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Changes in the Lipid Composition and Fine Structure of *Saccharomyces cerevisiae* During Ascus Formation

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Eighty to ninety percent of vegetative cells of *Saccharomyces cerevisiae* DCL 740 incubated in KCl-acetate medium form asci, the majority of which are four-spored. Ascospores are visible in asci after about 24 hr, and spore formation is complete after about 48 hr. The dry weight of the cells increases by about 75% during 48 hr of incubation, while the lipid content of the cells increases by a factor of four. The increase in lipid content is attributed mainly to an increased synthesis of sterol esters and triacylglycerols and to a lesser extent of phospholipids. The phospholipid and sterol compositions do not change appreciably, but there is a marked increase in the proportion of unsaturated fatty acid residues in ascan lipids. Uniformly labeled ^{14}C-acetate is incorporated mainly into sterol esters and triacylglycerols and phospholipids. Pulse-labeling by adding acetate-U-^{14}C to sporulating cultures and harvesting after a further 6 hr of incubation reveal two main periods of acetate incorporation, namely between 0 and 18 hr, and between 24 and 30 hr. Electron micrographs of thin sections through developing asci show that the principal changes in fine structure occur between 18 and 24 hr and include the appearance of numerous electron-transparent vesicles which become aligned around the meiotic nucleus, and the laying down of extensive endoplasmic reticulum membranes. Changes in fine structure are discussed in relation to the alterations in lipid content and composition of asci.

Many of the changes in composition that occur when diploid cells of *Saccharomyces cerevisiae* form asci containing one to four haploid ascospores have been described (see Fowell [14] for a review). These include an increase in cell volume and dry weight (8) which is accompanied by an increase in the contents of deoxyribonucleic acid (DNA) and carbohydrate (8). The contents of total ribonucleic acid (RNA) and protein (8) also change, although in a variable fashion. Moreover, it has been reported that yeast asci are richer in lipid than vegetative cells (17, 18, 32), and fat globules (23) are claimed to have been detected in electron micrographs of thin sections through asci. On the whole, however, the changes in lipid content and composition that accompany ascus formation in *S. cerevisiae* are poorly described especially in view of the obviously extensive synthesis of membranes that takes place. The present paper describes changes in the lipid composition during ascus formation in a strain of *S. cerevisiae*, and also the extent to which acetate, which is the preferred carbon source during ascus formation, is incorporated into cell lipids. Changes in lipid composition are related to morphological events that occur during ascus development as revealed by electron microscopy.

MATERIALS AND METHODS

Organism. The yeast used in this study was a strain of *S. cerevisiae* DCL 740 kindly provided by R. R. Fowell. It was maintained on slopes of malt wort-agar as described by Dixon and Rose (10). Stock cultures were stored at 4 C. This strain of yeast produces a high proportion (80–90%) of four-spored asci. When the sporulating ability of the yeast declined, single-cell isolates were obtained by plating on malt wort-agar medium. The majority of these isolates produced a high proportion of asci.

Growth of cells. Cells were grown in a presporulation medium containing 1.3% nutrient broth (Ox-

oid), 5% glucose, and 1% yeast extract (Oxoid); pH 6.3 (14). Portions (1 liter) of medium were dispensed into 21 round, flat-bottomed flasks which were plugged with cotton and sterilized at 115 C for 15 min. Batches of medium were inoculated with a loopful of cells from a slope culture and were incubated at 30 C as described by Patching and Rose (30), except that the magnetic stirrer was rotated only at about 200 to 300 rotations per min. After 40 hr, the culture was in the stationary phase of growth (about 4.75 mg dry weight or 1.50×10^8 cells/ml). The cells were then harvested by centrifugation at $12,000 \times g$ at 3 C, washed twice with water, and resuspended in water.

Production of asci. Sporulation of cells was induced by incubating vegetative cells in a sporulation medium containing 0.5% (w/v) sodium acetate and 1.0% (w/v) KCl (pH 7.0; 14). Batches of sporulation medium (1 liter) were dispensed into 2-liter round, flat-bottomed flasks and inoculated by adding an aqueous suspension of washed vegetative cells to a density of 0.44 mg dry weight (1.44×10^7 cells) per ml. Suspensions were incubated at 25 C with rapid stirring (30). Production of asci was followed by removing portions of suspension and counting the numbers of two-, three-, and four-spored asci by using a hemocytometer slide. A cell with an attached bud was counted as one cell. At least 300 cells or asci were counted. Ascospores were visible in asci after about 24 hr of incubation, and spore formation was complete after about 48 hr. Nevertheless, the suspensions were incubated for up to 120 hr to allow the spores to ripen. Asci and cells were harvested from the suspension by centrifugation at $12,000 \times g$ at 3 C. They were washed twice with water, freeze-dried, and stored at −20 C in the presence of a desiccant.

Dry weight measurements. Dry weight measurements were made on cells and asci that had been freeze-dried or dried to constant weight at 80 C under reduced pressure. These methods gave virtually identical values for vegetative cells, but not with developing asci (see Table 1).

Extraction of lipids. Lipids were extracted from vegetative cells and asci by a modification of the procedure used by Letters (21). Portions (500–800 mg) of freeze-dried material were extracted with 15 ml of ethanol at 80 C for 15 min. The residue was extracted at room temperature (18–22 C) with 3×30 ml of chloroform-methanol (1:1, v/v) and made up to 2 ml. The total lipid content of portions of this extract were then determined as described by Hunter and Rose (20).

Analysis of phospholipids. Total phospholipid content of the lipid extract was determined by assaying the phosphorus content of a 25-μliter portion of the extract by the method of Bartlett (1) or Chen et al. (7). Values for the phosphorus content were converted to phospholipid by multiplying by 25 (which assumes an average molecular weight of 800 for a phospholipid). Individual phospholipids in the extract were separated by two-dimensional thin-layer chromatography using plates (20 by 20 cm) coated with a layer (0.25 mm) of Kieselgel PF_{254} (Merck). Plates were developed in the first direction with chloroform-methanol-ammonia (0.88 specific gravity) (65:35:5, v/v/v), and in the second direction with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5, v/v/v/v/v). Individual phospholipids were identified by using standard preparations and specific spray reagents (9).

Analysis of neutral lipids. Neutral lipids in extracts were separated quantitatively by two-dimensional thin-layer chromatography. Plates (20 by 20 cm), with a layer (0.75 mm) of Kieselgel $PF_{366+254}$ (Merck) were washed overnight in chloroform. Plates were developed with diisopropyl ether-acetic acid (96:4, v/v) to a distance 9 cm above the origin and then in the same direction with petroleum spirit (40–60 C)-diethyl ether-acetic acid (90:10:1, v/v/v) to a distance 16 cm above the origin. Bands of lipid were located with an ultraviolet lamp and identified with simultaneously run standards. Phospholipids located at the origin were eluted from the silica gel with 2×3 ml of chloroform-methanol-water (5:5:1, v/v/v) followed by 3 ml of methanol and 3 ml of methanol-acetic acid-water (95:1:5, v/v/v). The phospholipid content of the extract was assayed as already described. Sterols and sterol esters were eluted from the silica gel with 3×3-ml portions of chloroform-methanol (4:1, v/v) and assayed by a modification of the Liebermann-Burchard reaction described by Moore and Baumann (28). Gas-liquid chromatography revealed that the extracts contained only very small amounts of sterols that lack the 5,7-diene grouping. Consequently, only 5,7-diene sterols, which are the fast-reacting sterols in the Liebermann-Burchard assay, were determined. Sterol contents were calculated from a standard curve prepared with ergosterol. Sterol esters were dissolved in 0.1 ml of benzene and saponified by refluxing for 2 hr with 0.4 ml of 10% (w/v) KOH in 90% (v/v) ethanol in a tube fitted with a cold-finger condenser. The sterols liberated were assayed as already described. Diacylglycerols and triacylglycerols were eluted from the silica gel by 2×3-ml portions of chloroform followed by 2×3-ml portions of diethyl ether. The first ether extraction was performed in a cold Rotary Evapomix (Buchler Instruments Inc.) without a vacuum for 10 min. Diacylgycerols and triacylglycerols were assayed by a chromotropic acid method (39). Contents are expressed as dipalmitin- and tripalmitin-equivalent, respectively, derived from a standard curve prepared with tripalmitin. Free fatty acids were eluted from the silica gel by 2×3-ml portions of diethyl ether-methanol (9:1, v/v) followed by 2×3-ml portions of chloroform. They were assayed by the method of Heinen and de Vries (19), and contents were related to oleic acid-equivalent by using a standard curve. Squalene was not eluted from the silica gel before being assayed by the method of Trappe (38), which involves bromination of squalene and iodometric titration of the unreacted bromine.

Sterols were extracted from freeze-dried cells by hydrolysis followed by saponification. Yeast (100 mg) was refluxed with 30 ml of 0.33 N HCl for 1 hr. The supernatant liquid was extracted with 3×30 ml of diethyl ether, and the residue was refluxed with 5 ml of 7 N KOH for 2 hr at 130 C. The reaction mixture was extracted with 2×25 ml of cyclohexane with

shaking. The diethyl ether and cyclohexane extracts were pooled and analyzed for total sterol by the Liebermann-Burchard method (38) and for ergosterol by the ultraviolet extinction method of Shaw and Jefferies (36).

Gas-liquid chromatography. Samples were analyzed using a Pye series 104 model 64 chromatograph, with flame ionization detectors. Sterols were prepared for gas-liquid chromatography by dissolving the lipid extract in 1 ml of benzene and refluxing with 4 ml of 1.78 M KOH in 90% (v/v) ethanol for 2 hr. Water (10 ml) was added, and the *p*H value was adjusted to 1.0 with HCl. The mixture was then extracted with 3 × 10 ml of diethyl ether, and the extracts were dried over sodium sulfate before being concentrated in vacuo. Free sterols were purified by thin-layer chromatography with petroleum spirit-diethyl ether-acetic acid (70:30:2, v/v/v) and eluted from the silica gel with 150 ml of diethyl ether. Trimethylsilyl ethers were prepared by dissolving the sterols in 1 ml of dry pyridine, adding 0.2 ml of hexamethyl disilazane and 0.1 ml of trimethyl chlorosilane (37). The mixture was shaken and left at room temperature for 15 min. Excess reagents were evaporated off under a stream of nitrogen gas, and the products were taken up in chloroform. Alternatively, the reaction mixture was partitioned between petroleum spirit and water, and the petroleum spirit extract was concentrated and redissolved chloroform. Trimethylsilyl ethers of the sterols were separated on 3% OV 17 supported by 100-200 mesh Gas Chrom Q in a 9 ft by ¼ inch (ca. 2.7 m by 0.6 cm) stainless-steel column. The column temperature was 225 C with a nitrogen gas flow rate of 70 ml/min; the detector oven temperature was 300 C.

Methyl esters of fatty acids were prepared by refluxing 10 to 20 mg of extract in 0.5 ml of dry benzene with 4 ml of methanolic HCl (5%, w/v) for 2 hr. A tube of anhydrous $CaCl_2$ was fitted to the condenser. After adding 10 ml of water, the methyl esters were extracted with 3 × 10 ml of diethyl ether. These extracts were dried over $CaCl_2$ before being concentrated. The methyl esters were purified by thin-layer chromatography with petroleum spirit-diethyl ether-acetic acid (90:10:1, v/v/v) and were eluted from the silica gel with 150 ml of petroleum spirit-diethyl ether (1:1, v/v). The eluate was concentrated and redissolved in chloroform before the esters were separated on 15% polyethylene glycol succinate (PEGS) on 85-100 mesh Universal B, or 15% EGSS-X on 100-120 mesh Gas Chrom P. The PEGS was packed in a 5 ft by ¼ inch (ca. 1.5 m by 0.6 cm) glass column and maintained at 155 C; the detector oven was set at 200 C with a nitrogen gas flow of 60 ml/min. The EGSS-X was packed in a 5 ft by ¼ inch (ca. 1.5 m by 0.6 cm) stainless-steel column, maintained at 175 C with a nitrogen gas flow rate of 50 ml/min and the detector oven at 200 C.

Radioactive counting methods. ^{14}C activity in cells and extracts was measured by using a Beckman model CPM 200 liquid scintillation spectrometer (Beckman Instruments Limited, Glenrothes, Fife, Scotland). Yeast cells were removed from 5 ml of suspension containing acetate-U-^{14}C by filtering through Sartorius or Millipore membrane filters (1.2-μm pore size; 2.5-cm diameter) and washed with 3 × 10 ml of water, and the cells plus filters were dried to a constant weight in a vacuum desiccator. They were then placed in a scintillation vial containing 5 ml of scintillation liquid (toluene-2-methoxyethanol-2,5-diphenyloxazole; 3:2:0.003, v/v/w). Bands of silica gel containing various classes of lipid extracted from cells grown in the presence of acetate-U-^{14}C were scraped off thin-layer plates and transferred to scintillation vials containing 5 ml of scintillation liquid (toluene-2,5-diphenyloxazole; 1:0.003, v/w). All samples were counted up to 100 min or to a 2 σ statistical counting error usually equal to 1% or 2%. Readings were corrected for average background count by using blank areas of silica gel from thin-layer plates.

Electron microscopy. Vegetative cells and asci were fixed in 1% (w/v) aqueous potassium permanganate. Preliminary experiments showed that asci could be satisfactorily fixed while suspended in water, but with vegetative cells best results were obtained by first freeze-drying the cells and then resuspending them in water before chemical fixation. The optimal times for fixation were established with each developmental stage examined: vegetative cells, 4.0 hr; 6-hr asci, 5.5 hr; 12-hr asci, 5.0 hr; 18-hr asci, 4.5 hr; 24-hr asci, 4.0 hr. Fixed material was washed in water and suspended in liquid 1% (w/v) agar which, after setting, was cut into 1-mm^3 blocks. These blocks were dehydrated in a graded ethanol-water series, soaked in propylene oxide, and embedded in Epon. Blocks were stained for 1 hr during dehydration in 2% (w/v) uranyl acetate in 70% (v/v) ethanol. Sections were cut with a diamond knife on an LKB ultramicrotome, stained in lead citrate (33), and viewed with an AEI EM 6M electron microscope.

Chemicals. Standard lipids were supplied by Sigma Chemical Co., London, England; standard mixtures of fatty acid methyl esters and 15% EGSS-X on Gas Chrom P (manufactured by Applied Science Laboratories, Inc.) by Field Instruments Ltd., Richmond-upon-Thames, Surrey, England; and PEGS (15% on Universal B) and 3% OV-17 on Gas Chrom Q by Phase Separations Ltd., Queensferry, Flintshire, Wales. Silica gels manufactured by E. Merck A. G. were from Anderman and Co. Ltd., London, England. The sodium salt of acetic acid-U-^{14}C was obtained from The Radiochemical Centre, Amersham, Bucks., England. All other chemicals were analytical grade or of the highest purity available commercially. Chloroform and methanol were redistilled before use.

RESULTS

Total lipid contents of developing asci. Vegetative cells do not bud in sporulation medium, although as previously reported (8) they increase in dry weight and volume as ascospore formation proceeds. Initially, cells and developing asci were dried by freeze-drying, and it was shown that freeze-dried cells do not lose weight when dried at 80 C under

reduced pressure. However, as shown in Fig. 1, freeze-dried asci retained water, the amount of which increased as the asci developed. This increase in water retention occurred in two distinct periods, namely between 0 and 12 hr, and between 30 and 48 hr. The increase in dry weight is accompanied by an increase in the content of total lipid (Fig. 1), which is more rapid than the increase in dry weight. Values for the lipid content of vegetative cells showed good agreement between batches, but, as ascus formation proceeded, values for the lipid content varied somewhat between batches.

Changes in the lipid composition of developing asci. The increase in lipid content of cells during the first 24 hr of incubation in sporulation medium can be attributed mainly to an increased production of sterol esters, triacylglycerols, and phospholipids (Table 1). During this period, there were relatively small increases in the contents of free sterols, diacylglycerols, free fatty acids, and squalene. The phospholipid, diacylglycerol, and free fatty-acid contents of lipids from asci after 120 hr of incubation differed little from those in cells incubated for only 24 hr, but the contents of sterols, sterol esters, and particularly of triacylglycerols were much greater.

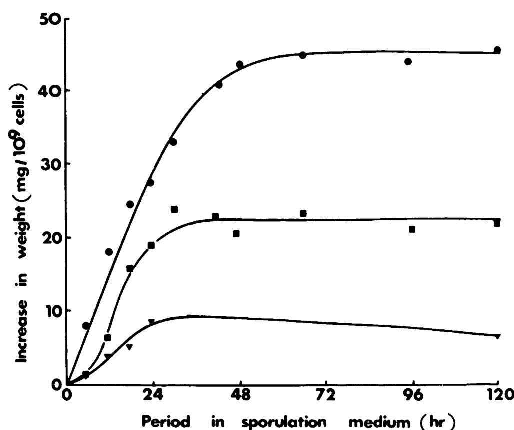


FIG. 1. *Increase in weight (freeze-dried cells, ●; heat-dried cells, ■) and content of total lipids (▼) of Saccharomyces cerevisiae DCL 740 during ascus formation. At zero time, the value for freeze-dried cells was 30.3 mg/10⁹ cells, and for heat-dried cells, 30.7 mg/10⁹ cells. The lipid content of cells at zero time was 2.31 mg/10⁹ cells.*

Vegetative cells contain large proportions of phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine, and smaller proportions of phosphatidic acid, bisphosphatidylglycerol, and *N,N*-dimethyl-phosphatidylethanolamine. The relative proportions of these phospholipids did not change appreciably during development of the asci up to 120 hr. There was a progressive increase in the proportion of unsaturated acids during ascus development, due mainly to an increased synthesis of $C_{16:1}$ and $C_{18:1}$ acids. Vegetative cells were grown in the presporulation medium at 30 C and incubated in the sporulation medium at 25 C. Growth temperature is known to influence the fatty acid composition of microorganisms (12), lower incubation temperatures favoring synthesis of unsaturated acids. Table 2 shows for comparison the fatty acid composition of cells grown in a presporulation medium at 25 C; when transferred to the sporulation medium, these cells gave rise to 5 to 10% fewer asci compared with cells that had been grown at 30 C. Nevertheless, the proportion of unsaturated fatty acids (68.1%) in vegetative cells grown in presporulation medium at 25 C is much lower than the value (77.9%), after 24 hr of incubation in sporulation medium, obtained for asci developing from cells grown in presporulation medium at 30 C.

Gas-liquid chromatography of sterols obtained from cells and asci either by saponification or by extraction with chloroform-methanol (Table 3) revealed two major components, with trace amounts of two others. One of the major components has a retention time (2.52 relative to cholestane) identical with that of ergosterol and was tentatively identified as such. The other major sterol, which is present in amounts equal to about half that of ergosterol, was at first tentatively identified by its relative retention time (3.00) as the tetraethenoid sterol

TABLE 1. *Changes in the lipid composition of Saccharomyces cerevisiae DCL 740 during ascus formation*[a]

Period in sporulation medium (hr)	Phospholipid	Sterol	Sterol ester	Diacylglycerol	Triacylglycerol	Free fatty acid	Squalene
0	0.80 ± 0.09	0.05 ± 0.01	0.08 ± 0.03	0.03 ± 0.01	0.14 ± 0.02	0.06 ± 0.02	0.01 ± 0.00
24	2.17 ± 0.13	0.09 ± 0.01	1.11 ± 0.28	0.21 ± 0.07	1.61 ± 0.23	0.08 ± 0.01	0.33 ± 0.04
120	2.24 ± 0.17	0.50 ± 0.09	2.92 ± 0.48	0.23 ± 0.10	5.24 ± 1.03	0.09 ± 0.04	0.25 ± 0.04

[a] Values quoted are milligrams per 10^9 cells with 95% confidence limits. Values for sterol ester were calculated assuming esterified sterol to be ergosterol palmitate.

TABLE 2. *Fatty acid composition of lipids of Saccharomyces cerevisiae DCL 740 during ascus formation*

Acid	Fatty acid composition[a]			
	0 hr	24 hr	120 hr	0[b] hr
12:0	3.47 ± 0.77	0.56 ± 0.19	tr[c]	0.77 ± 0.32
13:1	0.87 ± 0.02	tr	tr	tr
14:0	1.48 ± 0.29	1.41 ± 0.70	1.03 ± 0.02	1.29 ± 0.40
15:0	1.04 ± 0.08	0.60 ± 0.04	0.74 ± 0.09	tr
16:0	25.74 ± 2.98	15.57 ± 0.75	8.43 ± 0.73	23.03 ± 2.04
16:1	39.00 ± 0.93	44.24 ± 1.80	50.73 ± 1.68	31.40 ± 2.34
18:0	4.76 ± 1.02	4.95 ± 0.44	3.13 ± 0.86	6.80 ± 0.24
18:1	23.82 ± 2.16	33.32 ± 0.96	35.45 ± 0.58	36.71 ± 3.38
Percent saturated acids	35.45	23.09	13.70	31.89
Percent unsaturated acids	63.69	77.90	86.18	68.11

[a] Values quoted are the molar percentages of the total fatty acid (with 95% confidence limits) after 0, 24, or 120 hr in sporulation medium at 25 C.

[b] Cells grown in presporulation medium at 25 C instead of 30 C.

[c] tr indicates that the percentage of fatty acid was less than 0.50%. The following acids were also present at less than 0.5%: 7:0, 8:0, 9:0, 10:0, and 13:0.

TABLE 3. *Gas-liquid chromatographic determination of proportions of individual sterols (free and esterified) in developing asci of Saccharomyces cerevisiae DCL 740*

Retention time of sterol relative to cholestane[a] (hr)	Sterols extracted from cells by using:	Sterol content in cells incubated in sporulation medium[b]		
		0 hr	24 hr	120 hr
1.70	$CHCl_3$-MeOH	tr[c]	tr	tr
1.95	$CHCl_3$-MeOH	tr	tr	tr
2.52	$CHCl_3$-MeOH	62.0 ± 7.2	66.7 ± 3.0	60.6 ± 5.3
	Saponification	72.5 ± 6.5	63.9 ± 5.8	63.7 ± 8.4
3.00	$CHCl_3$-MeOH	35.2 ± 8.6	33.3 ± 3.0	39.4 ± 5.4
	Saponification	27.8 ± 6.6	34.4 ± 4.8	36.3 ± 8.4

[a] Sterols were separated by gas-liquid chromatography by using a column of OV-17.

[b] Values quoted are percent of the total sterol with 95% confidence limits.

[c] tr indicates trace (less than 0.50% of the total).

described by Longley, Rose, and Knights (22) and Hunter and Rose (20). However, this sterol does not exhibit the absorption maximum at 233 nm reported by Longley et al. (22), and we were therefore unable to identify it. Table 3 also shows that the proportions of the two major sterols differ only slightly in vegetative cells and in cells incubated in sporulation medium for 24 and 120 hr. No significant differences in the proportions of the two major sterols were recorded when the saponification assay method was used instead of extraction with chloroform-methanol. Sterols in the extracts, which include those present in cells in the free and esterified form, were assayed by using the Liebermann-Burchard and Shaw and Jefferies methods (Table 4). The Shaw and Jefferies method assays only ergosterol. The amounts of sterol assayed by this method (Table 4), when expressed as a percentage of the sterol assayed by the Liebermann-Burchard method, are very similar to the percentage of ergosterol assayed by gas-liquid chromatography.

Incorporation of radioactive acetate into lipid fractions. When acetate-*U*-^{14}C was included in the sporulation medium, about half of the label incorporated into cells was recovered in the lipids (Fig. 2). This percentage was considerably greater than that reported by the Espositos and their colleagues (11), who used acetate-2-^{14}C. Table 5 shows that the amount of acetate carbon in each of the major lipid fractions during incubation of cells in sporulation medium containing acetate-*U*-^{14}C. During ascus formation, the amounts of carbon, derived from acetate, incorporated into various lipid fractions, increased. However, in lipids from asci harvested after 120 hr of incubation, the contribution of acetate carbon to each of the fractions varied (compare Tables 1 and 5). Over 90% of the weight of sterol esters can be accounted for by acetate carbon, whereas only

50% of the weight of triacylglycerols and 30% of the total phospholipid are similarly accountable. When labeled acetate was added to sporulating cultures over 6-hr periods (Table 6), there was an initial rapid incorporation of label into all of the fractions over the first 6 hr. This rate of incorporation then progressively decreased up to 24 hr. From 24 to 30 hr, there was a further marked increase in incorporation into each of the fractions, but immediately after this period the rate of incorporation fell rapidly.

TABLE 4. *Total sterol contents of developing asci of Saccharomyces cerevisiae DCL 740*

Period in sporulation medium (hr)	Assay method	Sterol content in extracts obtained by[a]	
		Saponification	Extraction with $CHCl_3$-MeOH
0	Liebermann-Burchard	0.16 ± 0.04	0.10 ± 0.03
	Shaw & Jefferies	0.12 ± 0.05	
24	Liebermann-Burchard	0.79 ± 0.05	0.81 ± 0.19
	Shaw & Jefferies	0.32 ± 0.04	
120	Liebermann-Burchard	1.82 ± 0.76	2.23 ± 0.28
	Shaw & Jefferies	1.17 ± 0.42	

[a] Values quoted are milligrams per 10^9 cells with 95% confidence limits.

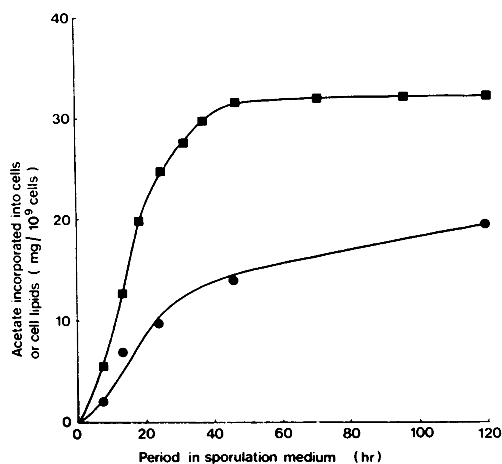


FIG. 2. *Time-course of incorporation of acetate into cells (■) and into cell lipids (●) during incubation of Saccharomyces cerevisiae DCL 740 in sporulation medium containing acetate-U-^{14}C.*

Changes in fine structure during ascus development. Few changes occurred in the fine structure of developing asci during the first 12 hr of incubation, with the exception of an increase in the number of mitochondria, alignment of endoplasmic reticulum around the cell periphery, and fragmentation of the vacuole (Fig. 3, 4, and 5). Incubation for another 6 hr led to more dramatic changes. Fragmentation of the central vacuole gave rise to many dispersed vacuoles, but the most notable features were the presence of numerous electron-transparent vesicles and the formation of extensive endoplasmic reticulum membranes (Fig. 6). The process of ascospore formation was rapid and apparently asynchronous since a variety of developmental stages were found in cells harvested after 24 hr of incubation in sporulation medium (Fig. 7-11). During this phase, the nucleus changed shape extensively, probably reflecting a state of division (compare Fig. 7 and 8). As this process continued, a double, delimiting membrane extended around the extremity of each lobe of the nucleus (Fig. 8). Electron-transparent vesicles became aggregated around these delimiting membranes, especially when adjacent lobes were close together (Fig. 8 and 9). Subsequently, many of the vesicles became enclosed within the delimited ascospores (Fig. 9, 10, and 11), around which walls were developed (Fig. 10 and 11).

DISCUSSION

Although it has been claimed that asci of *S. cerevisiae* are richer in lipids than vegetative cells (17, 18, 23, 32), these claims are based entirely on cytological observations. The present paper reports for the first time the extent

TABLE 5. *Incorporation of acetate carbon into different lipid fractions of Saccharomyces cerevisiae DCL 740 during ascus formation*[a]

Period in sporulation medium (hr)	Phospholipid	Sterol	Sterol ester	Diacylglycerol	Triacylglycerol	Free fatty acid	Total
6	0.36 ± 0.02	0.05 ± 0.00	0.27 ± 0.03	0.04 ± 0.00	0.21 ± 0.01	0.03 ± 0.01	0.98 ± 0.04
12	0.58 ± 0.05	0.08 ± 0.01	0.74 ± 0.16	0.14 ± 0.01	0.81 ± 0.23	0.11 ± 0.01	2.51 ± 0.04
24	0.72 ± 0.06	0.13 ± 0.00	1.21 ± 0.10	0.07 ± 0.02	0.92 ± 0.07	0.13 ± 0.03	3.15 ± 0.12
48	1.17 ± 0.41	0.21 ± 0.04	1.82 ± 0.15	0.22 ± 0.02	1.37 ± 0.14	0.39 ± 0.09	5.19 ± 0.64
120	0.78 ± 0.02	0.43 ± 0.04	2.80 ± 0.07	0.17 ± 0.04	2.58 ± 0.07	0.29 ± 0.03	7.05 ± 0.19

[a] Values quoted are milligrams of carbon incorporated into each lipid fraction from 10^9 cells, with 95% confidence limits, when acetate-U-^{14}C was included in the sporulation medium.

TABLE 6. *Pulse-labeling of lipid fractions of Saccharomyces cerevisiae DCL 740 following addition of acetate-U-^{14}C to sporulation cultures*[a]

Period during which ^{14}C-acetate was in sporulation culture[b]	Phospholipid	Sterol	Sterol ester	Diacylglycerol	Triacylglycerol	Free fatty acid	Total
6-12	0.34 ± 0.03	0.38 ± 0.23	0.36 ± 0.02	0.04 ± 0.01	0.61 ± 0.06	0.05 ± 0.01	1.46 ± 0.04
12-18	0.33 ± 0.02	0.03 ± 0.17	0.30 ± 0.09	0.03 ± 0.04	0.62 ± 0.16	0.09 ± 0.01	1.23 ± 0.20
18-24	0.24 ± 0.02	0.03 ± 0.00	0.30 ± 0.02	0.03 ± 0.00	0.38 ± 0.04	0.04 ± 0.02	1.01 ± 0.07
24-30	0.42 ± 0.03	0.10 ± 0.01	0.89 ± 0.02	0.10 ± 0.01	0.90 ± 0.09	0.16 ± 0.01	2.57 ± 0.10
30-36	0.14 ± 0.02	0.02 ± 0.01	0.19 ± 0.09	0.04 ± 0.01	0.18 ± 0.02	0.01 ± 0.01	0.59 ± 0.06
36-42	0.16 ± 0.01	0.03 ± 0.00	0.23 ± 0.02	0.04 ± 0.01	0.23 ± 0.01	0.04 ± 0.01	0.72 ± 0.08
114-120	0.05 ± 0.01	0.02 ± 0.00	0.10 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.00	0.28 ± 0.01

[a] Values quoted are milligrams of carbon incorporated into each fraction from 10^9 cells with 95% confidence limits.

[b] Labeled acetate was added to the sporulating culture at the time indicated, and cells and asci were harvested 6 hr later.

and nature of the changes in lipid composition that occur when diploid cells of *S. cerevisiae* form asci containing four haploid ascospores.

The main lipids in the plasma membrane of *S. cerevisiae* are phospholipids and sterols (K. Hunter, thesis, University of Bath). Calculations show that the formation of four ascospores in asci of *S. cerevisiae* DCL 740 (based on average dimensions from our electron micrographs) involves the formation of approximately 80 to 100% more membrane as a result of the formation of a plasma membrane and a spore membrane in each ascospore. The increase in the content of free sterol during the first 24 hr of ascus development could therefore be accounted for very approximately by the formation of new membranes. However, over the same period, the increase in phospholipid content of developing asci is greater than that required to make additional plasma membrane and spore membrane. Some of this phospholipid may be incorporated into newly formed mitochondria, as well as into membraneous structures that are retained in the ascan epiplasm. Some may also be present in membranes that enclose the newly synthesized electron-transparent vesicles. The electron micrographs suggest that a membrane of this type may be present.

The really dramatic changes in the lipid composition of developing asci are in the contents of sterol esters and triacylglycerols. Little is known of the cellular location and physiological significance of these lipids in yeast, but it has been shown that sterol esters and triacylglycerols are mainly located in *S. cerevisiae* in low-density structures (G. E. Wheeler, *unpublished observations;* K. Hunter, thesis, University of Bath) similar to those found in liver cells (25). It seemed possible that the increased synthesis of sterol esters and triacylglycerols might be associated with the formation of additional electron-transparent vesicles during ascus development. Our analytical data show that the contents of both classes of lipid increase steadily during ascus development, whereas the electron micrographs reveal that the formation of electron-transparent vesicles is rapid during the first 24 hr incubation, after which it levels off; indeed, it appears that there may be less vesicular material in asci at later stages of development. Clearly, therefore, the appearance of vesicles cannot be closely correlated with the contents of sterol esters and triacylglycerols in asci, and it is presumed that, if the vesicles do contain these classes of lipid, some must also be located in other ascan structures during the later stages of development.

It is possible only to speculate on the physiological role of the increased production of sterol esters and triacylglycerols some of which may be in vesicles. Vesicles of the type shown in the present study to be formed during ascus development have been implicated in secretion of enzymes (3) and with the extrusion of cell wall material (35) in vegetative cells of *S. cerevisiae*, although in neither study were the vesicles isolated or their chemical compositions determined. One possible role for the vesicles in ascus development is in the formation of the spore wall either by supplying previously synthesized wall material or by acting as a repository of wall-synthesizing enzymes. This notion is supported by two findings: firstly, the very rapid incorporation of acetate into sterol esters and triacylglycerols during the period (24-30 hr) during which spore walls are being formed;

FIG. 3. *Vegetative cell of Saccharomyces cerevisiae DCL 740. The nucleus (N) and the tonoplast of the tangentially sectioned vacuole (V) occupy the central region of the cell. A few mitochondria (M) and membrane profiles of endoplasmic reticulum (ER) occur around the periphery of the cell. Vertical bar represents 1 μm.*

FIG. 4. *Developing ascus after 6 hr of incubation in sporulation medium showing few structural changes from the vegetative phase except for an increase in the number of mitochondria (M) and the alignment of endoplasmic reticulum (ER) around the periphery of the cell. Vertical bar represents 1 μm.*

FIG. 5. *Developing ascus after 12 hr of incubation in sporulation medium. The vacuole (V) is highly lobed as seen in tangential section, and a few electron-transparent vesicles (TV) bounded by a dense layer are seen in the cytoplasm. Vertical bar represents 1 μm.*

FIG. 6. *An ascus after 18 hr of incubation in sporulation medium showing dramatic changes in cellular fine structure. Numerous electron-transparent vesicles (TV) can be seen throughout the cell, an extensive membrane system is present (ER), and the vacuole has now fragmented, resulting in several scattered vacuoles (V) some of which contain small electron-transparent vesicles. Vertical bar represents 1 μm.*

FIG. 7. *An ascus after 24 hr of incubation in sporulation medium. Numerous electron-transparent vesicles (TV), vacuoles (V), and endoplasmic reticulum profiles (ER) are present throughout the cell. Verticle bar represents 1 μm.*

FIG. 8. *An ascus after 24 hr of incubation in sporulation medium. The highly lobed nucleus (N) is apparently undergoing division, and the spore-delimiting membranes (SDM) can be seen enveloping the lobes of the nucleus in what are assumed to be the polar regions of the intranuclear spindle. The spindle microtubules are not preserved by potassium permanganate fixation. Electron-transparent vesicles (TV) are aligned around and between the lobes of the nucleus. Vertical bar represents 1 μm.*

FIG. 9. *Another ascus after 24 hr of incubation in sporulation medium. Ascospore delimitation is nearing completion. Electron-transparent vesicles (TV) are enclosed within the spore-delimiting membranes (SDM). Smaller vesicles with electron-transparent centers and thick, dense bounding layers are closely aligned between the delimiting spores. Vertical bar represents 1 μm.*

FIG. 10. *Another ascus after 24 hr of incubation in sporulation medium. Delimitation of ascospores (AS) has just reached completion. Electron-transparent vesicles (TV) are present both within the spores and in the ascus cytoplasm. Vertical bar represents 1 μm.*

FIG. 11. *Another ascus after 24 hr of incubation in sporulation medium. Four ascospores (AS) are visible, and around each an ascospore wall (ASW) has developed. Vertical bar represents 1 μm.*

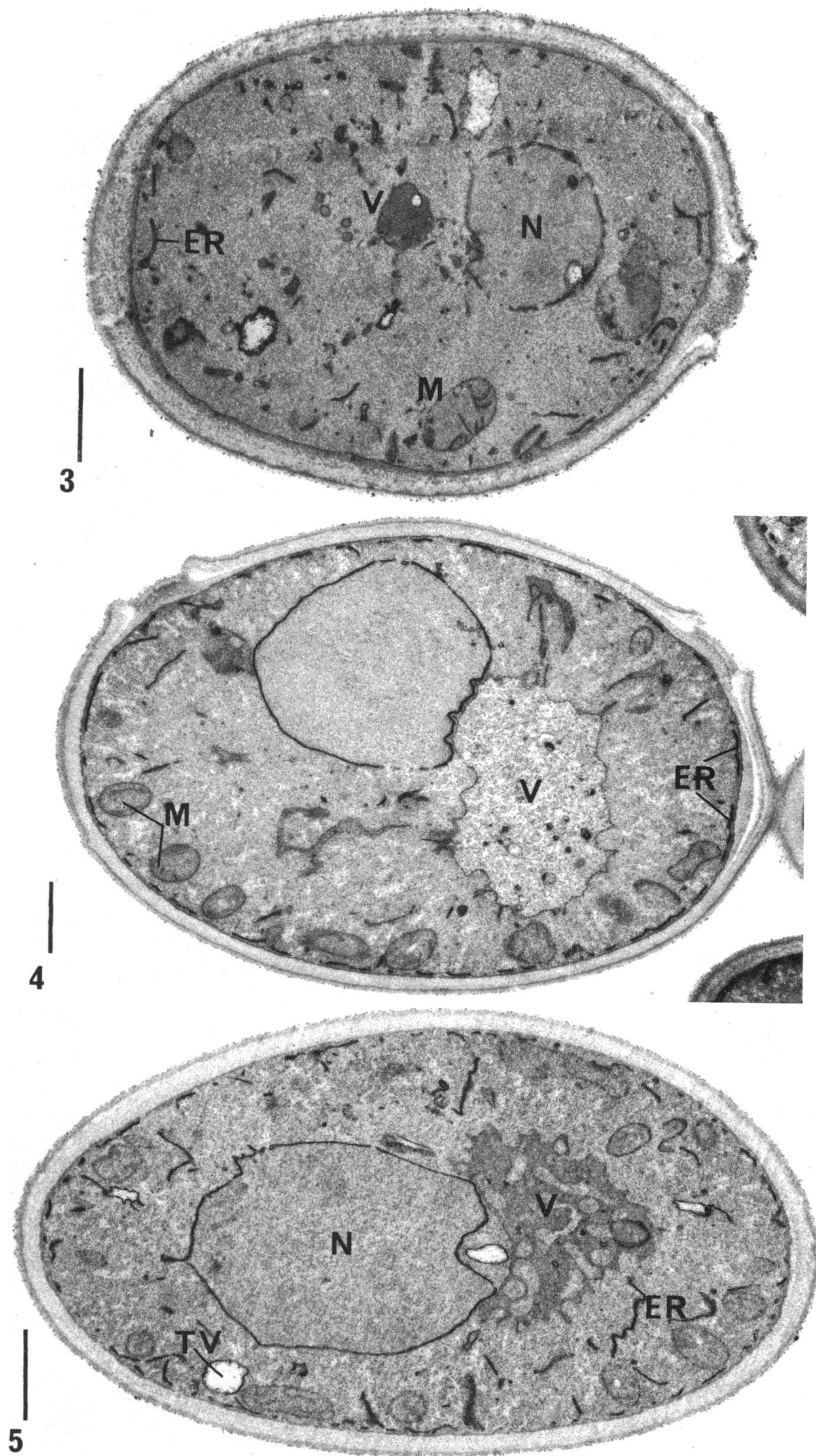
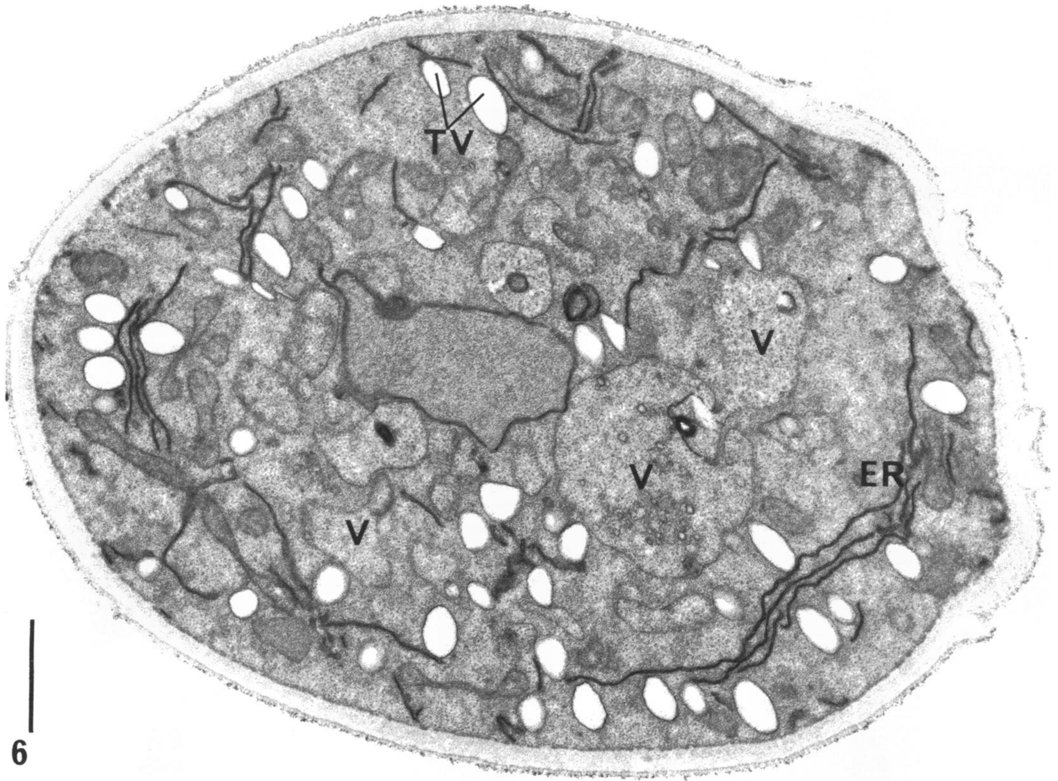


FIG. 3–5



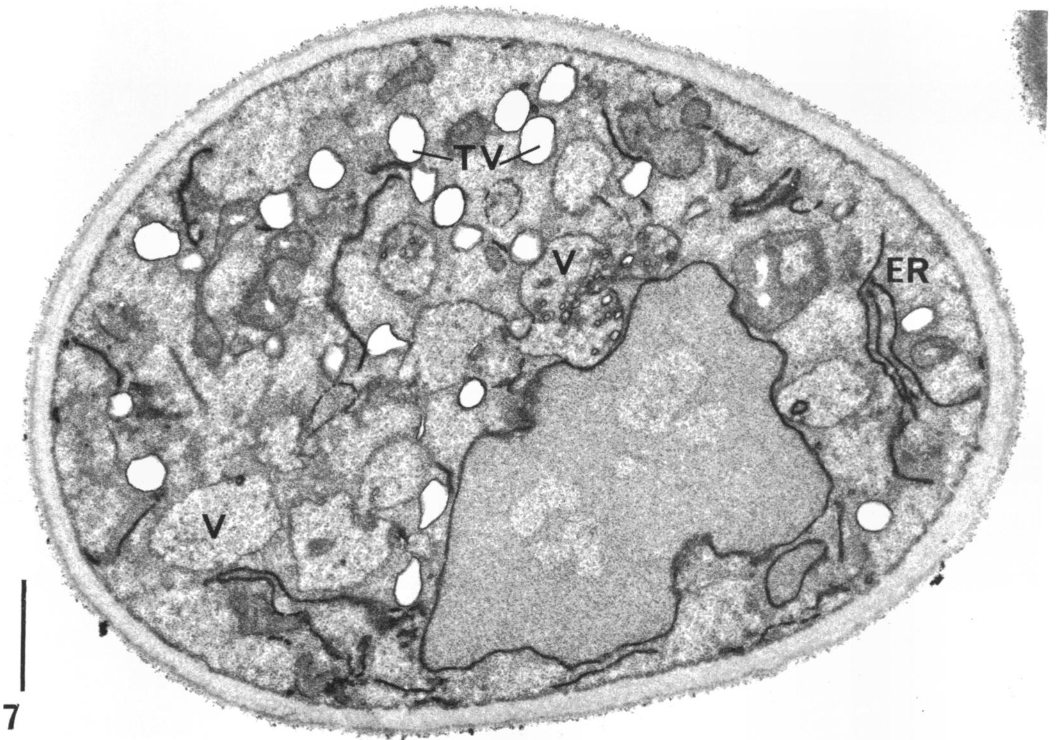
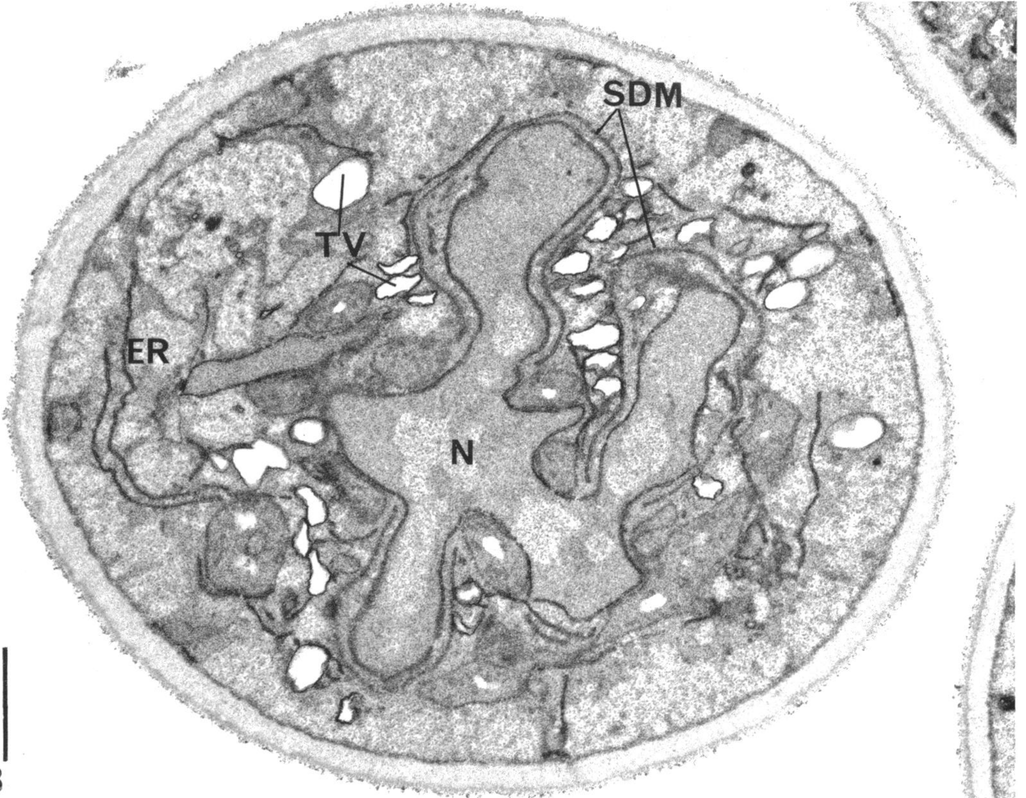


FIG. 6 AND 7



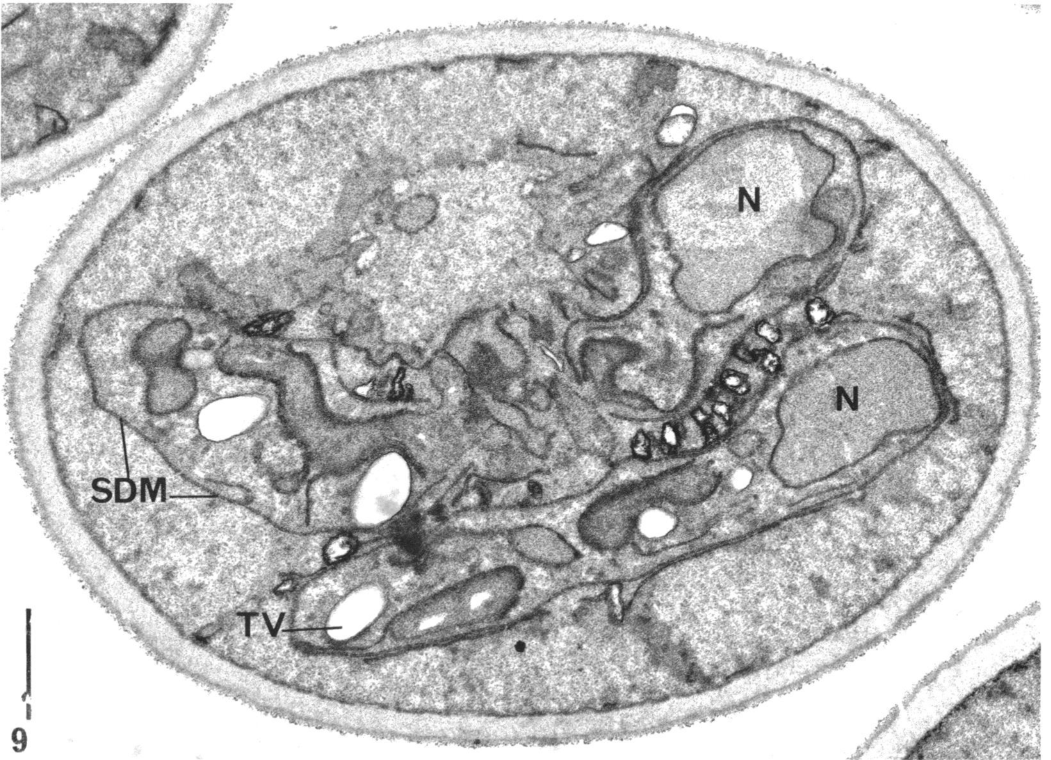
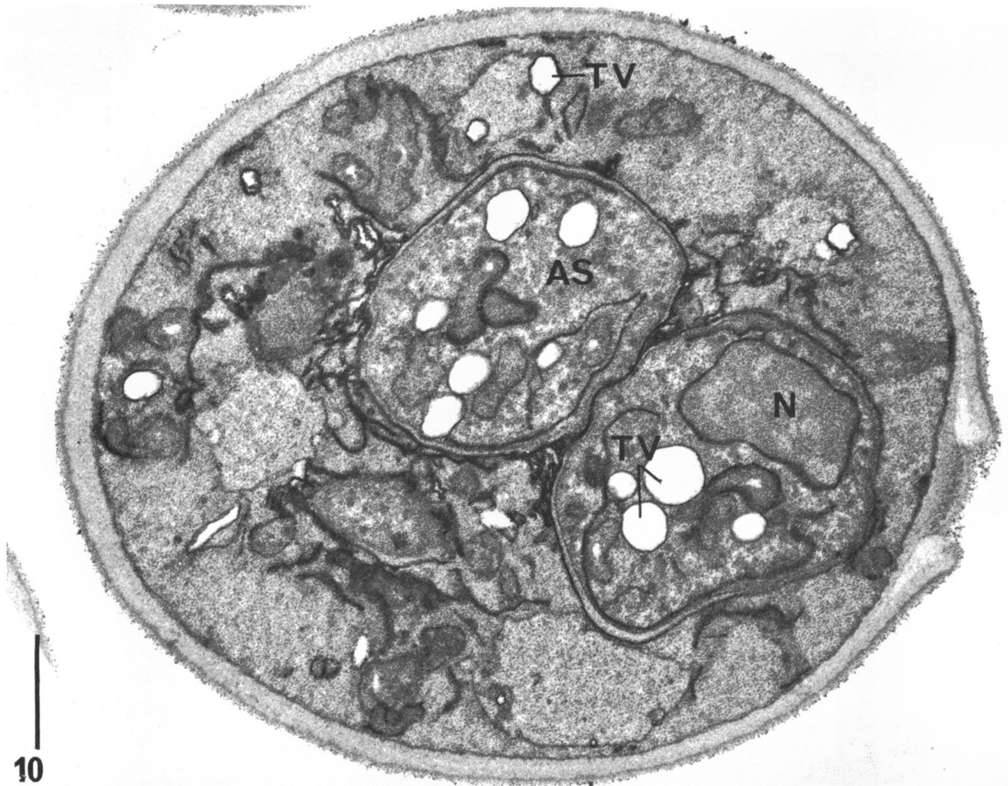


FIG. 8 AND 9



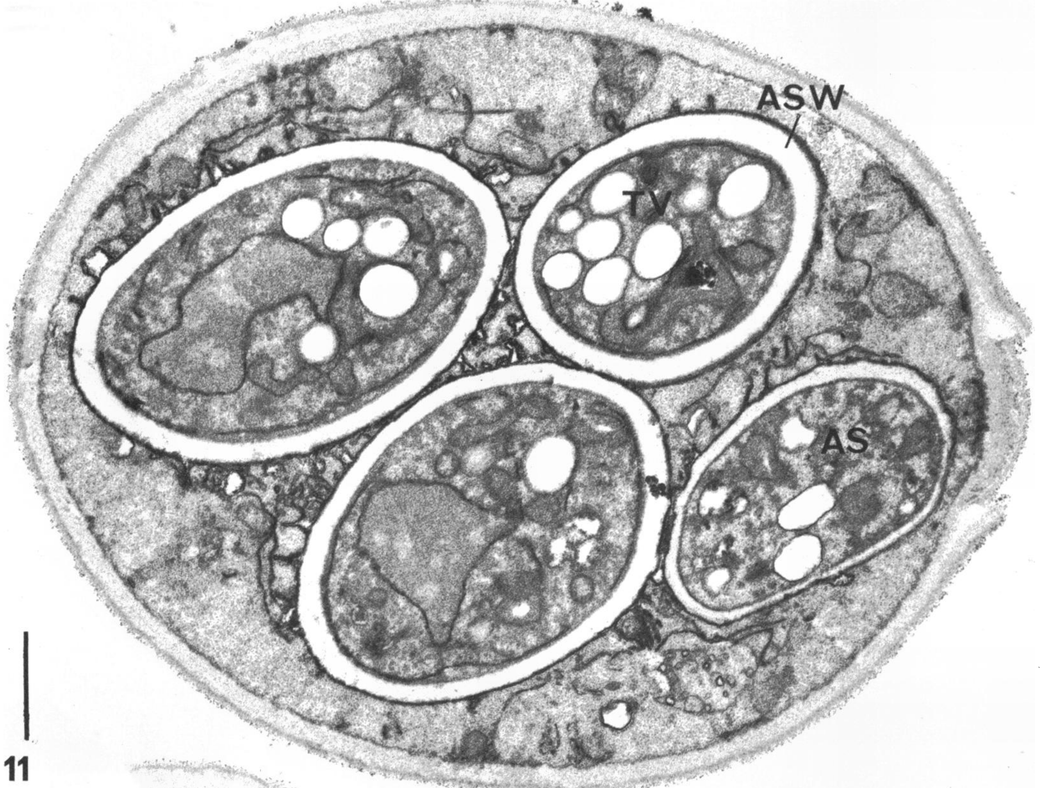


FIG. 10 AND 11

secondly, the location of the electron-transparent vesicles in asci at the time of spore delimitation. A similar association between osmiophilic (lipid) vesicles and "prospore wall" was demonstrated by Moens (26) in glutaraldehyde-fixed cells of *S. cerevisiae* although Moens does not discuss the possibility of a role for these vesicles in wall synthesis. Although our data do not indicate the preferential location, if any, of unsaturated fatty acids in ascan lipids, it is conceivable that these acids are concentrated in sterol esters and triacylglycerols where the extra mobility which unsaturated fatty acids confer on lipids (12) might be important in vesicles which transport wall material or enzymes. However, the increased synthesis of sterol esters and triacylglycerols, both of which are hydrophobic in nature, might be expected to lower the water-retaining capacity of asci, a suggestion which makes it difficult to explain the observed increase in water-retaining capacity of asci as development proceeds.

Electron micrographs obtained in the present study also show that the nucleus becomes highly lobed during meiosis. This finding is in agreement with the observations of Moens and Rapport (27) on "uninuclear meiosis," in which meiosis I and meiosis II are thought to take place without rupture of the nuclear membrane. These observations have recently been confirmed by the elegant freeze-etched micrographs published by Conti and his colleagues (16). Also worthy of mention is the presence of double, delimiting membranes, and their alignment around the meiotic nucleus. The inner of these membranes would appear to form the ascospore plasma membrane. By analogy with other ascomycetes (2, 6, 15, 24, 31), it is presumed that the spore wall is formed by insertion of material between these membranes. This essentially supports the findings of Lynn and Magee (23) with *S. cerevisiae* and Black and Gorman with *Hansenula wingei* (4). The outer membrane presumably gives rise to the proteinaceous spore membrane (5).

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LITERATURE CITED

1. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466-468.
2. Beckett, A., R. Barton, and I. M. Wilson. 1968. Fine structure of the wall and appendage formation in ascospores of *Podospora anserina*. J. Gen. Microbiol. **53**:89-94.
3. Beteta, P., and S. Gascón. 1971. Localization of invertase in yeast vacuoles. FEBS Lett. **13**:297-300.
4. Black, S. H., and C. Gorman. 1971. The cytology of *Hansenula*. III. Nuclear segregation and envelopment during ascosporogenesis in *Hansenula wingei*. Arch. Mikrobiol. **79**:231-248.
5. Briley, M. S., R. F. Illingworth, A. H. Rose, and D. J. Fisher. 1970. Evidence for a surface protein layer on the *Saccharomyces cerevisiae* ascospore. J. Bacteriol. **104**:588-589.
6. Carroll, G. C. 1967. The ultrastructure of ascospore delimitation in *Saccobolus kerverni*. J. Cell Biol. **33**:218-224.
7. Chen, P. S., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756-1758.
8. Croes, A. F. 1967. Induction of meiosis in yeast. I. Timing of cytological and biochemical events. Planta **76**:209-226.
9. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the determination of phospholipids on thin-layer chromatograms. J. Lipid Res. **5**:126-127.
10. Dixon, B., and A. H. Rose. 1964. On the synthesis of ornithine carbamoyltransferase in biotin-deficient *Saccharomyces cerevisiae*. J. Gen. Microbiol. **34**:229-240.
11. Esposito, M. S., R. E. Esposito, M. Arnaud, and H. O. Halvorson. 1969. Acetate utilization and macromolecular synthesis during sporulation of yeast. J. Bacteriol. **100**:180-186.
12. Farrell, J., and A. H. Rose. 1967. Temperature effects on microorganisms. Annu. Rev. Microbiol. **21**:101-120.
13. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **226**:497-509.
14. Fowell, R. R. 1969. Sporulation and hybridization of yeasts, p. 303-383. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts, vol. 1. Academic Press Inc., London.
15. Greenhalgh, G. N., and L. V. Evans. 1968. The developing ascospore wall of *Hypoxylon fragiforme*. J. Roy. Microsc. Soc. **88**:545-556.
16. Guth, E., T. Hashimoto, and S. F. Conti. 1972. Morphogenesis of ascospores in *Saccharomyces cerevisiae*. J. Bacteriol. **109**:869-880.
17. Hashimoto, T., S. F. Conti, and H. B. Naylor. 1958. Fine structures of microorganisms. III. Electron microscopy of resting and germinating ascospores of *Saccharomyces cerevisiae*. J. Bacteriol. **76**:406-416.
18. Hashimoto, T., S. F. Conti, and H. B. Naylor. 1959. Studies on the fine structure of microorganisms. IV. Observations on budding *Saccharomyces cerevisiae* by light and electron microscopy. J. Bacteriol. **77**:344-354.
19. Heinen, W., and H. de Vries. 1966. A combined micro- and semi-micro colorimetric determination of long-chain fatty acids from plant cutin. Arch. Mikrobiol. **54**:339-349.
20. Hunter, K., and A. H. Rose. 1972. Lipid composition of *Saccharomyces cerevisiae* as influenced by growth temperature. Biochim. Biophys. Acta **260**:639-653.
21. Letters, R. 1968. Phospholipids of yeasts, p. 303-319. *In* A. K. Mills (ed.), Aspects of yeast metabolism. Blackwells Scientific Publications, Oxford.
22. Longley, R. P., A. H. Rose, and B. A. Knights. 1968. Composition of the protoplast membrane from *Saccharomyces cerevisiae*. Biochem. J. **108**:401-412.
23. Lynn, R. R., and P. T. Magee. 1970. Development of the spore wall during ascospore formation in *Saccharomyces cerevisiae*. J. Cell Biol. **44**:688-692.
24. Mainwaring, H. R. 1972. The fine structure of ascospore

wall formation in *Sordaria fimicola*. Arch. Mikrobiol. **81**:126-135.
25. Margolis, S. 1969. Structure of very low and low density lipoproteins, p. 369-424. *In* E. Tria and A. M. Scanu (ed.), Structural and functional aspects of lipoproteins in living systems. Academic Press Inc., London.
26. Moens, P. B. 1971. Fine structure of ascospore development in the yeast *Saccharomyces cerevisiae*. Can. J. Microbiol. **17**:507-510.
27. Moens, P. B., and E. Rapport. 1971. Spindles, spindle plaques, and meiosis in the yeast *Saccharomyces cerevisiae* (Hansen). J. Cell Biol. **50**:344-361.
28. Moore, P. R., and C. A. Baumann. 1952. Skin sterols. I. Colorimetric determination of cholesterol and other sterols in skin. J. Biol. Chem. **195**:615-621.
29. Neudoerffer, T. S., and C. H. Lea. 1966. Antioxidants for the (thin layer) chromatography of lipids. J. Chromatogr. **21**:138-140.
30. Patching, J. W., and A. H. Rose. 1969. The effects and control of temperature, p. 23-28. *In* J. R. Norris and D. W. Ribbons. (ed.), Methods in microbiology, vol. 2. Academic Press Inc., London.
31. Plurad, S. B. 1972. Fine structure of ascosporogenesis in *Nematospora coryli* Peglion, a pathogenic yeast. J. Bacteriol. **109**:927-929.
32. Pontefract, R. D., and J. J. Miller. 1962. The metabolism of yeast sporulation. IV. Cytological and physiological changes in sporulating cells. Can. J. Microbiol. **8**:573-584.
33. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208-212.
34. Roth, R. 1970. Carbohydrate accumulation during the sporulation of yeast. J. Bacteriol. **101**:53-57.
35. Sentandreu, R., and D. H. Northcote. 1969. The formation of buds in yeast. J. Gen. Microbiol. **55**:393-398.
36. Shaw, W. H. C., and J. P. Jefferies. 1953. The determination of ergosterol in yeast. Part III. Corrections for irrelevant absorption in solutions of ergosterol. Analyst **78**:519-523.
37. Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells. 1963. Gas-liquid chromatography of trimethylsilyl derivatives of sugars and related substances. J. Amer. Chem. Soc. **85**:2497-2507.
38. Trappe, W. 1938. Modifikation der Jodzahlbestimmungsmethode nach H. P. Kaufmann fur kleine und kleinste Fettmengen. Biochem. Z. **296**:180-185.
39. Van Handel, E., and D. B. Zilversmit. 1957. Micromethod for the direct determination of serum triglycerides. J. Lab. Clin. Med. **50**:152-157.