

# Cellular Localization of Acetyl-Coenzyme A Synthetase in Yeast

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In cells of *Saccharomyces cerevisiae* grown with glucose in standing cultures, the microsomal fraction had the highest specific activity for acetyl-coenzyme A synthetase and contained the greatest fraction of the total activity regardless of when the cells were harvested during growth. The addition of acetate did not affect the distribution of the enzyme, nor did subsequent aeration of such cells in phosphate buffer even in the presence of glucose, acetate, or succinate. In cells grown aerobically, however, the microsomal fraction had the highest specific activity and the greatest fraction of the total activity only until the cells reached the stationary phase. After this time, most of the activity was associated with the mitochondrial fraction. Finally, 3 or 4 days after inoculation, this fraction appeared to lose most of the enzyme to the microsomal and soluble fractions. Chloramphenicol, at concentrations that interfered with respiration but not with fermentation, prevented the association of acetyl-coenzyme A synthetase with the mitochondrial fraction in aerated cells, but it did not appreciably affect the large increases in enzyme activity observed during aerobic incubation. Cells grown with glucose under strict anaerobic conditions contained barely detectable amounts of acetyl-coenzyme A synthetase.

The formation of acetyl-coenzyme A (acetyl-CoA) from acetate is known to follow one of two routes (13). In many bacteria, the activation of acetate involves the formation of acetyl phosphate and the subsequent transfer of the acetyl group, via the enzyme phosphotransacetylase, to CoA. The direct formation of acetyl-CoA via the enzyme acetyl-CoA synthetase has been reported in *Rhodospirillum rubrum* (10), *Euglena gracilis* (1), yeasts (4, 14), and higher plants and animals (1, 3, 5, 6, 11, 18-20, 22).

No evidence concerning the probable intracellular location of this enzyme has been presented to date in reports dealing with acetyl-CoA synthetase in microorganisms. However, Miller and Bonner (18), working with various plant sources, reported that mitochondrial preparations of oat seedlings and of avocados had about five times the specific activity found in whole spinach leaves. Furthermore, they observed no activity in the extramitochondrial fractions of these same tissues. Similarly, evidence for the mitochondrial localization of this enzyme in animal cells has also been presented by several investigators. Hele (11) studied the enzyme from beef heart mitochondria. Carey and Greville (6) reported that the mitochondrial fraction of chick embryos was

comparatively rich in this enzyme; Schubert (20) found that in the rat brain the mitochondrial fraction had the highest specific activity. In a series of recent papers, Webster (22) reported the purification and crystallization of the enzyme from bovine heart mitochondria.

During studies on the subcellular sites of enzymes involved in lipid synthesis in *Saccharomyces cerevisiae*, we obtained indirect evidence suggesting that acetyl-CoA synthetase was present in the "microsomal" fraction of the cells (16). The cells used in those studies were grown in standing cultures, harvested, washed, and then aerated for 2.5 hr before being used in cell-free extracts [since these cells (15) proved to be most active in lipogenesis]. In this paper, direct assays were performed for the enzyme in such cells. Furthermore, it was of interest to determine whether the intracellular distribution of the enzymes was constant under various conditions of growth. There are large differences between the specific activity of this enzyme in anaerobically and aerobically grown cells. In addition, the data suggest that during aerobic, but not anaerobic, growth on glucose, the enzyme moves from the microsomal fraction into the mitochondrial fraction. Finally, it appears that the enzyme leaves

the mitochondria when the cells become older, but while they are still viable.

#### MATERIALS AND METHODS

*Organism used and preparation of cellular fractions.* *S. cerevisiae* strain LK2G12 was used throughout this study. Cells were grown in a medium containing 2% peptone, 2% glucose, and 1% yeast extract. In this medium, the carbon source limits the final yield of cells up to a glucose concentration of about 4%. Aerobic cells were obtained by inoculating 500 ml of medium per 2-liter flask and incubating at 30 C on a rotary shaker. Strictly anaerobic cells were grown in 2-liter flasks containing 1.7 liters of medium, which included 3 mg of ergosterol and 3 mg of oleic acid. The flasks were autoclaved, then immediately gassed with nitrogen, and incubated with a bunsen valve. Standing cultures were grown as above, but without added ergosterol or oleic acid, and were not gassed with nitrogen. Further information on the cultivation, harvesting, washing, and disruption (by using a French pressure cell) of cells has been given previously (15-17). The disrupted cells were centrifuged at  $3,300 \times g$  for 10 min to sediment the whole cells and debris, and then at  $15,000 \times g$  for 30 min to sediment a pellet herein referred to as the "mitochondrial" fraction. The supernatant fluid from this procedure was then centrifuged at approximately  $100,000 \times g$  for 60 min to obtain the microsomal (17) and soluble supernatant fractions.

*Assay of acetyl-CoA synthetase.* The method of Berg (4) was slightly modified for use in these studies. Enzyme was incubated at 37 C with the following additions per ml: CoA, 0.1  $\mu$ mole; adenosine triphosphate (ATP), 10  $\mu$ moles;  $MgCl_2$ , 10  $\mu$ moles; potassium phosphate, 100  $\mu$ moles; potassium acetate, 10  $\mu$ moles; glutathione, 10  $\mu$ moles; and hydroxylamine, 200  $\mu$ moles. The rate of acetylhydroxamic acid formation was approximately 1.2 times greater at 37 C than at 30 C; therefore, the standard assay was performed at the higher temperature. The enzyme could be kept frozen for several days without appreciable loss of activity.

Proteins were determined by the microbiuret procedure with the reagent described by Zamenhof (23). ATP and CoA were purchased from Boehringer Mannheim Company, New York, N.Y.

#### RESULTS

*Some properties of the enzyme.* A crude homogenate was used for determining the optimal conditions for the assay. The reaction depended upon added CoA, saturation occurring at approximately 0.2 mM. Similarly, ATP was required, saturation occurring at about 8 mM. Both magnesium and manganese were tested for their ability to serve as metal cofactors and were equivalent at a concentration of approximately 10 mM. Jones et al. (14) used potassium fluoride, presumably to inhibit adenosine triphosphatase activity, when they originally described this

enzyme from yeast, but in our preparations it was unnecessary. The addition of fluoride at the concentration used in their investigations (50 mM) inhibited acetylhydroxamic acid formation by about 25% (Fig. 1). We added inorganic phosphate because relatively high concentrations increased the rate of color formation (Fig. 2). An optimal concentration (0.1 M) doubled the rate. Glutathione, although not required for the reaction, was added because it was somewhat stimulatory. Under the conditions of the standard assay, the reaction proceeds at a linear rate for at least 60 min provided the rate of acetylhydroxamate production is kept below 16 nmoles/min. The zero-order kinetics allowed ready comparisons to be made between different preparations.

*Distribution of enzyme in cells grown anaerobically, aerobically, and in standing cultures.* Cells from standing cultures (harvested after 48 hr of incubation) have been used in many of our previous investigations; earlier experiments (16) involving an indirect assay suggested that the enzyme was present in the microsomal fraction. This fraction did indeed have the highest specific activity (Table 1). Also, about three-fourths of the total activity of these extracts was found in this fraction, with only minor amounts in the mitochondrial and supernatant fractions. Essentially, the same specific activities and distribution of enzyme activity were found in cells grown in standing cultures whether they were aerated for 2.5 hr after they were harvested (15), a procedure we have routinely used in our studies on lipogenesis. However, when cells were grown under strictly anaerobic conditions for the same length of time, crude extracts had much lower levels of activity. All the fractions from strictly anaerobic cells had considerably lower specific activities than the corresponding fractions obtained from standing

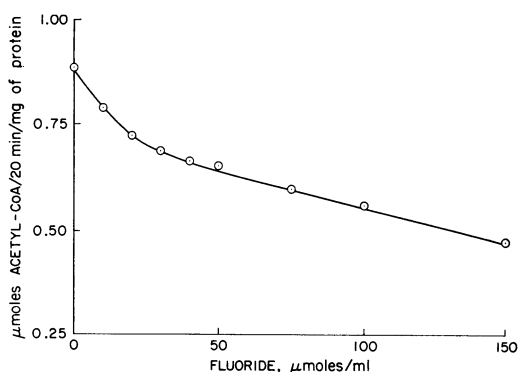


FIG. 1. Effect of fluoride on acetyl-CoA formation. A crude homogenate of cells from a standing culture was assayed with potassium fluoride additions.

cultures. Indeed, the activity of such strictly anaerobic cells was so low that the results of assays were on the border line of significance. For this reason, in later experiments, aerobic cultures were compared with standing cultures. The activity in preparations from cells harvested after 48 hr of aerobic growth was greater than in extracts from standing cultures and much greater than that found in strictly anaerobic cells. Furthermore, the distribution of enzymes in the aerobic cells differed significantly from that observed in standing cultures in that about 80% of the total activity was found in the mitochondrial fraction and only 20% in the microsomal fraction.

*Effects of aeration on the activity and distribution of the enzyme.* Since the results cited above indicated a relationship between aeration and the activity and distribution of the enzyme, it was of interest to determine whether anaerobically grown cells would be affected by subsequent aeration. For this purpose, resting cellular suspensions obtained from standing cultures were aerated in 0.1 M phosphate buffer for 24 hr after they were harvested. These cells showed no

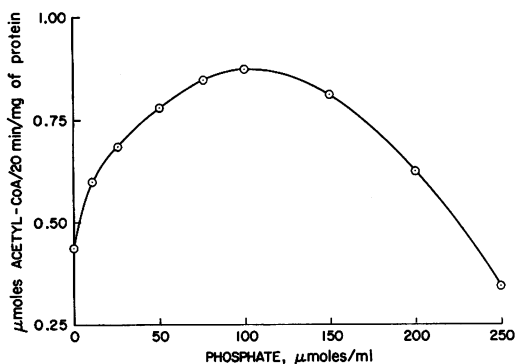


FIG. 2. Effect of phosphate on acetyl-CoA formation. Method as in Fig. 1, but without added fluoride.

changes in the distribution of the enzyme among the major cellular fractions, whether they had been aerated in the absence of an energy source or in the presence of 2% glucose, 0.5% succinate, or 0.5% acetate.

In contrast, when cells were grown under aerobic conditions and were tested for acetyl-CoA synthetase, a rather complex behavior was observed. First of all, there was a steady increase, with time of incubation, in the specific activity of the enzyme. Furthermore, wide variations occurred in the cellular distribution of the enzyme during incubation. During the exponential phase of growth, most of the enzyme sedimented with the microsomal fraction, and very little was found in the mitochondrial fraction. After the cells entered the stationary phase, however, large increases in the activity of the mitochondrial fraction were observed (Table 2). After 24 hr of incubation, approximately 80% of the total enzyme activity was in the microsomal fraction, and only negligible amounts in the mitochondrial material. However, after the cells entered the stationary phase, there was a sharp increase in the amount of enzyme that sedimented with the mitochondria, so that at 48 hr about 70% of the activity was now in this fraction, and the per cent of total activity in the microsomal material decreased correspondingly. Upon prolonged incubation, the specific activity of the mitochondrial fraction, as well as its total enzymatic activity, fell to a relatively low level once again. In other experiments of this type, plate counts made at intervals throughout the 96-hr growth period showed no decrease in the number of viable cells over the entire period of incubation, thus ruling out general cellular degradation as an explanation of the effects noted upon prolonged incubation.

Cells grown aerobically with 0.5% sodium acetate as the carbon source, instead of 2% glucose, gave results similar to those of glucose-

TABLE 1. Acetyl-CoA synthetase activity of strict anaerobic, standing, and aerobic cultures of *S. cerevisiae*

Fraction	Strict anaerobic cells				Standing-culture cells				Aerobic cells			
	Specific activity <sup>a</sup>	Total protein	Total activity <sup>b</sup>	Per cent of total activity	Specific activity	Total protein	Total activity	Per cent of total activity	Specific activity	Total protein	Total activity	Per cent of total activity
		mg				mg				mg		
Mitochondrial . . . . .	0.05	254	12	9	0.27	151	42	5	2.10	740	1,560	78
Microsomal . . . . .	0.04	740	33	27	0.78	900	708	76	1.02	414	423	20
Soluble supernatant . . . . .	0.08	1,010	78	63	0.24	750	177	19	0.05	1,080	57	3

<sup>a</sup> Expressed as micromoles of acetyl-CoA produced per milligram of protein in 20 min.

<sup>b</sup> Expressed as specific activity  $\times$  total protein.

TABLE 2. *Acetyl-CoA synthetase activity in S. cerevisiae grown aerobically*

Incubation time	Growth <sup>a</sup>	Mitochondrial fraction		Microsomal fraction		Supernatant fraction	
		Specific activity <sup>b</sup>	Per cent of total activity	Specific activity	Per cent of total activity	Specific activity	Per cent of total activity
hr							
6	0.9	0.10	2	0.75	80	0.22	18
12	2.4	0.15	2	0.50	75	0.20	23
24	2.7	0.22	7	1.20	84	0.10	8
48	2.8	2.25	72	1.15	18	0.10	10
72	2.8	2.70	44	2.10	38	0.70	18
96	2.8	0.66	8	2.85	60	1.15	32

<sup>a</sup> Expressed as log of Klett reading of suspension at 660 nm; 1 mg (dry wt) per ml equivalent to 215 Klett units.

<sup>b</sup> Expressed as micromoles of acetyl-CoA produced per milligram of protein in 20 min.

grown cells. The microsomal fraction obtained from acetate-grown cells harvested in the exponential and early stationary phases (*i.e.*, up to approximately 24 hr) contained about 70% of the total activity, whereas the mitochondrial fraction contained less than 10%. Then, as with glucose-grown cells, there were dramatic increases (to about 80%), in the total enzyme activity observed in the mitochondrial fraction of cells harvested between 24 and 48 hr.

A striking feature of these growth experiments with glucose or acetate is that the total activity of this enzyme continued to increase in cultures well after the time at which the cells enter the stationary phase. The greatest increase in enzyme activity occurs after the cells have stopped multiplying. In Fig. 3 are plotted the results of two experiments, one with glucose and the other acetate. The total enzyme activity of the crude homogenates (containing mitochondria, microsomes, and soluble supernatant fluid) is shown as a function of the protein content of these extracts.

Since the experiments described above indicated that the distribution of this enzyme changed significantly in aerobic cultures after the cells entered the stationary phase, standing cultures were also incubated for prolonged periods to see whether a similar phenomenon occurred. Washed cells were fractionated and assayed daily over a 72-hr period, but no change in distribution of enzyme was observed. Other standing cultures were incubated in the presence of 0.1% acetate, in addition to glucose, to test the possibility that the accumulation of acetate [which is known to occur when these cells are metabolizing glucose aerobically (9)] might be responsible for an induction of this enzyme in aerobic cultures. However, the addition of acetate under these conditions did not increase the specific activity of

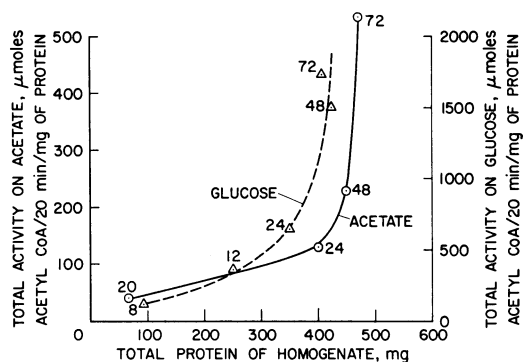


FIG. 3. Total activity of acetyl-CoA synthetase in extracts of *S. cerevisiae* growing aerobically with glucose or 0.5% acetate. Cells were grown on a shaker, and at various times, assayed for total protein and total enzyme activity in crude homogenates. Numbers refer to hours of incubation at time of preparation of extracts.

the enzyme in any of the fractions (above that found in the absence of acetate), and still yielded cells with the preponderance of activity in the microsomal fraction.

*Effects of chloramphenicol on the distribution of acetyl-CoA synthetase.* Huang et al. (12) recently reported that cells of *S. cerevisiae* grown in the presence of chloramphenicol under aerobic conditions had virtually no respiratory activity. They showed that these cells contained lowered amounts of cytochromes *aa*<sub>3</sub> and *b* and reduced respiratory activity. Furthermore, typical mitochondrial profiles were not observed in electron micrographs. They suggested that chloramphenicol might have a specific inhibitory effect upon the synthesis of functional mitochondria [see also Clark-Walker and Linnane (7)]. Since, in our

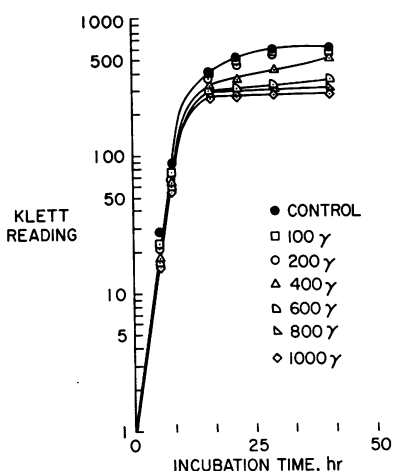


FIG. 4. Growth rates of *S. cerevisiae* in the presence of chloramphenicol. Cells were cultured aerobically with glucose and additions, as shown; chloramphenicol concentrations refer to micrograms per milliliter.

experiments, the acetyl-CoA synthetase seemed to become associated with the mitochondrial fraction during aerobic growth, a study was made of the effects of chloramphenicol on the activity and distribution of this enzyme.

Preliminary experiments showed that concentrations of chloramphenicol well below the 4 mg/ml used by Huang et al. (12) significantly inhibited the respiratory activity of this strain, but had no apparent effect on the growth rate of the cells. In Fig. 4 are shown growth curves of cultures exposed to several concentrations of chloramphenicol up to a maximum of 1,000  $\mu\text{g}/\text{ml}$ . In experiments of this type, it was observed that respiratory rates of this strain of *S. cerevisiae* were inhibited by about 50% at a chloramphenicol concentration of 400  $\mu\text{g}/\text{ml}$ , and were virtually completely inhibited at about 800  $\mu\text{g}/\text{ml}$  and above. Chloramphenicol had no deleterious effect on the fermentation rates of these cells. Indeed,  $\text{CO}_2$  evolution seemed to be consistently elevated in chloramphenicol-treated cells (Table 3).

Cells were grown with aeration for a period of 75 hr in two different concentrations of chloramphenicol, one in which respiration was maximally inhibited (800  $\mu\text{g}/\text{ml}$ ), and the other in which it was partially inhibited (400  $\mu\text{g}/\text{ml}$ ). At various times, samples were removed, washed, and tested for oxygen uptake and for carbon dioxide evolution. Simultaneously, additional samples of each culture were fractionated and assayed for acetyl-CoA synthetase in the major cellular subfractions. Table 3 tabulates the results obtained with whole cells. The initial growth rates of both chloramphenicol-treated cultures were

similar to that of the control. Whereas the control culture reached the stationary phase at about 24 hr, growth in the culture containing 400  $\mu\text{g}$  of chloramphenicol per ml slowed down between 24 and 48 hr before reaching the stationary phase; growth in the culture containing 800  $\mu\text{g}$  per ml was very slight after the initial exponential phase. The data in Table 3 also illustrate the partial inhibition of respiration at 400  $\mu\text{g}/\text{ml}$  and the severe inhibition at the higher concentration of chloramphenicol. In addition, the rates of fermentation of cells grown in the presence of chloramphenicol were comparable to those of the control.

When the cells were homogenized and assayed for acetyl-CoA synthetase at the intervals noted, it was observed that chloramphenicol had no pronounced effect on the overall specific activity of the enzyme in the crude homogenates. On the other hand, chloramphenicol treatment caused profound changes in the intracellular distribution of the enzyme. Figure 5 shows the distribution of acetyl-CoA synthetase in the three extracts. In the control, as usual, the bulk of the activity was found in the microsomal fraction until the cells entered the stationary phase. Thereafter, the mitochondrial fraction contained most of the enzyme, and upon prolonged incubation, the enzyme was again found in increasing yields in the microsomal fraction. The cells grown in 400  $\mu\text{g}$  of chloramphenicol per ml also yielded a mitochondrial fraction rich in this enzyme, but this enrichment was delayed by an additional period of about 24 hr, compared to that of the controls, approximating the delay in reaching the stationary phase. At the chloramphenicol concentration that maximally inhibited respiration, the mitochondrial fraction never accounted for more than a small portion of the total activity of the crude homogenate, whereas the microsomal fraction contained 80 to 90% of the activity throughout the experimental period.

#### DISCUSSION

The studies cited here indicate wide variations in the amount and in the cellular distribution of acetyl-CoA synthetase in *S. cerevisiae*. Cells grown under strictly anaerobic conditions contain barely detectable amounts of the enzyme, as assayed by the methods used in this paper, whereas standing cultures yielded extracts with higher specific activities for this enzyme. In the latter extracts, the microsomal fraction consistently contained most of the total activity, as well as the highest specific activity. This was true regardless of when the cells were assayed over a period of several days. The addition of acetate to such standing cultures also apparently had no effect on

TABLE 3. Effect of chloramphenicol on growth, respiration, and activity of acetyl-CoA synthetase of *S. cerevisiae* during aerobic incubation

Incubation time	Growth <sup>a</sup>			Respiration <sup>b</sup>			Fermentation <sup>c</sup>			Acetyl-CoA synthetase <sup>d</sup>		
	Control	CAP <sup>e</sup>		Control	CAP		Control	CAP		Control	CAP	
		400	800		400	800		400	800		400	800
hr												
10	151	141	130	22	11	2	458	480	498	0.56	0.55	0.67
12	345	315	300	47	23	3	476	502	546	0.45	0.76	0.80
24	560	410	334	74	21	0	320	334	378	1.36	1.08	0.89
36	620	525	375	65	40	0	256	3,0	358	1.49	1.46	0.99
48	620	620	395	65	36	0	227	243	331	1.11	1.30	1.05
72	620	620	455	58	32	10	198	194	220	1.25	1.01	1.05

<sup>a</sup> Expressed as Klett reading of cellular suspension at 660 nm (see Table 2).

<sup>b</sup> Rate of O<sub>2</sub> uptake of washed cells, expressed as microliters per milligram (dry weight) per hour, determined manometrically in air at 30 C with 0.11 M glucose as substrate.

<sup>c</sup> Rate of CO<sub>2</sub> evolution of washed cells, expressed as microliters of CO<sub>2</sub> per milligram (dry weight) per hour, determined manometrically under He at 30 C with 0.11 M glucose as substrate.

<sup>d</sup> Expressed as micromoles of acetyl-CoA synthesized per milligram of protein (of crude total homogenate) in 20 min under standard conditions.

<sup>e</sup> CAP, chloramphenicol; numbers refer to micrograms per milliliter.

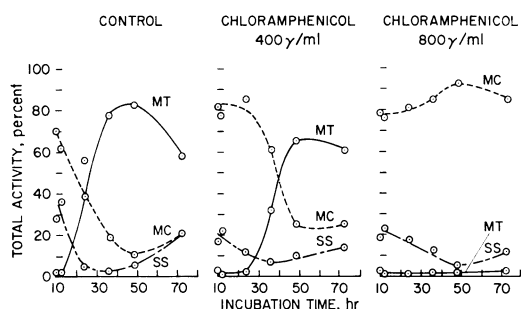


FIG. 5. Effect of chloramphenicol on acetyl-CoA synthetase distribution. Cells were grown with aeration in the presence and absence of chloramphenicol, as indicated. At the intervals shown, samples of each suspension were removed, washed, and disintegrated. After differential centrifugation, the fractions were assayed for acetyl-CoA synthetase. MT refers to the mitochondrial fraction; MS, the microsomal fraction; and SS, the soluble supernatant fraction.

the specific activity and distribution of the enzyme.

The highest activities of acetyl-CoA synthetase were observed in aerobically grown cells. Subsequent aeration of cells grown in standing cultures did not materially affect the activity and distribution of the enzyme. Why growth under aerobic conditions is necessary to elicit the highest specific activities is not known. Nor is it evident why, under aerobic conditions, the apparent total activity per cell continues to increase well after the cessation of growth. Whether this is a reflection of increases in enzyme protein cannot be

stated at this time. It is possible that over the course of incubation, the conformation of acetyl-CoA synthetase changes with time, causing the same protein molecule to be more active. The production of activators or of allosteric modifiers during growth under aerobic conditions is also possible. Increases in enzyme activity observed under these conditions may also be the result of the formation of several distinct acetyl-CoA synthetases. During the early part of growth, for example, a synthetase of relatively low activity may be formed, and later another synthetase of higher specific activity may appear in these extracts. Clearly, detailed studies must be carried out on purified synthetase, obtained from cells at different stages of growth and, indeed, from different cellular components, to decide among these possibilities.

Observations on the cellular distribution of the synthetase indicate that this enzyme, in standing cultures, is largely attached to some component of the microsomal fraction of the cells. Earlier studies with a different assay system (16) resulted in the conclusion that this enzyme is probably localized in the membrane component of this fraction. The microsomal fraction contains most of the enzyme also in aerobically grown cells, at least until after the cells enter the stationary phase. Vanderwinkel (21), in a study of yeast oxidative metabolism, also noted little or no acetyl-CoA synthetase activity in the mitochondrial fraction of cells grown on glucose or on acetate and harvested when they had just reached the stationary phase.

The results with aerobic cells suggest that on continued incubation in air, the enzyme moves from the microsomal to the mitochondrial fraction. Other interpretations, based on variations either in mitochondrial size or fragility, would appear to be untenable, since the mitochondrial enzymes, succinic dehydrogenase, and cytochrome oxidase, do not show this change in intracellular distribution (H. P. Klein and L. Jahnke, *unpublished data*).

When cells were treated with chloramphenicol, there was no marked inhibition in the total activity of this enzyme per cell, although chloramphenicol in some way prevented acetyl-CoA synthetase from becoming associated with the mitochondrial material. If the contentions of Clark-Walker and Linnane (7, 8) are correct, and chloramphenicol inhibits the mitochondrial protein-synthesizing machinery, our results would indicate that acetyl-CoA synthetase is not synthesized by the mitochondria even in aerobic cells. These investigators contend that chloramphenicol primarily affects the formation of the inner-membrane system of the mitochondria of *S. cerevisiae*, because they found that mitochondrial profiles of chloramphenicol-treated cells contained few or no cristae. Since our data indicate that acetyl-CoA synthetase is not made in the mitochondria, in that its formation is not inhibited by chloramphenicol as are several known inner-membrane enzymes such as succinic dehydrogenase and cytochrome oxidase (8), one might conclude that the enzyme normally attaches to the outer membrane of mitochondria late in the growth cycle. However, chloramphenicol treatment apparently (8) does not affect the outer membranes of yeast mitochondria, at least as far as their morphological appearance in the electron microscope is concerned. Thus, if this membrane were the normal site of association of acetyl-CoA synthetase, chloramphenicol-treated cells should still yield a mitochondrial fraction rich in this enzyme, and this was not found to be the case. Of course, this contention assumes that in chloramphenicol-grown cells of *S. cerevisiae* morphologically intact outer mitochondrial membranes are physiologically normal, a point which has not yet been established. The site of association of acetyl-CoA synthetase with the mitochondrial fraction cannot be identified unequivocally until clean mitochondria are isolated and then are separated into inner- and outer-membrane fractions, as has been demonstrated for several mammalian systems (2).

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