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Studies on Acetyl-Coenzyme A Synthetase of Yeast: Inhibition by Long-Chain Acyl-Coenzyme A Esters

T. SATYANARAYANA AND HAROLD P. KLEIN

Planetary Biology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California, 94035

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Long-chain acyl-coenzyme A (CoA) compounds (palmityl, stearyl, and oleyl) were found to be potent inhibitors of acetyl-CoA synthetase (ACS) of *Saccharomyces cerevisiae* strain LK2G12 from aerobic, but not from nonaerobic, cells. The effectiveness of the inhibitors of the aerobic enzyme was in the following order: palmityl-CoA < stearyl-CoA < oleyl-CoA. Short-chain acyl-CoA compounds (propionyl, butyryl, and valeryl) and long-chain fatty acids had no effect on ACS from either source. The inhibition by oleyl-CoA was found to be dependent on enzyme concentration, whereas the inhibition by palmityl- and stearyl-CoA was independent of ACS concentration. Inhibition by palmityl-CoA was noncompetitive with respect to both acetate and CoA, and with increasing concentration of inhibitor the pattern was sigmoidal, with a Hill value of 3.24. At maximally inhibitory concentrations of palmityl-CoA, a small amount of enzyme activity remained. This noninhibitable enzyme in aerobic cells was shown not to be of nonaerobic origin.

Acetyl-coenzyme A (CoA) synthetase (acetate:CoA ligase [adenosine monophosphate], EC 6.2.1.1) catalyzes the formation of CoA esters of short-chain fatty acids. During studies on the subcellular sites involved in the synthesis of lipids in *Saccharomyces cerevisiae*, we obtained evidence for a bimodal distribution of acetyl-CoA synthetase (ACS) in aerobically grown cultures of this yeast (8). The ACS was localized in the microsomal fraction obtained from cellular homogenates in the initial stages of the growth cycle, but shifted to the mitochondrial fraction when the cells reached the stationary phase of growth. On the other hand, in nonaerobic cells (that is, in cells obtained from standing cultures), the ACS was always localized in the microsomal fraction of these cells at all stages of growth.

By using solubilized preparations of ACS it was found (D. L. DeVincenzi and H. P. Klein, Fed. Proc., p. 872, 1970) that the enzyme obtained from aerobic cells differed from that of nonaerobic cells in a number of ways. The aerobic ACS exhibited K_m values tenfold lower for acetate and three- to fourfold lower for adenosine triphosphate, and it utilized propionate as a substrate.

These observations suggested that ACS activity from aerobic and nonaerobic cells represented two different proteins. The work reported in the present investigation supports this view because long-chain acyl-CoA esters had markedly different effects on the ACS obtained from aerobic and nonaerobic cells.

MATERIALS AND METHODS

Organism. *S. cerevisiae* strain LK2G12 was used in this investigation. Stock cultures were stored on slants containing 2% peptone, 2% glucose, 1% yeast extract, and 2% agar. Liquid media were of the same composition, with the omission of agar. For aerobic cells, 500 ml of medium in a 2-liter Erlenmeyer flask was inoculated and then incubated on a gyratory incubator-shaker at 30 C. Nonaerobic cells were grown in 1.7 liters of the same medium contained in a 2-liter flask (7).

Preparation of cellular fractions. After incubation, the cells were collected on glass-fiber filter paper (9-cm circles), washed with water and then washed with 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.4, containing 0.002 M $MgCl_2$. Cells were homogenized in the same buffer by using glass beads in a Braun homogenizer as described by Schatz (14). Cellular fractions were obtained as described earlier (6-8). ACS was solubilized from subcellular particulates by stirring the fractions in 0.1 M phosphate

buffer, pH 7.4, for 20 min and then centrifuging at 100,000 × *g* for 1 h, as described previously (De Vincenzi and Klein, Fed. Proc., p. 872, 1970).

Enzyme assay. In previous studies (6), we have utilized the method of Berg (1) as the standard assay for ACS activity. This method, however, includes hydroxylamine in the assay mixture, and it seemed possible that long-chain acyl-CoA compounds might be converted to their corresponding hydroxamates during incubation. If this were the case, any observed effects might then be attributable to acyl-hydroxamates rather than to acyl-CoA esters. Several lines of evidence alleviated this concern. First, it was found that solutions of long-chain acyl-CoA compounds, up to concentrations of 5×10^{-5} M, gave no color development upon addition of $FeCl_3$ under the conditions of the standard assay. Because long-chain acyl-hydroxamates and acetylhydroxamate produce ferric complexes with equivalent colors (9), it can be concluded that the long-chain acyl-CoA compounds added during the standard assay are not readily converted to their hydroxamates. Second, the addition of stearyl hydroxamate to aerobic ACS preparations caused no significant inhibition under standard assay conditions over a concentration range (10^{-5} to 10×10^{-5} M) in which stearyl-CoA proved to be very inhibitory. Finally, when aerobic ACS preparations were incubated with palmityl-CoA in the absence of hydroxylamine, the levels of inhibition were at least as great as those seen with the standard assay procedure (Table 1). In the absence of added hydroxylamine, however, 100 times as much CoA was used, because this compound was then required in stoichiometric amounts. On the basis of the results described above, and because it was far more economical to do so, we used the standard assay throughout this study.

In the case of enzyme obtained from aerobically grown cells, the standard assay mixture contained 10 μmol of potassium acetate and, in the case of ACS from nonaerobic cells, 100 μmol of acetate. All assays for ACS were performed after solubilization of the bound enzyme.

Other methods. Protein was measured by the procedure of Lowry et al. (10). ATP and CoA were purchased from Boehringer Mannheim Co., New York. Glass beads (0.45–0.50 mm) were obtained from B. Braun Melsungen Co. Long-chain acyl-CoA compounds were purchased from P. L. Biochemicals Co., Milwaukee, Wis. Stearylhydroxamic acid was obtained from Eastman Kodak Co., Rochester, N.Y. All chemicals were at least 90 to 95% pure as indicated by the manufacturers. Other chemicals used in this study were of reagent grade.

RESULTS

Inhibition of ACS by palmityl-CoA. Figure 1 describes the effect of increasing concentrations of palmityl-CoA on the ACS obtained from either the mitochondrial fraction of aerobic cells or from the microsomal fraction obtained from nonaerobic cells. The pattern of inhibition of the aerobic enzyme was sigmoidal, and at 5×10^{-5} M palmityl-CoA approximately 90% of the initial activity of the aerobic enzyme was inhibited. No inhibition was observed with ACS obtained from nonaerobic cells.

In these assays, substantially higher amounts of protein were used when nonaerobic preparations were assayed because these had a lower specific activity than did aerobic preparations (7). Accordingly, it seemed possible that other proteins in the nonaerobic preparations might be binding palmityl-CoA and thus reducing it to noninhibitory concentrations. Since bovine serum albumin and hydroxylamine nonspecifically bind long-chain acyl-CoA derivatives (16), bovine serum albumin was included in some of the assays for aerobic ACS, bringing the total protein level of the aerobic preparations to that of the nonaerobic ones. Under these conditions aerobic ACS was still inhibited (Fig. 1). It is interesting to note that serum albumin stabilized the enzyme during the assay period and hence yielded somewhat higher activity.

The possibility that preparations of nonaerobic cells might contain deacylase activity was also tested with the method of Grunert and Phillips, which measures free sulfhydryl groups (5). No deacylase activity was detected in these preparations.

The Hill plot of the data in Fig. 1 for ACS activity in the presence of varying concentrations of palmityl-CoA is given in Fig. 2, from which a Hill coefficient was calculated to be 3.24.

TABLE 1. *Inhibition of acetyl-CoA synthetase of aerobic S. cerevisiae by palmityl-CoA in the presence and absence of hydroxylamine*[a]

Palmityl-CoA ($\times 10^{-5}$ M)	Inhibition (%)	
	With hydroxylamine (standard assay)	Without hydroxylamine
0	0	0
0.5	3	11
1.0	9	33
1.5	18	55
2.0	33	66
2.5	48	71
3.0	64	
3.5	75	

[a] Incubation conditions were as in Fig. 1, except that without added hydroxylamine 10 μmol of CoA were used, and after 20 min of incubation the reaction was terminated by heating in a boiling-water bath for 5 min. Then, after cooling, 200 μmol of neutralized hydroxylamine was added and the tubes were again incubated for 20 min before proceeding.

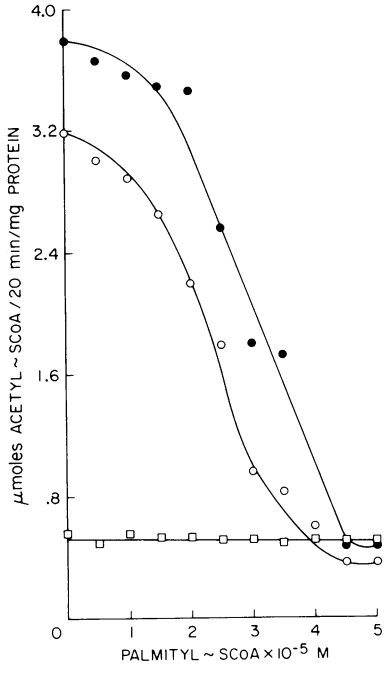

FIG. 1. *Effect of palmityl-CoA on acetyl-CoA synthetase (ACS) activity of S. cerevisiae. Solubilized preparations were obtained from the mitochondrial fraction of aerobic cells (○, ●) or from the microsomal fraction of nonaerobic cells (□). The incubation system contained in 1 ml: CoA (0.1 μmol); ATP (10 μmol); glutathione (10 μmol); $MgCl_2$ (10 μmol); potassium phosphate, pH 7.4, (100 μmol); neutralized hydroxylamine (200 μmol); enzyme (75 μg of nonaerobic ACS or 9.5 μg of aerobic ACS); and acetate (10 μmol in aerobic and 100 μmol in nonaerobic assays). ●, Results using 65.5 μg of bovine serum albumin in addition to the aerobic ACS. The reaction was started by addition of the enzyme and then incubated for 20 min at 37 C.*

The order of effectiveness of these acyl-CoA compounds in inhibiting aerobic preparations was: palmityl-CoA < stearyl-CoA < oleyl-CoA (Table 2). (The apparent slight inhibition seen with nonaerobic preparations using 5×10^{-5} M of these compounds could be the result of the detergent-like properties attributed to these long-chain acyl-CoA compounds at high concentrations [12].)

When varying amounts of protein containing ACS were incubated with constant levels of these acyl-CoA compounds, it was found that with palmityl-CoA and stearyl-CoA the percentage of inhibition remained constant over a wide range of protein concentrations. With

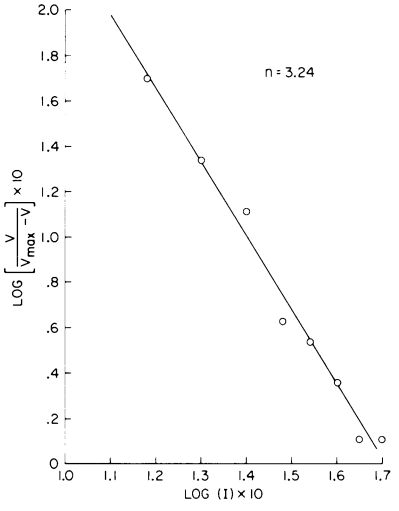

FIG. 2. *Hill plot of the data shown in Fig. 1 for aerobic acetyl-CoA synthetase in the presence of added palmityl-CoA.*

The inhibition of ACS by palmityl-CoA is noncompetitive with respect to acetate and CoA (Fig. 3, 4). K_m values of 2.08×10^{-4} M and 2.38×10^{-4} M for acetate and CoA, respectively, can be calculated.

Effects of palmityl-, stearyl-, and oleyl-CoA on ACS activity from aerobic and nonaerobic cells. In addition to palmityl-CoA, stearyl-CoA and oleyl-CoA also were inhibitory for preparations obtained from aerobic cells.

In Table 2, data are presented comparing the effects of these compounds on preparations obtained from 48-h-old cells grown with and without aeration. ACS obtained from aerobic preparations (either from the mitochondrial or microsomal fractions) was substantially inhibited. Nonaerobic preparations were essentially unaffected.

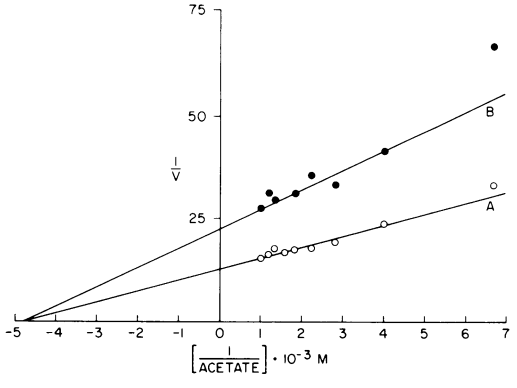

FIG. 3. *Double reciprocal plot of acetyl-CoA synthetase activity as a function of acetate concentration without (A) and with (B) palmityl-CoA (2.5×10^{-5} M).*

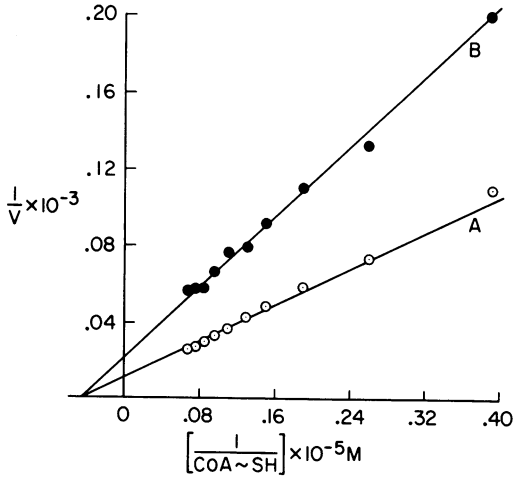

FIG. 4. *Double reciprocal plot of acetyl-CoA synthetase activity as a function of CoA concentration without (A) and (B) palmityl-CoA (2.5×10^{-5} M).*

oleyl-CoA, however, inhibition was dependent on protein concentration (Fig. 5). For this, levels of acyl-CoA were selected which would give, in routine assays, approximately 25% inhibition with palmityl-CoA, 60% with stearyl-CoA, and 90% with oleyl-CoA. The data obtained with palmityl-CoA and stearyl-CoA suggest that there is a direct interaction between the enzyme and the inhibitors, without significant binding to other proteins. However, the effectiveness of oleyl-CoA as an inhibitor rapidly fell off at higher protein concentrations, from which it would appear that this compound is binding to other proteins in the preparation.

Effects of short-chain acyl-CoA compounds and free fatty acids on ACS. Neither palmitate nor stearate had any observable effect on the ACS from either the microsomal or mitochondrial fractions of aerobic cells (Table 3), even at final concentrations up to 10^{-4} M. Several short-chain acyl-CoA compounds were also tested for their effectiveness as inhibitors. It is evident that these had no significant effects on ACS of either aerobic or nonaerobic preparations (Table 4). The spread of values between 96 and 119% of the control activity is very likely due to the fact that these low-molecular-weight acyl-CoA compounds themselves formed soluble hydroxamates, thus reducing the precision of the method (Table 4).

Nature of the noninhibitable ACS of aerobic cells. Because it was never possible to inhibit completely the ACS of aerobic cells (10 to 20% of the original enzyme activity always remained, even at the highest levels of acyl-CoA compounds used), it seemed possible that the residual, noninhibited ACS might consist of the nonaerobic variety of this enzyme. Because the two forms of ACS had very different K_m values for acetate, it was feasible to distinguish between them. Therefore, an experiment was carried out to ascertain whether the residual ACS activity under maximum inhibition by palmityl-CoA would give acetate saturation curves characteristic of aerobic or of nonaerobic preparations. ACS from the mitochondrial fraction of an aerobic preparation was assayed at various levels of acetate in the presence and absence of 4×10^{-5} M palmityl-CoA, a concen-

TABLE 2. *Effect of acyl-CoA esters on aerobic and nonaerobic acetyl-CoA synthetase of S. cerevisiae grown for 48 h*

Addition	Concn ($\times 10^{-5}$ M)	Inhibition of ACS activity[a] (%)			
		Aerobic preparation		Nonaerobic preparation	
		Mitochondrial[b] fraction	Microsomal[c] fraction	Mitochondrial[d] fraction	Microsomal[e] fraction
None		0	0	0	0
Palmityl-CoA	1.25	9	15	4	0
	2.50	24	27	4	0
	3.75	50	44	4	0
	5.00	67	69	8	0
Stearyl-CoA	1.25	24	20	0	1
	2.50	50	44	0	4
	3.75	65	70	7	7
	5.00	70	82	22	6
Oleyl-CoA	1.25	7	8	0	0
	2.50	49	45	1	0
	3.75	84	84	7	0
	5.00	89	93	10	0

[a] The terms mitochondrial and microsomal are used here merely in an operational sense, i.e., the particulate fractions sedimenting at 15,000 × *g* in 30 min and at 100,000 × *g* in 60 min, respectively. In these experiments, in order to compare the activity of mitochondrial and microsomal fractions, the protein levels used in these assays were held constant, but the period of incubation was varied in order to obtain approximately the same level of activity in the assays.

[b] 31.2 μg of protein, incubation for 15 min. Specific activity (7), 1.41 U.

[c] 31.0 μg of protein, incubation for 25 min. Specific activity, 0.73 U.

[d] 135.3 μg of protein, incubation for 40 min. Specific activity, 0.13 U.

[e] 133.3 μg of protein, incubation for 15 min. Specific activity, 0.36 U.

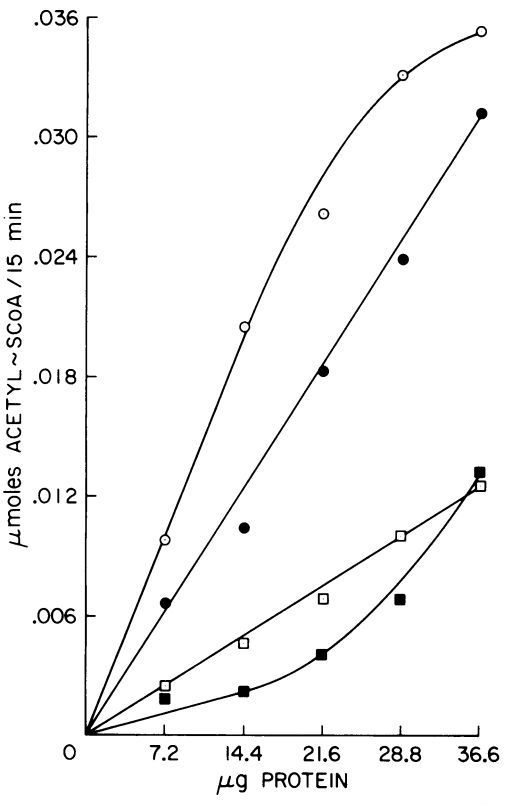

FIG. 5. *Effect of protein concentration on inhibition by acyl-CoA esters. Symbols:* ○, *control;* ●, *with palmityl-CoA* (2.13×10^{-5} *M*); □, *with stearyl-CoA* (3.25×10^{-5} *M*); ■, *with oleyl-CoA* (2.75×10^{-5} *M*).

TABLE 3. *Effect of free fatty acids on acetyl CoA synthetase of S. cerevisiae*[a]

Addition	Concn (μM)	Activity (%)	
		Aerobic[b]	Non-aerobic[c]
None		100	100
Sodium palmitate	25	100	98
	50	105	98
	100	106	96
Sodium stearate	25	105	104
	50	103	111
	100	98	106

[a] 48-h-old cells were harvested and fractionated. Aerobic mitochondrial and nonaerobic microsomal fractions were tested for acetyl-CoA synthetase activity with the additions shown.

[b] 9.5 μg of protein, incubation for 20 min. Spec act (7), 2.85 U.

[c] 75.0 μg of protein, incubation for 20 min. Spec act, 0.53 U.

tration that inhibited the ACS approximately 90%. In contrast to the ACS from nonaerobic cells, which is very sensitive to acetate concentrations below 2×10^{-2} M, the residual ACS activity found in the presence of palmityl-CoA was unaffected by corresponding decreases in the acetate concentrations (Fig. 6). This suggests that aerobic cells do not simultaneously contain a small amount of the nonaerobic form of enzyme, but rather that a small fraction of aerobic ACS may be bound in such a way as to make it unaccessible to the acyl-CoA inhibitors.

DISCUSSION

Data presented in this paper show that long-chain fatty acid CoA esters are potent inhibitors of the acetyl-CoA synthetase of *S. cerevisiae* obtained from cells grown under aerobic conditions. On the other hand, with preparations from nonaerobic cells, these compounds were virtually without effect at the concentrations tested.

The mechanism of action of the long-chain acyl-CoA inhibitors on the aerobic ACS is unknown. A number of enzymes concerned with lipogenesis or ketogenesis have been shown to be inhibited by these compounds. Among these are: acetyl-CoA carboxylase (11, 19), citrate synthetase (15), glucose-6-phosphate dehydrogenase (4), nicotinamide nucleotide transhydrogenase (13), and other enzymes (16). Possible regulatory mechanisms have been proposed (18) for the action of these compounds based on

TABLE 4. *Effect of short-chain acyl-CoA esters on acetyl CoA synthetase of S. cerevisiae*[a]

Addition	Concn (μM)	Activity (%)	
		Aerobic[b]	Non-aerobic[c]
None		100	100
Propionyl-CoA	25	96	111
	50	107	115
	100	103	107
Butyryl-CoA	25	108	112
	50	101	118
	100	117	109
n-Valeryl-CoA	25	110	111
	50	113	119
	100	118	109

[a] 48-h-old cells were harvested and fractionated.

[b] 9.5 μg of protein, incubation for 20 min. Spec act (7), 2.75 U.

[c] 75.0 μg of protein, incubation for 20 min. Spec act, 0.51 U.

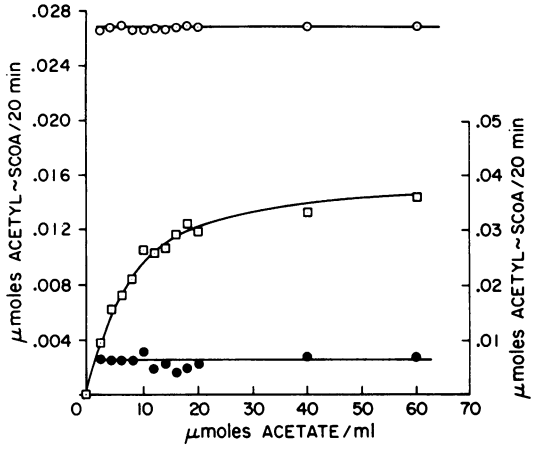

FIG. 6. *Acetate saturation curves in the presence and absence of inhibitory concentration of palmityl-CoA. Scale at left refers to aerobic preparations; scale at right refers to nonaerobic preparation. Symbols:* O, *acetyl-CoA synthetase activity from aerobic mitochondrial fraction;* ●, *same with palmityl-CoA* (4.0×10^{-5} *M*); □, *acetyl-CoA synthetase activity from microsomal fraction of nonaerobic cells.*

the kinetic aspects of the inhibitions or on the high levels of CoA esters found during diabetes or starvation (17). In all of these systems, inhibition by acyl-CoA compounds was shown to be competitive with respect to one of the components in the assay system, or else their actions were attributed to inactivation of the enzyme because of the detergent-like properties of these esters. In the case of the ACS of aerobically grown yeast cells, however, inhibition by palmityl-CoA was noncompetitive with either acetate or CoA. Irreversible inactivation due to the detergent-like properties of long-chain CoA esters (12) does not seem tenable as a mechanism in view of the fact that the corresponding free fatty acids are not inhibitory even at higher concentrations. Furthermore, the inhibitory effects can be reversed by simply diluting the inhibitory preparations. A mechanism of action that cannot be ruled out by these studies involves a regulatory role for long-chain acyl-CoA esters. This possibility is supported by the findings that palmityl-CoA gives an inhibition pattern typical of many regulatory enzymes: the response to increasing concentrations of inhibitor is sigmoidal, with a Hill coefficient of 3.24. On the basis of earlier work (DeVincenzi and Klein, Fed. Proc., p. 872, 1970), in which we estimated the molecular weight of this ACS to be approximately 130,000, it may be postulated tentatively that the ACS is, in reality, a tetrameric enzyme with a subunit molecular weight of about 30,000, because on this basis the Hill coefficient would indicate one binding site for palmityl-CoA on each subunit.

There appears to be no obvious reason why aerobic, but not nonaerobic, cells would be regulated in their metabolism of acetate by the long-chain acyl-CoA compounds tested here. However, the data are consistent with such a role. Under nonaerobic conditions, the rate of formation and the absolute amount of acetate formed during glucose degradation are very low (3). Because the K_m of the ACS of such cells for acetate is high, there would be a considerably lower rate of formation of acetyl-CoA than in aerobic cells. In nonaerobic cells, then, the rate of synthesis of lipids, including fatty acids, would be relatively slow. These factors undoubtedly play a significant role in the final low total lipid content of nonaerobic yeast cells (6). On the other hand, under aerobic conditions this yeast strain rapidly degrades glucose to ethanol and, after the glucose has essentially disappeared, the ethanol is oxidized to acetate (3). Under these conditions, considerably more acetate would be available for conversion to acetyl-CoA. We presume that substantially larger amounts of acetyl-CoA would then be condensed to long-chain acyl-CoA compounds. At the same time, we presume that under these conditions, the availability of *sn*-glycerol 3-phosphate for glyceride synthesis will be inadequate, and then, unless the acyl-CoA compounds are removed by immediate condensation to phosphatides or other glycerides, these esters (or their deacylated derivatives) may accumulate to levels that would be deleterious to functioning of vital enzymes or structures (e.g., mitochondria [2, 20]) in the aerobic cells. Accumulation of long-chain acyl-CoA compounds could then be prevented by a feedback inhibition of acetyl-CoA formation.

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