol colonies from a mutagenized culture of wild type yeast cells which had grown on media containing [^{14}C]cholesterol. Mutants were found which would take up

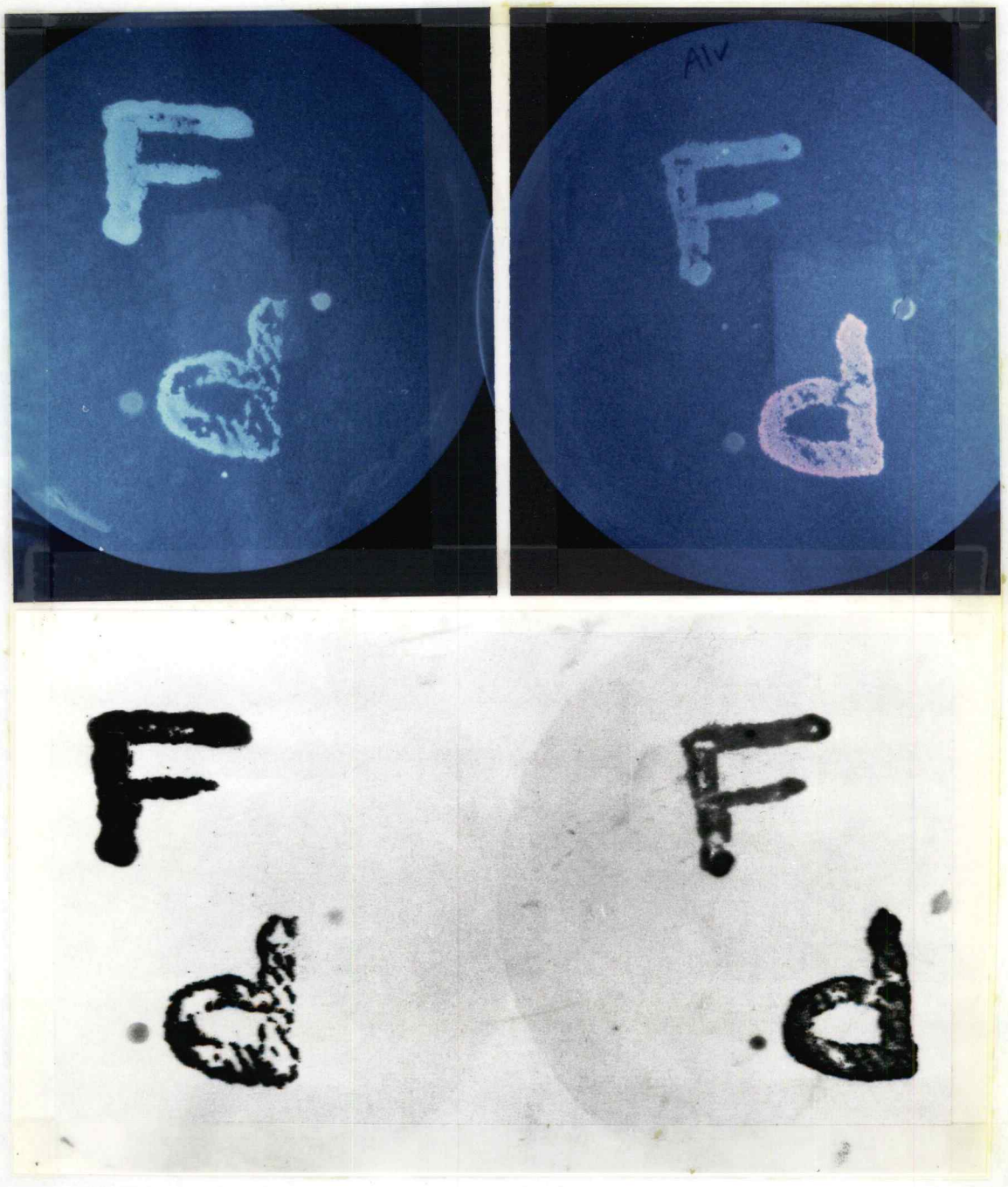

Figure 2.6. ALA-dependent fluorescence and ALA-insensitive sterol accumulation by strain PFY3A. Strains PFY3A and FY3 were replica inoculated from one original plate, onto media containing [^{14}C]cholesterol,(left), and [^{14}C]cholesterol plus ALA (right), on separate nitrocellulose membranes. These cells were then transferred to paper discs and photographed under long wavelength UV illumination (upper half of figure), and autoradiographed by direct exposure to X-ray film (lower half of figure). The "F" cells are FY3, and the "P" cells are PFY3A.

exogenously supplied cholesterol, whereas the unmutagenized parent strain would not. The strains were then screened for the ability to grow on minimal medium and on media containing glycerol as sole energy source. The strain TL-Upc27 is one strain obtained in this manner, and one whose phenotype was examined in more detail to determine if other distinguishing traits accompanied its ability to accumulate exogenous sterol.

Defects in sterol biosynthesis can be revealed by chromatographic analysis of non-saponifiable lipids (9). Since no requirement for sterol was found in TL-Upc27, any defect in sterol biosynthesis would necessarily have been either late in the ergosterol biosynthetic pathway or leaky and occurring in an early step. Strain TL-Upc27 accumulated radioactivity from [1-^{14}C]acetate into non-saponifiable lipids which migrated with the same R_f as those of the parent, wild-type strain in a TLC system which separated sterol precursors (Fig. 2.7). This was an indication that defects in the early portion of the sterol biosynthetic pathway were not present in strain TL-Upc27. Gas chromatographic analysis also revealed no differences in the non-saponifiables from strain TL-Upc27 and those of the wild-type strain (data not shown), whereas mutants defective in late steps of the ergosterol biosynthetic pathway are readily detected in this manner.

These data indicate that TL-Upc27 differed from the wild type in its ability to accumulate exogenous sterol or inability to exclude it. Because our data indicate that heme biosynthesis is involved in this process, and since crosses between wild type and FY3 yielded

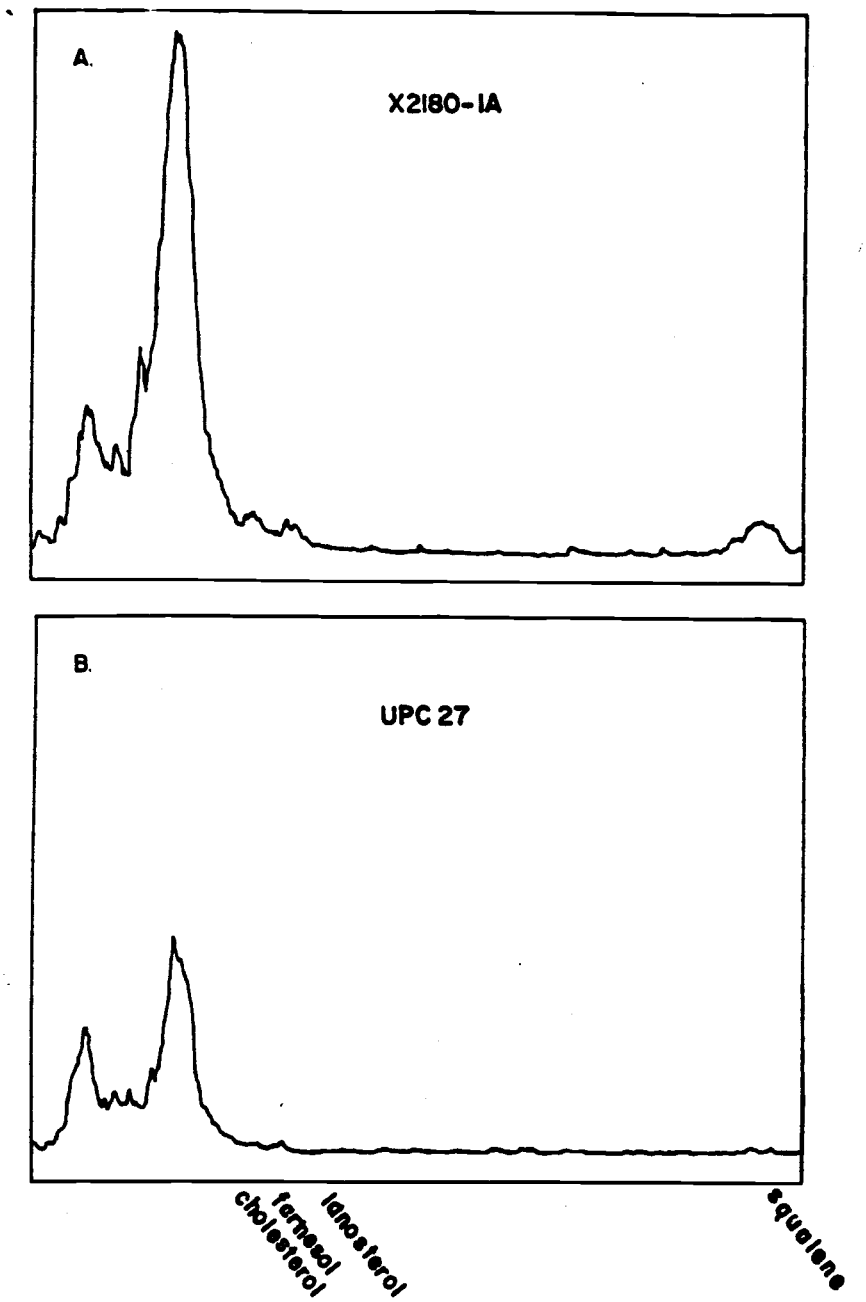

Figure 2.7. Sterol intermediates accumulated by strain TL-Upc27 and wild type parent, X2180-1A. Radiochromatogram scans of non-saponifiable lipids from (A.) strain X2180-1A and (B.) strain TL-Upc27 grown in media containing ^{14}C-acetate (as described in the text). Direction of solvent migration is from left to right.

viable erg7 spores only in a hem1 background, we presumed that a defective sterol uptake control mechanism, such as that of strain TL-Upc27, may allow the viability of erg7 spores in a HEM1 genetic background. This prediction was tested by screening for HEM1 erg7 strains among haploids produced by sporulation of a diploid heterozygous for hem1, erg7, and upc1 (sterol uptake control) alleles. This diploid was obtained by mating strains TL-Upc27 and FY3. Screening was carried out by testing viable haploid meiotic progeny for their ability to grow without lipid supplements, with ALA in place of lipid supplements, with ergosterol as the only lipid supplement, or with ergosterol and unsaturated fatty acids. Segregants were obtained which required only sterol supplementation for growth.

Strain TY27-1 is one of the strains found in this analysis to show the Hem+ Erg phenotype. Strain TY27-1 was found to incorporate [^{14}C]acetate into nonsaponifiable lipids with the same R_f in TLC as those lipids obtained from FY3 grown in the presence of ALA (Fig. 2.8). This was expected of a HEM1 erg7 strain since ALA suppresses the effect of the hem1 mutation of FY3 but not the erg7 defect.

Because this phenotype was never obtained from a cross between FY3 and a wild-type strain, we assumed that the upc1 allele of strain TL-Upc27 was present in TY27-1 and permitted its viability, as the hem1 allele had allowed the viability of FY3. To confirm this, TY27-1 was crossed with the wild-type strain, X2180-1B, and tetrad analysis was performed by testing the lipid requirements and sterol uptake capacity of haploid segregants (table 3). This analysis

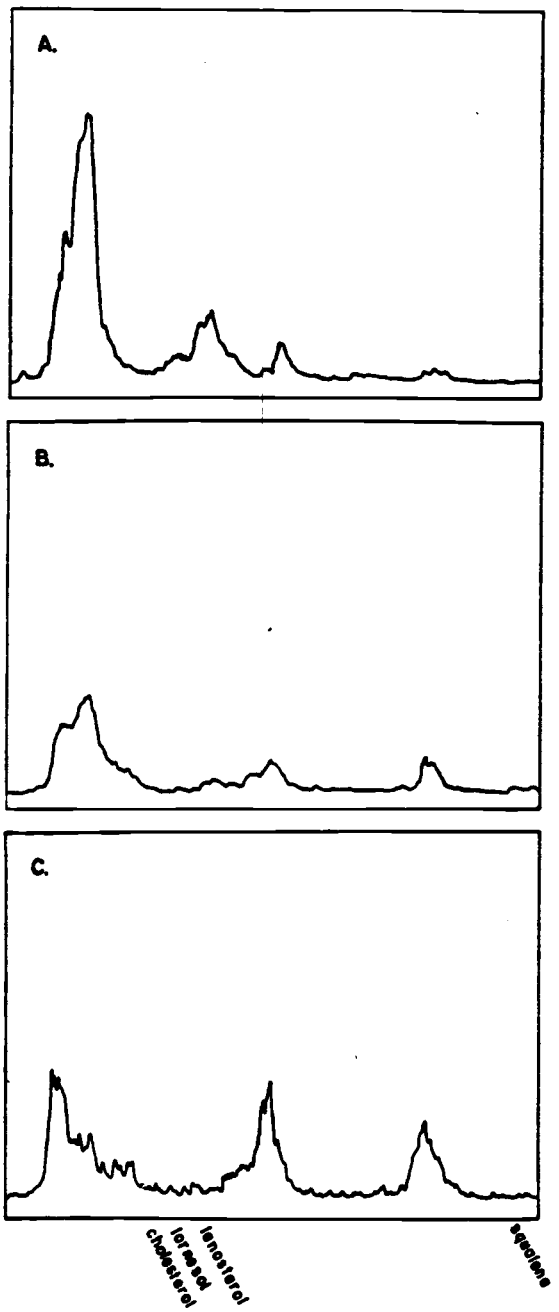

Figure 2.8. Sterol intermediates accumulated by strains FY3 and TY27-1. Conditions were the same as in Fig. 2.7 except sterol and unsaturated fatty acids were included in the growth medium. (A.) strain FY3, (B.) strain FY3 plus ALA, (C.) strain TY27-1.

Table 3. Tetrad analysis of TY27 X X2180-1B cross.

Tetrad	Spore	Growth on Test Media[b] MIN	ALA	ERG	ERG,UFA's	Sterol Uptake	Phenotype
	a	+	+	+	+	-	wt[c]
1	b	+	+	+	+	+	Upc
	c						NV[d]
	d						NV
	a						NV
2	b	+	+	+	+	+	Upc
	c	+	+	+	+	-	wt
	d						NV
	a						NV
3	b	+	+	+	+	+	Upc
	c	+	+	+	+	-	wt
	d	-	-	+	+	+	Erg
	a						NV
4	b	-	-	+	+	+	Erg
	c	+	+	+	+	-	wt
	d	+	+	+	+	-	wt
	a						NV
5	b						NV
	c	+	+	+	+	+	Upc
	d	+	+	+	+	-	wt
	a						NV
6	b						NV
	c	+	+	+	+	+	Upc
	d	+	+	+	+	-	wt
	a						NV
7	b	+	+	+	+	+	Upc
	c	-	-	+	+	+	Erg
	d	+	+	+	+	-	wt
	a						NV
8	b						NV
	c	+	+	+	+	+	Upc
	d	+	+	+	+	+	Upc
	a						NV
9	b	-	-	+	+	+	Erg
	c	+	+	+	+	-	wt
	d	+	+	+	+	+	Upc
	a						NV
10	b						NV
	c	+	+	+	+	-	wt
	d	+	+	+	+	+	Upc

[a] Segregants were replica plated onto test media from a master grid and scored after 3 days. Sterol uptake was assessed by autoradiography of master grid filter replate (see the text). Parental strains were used as standards for comparison.

[b] Media: MIN, yeast nitrogen base Casamino Acids media (see the text with no lipid supplements; ALA, MIN plus ALA; ERG, MIN plus 20 µg/ml ergosterol; ERG,UFA's, MIN plus 20 µg/ml ergosterol and 50 µg/ml unsaturated fatty acids.

[c] wt, Wild type.

[d] NV, spore was not viable.

showed that only strains requiring sterol as the sole lipid supplement, or strains without any lipid requirement were obtained, indicating that only the erg7 auxotrophic marker of FY3 was inherited by TY27-1. It was also found that 10 of the 20 strains with no lipid requirement displayed the Upc phenotype, thereby showing that the proposed upc1 allele was present in TY27-1, segregated independently of the erg7 allele, and in a 2:2 ratio.

Although there was generally poor viability of the Erg spores, (only 4 of 10 expected upc1 erg7 spores), the data are consistent with the hypothesis that the upc1 allele must be present for viability of Erg spores. Tetrads 3,7,8, and 9 are the best examples of this in which both of the expected upc1 alleles would be addounted for, and where recombination occurred to produce one or two Upc Erg^+ spores, and corresponding numbers of the predicted erg7 spores were not viable.

DISCUSSION

The uptake of sterol by Saccharomyces cerevisiae occurs under conditions which are known to limit sterol synthesis, such as anaerobic conditions and defects in the ergosterol biosynthetic pathway (1,8,13,17). Why sterols are not taken up under other circumstances in order to spare the metabolic expense of synthesizing them is not clear. If exogenous sterol only enters the cells when synthesis is precluded, it would seem possible that there is no active control of sterol exclusion by yeast at all, but rather that

sterol is excluded merely by physical displacement by the sterol synthesized endogenously. This would mean that endogenous sterol has an advantage in entering the membranes and that sterol uptake is dependent upon the level of endogenous production. Our experiments, presented here, with a mutant strain incapable of synthesizing its sterol, indicate that sterol may be excluded by a mechanism independent of sterol synthesis but dependent upon heme synthesis. This information seems to obscure any physiological advantage of the mechanism which excludes exogenous sterol, because it suggests that control is even less directly related to the availability of sterol in the cell.

A model of sterol uptake control involving heme is consistent with some of the observations of sterol uptake and sterol auxotrophy in S. cerevisiae. Under anaerobic conditions, where sterol uptake must occur to permit growth, heme synthesis would be precluded by the oxygen requirements of coproporphyrinogen decarboxylation (10), and protoporphyrinogen oxidation (11). A heme-dependent system of control over sterol uptake might also be expected to limit the possible genetic backgrounds from which sterol auxotrophs can be isolated. As was mentioned above, tight, unconditional sterol auxotrophic mutants have previously only been isolated in hem backgrounds. There is good evidence, however, that enzymes of the early steps of ergosterol biosynthesis (i.e. prior to squalene synthesis), are vital to the cell for supplying metabolic intermediates used in systems other than sterol synthesis, thereby making a complete loss of these functions lethal to the cell (13).

Because relatively little is known about the mechanism of sterol uptake by yeast cells, data on sterol uptake control is still rather inconclusive. Most evidence suggests that control, or at least sterol exclusion, may be due to a displacement effect exerted by sterol already in the membrane. The discovery of mutants with no apparent defect in sterol biosynthesis or heme biosynthesis, but which will take up sterol under circumstances in which wild type strains do not, as well as the further characterization of the sterol uptake process, may provide a means of determining what processes are involved in relating heme synthesis, sterol synthesis, and sterol uptake. These studies are currently in progress.

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CONCLUSION

Our observations on sterol assimilation by S. cerevisiae reinforce ideas that sterol uptake control and the esterification and hydrolysis system function to regulate cellular sterol composition. The exact mechanisms by which these processes operate is still not known. The implications of our study of cholesterol esterification are that sterol content is regulated by activities of ester synthase and hydrolase. At this point, it is still not clear whether the esterification of sterol actively regulates membrane free sterol content or whether it acts opportunistically on its substrate and other properties built into the membrane regulate how much sterol will be included. The dispensibility of steryl ester synthase activity is an interesting idea. Mutants in this function might be readily obtained with the proper isolation technique. Specific inhibitors of steryl ester synthesis should also be without deleterious effect on yeast cells. Alternatively, cells with excess ester synthase should be relatively unimpaired. A genetic approach, through the search for such mutant phenotypes, emerges as a worthwhile approach to this problem. By improving our understanding of how sterol metabolism normally occurs, logical strategies for the isolation of steryl ester synthase and steryl ester hydrolase mutants should become evident.

The sterol uptake control mutants and the isolation procedure used to obtain them have provided useful tools in studying the phenomenon of sterol uptake. They stand to tell us whether sterol is

excluded by membrane composition alone or if a cell uptake protein is involved. They may ultimately be designated sterol "exclusion" mutants if the former is correct. This provides for interesting speculation as to how a sterol exclusion-defective phenotype arises. Some intriguing possibilities include a down-regulated sterol synthetic rate, or perhaps a defective system of sterol delivery to the sites of membrane biogenesis. Continuation of the work presented here should allow us to answer some of these questions which are basic to eukaryotic cell physiology.

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