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Nature of Particles Involved in Lipid Synthesis in Yeast

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ABSTRACT

KLEIN, HAROLD P. (Ames Research Center, Moffett Field, Calif.). Nature of particles involved in lipid synthesis in yeast. J. Bacteriol. **90**:227–234. 1965.—Mitochondria-free homogenates of *Saccharomyces cerevisiae* yielded several particulate layers upon centrifugation at 100,000 × *g*. Electron microscopy revealed that membranes are present only in the uppermost ("fluffy") layer, which is inactive in lipid synthesis. The membrane-free material of the middle ("red") layer stimulated the synthesis of fatty acids and of nonsaponifiable lipids. In addition, this fraction appeared to be rich in the enzyme systems responsible for desaturating fatty acids and for converting squalene to sterols. The purified particles contained protein and ribonucleic acid (approximately 65:35), and further resembled ribosomal material in that they sedimented almost entirely as an 80*S* particle in tris(hydroxymethyl)aminomethane-magnesium buffer. Various treatments that dissociated the 80*S* material did not affect the lipogenic capabilities of this particle fraction.

During the past 10 years, the mechanisms involved in the biosynthesis of lipids have been investigated at an ever-increasing pace. As a result, a considerable amount of information is now available about these processes from studies with mammalian preparations, plants, and microorganisms. In several cases, investigators have reported that optimal formation of fatty acids or of nonsaponifiable lipids, or both, occurs when subcellular particles are added to the soluble portion of the cellular contents (Bucher and McGarrahan, 1956; Klein, 1957; Popják et al., 1960; Abraham, Matthes, and Chaikoff, 1963), although the biosynthesis of fatty acids has been reported to take place exclusively, or primarily, in the soluble fraction of the cells of various organisms and tissues (Brady, Mamoon, and Stadtman, 1956; Langdon, 1957; Porter and Tietz, 1957; Ganguly, 1960; Dils and Popják, 1962; Cheniae, 1963; Lennarz, 1963; Vagelos et al., 1964).

Microsomes were reported to stimulate fatty acid synthesis in rat-liver preparations (Abraham et al., 1959; Fletcher and Myant, 1961), and this fraction of the cell appears to be involved in the cyclization of squalene to sterols (Tchen and Bloch, 1955; Popják et al., 1958). In addition, it was suggested that microsomes contain the system that desaturates fatty acids in rat liver and yeast systems (Bloomfield and Bloch, 1960). In all of these cases, the localization of the enzyme systems responsible for these effects has been vague. However, there are references to the "membranous" portion of the microsomal material as being the site of these processes (Bucher and McGarrahan, 1956; Hibbitt, 1964).

We have been studying a system derived from *Saccharomyces cerevisiae* that requires a particulate fraction for maximal synthesis of both fatty acids and sterols (Klein and Booher, 1956; Klein, 1957, 1960). We have reported briefly (Klein, 1963) that, after removal of the mitochondria from these yeast preparations, the residual particulate material can be fractionated into three subfractions, only one of which is active in these lipogenic processes. Furthermore, it was shown that the active fraction consisted almost entirely of ribonucleic acid (RNA) and protein, with approximately 35% RNA and 65% protein. It is the purpose of this report to give further details concerning the "purification" of the active fraction, as well as additional information concerning the nature of this active material.

MATERIALS AND METHODS

S. cerevisiae (strain LK 2G12) was grown in standing cultures at 30 C for 48 hr (Klein, 1957). After being harvested, the cells were washed twice with 0.1 M potassium phosphate buffer (*p*H 7.0), suspended in this buffer containing 1% glucose, and then stirred with a magnetic stirrer for 2.5 hr at 30 C. The cells were harvested and washed twice with cold distilled water, after which they were suspended in tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5, 0.001 M) containing 0.001 M $MgCl_2$ [1 ml of buffer per gram (wet weight) of cells]. The suspension was subjected to treatment

in a French pressure cell at 5 to 6 tons per square inch. After cell disruption, the suspension was centrifuged at 2,300 × *g* for 10 min to remove unbroken cells and cell debris. The supernatant fraction was again spun in the same manner, and the resultant small pellet was discarded. The crude homogenate was centrifuged at 14,500 × *g* for 30 min, after which the supernatant liquid was carefully removed to avoid removing any of the large particle pellet (containing mitochondrial material; Klein, 1957), or the fatty material that layered on top of the homogenate.

To obtain purified small particles, the mitochondria-free homogenate was further centrifuged, and the particle pellet was mechanically separated, as previously described (Klein, 1963). A more convenient method of obtaining the particles was to centrifuge the homogenate at 40,000 rev/min for 1 hr in the Spinco (model L) centrifuge and carefully remove the supernatant fraction from the surface of the pellet. The particulate material was then suspended in the Tris-Mg buffer used above, and the resultant suspension was homogenized in a Potter Elvehjem tissue homogenizer. After this, the suspension was brought to 40,000 rev/min in the Spinco centrifuge, after which the centrifuge motor was immediately turned off to allow the rotor to slow down and come to rest. At this point, a small pellet was obtained and discarded. The supernatant fraction of the treatment was centrifuged once more at 40,000 rev/min for 1 hr, after which the pellet material was retained. The latter was suspended in Tris-Mg buffer to give a protein concentration of 30 to 50 mg of protein per ml.

Methods for isolating major lipid fractions were

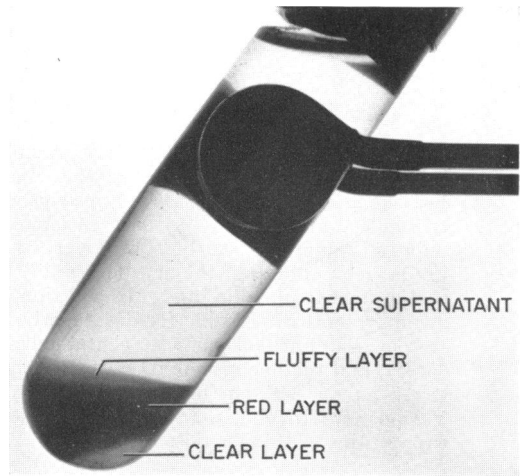

FIG. 1. *Photograph of material obtained from mitochondria-free homogenate after centrifugation at 100,000 × g for 90 min.*

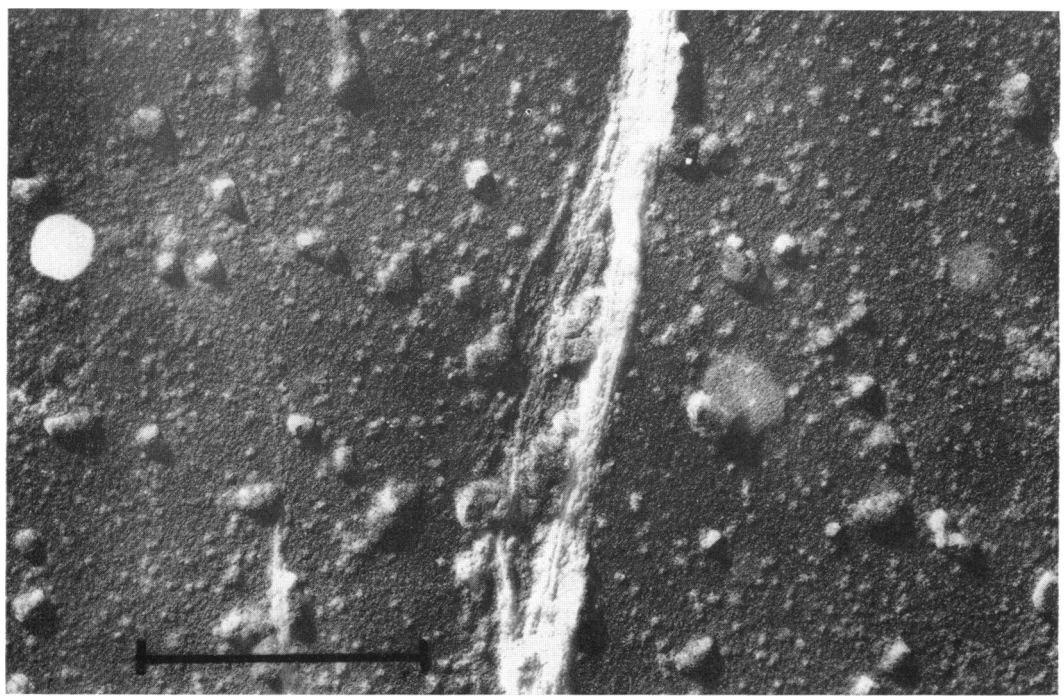

FIG. 2. *Appearance of "fluffy" layer of crude small-particle fraction. Line denotes 1 μ.*

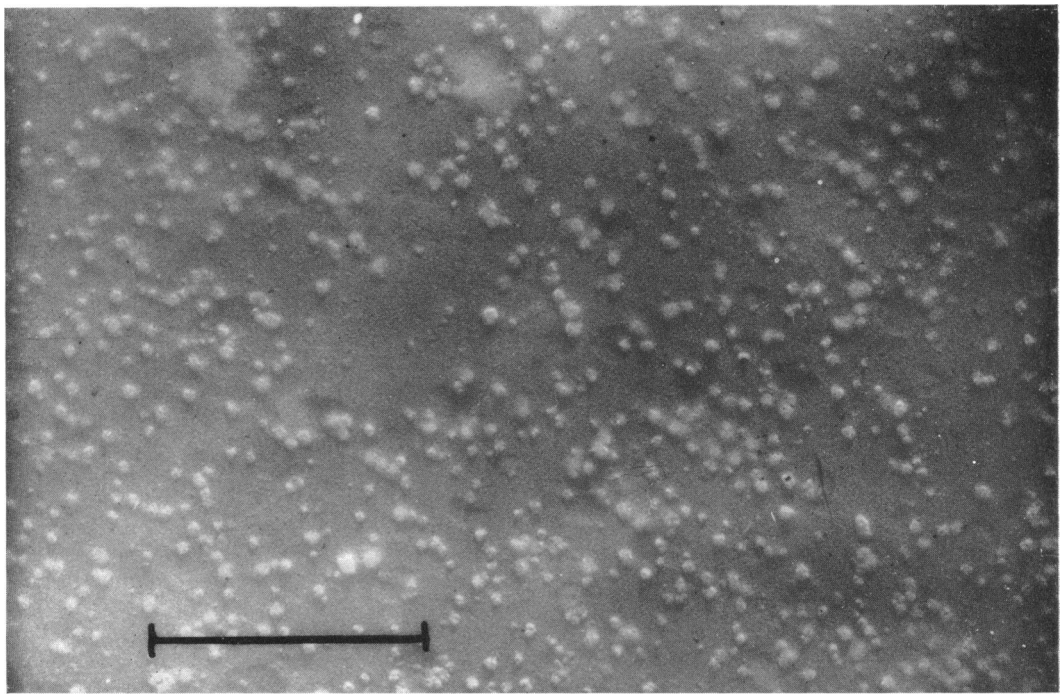

FIG. 3. *Appearance of "red" layer of crude small-particle fraction. Line denotes 1 μ.*

given in other publications (Klein, 1957, 1960). These procedures were followed in the present studies, except that, after hydrolysis of the incubated suspensions, the hydrolysates were first acidified and extracted with petroleum ether to remove both fatty acids and nonsaponifiable lipids. Acidification prior to extraction of the nonsaponifiable lipids was desirable, because, in these extracts, squalene was bound in some unknown manner, making it almost entirely water-soluble unless first treated for brief periods with acid (Den and Klein, *unpublished data*).

For the separation of fatty acids, the Aerograph gas chromatograph (model A-90-P) was used. Unknown fatty acids were first methylated as described by Metcalfe and Schmitz (1961) and then chromatographed on a column composed of 20% diethylene glycol succinate on a support of Chromosorb W at a temperature of 170 C. Presumptive identification of the fatty acids was achieved by comparing retention times of individual methyl esters to that of known compounds. Individual fatty acids were collected in scintillation fluid [toluene, 1 liter; dioxane, 1 liter; ethyl alcohol, 0.6 liter; naphthalene, 130 g; diphenyl oxazole, 13 g; 1,4 - bis[2 - (5 - phenyloxazolyl)] - benzene (POPOP), 0.25 g] for radioactivity determinations.

Radioactivity was usually measured with a Packard scintillation counter (model no. 500 C). In some experiments, infinitely thin samples were plated on metal discs and counted with a Packard-Wood end window gas-flow counter equipped with a Baird automatic scaler (model 1090).

For analyses in the analytical ultracentrifuge, particles were suspended in Tris-Mg buffer to a

TABLE 1. *Synthesis of fatty acids and nonsaponifiable lipids in the presence of purified small-particle fraction**

Amt (mg) of protein in		Acetate incorporation into lipids	
Supernatant fraction	Particles†	Fatty acids	Nonsaponifiables
		count/min	*count/min*
31.7	0	6,500	700
31.7	5.1	48,630	4,430
31.7	10.2	94,220	5,980

* Cells were prepared in Tris-Mg buffer (*p*H 7.3) and fractionated as described in Materials and Methods. Fractions were incubated for 2 hr at 30 C with the following additions: NADP, 0.5 μmole; isocitrate, 5.5 μmoles; phosphate, 100 μmoles; isocitric dehydrogenase, 50 units; coenzyme A, 0.1 mg; $MnCl_2$, 3 μmoles; $KHCO_3$, 60 μmoles; sodium acetate-*1*-C^{14}, 3 μmoles, 2×10^6 count/min; ATP, 5 μmoles; in a total volume of 2.0 ml.

† Particle fraction contained, per milliliter, 51.2 mg of protein and 30.0 mg of RNA.

concentration of between 1 and 2 mg of protein per ml, and examined in a Spinco model E instrument.

For electron microscopy, samples of the particle fractions were diluted in buffer, sprayed on Formvar-coated grids, air-dried, and then subjected to shadowcasting with platinum at an angle of 5 to 10°. The microscope used was a Hitachi model HV-11.

Chemical reagents were chemically pure. Adenosine triphosphate (ATP, disodium salt), nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), isocitric dehydrogenase (type IV), and coenzyme A were products of the Sigma Chemical Co., St. Louis, Mo.

RESULTS

Electron microscopy. In previous studies, it was reported (Klein, 1963) that the crude, small-particle pellet obtained from mitochondria-free homogenates yields three subfractions upon centrifugation at 100,000 × *g*. Figure 1 shows the appearance of the sedimented material after such centrifugation. The loose, "fluffy" layer at the top was rich in lipids and proteins, poor in ribonucleic acid (RNA), and inactive in stimulating lipogenesis (Klein, 1963). When samples of this layer were removed and examined in the electron microscope, numerous membranous elements were seen in the preparations (Fig. 2). Neither of the other fractions contains such elements.

Of the remaining two fractions, the middle, or "red" fraction, contained the bulk of activity in the synthetic reactions under consideration. Electron-microscopic examination of this material (Fig. 3) revealed particles ranging in size from 20 to 80 mμ. This fraction was virtually free from lipids, and contained protein and RNA as the only two major constituents (Table 1; Klein, 1963).

The procedure described in Materials and Methods effectively removes the "fluffy" and "clear" layers from the crude small-particle pellet and yields a product indistinguishable from the "red" fraction (henceforth called the purified small-particle fraction).

Ultracentrifugal patterns. When the purified small-particle fraction was subjected to ultracentrifugal procedures to determine the number of homogeneously sedimenting components, and to estimate the *S* value of these components, the preparation showed a single large component (Fig. 4). This peak sedimented with an *S* value of 85. The more slowly moving single component

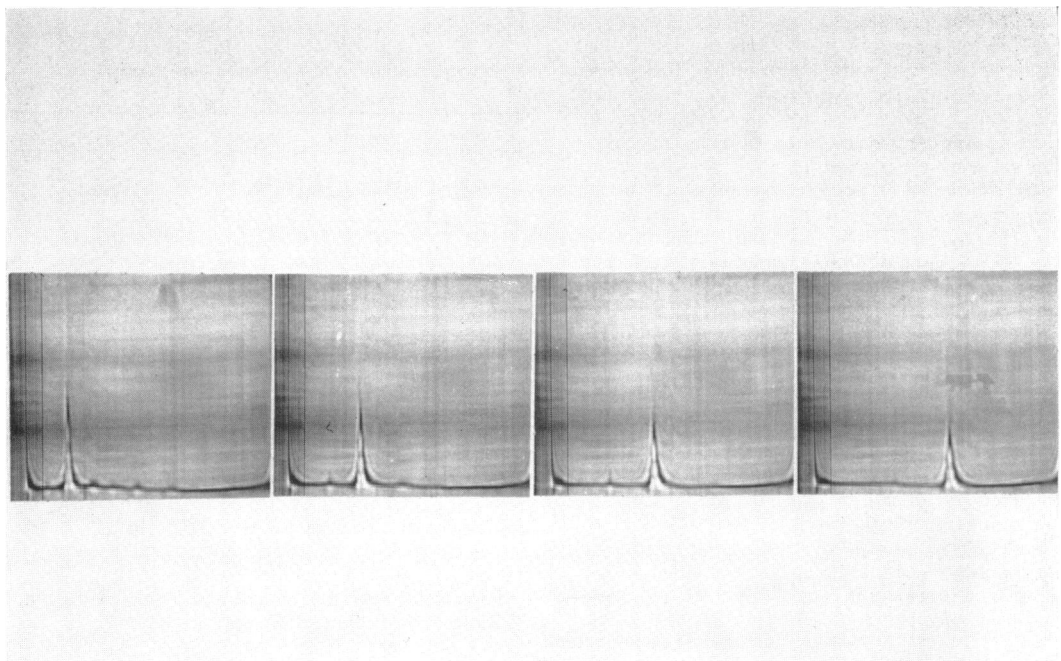

FIG. 4. *Schlieren patterns of purified small-particle fraction in Tris-Mg buffer. Sedimentation is from left to right; temperature, 9.8 C. Pictures were taken at 2-min intervals at 56,100 rev/min (bar angle, 60°); first picture was taken 15 sec after reaching constant speed.*

yielded an S value of 49. In addition, several minor components were noted, which were present in low concentrations and consisted of polyribosomes or other cellular fragments. In any case, the major peak accounted for approximately 90% of the total sedimenting material. From sedimentation studies of this kind, as well as from electron microscopy and chemical analysis, it is reasonable to conclude that the major component of this particle fraction consists of ribosomes (Chao, 1957). The factors active in lipogenesis were also contained in this particulate fraction (Tables 1 and 2; Fig. 7). It was not evident whether the active particles were identical to, or distinguishable from, the ribosomes.

That the structural intergrity of ribosomes is not necessary for lipid synthesis in these preparations was reported earlier (Klein, 1960), when it was shown that crude particles did not lose lipogenic activity after standing in distilled water, in 0.01 M ethylenediaminetetraacetic acid (EDTA), or in phosphate solutions at concentrations as high as 0.1 M. The release of over 80% of the bound RNA also resulted in no significant loss of activity. These findings were further substantiated by results obtained with the analytical ultracentrifuge. Cells were disrupted and processed, as described in Materials and Methods, and the resultant purified small particles were obtained. Half of the particles were suspended immediately in the Tris-Mg buffer; the other half in 0.1 M phosphate buffer (*p*H 7.5). Both particle preparations were active in stimulating acetate incorporation into lipids. However, particles prepared in the phosphate buffer showed several components, with major peaks sedimenting at S values of 42, 27, and 4, and with no trace of the original 84S particles (Fig. 5). In other experiments, it was demonstrated that the purified small particles prepared in Tris-Mg buffer underwent considerable degradation during the customary incubation period for lipid synthesis. When particles were added to the supernatant fraction plus the usual cofactors and incubated for 1 hr at 30 C, particle integrity was greatly affected, although this effect appears to be caused

TABLE 2. *Effect of purified small-particle fraction on fatty acids formed from acetate**

Fatty acid chain length	Supernatant fraction		Supernatant fraction + particles	
	Per cent of counts	Ratio of unsaturated† to saturated	Per cent of counts	Ratio of unsaturated† to saturated
$<C_{12}$	2.5	—	1.8	—
C_{12}	4.8	0.04	0.6	5.00
C_{14}	11.5	0.25	11.6	1.37
C_{16}	57.0	0.76	63.9	2.75
C_{18}	14.0	1.02	13.7	2.12
$>C_{18}$	7.4	—	4.3	—
Total	97.2		95.9	

* Cells were prepared in phosphate buffer (*p*H 7.0) and fractionated as described in Materials and Methods. Supernatant fraction (105 mg of protein) with and without added particles (38 mg of protein) was incubated at 30 C for 2 hr in a total volume of 12.4 ml, with the following additions (per milliliter): NADP, 0.4 μmole; isocitrate, 4 μmoles; isocitric dehydrogenase, 40 units; coenzyme A, 0.08 μmole; $MnCl_2$, 2.4 μmoles; $MgCl_2$, 0.8 μmole; $KHCO_3$, 24 μmoles; glutathione, 16 μmoles; sodium acetate-*1*-C^{14}, 1.6 μmoles, 1.4×10^6 count/min; ATP, 8.1 μmoles. After hydrolysis, fatty acids of the supernatant fraction contained a total of 1.22×10^6 count/min; those of the supernatant fraction + particles, 3.73×10^6 count/min.

† Unsaturated fatty acids considered here are all monounsaturated. No separation was attempted of individual acids below C_{12} or above C_{18}.

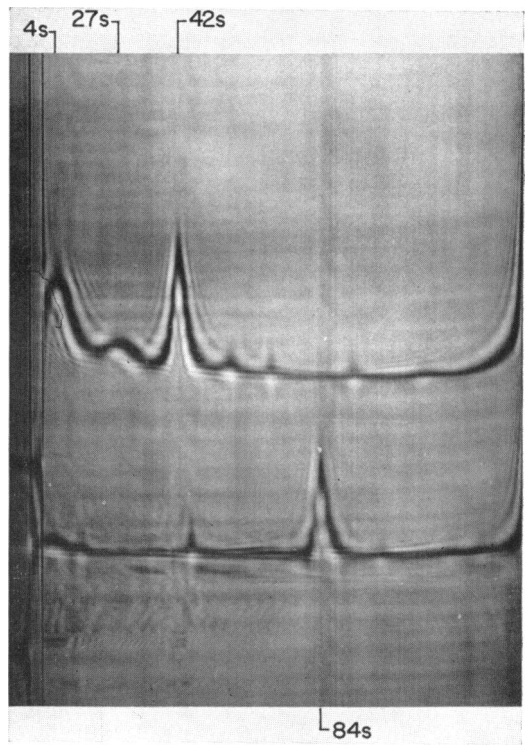

FIG. 5. *Schlieren patterns of purified small-particle fraction in 0.1 M phosphate buffer (above) and in Tris-Mg buffer (below). Picture was taken 4.5 min after reaching 56,100 rev/min (bar angle, 60°) at 19.5 C. Sedimentation from left to right.*

by the presence of the high phosphate concentrations used (Fig. 6). In all experiments of this type, the lipogenic activities measured here remained with the particle fraction, even after degradation of the major ribosomal component.

Physiological role(s) of the purified small-particle fraction. The purified small particles, when added back to the soluble supernatant fraction, in the presence of suitable cofactors, evinced all the biochemical properties shown by crude particle preparations of this yeast (Klein, 1960).

The addition of particles resulted in large stimulations in fatty acid synthesis (Table 1).

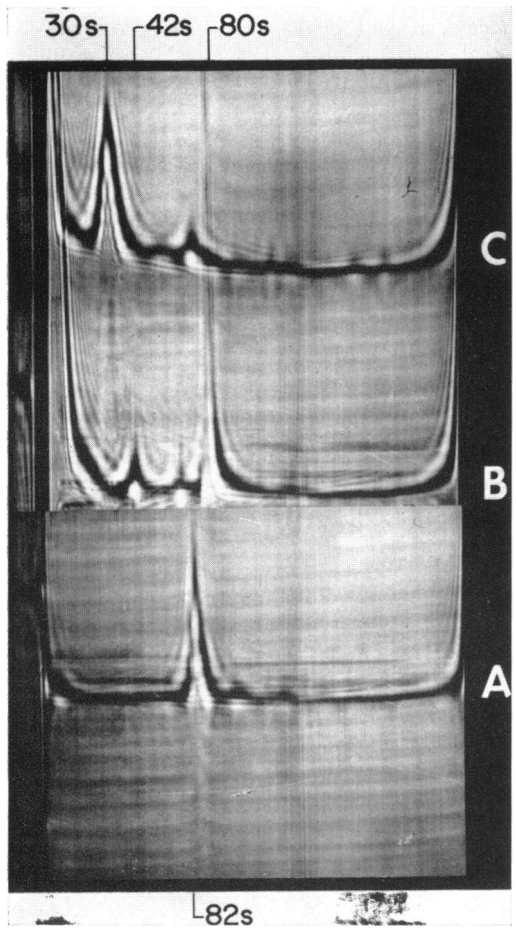

FIG. 6. *Schlieren patterns of purified small-particle fraction after incubation at 30 C for 60 min. (A) Particles suspended in Tris-Mg buffer. (C) Medium containing soluble supernatant fraction plus cofactors for lipogenesis (see Table 1). (B) Same as (C), but without phosphate. Pictures taken at 56,100 rev/min (bar angle, 50°) after 2 min at 19.2 C in (A), and after 3 min at 9.6 C in (B) and (C).*

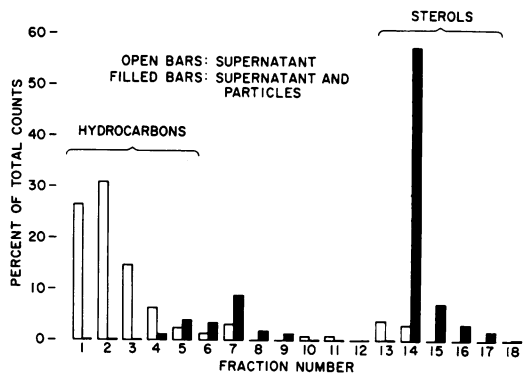

FIG. 7. *Effect of purified small-particle fraction on composition of nonsaponifiable lipids formed from acetate. A 0.5-ml amount of supernatant fraction (9 mg of protein), or this amount of supernatant fraction plus particles (10 mg of protein), was incubated in a total volume of 2 ml with cofactors, as in Table 2.*

Furthermore, gas chromatography of the fatty acids formed always showed considerably higher amounts of unsaturated fatty acids in the presence of particles (Table 2), thus indicating that the desaturation system described by Bloomfield and Bloch (1960) is in this particle fraction. (This effect of added particles is most striking in the case of the C_{14} and C_{16} acids. Why the effect is less pronounced with stearic acid is not clear.)

In addition to stimulating fatty acid synthesis, the purified small-particle fraction also increased the formation of nonsaponifiable lipids (Table 1). Of particular interest is the fact that particle-free soluble supernatant fraction synthesized virtually no sterols, and the addition of this particle fraction converted the nonsaponifiable lipid product mainly to sterols. Figure 7 shows the results obtained after chromatography on alumina (Klein, 1960) of the nonsaponifiable lipids obtained in the presence and absence of the particles. In the absence of particles, about 80% of the counts were found in hydrocarbons, with less than 10% in the sterol region. In contrast, the nonsaponifiable lipids formed in the presence of particles consisted primarily of sterols.

DISCUSSION

When this strain of *S. cerevisiae* is disintegrated in the French press, and the resultant homogenate is centrifuged to remove large subcellular particles, including mitochondria (Klein, 1957), it can be shown that several processes involved in the formation of lipids are either primarily catalyzed by the small-particle fraction or greatly stimulated in its presence (Klein, 1960). In the

present study, no evidence was obtained for the participation of membranous elements in any of these reactions. Indeed, all of the available results indicate that the various activities attributable to the crude small particles are associated in some manner with the ribosomal fraction of the yeast homogenates. It is also clear, however, that the integrity of the "80*S*" ribosomes is not required for this activity. On the basis of the available information, it is difficult to choose between two alternative explanations for these findings. It is possible that the ribosomal fraction is simply contaminated with small amounts of relatively active particulate materials of an undisclosed nature. The findings would be explained if such particles were present at concentrations below the limit of resolution of the analytical ultracentrifuge, as used in the experiments reported here. Another possibility is that the enzymatic activities associated with the purified small-particle fraction are bound to ribosomes. In this case, it would be necessary to assume that the various treatments to dissociate the "80*S*" particles (such as treatment with 0.1 M phosphate) have little effect on the specific ribosomes that carry the enzymes of interest here. Since considerable dissociation of the "80*S*" material can be demonstrated (Fig. 5 and 6) with no loss in activity, and, as mentioned above, since most of the bound RNA of the ribosomal fraction can also be solubilized without severe impairment of the activity of the particles that subsequently are sedimentable from such preparations, it would appear unlikely that ribosomes, *per se*, are involved. Before a choice can be made between these two alternatives, further fractionation of the particle-bound activities must be achieved. Such fractionation will also assist in answering the question of whether the several lipogenic activities of the purified small-particle fraction reside on one or several types of particles.

At present, it is not possible to do more than speculate about the relationship of the particles described here to the "fatty acid synthetase" described by Lynen (1961). He described a protein complex, presumably free from RNA, having a molecular weight of approximately 2.3×10^6. The sedimentation constant of the particles described by Lynen gave an *S* value of 43. These particles were obtained from a crude yeast homogenate which must have contained the ribosomal material, since the homogenates were obtained simply by filtering a crude broken-yeast suspension that had been obtained by shaking cells with glass beads (Lynen, Hopper-Kessel, and Eggerer, 1964*a*). As far as can be determined, the particulate nature of the "synthetase" described by Lynen became apparent after considerable processing, which included ammonium sulfate fractionation followed by adsorption on calcium phosphate gel, further ammonium sulfate precipitation, and treatment with alumina gel. The particles described in the present report are readily available simply by centrifugation at the proper gravitational level. It is possible that particles of the type described by Lynen sediment with the ribosomal material in our preparations. However, there are reasons to believe that the two kinds of active particles are not identical. For example, treatment of the particles described by Lynen with sodium desoxycholate resulted in complete loss of enzymatic activity (Lynen et al., 1964*b*). Preliminary experiments in this laboratory with the desoxycholate-lubrol treatment of Rendi and Hultin (1960) indicate that this procedure yields particles that still retain considerable lipogenic activity. Furthermore, there is no indication that the fraction described by Lynen is involved in the biosynthesis of nonsaponifiable lipids or in the desaturation of saturated fatty acids.

That there may be two separate systems to synthesize fatty acids in yeast is suggested by an investigation (Klein, *unpublished data*) in which it is shown that the purified small particles described here can actively carry out the synthesis of fatty acids, yielding largely unsaturated acids of shorter chain lengths (C_{12} to C_{16}), although the soluble supernatant fluid when fortified with the same cofactors gives a different spectrum of fatty acids—mainly saturated C_{16} and C_{18}—similar to the products of the "synthetase" described by Lynen.

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