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Variations in the Localization of Acetyl-Coenzyme A Synthetase in Aerobic Yeast Cells

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In cells of *Saccharomyces cerevisiae* growing aerobically for 24 hr, acetyl-coenzyme A synthetase [acetate: CoA ligase (AMP), EC 6.2.1.1] was localized principally in the microsomal fraction. On density gradients, the enzyme in such cells behaved as a low-density particle, readily separable from the soluble proteins. After 48 hr of incubation, the cells showed a bimodal distribution of enzyme, with most of the activity now sedimenting with the mitochondrial fraction and only a smaller amount with the microsomal fraction. By using density gradients, two forms of synthetase were obtained from these cells: one band denser and the other band less dense than the intact mitochondria. In all preparations containing synthetase activity, appreciable levels of phospholipids were also detected.

Upon differential centrifugation of homogenates obtained from several plant and animal tissues, the enzyme acetyl-coenzyme A (acetyl-CoA) synthetase [acetate: CoA ligase (AMP), EC 6.2.1.1] sediments primarily with the mitochondrial fraction, and on this basis the enzyme has been regarded as a mitochondrial enzyme in these sources (15, 23). However, recent investigations utilizing density gradient separations have suggested that this enzyme may not be directly associated with mitochondria. For example, Neidle et al. (16) were able to separate this enzyme from mitochondrial enzymes of rat brain preparations on density gradients. Similarly, acetyl-CoA synthetase could be separated from mitochondrial markers in extracts of *Tetrahymena* (11). In the yeast *Saccharomyces cerevisiae*, we earlier reported wide divergences in the distribution of this enzyme among the various cellular fractions, depending upon growth conditions (8). In cells grown in standing cultures, the greatest portion of enzymatic activity sedimented with the microsomal fraction at all stages of growth. In cells grown aerobically, however, the location of the enzyme depended on the stage of growth. In early cultures acetyl-CoA synthetase sedimented with the microsomal fraction, and, after the cells reached the stationary phase of growth, the enzyme was almost entirely sedimentable with the mitochondrial material. Because the previous study suggested the possibility that during the transition from exponential to stationary phase the acetyl-CoA synthetase might become bound to fully functional mitochondria in aerobically grown cells, the present study was directed at a critical assessment of this possibility. In addition, it was of interest to obtain information concerning the site to which the enzyme may bind when it is found in the microsomal fraction. In the present study, aerobic cells were fractionated at different stages of growth, and the resultant cellular fractions were subjected to density gradient analyses.

MATERIALS AND METHODS

S. cerevisiae strain LK2G12 was grown aerobically as described previously (7, 8) and was homogenized, at various intervals after inoculation, in a Braun homogenizer (4, 6, 14) by the method of Schatz (19). Under the conditions of these experiments, the exponential phase of growth extends until about 18 hr after inoculation, and cells remained completely viable in the stationary phase until at least 96 hr (8). Crude homogenates, contained in a buffer consisting of 250 mM mannitol, 2 mM tris(hydroxymethyl)aminomethane (Tris) *p*H 7.4, and 2 mM $MgCl_2$, were subsequently fractionated (7) into mitochondrial, microsomal, and soluble supernatant fractions.

Density gradient analyses were performed by using 1-ml suspensions of cellular fractions. When 3-ml fractions were to be collected from gradients, the sample to be centrifuged was suspended in 1 M sorbitol, 2 mM Tris, and 2 mM $MgCl_2$ (*p*H 7.4) and applied to the top of 25 ml of a linear gradient of 30 to 60% (w/w) sorbitol with a 3-ml cushion of 62% sorbitol prepared in the same buffer. Such gradients were centrifuged at an average force of 56,000 × *g* for 60 min. Equilibrium density gradients of this type run for extended periods (up to 16 hr under these conditions) did not change the position of the major fractions, and consequently the

shorter centrifugation period was routinely used. For those gradients in which 1-ml fractions were to be collected, a 1-ml sample suspended in the mannitol-Tris-$MgCl_2$ buffer was added to the top of 25 ml of a linear gradient of 15 to 40% (w/w) sorbitol with a cushion of 3 ml of 42% sorbitol prepared in the same buffer. These were centrifuged for 16 hr as above. All gradient centrifugations were carried out by using a model L Spinco centrifuge equipped with an SW25.1 rotor. Fractions were collected with an ISCO fraction collector.

Acetyl-CoA synthetase activity was determined by methods described previously (8). Specific activities of this enzyme are expressed as micromoles of acetyl-CoA formed per 20 min per mg of protein. Other enzymes of interest in this study were measured by the following procedures: succinic dehydrogenase (EC 1.3.99.1), by the method of Arrigoni and Singer (2), expressed as micromoles of succinate oxidized per minute; cytochrome oxidase (EC 1.9.3.1), expressed as micromoles of oxygen consumed per minute, by a modification of the method of Umbreit et al. (22); Nicotinamide adenine dinucleotide phosphate (NADP)-isocitrate dehydrogenase (EC 1.1.1.42), by the method of Sottocasa et al. (21); and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), as described by Kornberg and Horecker (10) and expressed as micromoles of reduced NADP produced per 5 min. Protein was determined as described by Zamenhof (24). The location of membranes was followed by determining the radioactivity of lipids isolated from various cellular fractions after growth in medium containing ^{14}C-choline as previously described (9).

RESULTS

Correlation of acetyl-CoA synthetase activity with succinic dehydrogenase and cytochrome oxidase. Cells of *S. cerevisiae* were homogenized at different times during aerobic growth, and the three fractions described above were obtained by differential centrifugation. As is seen in Fig. 1, the distribution of acetyl-CoA synthetase under these conditions showed variations depending on the stage of growth. Most of the enzymatic activity was found in the microsomal fraction at 24 hr (corresponding to the early stationary phase). Subsequently, however, enzyme activity became primarily associated with the mitochondrial fraction until late in the incubation period. During the course of incubation, the relative protein content of each of the cellular fractions remained approximately constant with time, indicating no particular change in fragility of the cellular fractions. Furthermore, it is apparent that the mitochondria were not significantly fragmented by this method of breakage at any stage of incubation, since about 85% of the total succinic dehydrogenase and cytochrome oxidase activities remained with the mitochondrial fraction throughout the experimental period. The small amounts of these enzymes found in the microsomal fraction are consistent with the findings of Matile and Bahr (13) and Schatz et al. (20), who

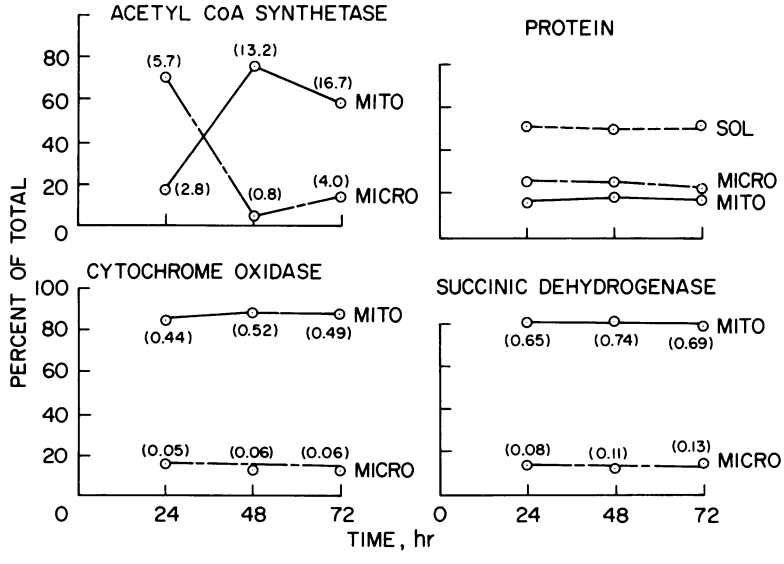

FIG. 1. *Distribution of acetyl-CoA synthetase and mitochondrial marker enzymes in cells of Saccharomyces cerevisiae. MITO, mitochondrial fraction (pellet obtained at 15,000 × g after 30 min); MICRO, microsomal fraction (pellet obtained from latter supernatant after centrifuging at 100,000 × g for 60 min); SOL, soluble supernatant (obtained after latter centrifugation). Figures in parentheses refer to specific activities of enzymes. Activities of mitochondrial marker enzymes and of acetyl-CoA synthetase were negligible in all SOL fractions and are omitted.*

have reported some fragmentation of yeast mitochondria by using this method of mechanical rupture.

Density gradient analyses of mitochondrial fraction obtained from cells in the stationary phase. When mitochondrial pellets from 48-hr-old cells were examined in the electron microscope, the mitochondrial fraction was found to be heterogeneous. Whereas most of the mass of this fraction could be accounted for as intact mitochondria, a number of other structures, notably membranes, were also present in such preparations (Fig. 2). Accordingly, to fractionate this material further, mitochondrial pellets were subjected to density gradient analysis on linear sorbitol gradients. The use of sucrose for this purpose proved to be of limited value since acetyl-CoA synthetase activity was poorly recovered from gradients prepared with that carbohydrate. For these experiments, cells were grown for 48 hr with aeration in the presence of ^{14}C-choline to label cellular membranes.

After obtaining the mitochondrial fraction, a portion of this material was applied to linear gradients as described above. Fractions (3 ml) were collected after centrifugation, and each fraction was assayed for protein, radioactive phospholipids, succinic dehydrogenase, and acetyl-CoA synthetase. Protein and phospholipids were distributed throughout the gradient (Fig. 3). Succinic dehydrogenase activity showed a peak in fraction 5, at a density of approximately 1.186 g/ml, whereas most of the synthetase activity sedimented to a region of higher density, with virtually no synthetase activity associated with the mitochondrial marker enzyme. A small amount of the acetyl-CoA synthetase appeared near the top of the gradient in a region of lower density. Results entirely analogous to these were obtained in other experiments using cytochrome oxidase as the mitochondrial marker enzyme.

Assays for NADP-isocitrate dehydrogenase. Since both cytochrome oxidase and succinic dehydrogenase are firmly bound to mitochondrial membranes (6, 12), there was still a possibility in these experiments that acetyl-CoA synthetase, bound to some other particulate entity within the mitochondria, was released from mitochondria during or after the disruption of the yeast cells. To test more critically whether the integrity of the mitochondria had been significantly reduced by the procedures used here, tests were performed for NADP-isocitrate dehydrogenase. This enzyme has a bimodal distribution in *S. cerevisiae* (17, 18), and Perlman and Mahler, using sphaeroplast preparations, have shown that about 15% of the total found in yeast homogenates is clearly localized in the mitochondria (18). In a series of assays for this enzyme, we found a similar bimodal distribution between the soluble and mitochondrial fractions, the latter accounting for 12 to 20% of the total. Further, when the mitochondrial fraction was subjected to density gradient analysis, the bulk of NADP-isocitrate dehydrogenase was recovered in the same region as succinic dehydrogenase (Fig. 4). Finally, when suspensions of the mitochondrial fraction were subjected to sonic treatment for 30 sec, or were simply resuspended in distilled water for 30 min, only traces of the initial NADP-isocitrate dehydrogenase could be sedimented by centrifugation at 15,000 $\times$ *g* for 30 min, whereas virtually all of the succinic dehydrogenase was still sedimentable.

These results all support the conclusion that the methods of preparation utilized in the current study do not lead to significant fragmentation of the mitochondria and, therefore, are consistent with the contention that the acetyl-CoA synthetase found in such preparations (Fig. 3) is not derived from the mitochondria as a result of mechanical damage.

Density gradient analyses of the microsomal fraction. Microsomal preparations obtained from either 24- or 48-hr-old cells, when subjected to density gradient analysis similar to that discussed above, showed only one band of acetyl-CoA synthetase activity. This band always sedimented to the same position as the minor, low-density component seen in mitochondrial preparations. In Fig. 5, data are presented for a microsomal preparation from cells that had been grown in the presence of ^{14}C-choline. The fractions were assayed for protein, synthetase activity, radioactive lipids, and succinic dehydrogenase activity. The latter enzyme was present in low specific activity in this cellular fraction (see also Fig. 1) and, since it probably represents fragmented mitochondrial material, it was followed on the gradient to ascertain whether the low-density component of acetyl-CoA synthetase might be carried on such fragments. In this case, the acetyl-CoA synthetase again banded slightly below the top of the gradient and was clearly separated from the residual succinic dehydrogenase activity. Although the synthetase was close to the top of the gradient, it did not quite coincide with the position of the soluble proteins. Since radioactive phospholipids spanned this region of the gradient, these results suggest that both the synthetase and dehydrogenase are associated with membrane-containing components of the cell.

Further resolution of the low-density component of acetyl-CoA synthetase. From the data presented above, it is evident that the low-density

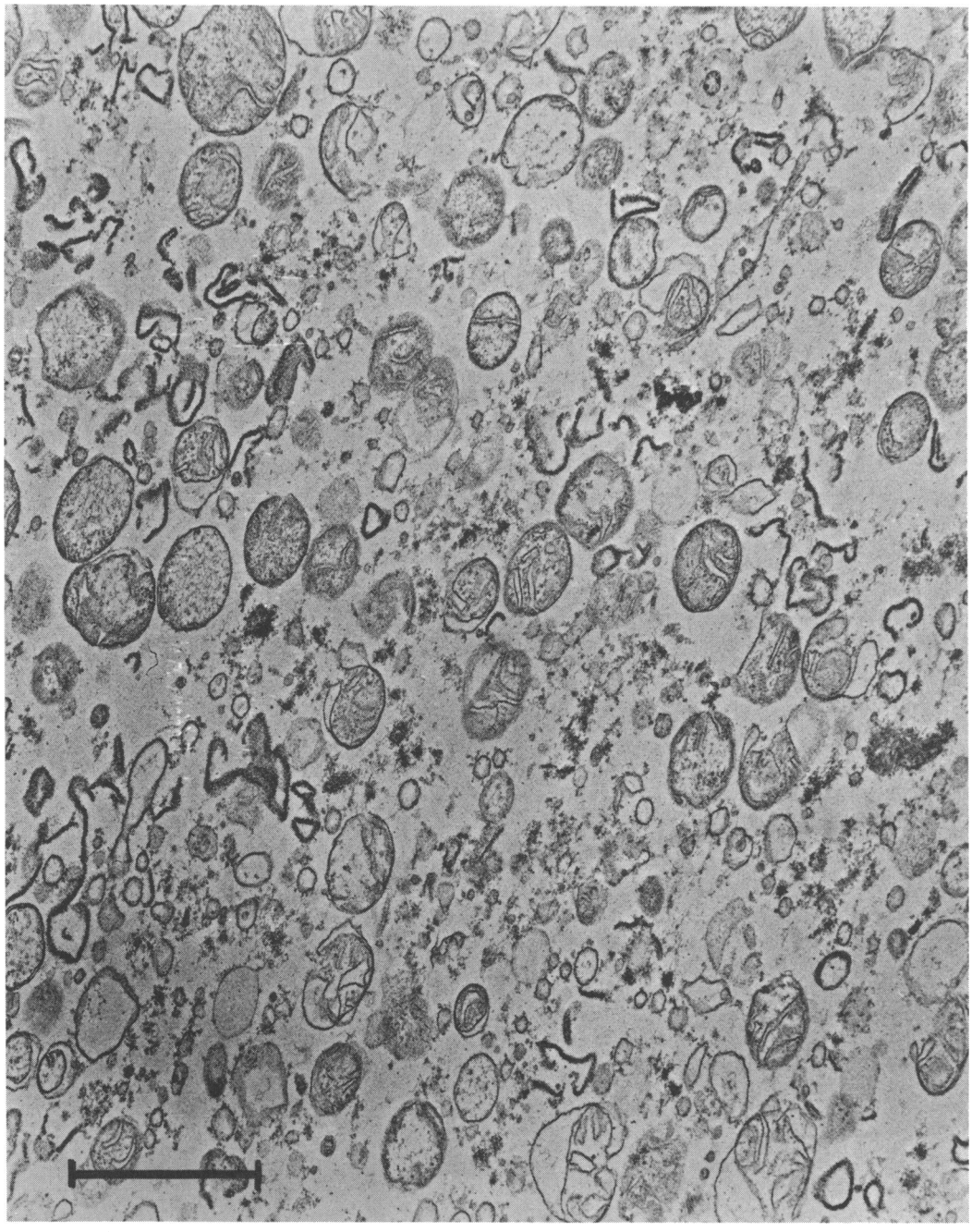

FIG. 2. *Electron micrograph of mitochondrial fraction of Saccharomyces cerevisiae cells grown for 48 hr. Pellet was obtained as in Fig. 1. For electron microscopy, the pellet was fixed with glutaraldehyde and osmium tetroxide, sectioned, and then stained with uranyl acetate and lead hydroxide (9). Line indicates 500 nm. MITO pellets at all stages of growth showed similar features.*

component of acetyl-CoA synthetase bands close to the top of linear 30 to 60% sorbitol gradients. To clarify the point whether this component is in fact soluble, gradients of lower density were prepared and subjected to centrifugation for 16 hr to achieve density equilibration, and 1-ml fractions were collected for a better resolution of the components. By using these procedures, the low-den-

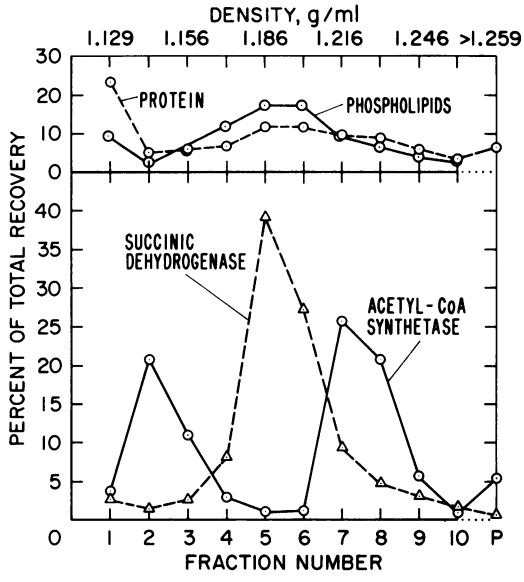

FIG. 3. *Distribtuion of acetyl-CoA synthetase and succinic dehydrogenase of the mitochondrial fraction from 48-hr cells of Saccharomyces cerevisiae on sorbitol gradient. A 1-ml amount containing 18 mg of protein was used in this analysis. Initial specific activities were: acetyl-CoA synthetase, 8.8; succinic dehydrogenase, 0.91. Fractions (3 ml) were collected after 60 min of centrifugation. P refers to a pellet that accumulated at the bottom of the tube. Recovery of each component analyzed was at least 75%.*

sity component of acetyl-CoA synthetase was readily resolved from the soluble material. In the experiment summarized in Fig. 6, a crude homogenate of 24-hr-old cells was treated as described. Assays were performed also for glucose-6-phosphate dehydrogenase, a soluble enzyme in this organism (20). It is apparent from Fig. 6a that the soluble proteins, including glucose-6-phosphate dehydrogenase, banded together near the top of the gradient. On the other hand, the acetyl-CoA synthetase came to equilibrium well below this band in the gradient, except for a small amount which appears to be soluble. The synthetase was also clearly separable from the ribosomal material which, in these gradients, banded at a density of approximately 1.13 to 1.15 g/ml.

The major synthetase band in these preparations could be made to move into the position of the glucose-6-phosphate dehydrogenase band on these gradients, taking advantage of the observation that treatment of suspensions of either mitochondrial or microsomal fractions with 100 mM phosphate buffer resulted in complete solubilization of acetyl-CoA synthetase (D. L. DeVincenzi and H. P. Klein, Fed. Proc., p. 872, 1970). For this purpose, a sample of the homogenate used in Fig. 6a was adjusted to 100 mM potassium phosphate (*p*H 7.4) and kept at 0 C for 20 min prior to density gradient analysis. As shown in Fig. 6b, all of the acetyl-CoA synthetase of the homogenate now appeared within the soluble protein peak.

DISCUSSION

From the data presented here, it appears that acetyl-CoA synthetase may be present in either of two cellular components in aerobically grown cells of *S. cerevisiae*. One of these sediments as a low-density particle on sorbitol gradients and the other is denser than the intact mitochondria of this organism. The denser component develops after the cells have reached the stationary phase of growth and may, in some way, be related to mitochondrial development, since chloramphenicol prevents the accumulation of the enzyme in the mitochondrial fraction (8). A similarly dense acetyl-CoA synthetase component appears to be present in preparation of rat brain according to Neidle et al. (16), who demonstrated that acetyl-CoA synthetase, which was present in crude mitochondrial fractions to the extent of 80% of the total activity of the brain homogenates, sedimented on sucrose gradients below succinic dehydrogenase. From his experiments with *Tetrahymena*, Levy (11) postulated that this enzyme was located primarily, if not exclusively, on peroxi-

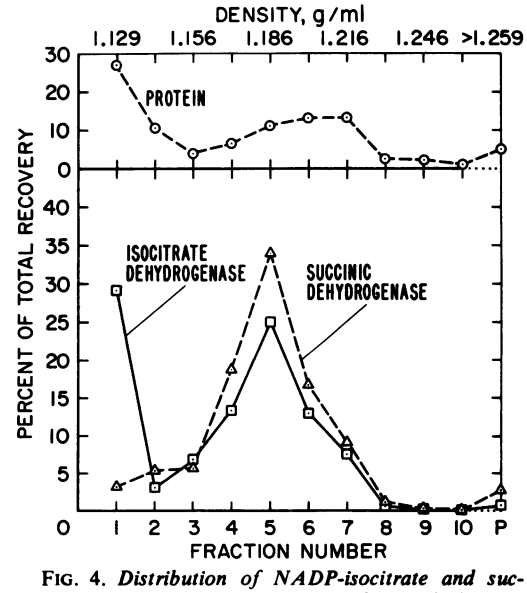

FIG. 4. *Distribution of NADP-isocitrate and succinic dehydrogenases of the mitochondrial fraction from 48-hr cells on sorbitol gradient. A 1-ml amount of the suspension containing 10.2 mg of protein was used. Fractions (3 ml) were collected after 60 min of centrifugation. Recovery of each component analyzed was at least 78%.*

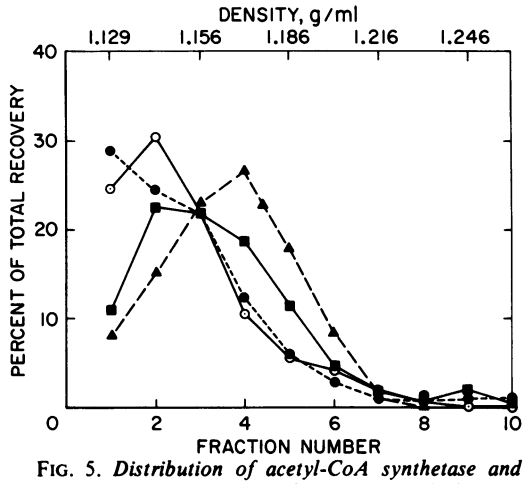

FIG. 5. *Distribution of acetyl-CoA synthetase and succinic dehydrogenase of the microsomal fraction from 24-hr cells of Saccharomyces cerevisiae on sorbitol gradient. A 1-ml amount containing 22.8 mg of protein was used. Initial specific activities were: acetyl-CoA synthetase, 6.3; succinic dehydrogenase, 0.05. Fractions (3 ml) were collected after 60 min of centrifugation. Symbols: synthetase (○); total protein (●); succinic dehydrogenase (▲); radioactive phospholipids (■). Recovery of each component analyzed was at least 75%.*

somes which, he claimed, band below mitochondria upon equilibrium density centrifugation. Whether particles equivalent to peroxisomes are present in *S. cerevisiae*, and whether acetyl-CoA synthetase may be associated with these particles in stationary-phase cells, is not clear. In this connection, Avers and Federman (3) reported finding particles termed "microbody-like" particles in many strains of *S. cerevisiae*. These particles, like the peroxisomes of *Tetrahymena*, were reported to be rich in catalase but devoid of cytochrome oxidase. On the other hand, Duntze et al. (5) found no evidence in yeast for a particulate fraction containing glyoxylate-shunt enzymes. Indeed, they reported that all of the key enzymes of this pathway in aerobic cells of *S. cerevisiae* are located in the soluble supernatants of homogenates.

The lighter component of acetyl-CoA synthetase in this strain of yeast is found to sediment principally with the microsomal fraction at all stages of incubation, although some of this material may also be trapped in the mitochondrial fraction. This component, which is considerably less dense that the mitochondria, does not have the characteristics of a soluble protein. It can be readily separated from glucose-6-phosphate dehydrogenase, a soluble enzyme with a molecular weight (120,000) approximately equal to that indicated for the acetyl-CoA synthetase (130,000) of this organism (DeVincenzi and Klein, Fed. Proc., p. 872, 1970).

No specific statement can be made at present concerning the nature of the particulate material with which either of the two synthetase components may be associated. Since the fractions in which the enzyme is found, even on gradients, always contain significant quantities of phosphatidyl choline, the major phospholipid of this strain of yeast (7, 9), it seems reasonable to propose that the light and dense components of acetyl-CoA synthetase are associated with membranes of different density.

The experiments described above indicate a unimodal distribution of the enzyme in the earlier stages of aerobic growth and a bimodal distribution later on. Aas and Bremer (1) concluded that acetyl-CoA synthetase in rat liver also has a bimodal distribution in which the major portion of the enzyme is mitochondrial and the minor

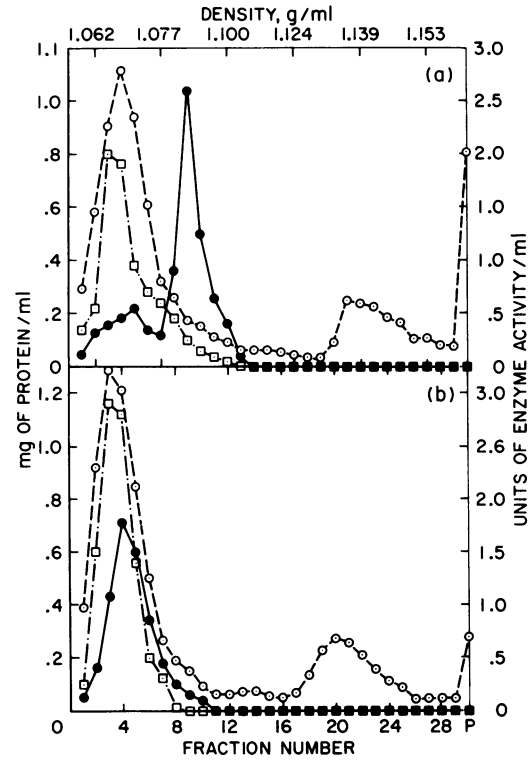

FIG. 6. *Distribution of acetyl-CoA synthetase, glucose-6-phosphate dehydrogenase, and proteins of crude homogenate of 24-hr cells of Saccharomyces cerevisiae. (a) Homogenate prepared in Tris-magnesium-mannitol buffer; (b) prepared in phosphate buffer. Both samples were kept at 0 C for 20 min, after which 1 ml containing 9.1 mg of protein was applied to separate gradients. Fractions (1 ml) were collected after 16 hr of centrifugation. Symbols: synthetase (●); total proteins (⊙); glucose-6-phosphate dehydrogenase (⊡). Recoveries of the components analyzed were over 80%.*

portion soluble. The apparent shift in localization of the acetyl-CoA synthetase during aerobic incubation of *S. cerevisiae* could imply that two different proteins are involved, one formed in the early phases of growth and associated mainly with the microsomal fraction and the other synthesized later and accumulated into some dense component sedimenting with the mitochondria. Alternatively, a single protein may be involved which could attach to membranes in the microsomal material in the early stages of growth and later, after dissociating from this site, to some element sedimenting with the mitochondria. Currently, the latter possibility seems more attractive, in view of the fact that solubilized synthetase preparations obtained from either the microsomal or mitochondrial fractions appear to be identical on the basis of *p*H optima, substrate specificity, mobility on gel electrophoresis, and behavior on Sephadex (DeVincenzi and Klein, Fed. Proc., p. 872, 1970). Although the available evidence supports the idea that only a single protein is involved, the apparent movement of acetyl-CoA synthetase from the microsomal to the mitochondrial fraction during aerobic incubation cannot be simply the transfer of preformed enzyme from one cellular compartment to another, because large increases in total synthetase activity are observed after the cells have reached the stationary phase (8; see also Fig. 1). The cells thus appear to continue synthesizing this enzyme well into the stationary phase.

ACKNOWLEDGMENT

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