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Metabolic Interconversion of Free Sterols and Steryl Esters in *Saccharomyces cerevisiae*†

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The interconversion of free and esterified sterols was followed radioisotopically with [*U*-^{14}C]acetate and [*methyl*-^{14}C]methionine. In pulse-chase experiments, radioactivity first appeared mainly in unesterified sterols in exponential-phase cells. Within one generation time, the label equilibrated between the free and esterified sterol pools and subsequently accumulated in steryl esters in stationary-phase cells. When the sterol pools were prelabeled by growing cells aerobically to the stationary phase and the cells were diluted into unlabeled medium, the prelabeled steryl esters returned to the free sterol form under several conditions. (i) During aerobic growth, the prelabeled sterols decreased from 80% to 45% esters in the early exponential phase and then returned to 80% esters as the culture reached the stationary phase. (ii) Under anaerobic conditions, the percentage of prelabeled steryl esters declined continuously. When growth stopped, ony 15% of the sterols remained esterified. (iii) In the presence of an inhibitor of sterol biosynthesis, which causes accumulation of a precursor to ergosterol, prelabeled sterols decreased to 40% steryl esters while the precursor was found preferentially in the esterified form. These results indicate that the bulk of the free sterol and steryl ester pools are freely interconvertible, with the steryl esters serving as a supply of free sterols. Furthermore, there is an active cellular control over what types of sterol are found in the free and esterified sterol pools.

A portion of the sterol pool of *Saccharomyces cerevisiae* is esterified to long-chain fatty acids, whereas the remainder is in the free hydroxyl form. Previous studies have shown that the steryl ester content of this organism is small in the expoential phase of growth but increases dramatically as the culture approaches the stationary phase (3). The proportion of total sterols that are esterified also increases during sporulation (13), under certain conditions of starvation (10), and during treatment with hypocholesteremic agents, which inhibit steps in the sterol biosynthetic pathway (7, 22).

Studies on the metabolism of steryl esters in yeast have been limited to those of Nagai et al. (17) and Parks and co-workers (3, 15). They have shown that some of the intermediates to ergosterol, the predominant sterol of yeast, made de novo during adaptation to oxygen are esterified. Both laboratories also presented evidence demonstrating that zymosteryl oleate does not serve as a substrate in the sterol methyl transferase reaction in vitro, implying that esterified precursors would have to be converted to free sterols before ergosterol synthesis could be completed. However, Nagai et al. (17) suggested, based on pulse-chase experiments, that the steryl esters become a separate pool and do not exchange into the general pool of free sterols. In contrast to this, Aries and Kirsop (2) have suggested that the steryl esters are a storage form of sterol which may be converted to free sterols during anaerobic growth, since the lack of oxygen precludes additional sterol biosynthesis.

Also of interest are reports by several groups that the sterol species found in the ester fraction differ somewhat from those in the free fraction (3, 7, 17, 22). Namely, precursors to ergosterol accumulate in the ester fraction, especially when the pathway is blocked by chemical agents. This accumulation may simply be the result of the inefficiency of steryl esters as substrates in the remaining steps of the pathway, as mentioned above, or be due to some cellular control.

The function and metabolism of sterols in *S. cerevisiae* have been studied by a number of different approaches including use of mutants (4, 14, 24), use of chemical agents affecting the biosynthetic pathway (7, 11, 22), and substitution of other sterols for ergosterol under anaerobic conditions (12, 18). Of uncertain consequence in these studies is the role of steryl esters in sterol metabolism. In this paper we present

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evidence that the free and esterified pools of sterols are readily interconverted and that the esterified pool serves as a means of controlling the type of sterol structures found in the free sterol fraction.

MATERIALS AND METHODS

Yeast strains and growth conditions. Most experiments were performed with *S. cerevisiae* 3416b (a haploid, *met ura ade*), although some of the initial experiments were performed with strain 3701b (a haploid, *ura*). Both strains were obtained from the University of Washington stock cultures. Rich broth medium contained 1% tryptone and 0.5% yeast extract, and minimal medium was yeast nitrogen base (Difco Laboratories) supplemented with the following at 50 μg/ml: tyrosine, phenylalanine, leucine, lysine, tryptophan, histidine, uracil, and alanine. Methionine was added at 100 μg/ml. Glucose (2%) was the carbon source. Aerobic cultures were grown on a shaker bath at 30°C. Semianaerobic growth was obtained in tubes completely filled with medium, flushed with argon, capped, sealed to exclude oxygen, and placed in a 30°C incubator without shaking. Growth was monitored turbidimetrically in a Klett-Summerson spectrophotometer at 540 nm and related to cell number by a standard curve.

Radioisotope labeling. Labeling with methionine was accomplished by adding 0.1 μCi of [*methyl*-^{14}C]methionine per ml to Wickerhams medium (final concentration of methionine, 0.01%). Acetate labeling was also in Wickerhams medium with the addition of [*U*-^{14}C]potassium acetate at 0.1 μCi/ml (final concentration, 0.1%). In pulse-chase experiments, the cells were removed from the labeled medium by filtration on membrane filters (Millipore Corp., type HA, 0.45-μm pore size) and washed three times with 10 ml of unlabeled medium.

Extraction of lipids. The sterol and steryl ester fractions were extracted from whole cells by a modification of a procedure previously reported from this laboratory (3). A cell pellet (usually from a 10-ml culture volume) was lyophilized in a tube, and the desiccated cells were powdered with a spatula. Dimethyl sulfoxide (2.5 ml) was added and the tubes were steamed for 1 h. After the tubes cooled, 2.5 ml of chloroform was added, mixed to form a single phase, and left overnight at 4°C. Another 2.5 ml of cholorform was then added and mixed, followed by 2.5 ml of distilled water. After the contents were mixed thoroughly, the tubes were centrifuged, the top phase (water-dimethyl sulfoxide) was drawn off, and the chloroform phase containing total cell lipids was washed twice with 2 M KCl. This procedure was found to be as effective in the extraction of total sterols as the procedures of Bailey and Parks (3) and Sobus and Holmlund (21), the extraction of broken cells by the procedure of Bligh and Dyer (5), and an acid labilization procedure developed in this laboratory for the total extraction of sterol (9).

Separation of lipids. Separation of the free and esterified fractions was obtained by thin-layer chromatography on silica gel plates (Silica Gel 60 F-254, 0.25 mm thick, manufactured by EM Laboratories). In most cases, the solvent system was cyclohexane-ethylacetate (85:15, vol/vol). When [^{14}C]acetate was used as the label, the lipids were separated by the solvent systems of Skipski et al. (20). The sterol and steryl ester bands were visualized in UV light and scraped from the plate and the lipids were eluted with three washes of chloroform-methanol (4:1, vol/vol). Radioactivity was determined by scraping the silica gel directly into scintillation vials and counting in a Beckman LS 8000 liquid scintillation counter. Proof that the label was in steryl esters was obtained by saponifying the sample and rerunning in the same chromatographic system. The radioactivity from the methionine label was found in the free sterol fraction, and the radioactivity from the acetate label was found in the free sterol and free fatty acid fractions.

Determination of sterols. The Lieberman-Burchard assay of sterols was performed by adding 2 ml of a cooled mixture of acetic anhydride and concentrated sulfuric acid (19:1, vol/vol) to 3 ml of a chloroform solution of the sterol or steryl ester sample. After 15 min at room temperature in the dark, the amount of sterol was quantitated at 680 nm in a Bausch and Lomb Spectrometer 20. UV spectroscopy was performed as described previously (11).

Chemicals. Radioactive compounds were from New England Nuclear Corp. 15-Aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (azasterol) was the generous gift of Eli Lilly and Co. Organic solvents were purchased from Mallinkrodt and redistilled before use.

RESULTS

Sterol pools during growth. Three different methods were used to assay the amounts of sterols and steryl esters as a function of growth in strain 3416b (Fig. 1). Incorporation of radioactive methionine (Fig. 1A and B) labeled the sterols in the C28 position and, thus, only indicated sterols past the methyl transferase step in the biosynthetic pathway. Radioactive acetate (Fig. 1C) was used to label all the carbons of the sterol structure (except for C28) as well as the esterified fatty acid. To correct for the additional label in the steryl ester fraction owing to the fatty acid carbons, the counts per minute incorporated was multiplied by 27/43, and this is what is presented in the figures. This procedure assumes that the fatty acids and sterols have the same specific activity, and this appears to be the case during the initial labeling as monitored by saponification of the steryl esters. This assumption may not be true in the pulse-chase experiments, and we are currently examining the metabolism of fatty acids in these strains. The Lieberman-Burchard assay (Fig. 1D) is sensitive to most sterol structures although they differ somewhat in absorption maxima and extinction coefficients. It should be noted that the chromatographic system we used removes the 4-mono- and 4,4-dimethyl sterols, which are about 5% of the total, from the free sterol fraction.

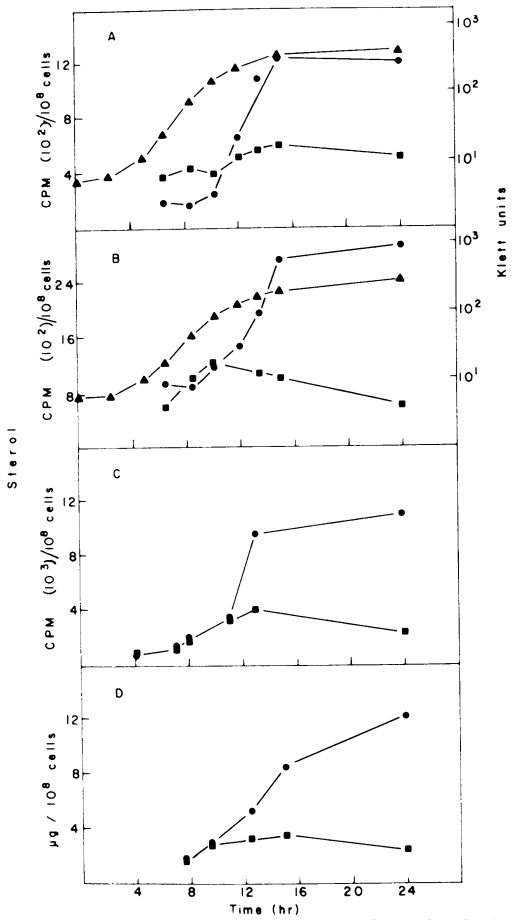

FIG. 1. *Free and esterified sterol pools during growth. For the incorporation of [methyl-^{14}C]methionine (A, B) and [^{14}C]acetate (C), the cultures were grown aerobically in 80 ml of either rich (A) or minimal (B, C) medium in a 250-ml flask, and 10-ml portions were taken as indicated. For the Lieberman-Burchard assay (D), 2 liters of culture was grown in minimal medium, 1 liter per 2-liter flask. Symbols: ■, free sterol; ●, steryl ester; ▲, Klett units.*

The comparison of the analytical methods was made to test the procedures used, especially for the radiolabeling since the strains used do not require acetate for growth. Furthermore, acetate labels many different cellular lipids which must be separated from the sterol fractions. The amount of free sterols relative to esterified sterols was slightly higher in the exponential phase when assayed by the radioactive label in comparison to the colorimetric assay. This was most pronounced when methionine was the label and can be explained by our previous observation that the free sterol fraction is enriched for the methylated species (3). Otherwise the three assays give the same overall view of steryl ester metabolism. All of the experiments we report were repeated one or more times and gave consistent results.

Interconversion of sterols and steryl esters. The interconversion of the free and esterified sterol pools was examined by pulse-chase experiments. In one experiment (Fig. 2), cells were labeled for 30 min with either [^{14}C]methionine or [^{14}C]acetate during the early log phase of growth (generation time, 90 min). Immediately after labeling, the radioactivity from [^{14}C]acetate was found to be distributed in the two pools of sterols in the same ratio as found in the exponential phase of growth in the continuous-label experiment (Fig. 1C). As growth proceeded, there was a redistribution of label into the steryl ester fraction, giving the same ratio of free to esterified sterols as revealed by continuous acetate labeling or the Lieberman-Burchard assay.

The pulse-chase experiment with [^{14}C]methionine was somewhat different. Initially, the label appeared to a greater extent in the free sterol fraction compared to the same point in growth in the continuous-label experiment. This is in keeping with the findings of Nagai et al. (17) and Bailey and Parks (3), who found that only free sterols are substrates for the methyl transferase enzyme. As with the acetate label, the methionine label eventually moved to the ester fraction. After 2 to 4 h of chase and throughout the remainder of the growth cycle, the distribution was identical to the acetate label. In both cases it is clear that free sterols made in the early exponential phase are later esterified when the steryl ester pool rises.

To demonstrate the reverse conversion, that of steryl esters into free sterols, a chase experiment was performed by labeling cells throughout growth and into the stationary phase, resulting in 70 to 80% of the label appearing in the ester fraction. This labeled culture was then used as the inoculum (1:100 dilution) for unlabeled medium (Fig. 3). As growth proceeded, the label in the steryl ester fraction declined, shifting into the free sterol fraction. By the time the culture reached the stationary phase, the label had returned to the steryl ester fraction. The label seemed to equilibrate in the two pools, showing much the same distribution of free and esterified sterol as found in the Lieberman-Burchard assay.

S. cerevisiae cannot synthesize sterols and unsaturated fatty acids under anaerobic conditions (1). When a chase experiment was performed under anaerobic conditions with a prelabeled stationary-phase culture as the inoculum the label shifted from the steryl ester to the free

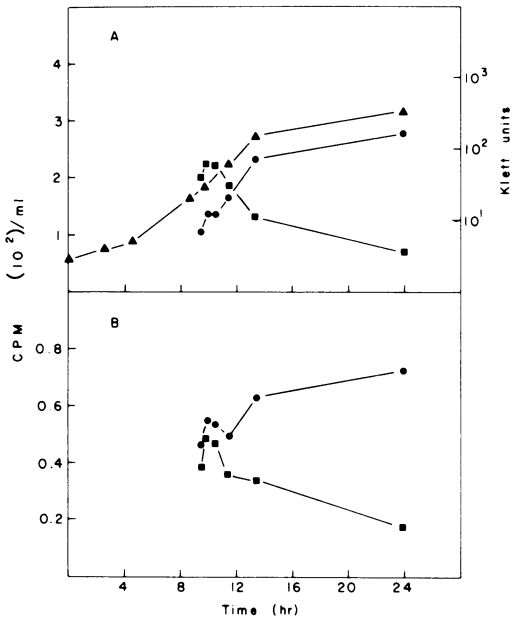

FIG. 2. *Pulse-chase of sterol fractions during growth. When cultures growing aerobically reached a density of 40 Klett units, either [methyl-^{14}C]methionine (A) or [^{14}C]acetate (B) was added. After 30 min, the cells were washed and suspended in minimal medium. The first portion was taken immediately after the wash. Symbols as in Fig. 1.*

sterol fraction as growth proceeded (Fig. 4). Unlike the aerobic experiment (Fig. 3), the amount of label in the steryl esters remained at the low level of 15 to 30% of the total label as culture growth ceased. Thus, steryl esters acted as sources of free sterols and perhaps fatty acids during anaerobic growth.

Effect of azasterol on sterol ester metabolism. Holmlund and co-workers have reported that one of the effects of the hypocholesteremic compounds trifluperdol and triparanol, in addition to inhibiting steps in the sterol biosynthetic pathway, is to increase the steryl ester content of cells two- to fourfold (7, 22). We examined the effect of the sterol analog 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (azasterol) on steryl ester metabolism as assayed by the incorporation of [^{14}C]acetate into the sterol and steryl ester fractions (Table 1). This agent has been shown to inhibit the Δ^{14} reductase step of the biosynthetic pathway, which results in the accumulation of ergosta-8,14-dien-3β-ol (ignosterol) (11). At azasterol levels of 25 ng/ml and below, cell growth was not slowed even though ergosterol synthesis was almost entirely inhibited. The amount of total sterols was nearly identical to that of uninhibited cells, although there was a slightly higher percentage of steryl esters. Increasing amounts of azasterol slowed and eventually inhibited growth entirely, although again ignosterol was produced by the cells. At these levels an increased amount of steryl ester was found per cell (but not per milliliter of culture). The increase in steryl ester per cell might be attributed to the slower growth

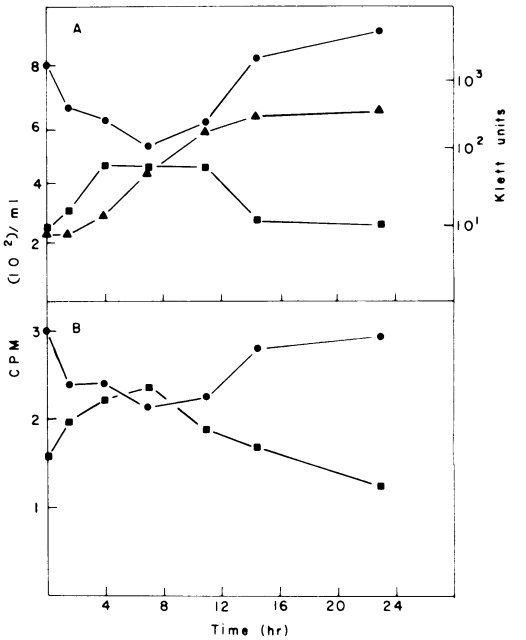

FIG. 3. *Chase of radioactivity during aerobic regrowth of a stationary-phase culture. Cultures were grown to the stationary phase in minimal medium with either [methyl-^{14}C]methionine (A) or [^{14}C]acetate (B). The cells were then washed and used as the inoculum (1:100 dilution) into unlabeled minimal medium. Symbols as in Fig. 1.*

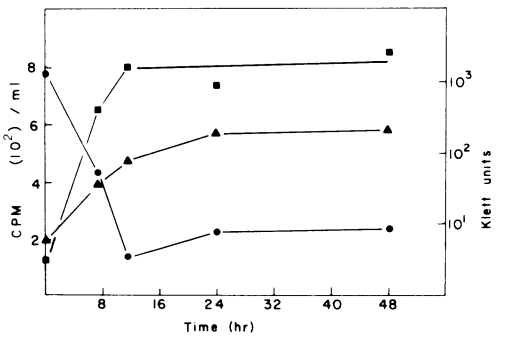

FIG. 4. *Chase of radioactivity during anaerobic regrowth of a stationary-phase culture. A prelabeled culture was prepared with [methyl-^{14}C]methionine as described in the legend of Fig. 3. The washed cells were used as the inoculum (1:100 dilution) for semi-anaerobic growth as described in the text. Symbols as in Fig. 1.*

TABLE 1. *Effect of azasterol on steryl ester metabolism*[a]

Azasterol concn (ng/ml)	Doubling time (min)	Final cell density (Klett units)	Total sterol (cpm/10^5 cells)		% Esterified	
			Late exponential	Stationary	Late exponential	Stationary
0	96	395	43.5	75.1	51	72
25	96	405	41.1	63.1	53	75
50	108	395	45.3	67.3	65	72
200	120	190	57.2	141.4	60	74

[a] Strain 3416b was grown aerobically in 50 ml of minimal medium with [^{14}C]acetate and azasterol. Ten-milliliter portions were taken for radioactive assay, and 30-ml portions were taken for spectrophotometric scans to verify the presence of ignosterol.

rate and not to the unusual sterol accumulated, although at low levels of azasterol there was more residual ergosterol synthesis (8.3% at 50 ng/ml versus 1.2% at 200 ng/ml). This could also explain the increase in steryl ester production observed by Holmlund and co-workers, since in their experiments growth was greatly reduced by the hypocholesteremic agents (7, 22).

Control of sterol species in the free and esterified fractions. Several groups have reported that the steryl ester fraction of *S. cerevisiae* is enriched with intermediates of the ergosterol biosynthetic pathway. We utilized azasterol inhibition of sterol synthesis to investigate the manner in which this distribution comes about. A culture was grown in [*methyl*-^{14}C]methionine to the stationary phase in the absence of azasterol so that 80% of the sterol of the cell was esterified. This culture was then used as the inoculum (as in Fig. 3) into medium containing a high amount of azasterol (10 μg/ml). The prelabeled sterols shifted into free sterol forms until only 40% remained esterified (Fig. 5). Spectrophotometric scans, which distinguish ignosterol (absorption maximum at 250 nm) from ergosterol (absorption maximum at 282 nm), verified that what ergosterol the cells contained was entirely in the free form. The same result was also obtained when a culture was challenged with 10 μg of azasterol per ml at the mid-exponential phase of growth; the ergosterol in the ester pool shifted into the free sterol fraction (results not shown). This establishes that there is an active cellular control over the types of sterol found in the free and esterified sterol pools.

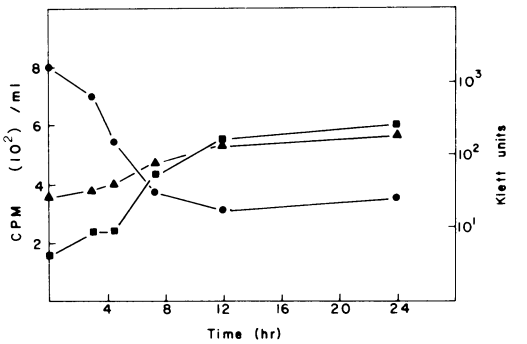

FIG. 5. *Chase of radioactivity during the aerobic regrowth in the presence of 10 μg of azasterol per ml. The experiment and symbols were as described in the legend to Fig. 3 for [methyl-^{14}C]methionine.*

DISCUSSION

The accumulation of steryl esters in *S. cerevisiae* occurs under a variety of conditions including stationary phase of growth, submaximal growth in poor medium, starvation for nutrients, and sporulation. Since a common feature of these conditions is the retardation of growth, it is possible that the biosynthesis of sterol is not tightly coupled to the cell growth rate. The amount of sterols beyond that required for cell growth might then be stored as steryl esters in the lipid droplets that have been shown to accumulate in yeast under most of these conditions (8, 16). This could also explain the increase in steryl esters found during inhibition of sterol synthesis by several chemical agents. This same suggestion has been made for human fibroblast cells in which a sudden influx of sterols results in the intracellular synthesis of sterol esters (6). Usually in these cells, however, the amount of sterols is tightly controlled and esters do not accumulate. An alternative explanation, given for the buildup of steryl esters during demyelination of nerve cells, is that steryl esters are made in response to an excess of fatty acids which would otherwise be toxic (19).

Yeast cells are known to accumulate a number of storage products, such as triacylglycerols, polyphosphates, and glycogen (23). The control of sterol biosynthesis in yeast, therefore, might be regulated to provide for an excess to fit into a storage economy. Since free sterols are components of the membrane whereas steryl esters are extramembranous lipids, esterification could

also serve as a means of regulating the amount of sterols in the membrane. We are currently examining these possibilities.

The pulse-chase experiments presented in this paper demonstrate that the free sterol and steryl ester pools are readily interconverted. As the distribution of label at all times during growth is nearly identical to the amount of sterols in each pool as determined by chemical assay, it appears that the two pools are in active equilibrium. About 50% of the label found initially in the free sterol fraction in the early exponential phase is converted to steryl esters by the time growth ceases. It does not seem likely that this percentage of sterols is specifically sequestered for esterification but, rather, that the sterols, which are distributed among the cell membranes, eventually become available for esterification. The results with anaerobic growth and azasterol inhibition indicate that at least 75% of the steryl ester pool is available for conversion to free sterols. Therefore, the steryl ester pool functions as an expandable reserve of free sterols, and perhaps fatty acids, for membrane biosynthesis. This reserve is utilized not only when the cell cannot synthesize more sterol (anaerobic conditions), but also when the cell is growing rapidly (aerobic conditions).

The results from the redistribution of sterol species after azasterol inhibition suggests another role of steryl esters: a means of segregating sterol structures suboptimal for cell growth. If the distribution of sterol species reported by Bailey and Parks (3) is also due to this active discrimination by the cell, it would appear that the cell is sensitive to very small differences in sterol structure (e.g., the presence of Δ^{22}). This discrimination can also be demonstrated by feeding cultures mixtures of sterols and measuring their incorporation into each fraction. We are currently exploring the structural specificity of this phenomenon. The most obvious consequence of this specificity is that, in experiments attempting to change the type of sterol by feeding or by inhibition with chemical agents, any residual amount of ergosterol is likely to be concentrated in the free sterol fraction and have a greater effect on cell properties than expected.

ACKNOWLEDGMENTS

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