

Sphingolipid Long-Chain-Base Auxotrophs of *Saccharomyces cerevisiae*: Genetics, Physiology, and a Method for Their Selection

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A selection method for sphingolipid long-chain-base auxotrophs of *Saccharomyces cerevisiae* was devised after observing that strains that require a long-chain base for growth become denser when starved for this substance. Genetic analysis of over 60 such strains indicated only two complementation classes, *lcb1* and *lcb2*. Mutant strains from each class grew equally well with 3-ketodihydrosphingosine, erythrodihydrosphingosine or threodihydrosphingosine, or phytosphingosine. Since these metabolites represent the first, second, and last components, respectively, of the long-chain-base biosynthetic pathway, it is likely that the *LCB1* and *LCB2* genes are involved in the first step of long-chain-base synthesis. The results of long-chain-base starvation in the *Lcb*⁻ strains suggest that one or more sphingolipids have a vital role in *S. cerevisiae*. Immediate sequelae of long-chain-base starvation were loss of viability, exacerbated in the presence of α -cyclodextrin, and loss of phosphoinositol sphingolipid synthesis but not phosphatidylinositol synthesis. Loss of viability with long-chain-base starvation could be prevented by also blocking either protein or nucleic acid synthesis. Without a long-chain-base, cell division, dry mass accumulation, and protein synthesis continued at a diminished rate and were further inhibited by the detergent Tergitol. The cell density increase induced by long-chain-base starvation is thus explained as a differential loss of cell division and mass accumulation. Long-chain-base starvation in *Lcb*⁻ *S. cerevisiae* and inositol starvation of *Inos*⁻ *S. cerevisiae* share common features: an increase in cell density and a loss of cell viability overcome by blocking macromolecular synthesis.

Sphingolipids are ubiquitous eukaryotic membrane constituents with a hydrophobic segment (ceramide) consisting of a long-chain base (LCB; sphingosine in animals, phytosphingosine (PHS) in fungi and plants) that is N fatty acylated and linked to various polar head groups, hundreds of which have been described (7). The polar head groups of certain fungal and plant sphingolipids are distinguished from those of animals in that they contain inositol, which is phosphodiester linked to ceramide, with the inositol further decorated with polar substituents (1, 13, 26, 29). In *Saccharomyces cerevisiae*, the phosphoinositol sphingolipids are located mostly in the plasma membrane (21) and consist of three classes: inositol-*p*-ceramide, mannose-inositol-*p*-ceramide, and mannose(inositol-*p*)₂ceramide [M(IP)₂C] (26, 29).

Cellular functions for animal sphingolipids and their catabolites are under active study (6, 8, 9). To explore the biosynthesis and function of these lipids in *S. cerevisiae*, we have begun a study of mutants that are defective in sphingolipid synthesis. One such mutant, unable to grow unless cultured with a sphingolipid LCB, has been briefly described (32). In this report, we describe a simple selection method that yields LCB auxotrophs that fall into two genetic complementation groups, with a pattern of LCB auxotrophy suggesting a defect in the initial step of LCB synthesis. Various sequelae of LCB starvation described with these mutants, such as depressed phosphoinositol sphingolipid synthesis and loss of viability, suggest that sphingolipid performs one or more vital functions.

(Abstracts of this work have appeared previously [22, 28].)

MATERIALS AND METHODS

Abbreviations. The following abbreviations are used in this report: DHS, DL-sphinganine or DL-dihydrosphingosine; 3-KDS, DL-3-ketosphinganine.

Materials. *N*-acetylerthro-DHS, 3-KDS, and its *N*-acetyl derivative were prepared as previously described (5, 24). Structures were confirmed by mass spectral and ¹H nuclear magnetic resonance analyses. Threo-DHS was separated from erythro-DHS in a mixture of the two isomers obtained from Sigma Chemical Co.; the biphenylcarbonyl derivatives were prepared and separated by high-performance liquid chromatography (HPLC) (4, 15), and the biphenylcarbonyl derivative of the threo isomer was hydrolyzed with 10% Ba(OH)₂ and dioxane (1:1) at 110°C for 18 h, diluted with water, and extracted with CHCl₃. The C₂₃ analog of threo-DHS was a gift of E. F. Jenny, CIBA-GEIGY, Basel, Switzerland. PHS was prepared from tetra-acetyl PHS (a generous gift of R. F. Vesonder, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., obtained from cultures of *Hansenula ciferri*) by hydrolysis with 1 N HCl in methanol-H₂O (82:18) at 80°C for 17 h and recrystallized from 95% ethanol and hexane. *N*-Palmitoylerthro-DHS and *N*-lignocerylerthro-DHS were from Serdary Research Laboratories, London, Ontario, Canada. *N*-2-hydroxyhexacosanyl-PHS and M(IP)₂C were prepared from baker's yeast cake (19, 29).

Strains. The *S. cerevisiae* strains used in this study are listed in Table 1. Strains BS232, BS238, and BS239 were selected by the density shift procedure after mutagenesis of strain SJ21R. The following strains were obtained from dissected tetrads (parental strains are indicated in parentheses): 1B1A and 1B2D (31A × W303-1B), X35C (1B1A × W303-1B), X2A1B, X45A, X45B, X45C, and X45D (1B2D × W303-1B). The following strains were derived from random

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TABLE 1. Genotypes and origins of yeast strains used in these studies

Strain	Genotype	Source or reference
MC6A	<i>MATa ino1-13 ino4-8</i>	S. A. Henry; 12
SJ21R	<i>MATa ura3-52 leu2-3,112 ade1 MEL1</i>	14
W303-1B	<i>MATα ade2-1 can1-100 ura3-1 his3-11,15 leu2-3,112 trp1-1</i>	R. Rothstein
<i>lcb1^a</i>	<i>MATa lcb1 ino1-13 ino4-8</i>	32
<i>lcb2^b</i>	<i>MATa lcb2 ino1-13 ino4-8</i>	32
BS239	<i>MATa lcb1 ura3-52 leu2-3,112 ade1</i>	This study
BS232	<i>MATa lcb2 ura3-52 leu2-3,112 ade1</i>	This study
BS238	<i>MATa lcb2 ura3-52 leu2-3,112 ade1</i>	This study
1B1A	<i>MATa lcb1 ino trp1-1</i>	This study
1B2D	<i>MATa lcb1 ino</i>	This study
X35C	<i>MATα LCB1 ino trp1-1 his3-11,15</i>	This study
X25D4	<i>MATa lcb2 ura3-1</i>	This study
X28D4	<i>MATa lcb1 leu2-3,112 his3-11,15</i>	This study
X25D3	<i>MATα lcb1 leu2-3,112 ura3-1 trp1-1</i>	This study
X2A1B	<i>MATa lcb1 ura3-1 trp1-1 his3-11,15</i>	This study
X24D5	<i>MATα lcb1 ura3-1 trp1-1 his3-11,15 leu2-3,112</i>	This study
X26D7	<i>MATα lcb1 ura3-1 his3-11,15 leu2-3,112</i>	This study
X45A	<i>MATa lcb1 ade2-1 ura3-1 trp1-1 inos</i>	This study
X45B	<i>inos</i>	This study
X45C	<i>ade2-1 leu2-3,112 his3-11,15</i>	This study
X45D	<i>MATα lcb1 ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15</i>	This study
X19D3	<i>MATα lcb2 ade2-1 ura3-1 trp1-1 leu2-3,112</i>	This study
X23D3	<i>MATα lcb1 ura3-1 leu2-3,112</i>	This study

^a *lcb1* strains derived from mutagenized MC6A: 12A, 13B, 15CS, 31A, 32A, 32B, and 36A.

^b *lcb2* strains derived from mutagenized MC6A: 1D, 7, 16A, 24C, and 19AAP3.

spores: X25D3 and X25D4 (16A × W303-1B), X28D7 (13B × W303-1B), X28D4 (12A × W303-1B), X24D5 (32A × W303-1B), X19D3 (24C × W303-1B), and X23D3 (15CS × W303-1B).

Genetic procedures. Standard yeast genetic procedures were used (25, 31). For experiments that involved dissection of tetrads, sporulation was carried out on a liquid medium consisting of 1% K acetate, 0.1% yeast extract, 0.05% glucose, and 0.05% Tergitol; LCBs were found to inhibit sporulation. In experiments in which random spores were selected and in complementation analysis, mating was carried out on filters (31) placed on complex medium with 2% agar for 6 to 16 h as necessary and washed with 1 M sorbitol, followed by plating on synthetic medium to select diploids. Diploid colonies were selected and cultured overnight on complete liquid complex medium; A number of cells equivalent to 2 absorbance U was washed with water, suspended in 15 μl of water, and plated on sporulation medium consisting of 2% agar, 1% K acetate, and 50 mg each of adenine, uracil, leucine, tryptophan, histidine, and inositol per liter. Tetrads were disrupted in 0.1 M KPO₄ (pH 7.5) containing 2 mg of Zymolyase 100000 (Miles Laboratories, Inc.) per ml and then subjected to mild sonic treatment; the spores were washed with 1 M sorbitol prior to plating on selective synthetic media.

Culture media and conditions. Cells were cultured at 30°C with shaking, and turbidity was monitored by *A*₆₅₀ (1 cm) measurement with a Zeiss PMQ-2 spectrophotometer. Complete synthetic medium consisted of 0.05 M sodium succinate (pH 5.0), 25 μM PHS, and (per liter) 16.7 g of Difco

Vitamin-Free Yeast Nitrogen Base; 40 g of glucose; 20 mg each of adenine and uracil; 50 mg of *myo*-inositol; 0.5 g of Tergitol-Nonidet P-40; 0.2 mg each of riboflavin and *p*-aminobenzoic acid; 2 μg each of biotin and folic acid; 0.4 mg each of niacin, thiamine HCl, pyridoxine HCl, and pantothenic acid; 30 mg each of the L-amino acids glycine, glutamine, isoleucine, serine, alanine, valine, phenylalanine, threonine, cysteine HCl, asparagine, proline, arginine, tyrosine, lysine 2HCl, leucine, histidine, methionine, and tryptophan. For leucine auxotrophs, the amount of leucine used was 150 mg/l. Complete complex medium consisted of 0.05 M sodium succinate (pH 5.0), 25 μM PHS, and (per liter) 10 g each of Difco Bacto-Peptone and Difco yeast extract, 40 g of glucose, 50 mg of *myo*-inositol, and 0.5 g of Tergitol. A 100 mM stock solution of LCB in 95% ethanol was added to a sterile 0.5% Tergitol solution in water prior to addition to the rest of the media. Starter cultures were obtained by growth for 24 h on complete synthetic medium containing 0.1% glucose and then stored at 4°C. The low glucose concentration extended the viability of starter cultures.

Density shift mutant enrichment procedure. Cells (25 *A*₆₅₀ U) from an exponential culture grown in complete synthetic medium were washed twice with water and suspended in 5 ml of 0.1 M sodium phosphate, pH 8.0. After addition of 0.125 ml of ethyl methanesulfonate, the cell suspension was shaken at 30°C for 30 min, centrifuged, suspended in 10 ml of 5% Na₂S₂O₃, shaken at 30°C for 10 min, washed twice with complete complex medium, and suspended in the same medium to give an absorbance of 0.25. This treatment resulted in 10% killing. This culture was allowed to grow in complete complex medium to an absorbance of 2.0 and then cultured for 5.5 h in synthetic medium minus LCB. The culture was then layered on 28% Na ditriazoate (Winthrop Laboratories) and centrifuged at 2,000 × *g* for 10 min. The pellet was then resedimented through 28% Na ditriazoate; this represents the first stage of enrichment. The resulting pellet was suspended in complete complex medium and cultured for three or four doublings, cultured for 5.5 h in synthetic medium minus LCB, and then sedimented through ditriazoate as before. A third stage of enrichment was used for some experiments.

Incorporation of [³H]inositol in alkali-labile and -stable lipids. [³H]inositol incorporation into cellular lipid in a culture containing 1 to 20 absorbance U was terminated by adding trichloroacetic acid to a final concentration of 5%, adding 50 absorbance U of unlabeled cells, and washing the culture twice with 5% trichloroacetic acid and once with water to remove nonlipid inositol. To the final pellet was added 4 ml of 0.1 N KOH in methanol-toluene (1:1) to transesterify the acyl ester lipids, converting the polar portions of the glycerol ester phospholipids to water-soluble products, leaving the sphingolipids unmodified and associated with cellular material. After 1 h at room temperature, 25 μl of glacial acetic acid was added, the mixture was centrifuged, and the supernatant was saved. The pellet was washed with 1 ml of methanol and centrifuged, and the methanol wash was combined with the previous supernatant; the pellet was washed once with 4 ml of 5% trichloroacetic acid. The combined supernatants and the trichloroacetic acid wash were evaluated for radioactivity separately and collectively constitute the deacylated glycerophosphoryl esters derived from phosphatidylinositol, phosphatidylinositolphosphate, and phosphatidylinositolbisphosphate. The sphingolipids were extracted (10) from the final pellet with 4 ml of ethanol-water-diethyl ether-pyridine-concentrated

TABLE 2. *LCB1* and *LCB2* mutants become dense with LCB starvation^a

Strain	Location ^b of cells after LCB starvation for:			
	0 h	2 h	4 h	6 h
BS239 (<i>lcb1</i>)	97-100	94-97	60-66	43-57
BS232 (<i>lcb2</i>)	97-100	91-97	57-64	43-60
SJ21 (wild type)	97-100	91-97	90-93	91-94

^a Percentages are shown; the top of the gradient corresponds to 100%.

^b After overnight culture in complete synthetic medium, an amount of cells equal to 25 *A*₆₅₀ U was washed twice in synthetic medium without LCB and then cultured in 25 ml of synthetic medium without LCB. At the indicated times, 5 *A*₆₅₀ U from each culture was concentrated in 1 ml of medium, layered onto a 7-ml linear 20 to 50% sodium ditriazoate gradient in a graduated centrifuge tube, and centrifuged in a swinging-bucket rotor at 2,000 × *g* for 10 min. The location of the band of cells was visually scored.

NH₄OH (15:15:5:1:0.018) at 60°C for 1 h and centrifuged warm, and the pellet was extracted with 2 ml of the same solvent for 30 min at 60°C. The combined supernatants containing the inositol sphingolipids were evaluated for radioactivity. The radioactive lipid fractions were converted to inositol equivalents based on the initial inositol specific radioactivity. Control experiments showed complete deacylation of ester lipids, retention of sphingolipid, and its subsequent complete extraction under the conditions noted above.

Viability, protein synthesis, and protein and DNA contents.

Cell viability was measured by diluting the cultures with complete medium (dilution of cells starved for LCB in water resulted in additional cell killing), plating on complete complex medium with 2% agar, and evaluating the colony number after 48 h at 30°C. [¹⁴C]leucine incorporation into protein in various cultures was measured by terminating the reaction with 5% trichloroacetic acid, adding 200 absorbance U of unlabeled carrier cells, washing the cells free of trichloroacetic acid-soluble radioactivity, scintillation counting of the final pellet, and converting the radioactivity to leucine equivalents based on the initial specific activity. Protein and DNA contents per particle were measured in a fluorescence-activated cell sorter by the fluorescence due to reaction with fluorescein isothiocyanate and propidium iodide, respectively, as described by Martegani et al. (18).

TABLE 3. LCB auxotroph enrichment from an artificial mixed culture^a

No. of rounds of enrichment	No. of colonies		% LCB auxotrophs
	Prototrophs	LCB auxotrophs	
0	78	15	16
1	17	57	77
2	3	168	98

^a Cultures of AW303-1B and X45A, in complete synthetic medium, were mixed in a 10:1 ratio of absorbance units, washed twice in synthetic medium minus LCB, and then cultured in the same medium for 5 h. At this time, an amount equal to 10 absorbance U was concentrated to 1 ml and centrifuged on a 28% sodium ditriazoate solution as described in Materials and Methods. The pellet was cultured in complete synthetic medium for 5 h, subjected to a second round of LCB starvation for 5 h, and then pelleted through a 28% ditriazoate solution. At each stage of enrichment, the percentage of auxotrophs was determined after plating the culture on complete medium and then replica plating it to medium minus LCB.

TABLE 4. Selection of LCB auxotrophs by the density shift method^a

No. of rounds of enrichment	No. of colonies tested	No. auxotrophic for:	
		DHS	PHS
2	1,060	20	20
3	1,520	50	50

^a Strain X35C was subjected to mutagenesis and three stages of the density shift method described in Materials and Methods. After stages 2 and 3, samples were plated on complete complex medium and the resulting colonies were replica plated on complex medium with no LCB, 100 μM erythro-DHS, or 25 μM PHS and scored for LCB auxotrophy. Each auxotroph grew with either erythro-DHS or PHS.

RESULTS

Enrichment of mutant *S. cerevisiae* strains auxotrophic for LCB.

We wished to find a simple selection method for obtaining a large number of LCB auxotrophs, since the original isolation procedure was nonselective and involved screening >10⁵ colonies by replica plating (32). An indirect mutant enrichment method was developed on the basis of our observation that LCB auxotrophs became denser than wild-type cells when starved for LCB as evaluated by sedimentation on a gradient of Na ditriazoate (Table 2). The utility of the procedure (see Materials and Methods) was verified by showing that LCB auxotrophs could be enriched by starting with an artificial mixture of wild-type cells and an LCB auxotroph (Table 3). Starting with mutagenized wild-type cells, about 2 to 3% of the cells after two or three rounds of enrichment proved to be LCB auxotrophs (Table 4).

Genetic analysis of LCB auxotrophs. LCB auxotrophs obtained from different parental strains by the density shift enrichment procedure fell into two complementation groups termed *LCB1* and *LCB2* (Table 5). LCB auxotrophs obtained from strain MC6A by the replica plating procedure also gave mutants that fell into *lcb1* or *lcb2*. Haploid *lcb1* and *lcb2* tester strains carrying opposite mating types and various auxotrophic mutations suitable for complementation analysis were produced by crossing the original mutants with strain W303-1B (Tables 1 and 5). Several additional LCB auxotrophs could not be scored owing to inability to mate with one or both tester strains.

Tetrad analysis was used to determine whether the two *lcb* complementation groups represent single nuclear genes. Strain 31A (*lcb1*) was mated with strain W303-1B and sporulated, and tetrads were dissected; all 14 tetrads yielded two *Lcb*⁻ and two *Lcb*⁺ spores. No linkage to the other markers scored, including *ade2*, *ura3*, *his3*, *leu2*, and *trp1*, was observed. Two *Lcb*⁻ segregants from the above-de-

TABLE 5. Summary of complementation analysis of LCB auxotrophs

Mutant enrichment procedure	Parent strain	<i>lcb</i> tester strains	No. of strains in complementation group:	
			<i>lcb1</i>	<i>lcb2</i>
None	MC6A	X23D3, X24D5, X19D3, X25D3	7	5
Density shift	X35C SJ21R	X28D4, X25D4 X24D5, X25D3	6 15	15 15

TABLE 6. LCB growth requirement of *lcb* mutant strains^a

Strain	Cell growth with the following LCB or derivative added:					
	None	3-KDS	DHS	PHS	<i>N</i> -Acetyl 3-KDS	<i>N</i> -Acetyl DHS
Wild-type MC6A	+	+	+	+	+	+
<i>lcb1</i> strains						
31A	-	+	+	+	-	-
X45A	-	+	+	+	ND	ND
<i>lcb2</i> strains 1D, 7, 19AAP3	-	+	+	+	ND	ND

^a Cells were cultured for 20 h on synthetic medium with the indicated LCBs added at 200 μ M, except for PHS, which was added at 25 μ M. Initial A_{650} , 0.04; -, no growth, +, final A_{650} of ≥ 10 ; ND, not determined.

scribed cross (strains 1B2D and 1B1A) were backcrossed to strain W303-1B, and the 10 (1B2D) and 4 (1B1A) tetrads obtained, respectively, all gave two *Lcb*⁻ and two *Lcb*⁺ spores. Analysis of 10 tetrads from a cross of strain 16a (*lcb2*) with strain W303-1B also gave 2:2 segregation for the *Lcb*⁻:*Lcb*⁺ phenotypes in each case; the same results were obtained from 2 tetrads analyzed from a cross of strain 24C (*lcb2*) with strain W303-1B. Tetrad analysis was also used to show that *LCB1* and *LCB2* are unlinked genetically. A cross of strains 26D7 (*lcb1*) and BS238 (*lcb2*) gave 10 tetratype (1 *Lcb*⁺:3 *Lcb*⁻), 5 nonparental ditype (2 *Lcb*⁺:2 *Lcb*⁻), and 5 parental ditype configurations in tetrads, indicating that the two genes are unlinked genetically. We conclude from these data that both *lcb1* and *lcb2* behave as single, separate nuclear genes. Furthermore, these genes are recessive, since the heterozygous diploids were *Lcb*⁺.

Growth responses of LCB auxotrophs to various LCBs and their derivatives. We tested *lcb1* and *lcb2* strains with various intermediates in the synthesis of PHS, the major LCB found in yeast sphingolipids, to distinguish these strains phenotypically. The PHS biosynthesis pathway probably has a minimum of three steps, as judged from results obtained with other organisms (27, 30); the last step has not been demonstrated in vitro and requires further study; however, molecular oxygen is the precursor of the 4 oxygen of PHS (16):

1. Palmitoyl coenzyme A + serine \rightarrow 3-KDS + coenzyme A + CO₂
2. 3-KDS + NADPH + H⁺ \rightarrow erythro-DHS + NADP⁺
3. Erythro-DHS + O₂ + ? \rightarrow PHS + ?

The product of each of these steps supported growth of strains bearing either an *lcb1* or an *lcb2* mutation (Table 6 and Fig. 1). The *N*-acetyl derivatives of the first two intermediates neither supported growth of the mutants nor inhibited the growth of wild-type cells, suggesting that these compounds either were not taken up by cells or were not converted to the parent LCB. These data are consistent with the hypothesis that step 1 is disrupted in both the *lcb1*- and *lcb2*-defective strains.

To define the specificity of the LCB requirement of an *Lcb*⁻ strain further, we examined the growth response to various isomers and homologs of LCBs and ceramides. For these studies, we used strain X45A (*lcb1*), obtained by backcrossing a mutant derived from wild-type MC6A twice with strain W303-1B, and the two parental wild-type strains (Table 7). Although the LCB isomer found in sphingolipid is of the erythro configuration, at low concentrations both the erythro and threo isomers of DHS support growth equally as well as does PHS. These observations were reported earlier

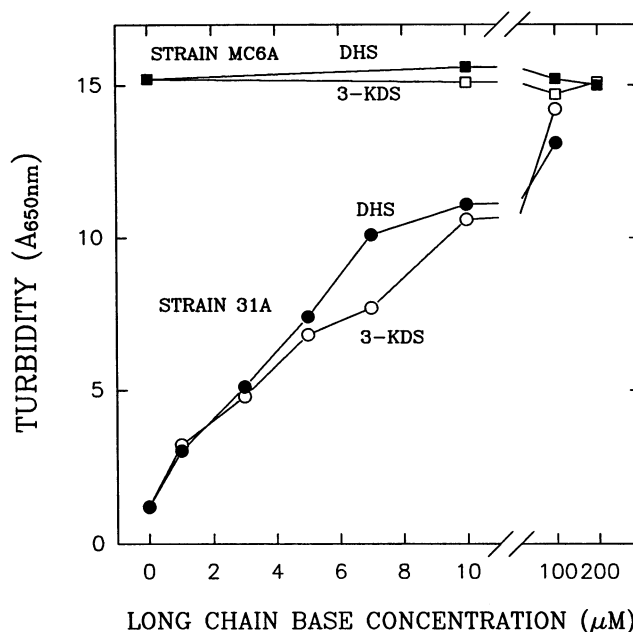


FIG. 1. Comparison of growth yields in the presence of 3-KDS or erythro-DHS. Cells from overnight cultures were washed twice with synthetic medium minus LCB and inoculated into synthetic medium with various concentrations of LCBs at an initial absorbance of 1.0 (strain MC6A) or 0.04 (strain 31A). Turbidity was monitored until maximum values were achieved: 24 h for strain MC6A and 35.5 h for strain 31A.

(32), but the experiments were flawed because subsequent analysis showed that the commercial sample of threo-DHS used was heavily contaminated with erythro-DHS. For the present experiment, threo-DHS was purified by HPLC (see Materials and Methods) and shown to be free of the erythro isomer, as judged by HPLC of the biphenylcarbonyl derivative (15). Sphingolipid derived from mutant cells grown for many generations with threo-DHS contains only PHS (data not shown), as judged by HPLC analysis (4); no standard was available for nonbiological isomers of PHS, but since erythro-DHS and threo-DHS can be easily distinguished by HPLC (15) it is safe to assume that the comparable isomers of PHS would also be separated. Our working hypothesis is that the threo isomer can be converted to the erythro isomer, possibly by a nonspecific dehydrogenase which oxidizes threo-DHS to 3-KDS.

We also compared the effects of three ceramides on the growth of the wild type and an *lcb1*-defective strain (X45A). None of the ceramides tested at a concentration of 25 μ M supported the growth of strain X45A. Since at this concentration the ceramides did not inhibit the growth of wild-type cells, we conclude that the failure of the *lcb1* strain to grow was not due to inhibition of growth (Table 7). It was not unreasonable to expect that a ceramide could support the growth of an *Lcb*⁻ strain, since exogenous ceramides have been shown to be incorporated into sphingolipids of cultured animal cells (20). The ceramide composed of PHS and the 2-OH C₂₆ fatty acid is the major component of the phosphoinositol-containing sphingolipid and might have been expected to serve as a precursor if it could diffuse across the membrane, a reasonable possibility in light of its fairly hydrophobic character; *N*-hexanoyl-DHS, with a shorter acyl chain, was also was ineffective. The major yeast sphin-

TABLE 7. Growth effects of LCBs and their N fatty acyl derivatives on an *lcb1* mutant^a and its parent strains^b

LCB and concn (μ M)	<i>A</i> ₆₅₀ U					
	X45A		MC6A		W303-1B	
	24 h	45 h	8 h	24 h	10 h	24 h
None	0.18	0.13	7.9	15.0	8.0	7.3
PHS						
25	3.6	4.5				
50	3.2	5.7		15.0	4.3	
100	4.0	7.5	5.4	14.7		4.5
Erythro-DHS						
25	3.3	3.6				
50	3.3	5.0		15.3	7.9	
100	4.7	6.0	7.5	14.9		7.3
Threo-DHS						
25	3.6	4.7				
50	3.8	6.0		14.5	3.4	
100	1.2	0.8	7.0	15.0		4.9
Threo-DHS (<i>C</i> ₂₃)						
25	0.4	0.2				
50	1.0	0.2		15.3	7.7	
100	1.2	0.8				
<i>N</i> -hexanoyl-DHS, 25	0.2	0.1		15.0 ^c	8.0 ^c	
<i>N</i> -tetracosanoyl-DHS, 25	0.2	0.2		15.1 ^c	8.1 ^c	
<i>N</i> -(2-hydroxyhexacosanoyl)-PHS, 25	0.2	0.2		15.2 ^c	7.9 ^c	
M(IP) ₂ C-III, 25	0.04	0.08		15.3 ^c	7.9 ^c	

^a Backcrossed twice to the wild type.

^b Cells were grown overnight in complete synthetic medium (0.1% glucose); washed three times with synthetic medium minus LCB; inoculated at initial absorbances of 0.2 (X45A), 0.35 (MC6A), and 0.4 (W303-1B) into synthetic medium with the indicated lipid supplements; and grown for the times indicated at 30°C. The *N*-acyl derivatives were dispersed in 0.5% tergitol prior to addition to the synthetic medium. M(IP)₂C-III (ceramide is 2-OH26:O-PHS).

^c 50 μ M ceramides.

golipid M(IP)₂C also did not support growth of the mutant or inhibit wild-type cells. While one could not expect this charged molecule to permeate the cell, it could be expected to insert itself into the outer leaflet of the plasma membrane.

LCBs are fairly toxic to animal cells, in the range of 25 to 50 μ M, in part owing to their action as inhibitors of protein kinase C (9). These compounds are relatively benign with respect to vegetative growth of yeast cells (Table 7 and Fig. 1); however, they do inhibit sporulation (data not shown). The three *C*₁₈ LCBs tested (Table 7) were moderately inhibitory to the growth of one of the wild-type strains (W303-1B), and 100 μ M threo-DHS inhibited growth of the mutant. That the *C*₂₃ homolog of threo-DHS did not support growth of the mutant and did not affect the growth of either wild-type strain suggests a fairly stringent LCB chain length specificity; small amounts of *C*₂₀ LCBs, along with the predominant *C*₁₈ homolog, have been observed in fungal sphingolipids (17, 26).

Exogenously added LCB is efficiently converted to sphingolipid by an *lcb1*-defective strain, as judged by the maximum growth yield attained in a stationary-phase culture with limiting amounts of either 3-KDS or DHS (Fig. 1). For example, at 5 μ M LCB, the growth increment due to LCB is about 6 *A*₆₅₀ U (Fig. 1) or about 1 *A*₆₅₀ U per 2.4 nmol (6 \div

2.5) of the natural D isomer. This compares with a sphingolipid content of about 1.5 nmol per *A*₆₅₀ U observed in exponential cultures of wild-type cells (data not shown). These data suggest efficient incorporation of LCB into sphingolipid without substantial catabolism.

Lcb⁻ strains die when starved for LCB. To study the physiological consequences associated with LCB starvation of *lcb* mutants, a dependable procedure for removing exogenous LCB from cells had to be developed because simply washing mutant cells grown with medium lacking LCB did not appear to remove all of the free LCB. For example, the turbidity of LCB-starved cultures continued to increase at the same rate as that of LCB-supplemented cultures for as long as 6 to 8 h, despite several washes of the cells with culture medium minus LCB (32). The procedure we finally adopted for removing LCB was to wash cells three times with culture medium containing 0.05% Tergitol and 20 mM α -cyclodextrin, a compound known to sequester hydrophobic substances in water. Washing or culturing of wild-type cells in α -cyclodextrin and Tergitol did not affect growth (data not shown).

When an Lcb⁻ mutant was starved for LCB in otherwise complete culture medium, exponential loss of viability ensued (Fig. 2). When the cells were washed with LCB-free medium containing Tergitol and α -cyclodextrin (Fig. 2, bottom), loss of viability began to occur more promptly than in cells just washed with LCB-free culture medium (Fig. 2, top). When LCB-starved cells were cultured in the presence of α -cyclodextrin, the rate of viability loss was significantly enhanced. Whether the latter finding was due to sequestration of persisting traces of LCB or to a sensitivity of sphingolipid-deficient cells to α -cyclodextrin is an open question.

The loss of viability of LCB-starved mutant cells is apparently related to unbalanced growth, since the viability loss was prevented when either protein or nucleic acid synthesis was blocked by omission of tryptophan or uracil in an Lcb⁻ mutant also auxotrophic for uracil and tryptophan (Table 8).

Differential changes in synthesis of inositol lipids and protein induced by LCB starvation. Further evidence for unbalanced growth induced by LCB starvation in an *lcb1* mutant strain was shown by the rapid diminution of phosphoinositol sphingolipid (OH stable) synthesis with little change in the synthesis of inositol acyl ester lipids (OH labile) or proteins until 4 to 6 h later (Fig. 3). It can be seen (Fig. 3) that control synthesis of protein and of the two lipid fractions in the presence of PHS occurred equally well with and without Tergitol; PHS is evidently polar enough not to require a detergent for dispersal at low concentrations. However, in the LCB-starved cells Tergitol significantly depressed protein synthesis, as well as the increase in the turbidity of the culture, an effect reminiscent of the α -cyclodextrin sensitivity noted above (Fig. 2). A similar differential effect of LCB starvation on the synthesis of the two classes of inositol lipids was also observed with an *lcb2* strain (Fig. 4).

LCB starvation: cell division, dry mass accumulation, and protein content. The increased density of LCB-starved *lcb* cells (Table 2) appears to result from an imbalance between cell division and mass accumulation, since cultures of LCB-starved cells showed that dry mass accumulated faster than particle numbers (Fig. 5). The effect was largest when LCB starvation was carried out in the absence of Tergitol (Fig. 5); evidently, cell division in LCB-starved cells is sensitive to Tergitol. The protein content per particle in the LCB-starved cultures, measured after staining with fluorescein isothiocy-

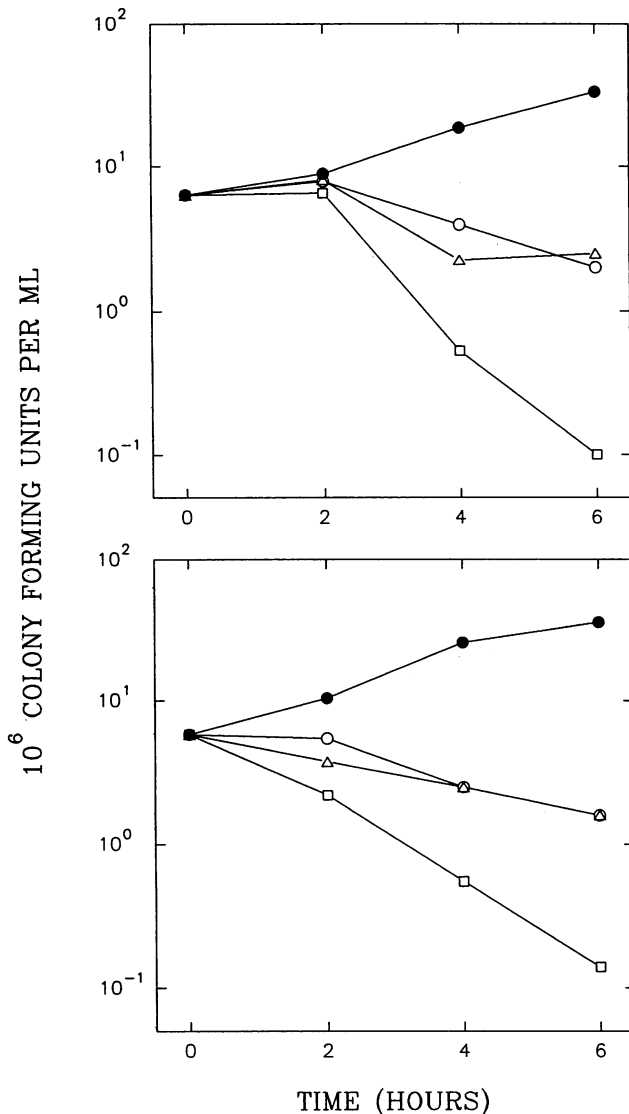


FIG. 2. Loss of viability results from LCB starvation of an *lcb1* strain and is enhanced by α -cyclodextrin. Cells of strain X45A grown in complete synthetic medium to an A_{650} of 3.0 were washed three times with either synthetic medium with no Tergitol and no LCB (top) or synthetic medium with no LCB but containing Tergitol and α -cyclodextrin (bottom). The washed cells were used at approximately 6×10^6 /ml to inoculate synthetic media containing Tergitol and $25 \mu\text{M}$ PHS (●) or synthetic medium containing no PHS either with Tergitol (Δ), without Tergitol (\circ), or with Tergitol plus α -cyclodextrin (\square). Cell viability was evaluated as described in Materials and Methods. The concentration of Tergitol was 0.05%, and that of α -cyclodextrin was 20 mM.

anate and evaluated in a fluorescence-activated cell sorter, exhibited a significant increase over that of cells cultured with LCB or over that of the LCB-starved culture containing α -cyclodextrin (see Fig. 7, bottom). Thus, direct measurement of increased dry mass and protein content per particle in LCB-starved cultures explains the observed increase in particle density.

LCB starvation: budding and DNA synthesis. LCB starvation also resulted in a mismatch between cell division and DNA synthesis. The percent budded cells in an LCB-starved

TABLE 8. Macromolecular synthesis is required for loss of viability associated with LCB starvation^a

Composition of growth medium	% Survival ^b after culture for:	
	4 h	8 h
Complete	266	1,284
-PHS	91	6
-PHS ₁ -tryptophan	83	107
-PHS ₁ -uracil	129	107
+PHS ₁ -tryptophan	65	74
+PHS ₁ -uracil	92	66

^a Cells from an overnight culture of strain X45A grown on complete synthetic medium containing 0.1% glucose were washed three times with synthetic medium minus LCB and twice with water, inoculated into synthetic media with the indicated compositions at 5×10^6 to 6×10^6 CFU/ml, and incubated at 30°C with shaking. Viability was scored as described in Materials and Methods.

^b At 0 h of culture, viability was 100% in all of the media used.

culture without Tergitol decreased sharply despite continued protein (Fig. 6) and DNA (Fig. 7) synthesis. Tergitol restricted budding off and protein synthesis in LCB-starved cultures (Fig. 6). Evidently, new bud initiation does not occur upon LCB starvation. By phase-contrast microscopy, no unusual morphology was observed with LCB starvation.

The DNA content per particle, monitored in a fluorescence-activated cell sorter, showed a significant number of particles with a 3N content in LCB-starved cultures, a result not observed in cultures containing LCB. These results suggest continued replication in the absence of cell division. Curiously, Tergitol did not influence the results, whereas α -cyclodextrin severely depressed the number of 3N cells (Fig. 7, top five panels) and inhibited the increased protein content per cell (Fig. 7, bottom panel). It is difficult to exclude totally the possibility that the particles with 3N DNA represent an aggregate of 1N and 2N particles that somehow formed during analysis; however, the cultures were sonically treated before analysis to break up aggregates of cells. Examination by light microscopy showed no aggregates.

DISCUSSION

We have isolated strains of *S. cerevisiae* that require a sphingolipid LCB for growth by a nonselective replica plating procedure and by a density enrichment method. Over 60 such strains that were genetically analyzed fell into only two complementation groups, *lcb1* and *lcb2*. Experiments comparing the specificity of the LCB requirement suggest that these strains are defective in the first step in LCB synthesis catalyzed by the enzyme serine palmitoyltransferase. This conclusion follows from the finding that 3-KDS, the product of the first step in LCB synthesis, supports the growth of both *lcb1* and *lcb2* strains. In fact, subsequent work (22, 23) showed that both *lcb1* and *lcb2* strains have barely detectable levels of serine palmitoyltransferase activity. Furthermore, the *LCB1* gene has been isolated and its predicted amino acid sequence also strongly suggests that the gene codes for a catalytic segment of serine palmitoyltransferase (2).

Although many *Lcb*⁻ mutants were selected by the density enrichment method described herein, none appeared to be blocked in the step 2 or 3 of the LCB-synthetic pathway. Among explanations for the lack of 3-KDS reductase mutants, blocked in step 2 of LCB synthesis, is the possible existence of one or more ketone reductases that nonspecif-

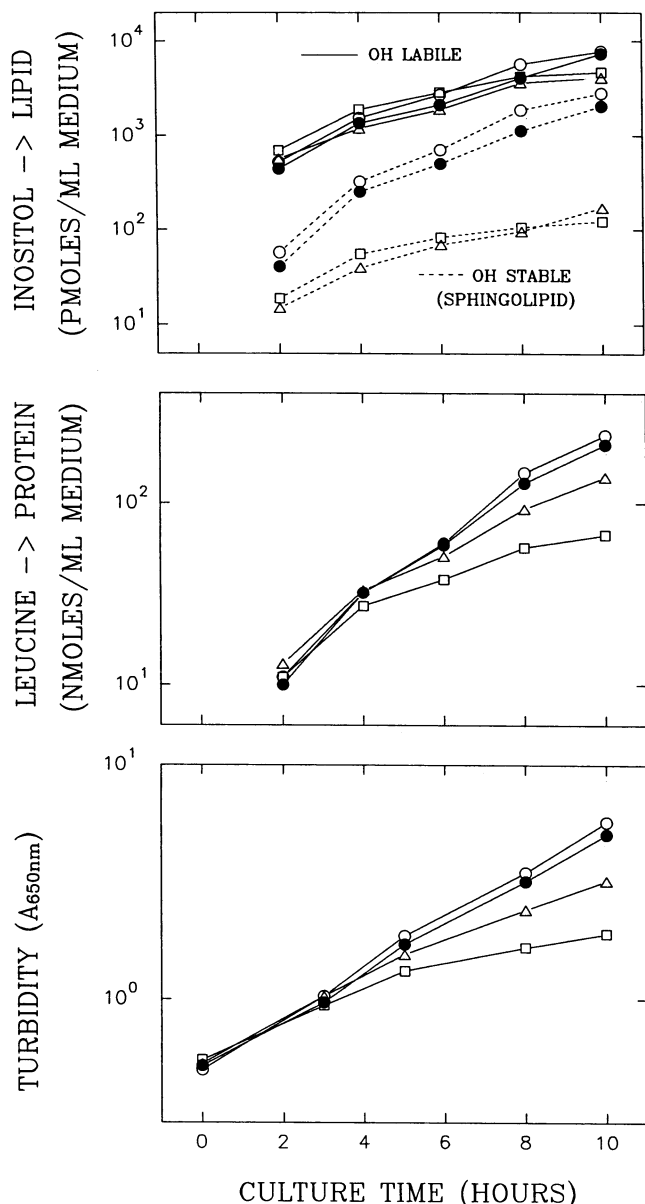


FIG. 3. LCB deprivation of an *lcb1* strain differentially affects the synthesis of acyl ester inositides, inositol sphingolipids, and protein. Cells of strain X45A cultured overnight on slightly modified complete synthetic medium (inositol, 10 mg/l; leucine, 300 mg/l) were washed three times with synthetic medium minus LCB and containing 20 mM α -cyclodextrin and once with synthetic medium minus LCB and inoculated into the modified synthetic medium with PHS plus (●) or minus (○) 0.05% Tergitol and without PHS plus (□) or minus (△) 0.05% Tergitol, containing either 11.6 μ Ci of [2 - 3 H] inositol per ml or 0.29 μ Ci of [1 - 14 C]leucine per ml. Aliquots of the cultures were evaluated for turbidity (bottom), leucine incorporation into protein (middle), and inositol incorporation into alkali-labile (————) and alkali-stable (-----) lipids (top) as described in Materials and Methods.

ically reduce 3-KDS. Alternatively, high intracellular levels of 3-KDS may be cytotoxic; LCBs are known to be toxic in cultured animal cells (9). It is likely that several polypeptides are involved in step 3 of the pathway, conversion of DHS to PHS. This step probably requires a mixed-function oxidase

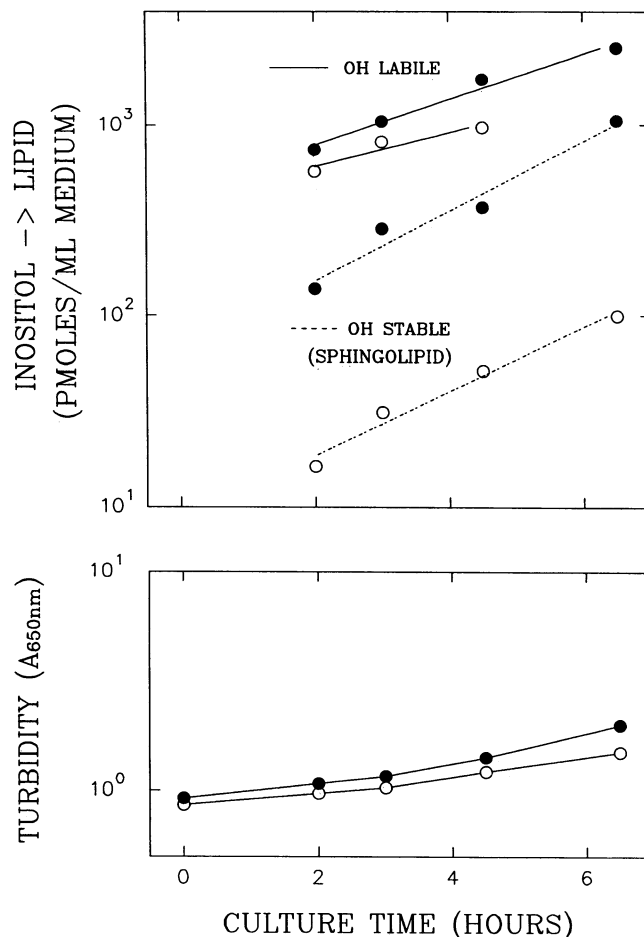


FIG. 4. LCB deprivation of an *lcb2* strain differentially affects synthesis of acyl ester inositides and inositol sphingolipids. Cells from a 36-h culture of strain 19AAP3 grown on synthetic medium containing 100 μ M erythro-DHS were washed three times with synthetic medium minus LCB and then incubated in synthetic medium containing 1 μ Ci of [3 H]inositol per ml with (●) or without (○) 100 μ M erythro-DHS. The culture was monitored for turbidity (bottom) and processed to evaluate the radioactive alkali-labile (————) and alkali-stable (-----) lipids (top) as described in Materials and Methods.

system (16). We found no mutants that displayed the phenotype expected of mutants blocked in this step, i.e., growth supported by PHS but not DHS. Possible explanations for a failure to find such mutants include cytotoxicity of intracellular DHS and the ability of the cell to make adequate sphingolipid from DHS instead of PHS, even though PHS is the predominant LCB found in wild-type yeast sphingolipids (26, 29).

Toxicity of accumulated endogenous LCBs has been offered above as an explanation for the failure to find certain mutant classes. This is a reasonable possibility in light of the fact that LCBs are potent protein kinase C inhibitors and toxic for animal cells at low concentrations (9). Our results obtained with exogenous LCB were ambiguous. We found that 100 to 200 μ M concentrations of exogenous LCBs that would be toxic for animal cells had little effect on growth of *S. cerevisiae* starting with $\geq 10^6$ cells per ml (Table 7 and Fig. 1). However, substantial growth inhibition was observed

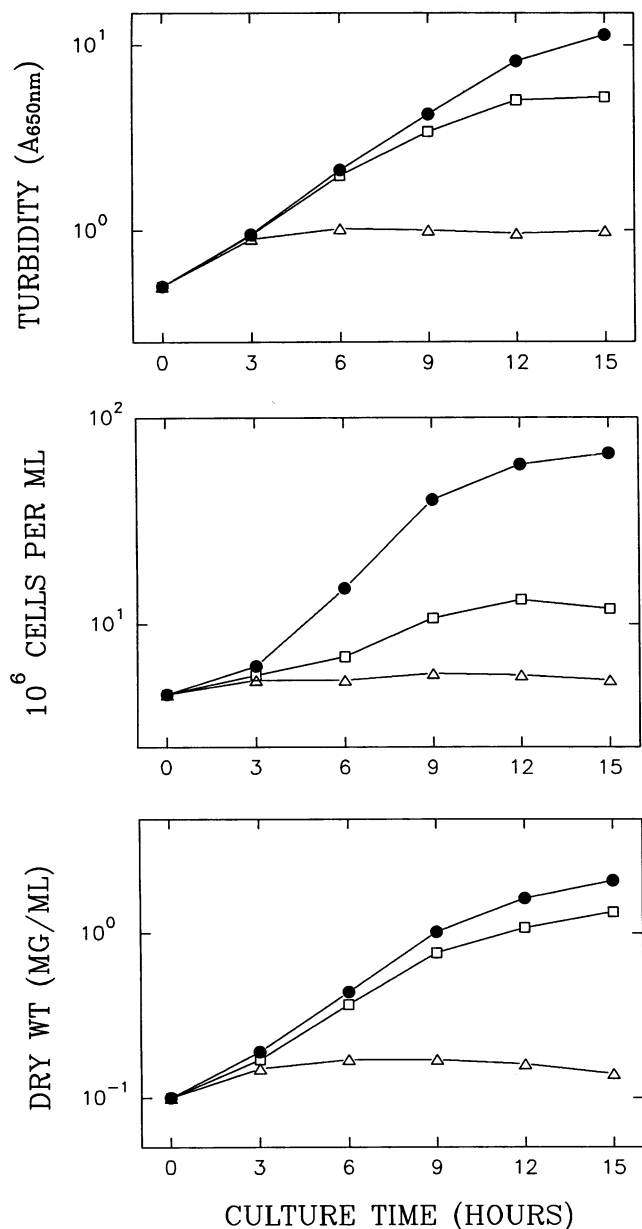


FIG. 5. Starvation for LCB has differential effects on turbidity, cell division, and dry mass (dry wt) accumulation in an *lcb1* strain. Cells of strain X45A grown on complete synthetic medium to an absorbance of 4.0 were washed three times with synthetic medium with no LCB and containing 20 mM α -cyclodextrin, washed once with synthetic medium without either LCB or tergitol, and inoculated into complete synthetic medium (●) and into medium minus LCB without (□) or with (△) 0.05% Tergitol. Cell numbers were evaluated in a Coulter counter.

with such concentrations of LCB starting with $\leq 10^5$ cells per ml (data not shown).

LCB starvation of the *Lcb*⁻ strains bears a striking resemblance to inositol starvation of inositol auxotrophs of *S. cerevisiae* (33) in terms of loss of viability, continued macromolecular synthesis, and increased cell density; loss of viability in both circumstances is also prevented by blocking macromolecular synthesis (3). The viability loss in

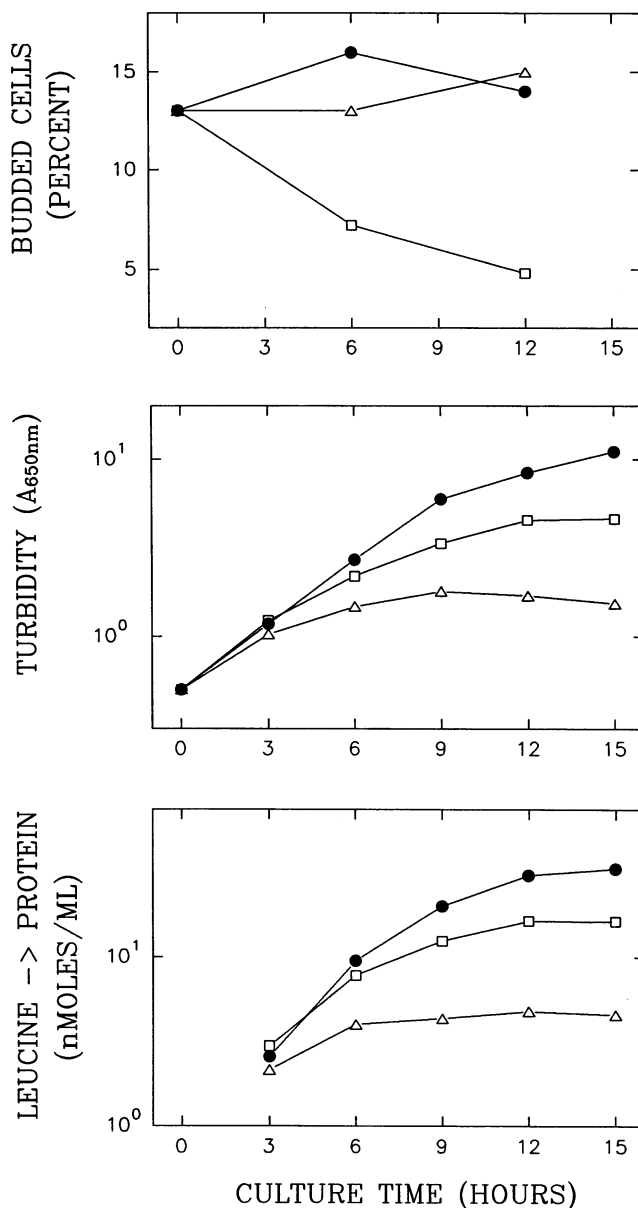


FIG. 6. Effect of LCB starvation on protein synthesis and cell budding in an *lcb1* strain. Strain X45A was grown, washed, and cultured as described in the legend to Fig. 5 (same symbols). All cultures contained 5 μ Ci of [¹⁴C]leucine per ml. Leucine incorporation into protein was monitored as described in Materials and Methods. The percentage of budded cells was evaluated from photographs taken under phase contrast from a series of fields with a total of 200 to 300 cells.

Inos⁻ and *Lcb*⁻ strains may arise from a common defect, namely, inability to synthesize inositol-containing sphingolipids. In any case, the loss of viability in *Lcb*⁻ strains suggests that one or more sphingolipids are vital to *S. cerevisiae*.

The sensitivity of LCB-starved cells to Tergitol and α -cyclodextrin suggests an altered plasma membrane; this is not surprising in view of the fact that phosphoinositol sphingolipids are a major class of yeast plasma membrane phospholipids (21). Tergitol sensitivity has also been reported for

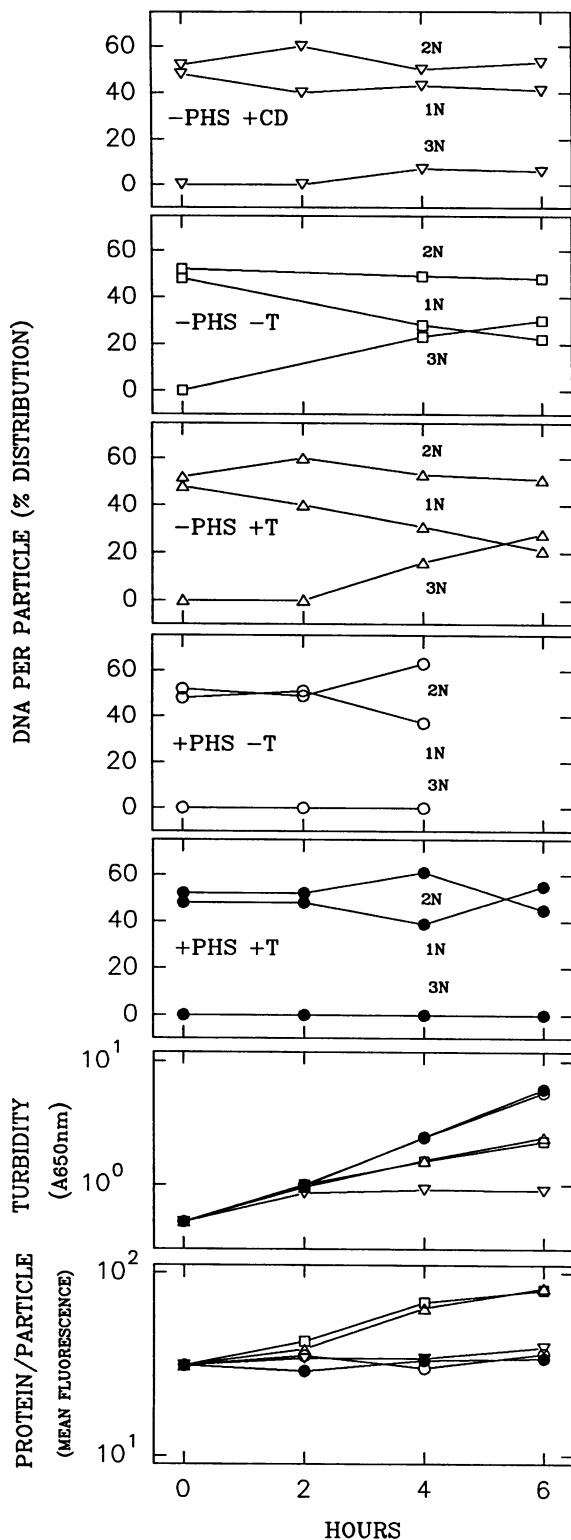


FIG. 7. Cellular protein and DNA contents as affected by starvation for LCB in an *lcb1* strain cultured in complex medium. Cells from a culture of strain X45A grown overnight in complex medium containing 25 μ M PHS were washed three times with complex medium without PHS but containing 20 μ M α -cyclodextrin (CD) and inoculated into complex media containing 25 μ M PHS with (●) or without (○) 0.05% Tergitol (T) and into complex medium with no PHS with (▲) or without (△) 0.05% Tergitol or with 20 μ M α -cyclodextrin (▼). Protein and DNA were measured in a fluorescence-activated cell sorter (see Materials and Methods). For DNA analysis, the percentages of the populations that were 1N, 2N, and 3N are plotted in the upper five panels.

fatty acid starvation in a fatty acid auxotroph of *S. cerevisiae* (11).

Further exploitation of these mutants should enhance our understanding of the metabolism and function of yeast sphingolipids. For example, we are currently studying *lcb1* suppressor strains derived from an *lcb1* mutant strain (4) to find out how they survive without making sphingolipids.

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