

CARDIOLIPIN CONTENT OF WILD TYPE AND MUTANT YEASTS IN RELATION TO MITOCHONDRIAL FUNCTION AND DEVELOPMENT

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ABSTRACT

The phospholipid composition of various strains of the yeast, *Saccharomyces cerevisiae*, and several of their derived mitochondrial mutants grown under conditions designed to induce variations in the complement of mitochondrial membranes has been examined. Wild type and petite (cytoplasmic respiratory deficient) yeasts were fractionated into various subcellular fractions, which were monitored by electron microscopy and analyzed for cytochrome oxidase (in wild type) and phospholipid composition. 90% or more of the phospholipid, cardiolipin was found in the mitochondrial membranes of wild type and petite yeast. Cardiolipin content differed markedly under various growth conditions. Stationary yeast grown in glucose had better developed mitochondria and more cardiolipin than repressed log phase yeast. Aerobic yeast contained more cardiolipin than anaerobic yeast. Respiration-deficient cytoplasmic mitochondrial mutants, both suppressive and neutral, contained less cardiolipin than corresponding wild types. A chromosomal mutant lacking respiratory function had normal cardiolipin content. Log phase cells grown in galactose and lactate, which do not readily repress the development of mitochondrial membranes, contained as much cardiolipin as stationary phase cells grown in glucose. Cytoplasmic mitochondrial mutants respond to changes in the glucose concentration of the growth medium by variations in their cardiolipin content in the same way as wild type yeast does under similar growth conditions. It is concluded that cardiolipin content of yeast is correlated with, and is a good indicator of, the state of development of mitochondrial membrane.

INTRODUCTION

The yeast, *Saccharomyces cerevisiae*, offers a unique opportunity for the study of mitochondrial biogenesis. It is a facultative anaerobe, being capable of growing anaerobically with glucose or related carbohydrate as carbon source, while aerobic growth may be supported by a wide variety of

carbon compounds, including lactate, glycerol or acetate. During anaerobic growth on a medium with a high glucose content, cytochromes are not formed and characteristic mitochondrial functions disappear, while during aerobic growth mitochondrial profiles and functions readily develop (Slonimski, 1953; Yotsuyanagi, 1962).

During aerobic growth in the presence of large repressive concentrations of glucose, mitochondrial functions are incompletely developed (Ephrussi et al., 1956; Utter, Duell, and Bernofsky, 1967). With glucose as carbon source, mitochondrial function is only fully expressed as the yeast culture passes into stationary phase, when all the medium glucose is exhausted. This situation clearly affords an excellent system in which to study the factors that regulate the emergence of typical mitochondrial activities. The availability in yeast of both cytoplasmic and chromosomal mutants (Ephrussi, 1953; Sherman and Slonimski, 1964), which affect mitochondrial function, furnishes yet a further advantage for the study of the mechanisms of mitochondrial formation in this organism. The mutants affecting mitochondrial function are readily identifiable by their inability to grow on a nonfermentable carbon source, e.g., glycerol. The lack of oxidative activities in anaerobic yeast is not associated with a complete absence of mitochondrial membrane profiles (Swift, Rabinowitz, and Getz, 1967; Swift, Rabinowitz, and Getz, 1968; Plattner and Schatz, 1969). This situation is reminiscent of that observed in the mitochondrial mutants, where recognizable (cytoplasmic mutants) and in some cases normal (chromosomal mutants) mitochondrial structures are seen without any evident terminal respiratory activity (Yotsuyanagi, 1962). Thus both in anaerobically grown yeast and in certain mutant yeasts, microscopically identifiable mitochondrial membranes and characteristic mitochondrial function do not develop in parallel.

For the study of mitochondrial membrane biogenesis and its regulation, the availability of a membrane marker that is independent of respiratory function would be of considerable value. The characterization of, and analytical procedures for, the structural or noncatalytic proteins are currently insufficiently refined for this purpose. We therefore turned our attention to the phospholipid components of these membranes. We were encouraged in this by the observation that the phospholipid, cardiolipin, is found to be particularly associated with mammalian mitochondrial membranes (Fleischer et al., 1967; Getz et al., 1968) and especially with the inner mitochondrial membrane (Parsons et al., 1967; McMurray and Dawson, 1969). We have embarked on an examination of the phospholipid

composition of whole yeast and its subcellular fractions in order to evaluate whether in yeast, also, cardiolipin is particularly associated with mitochondrial membranes. In this paper we also report on the phospholipid and cardiolipin content of several strains of yeast, diploid and haploid, normal with respect to respiratory status, and derived respiratory-deficient mutants. With one exception (i.e., loss of auxotrophy for lysine in $R_1 P_7 \rho^+$ vs. $scl_1 p_7 \rho^+$ —see Table I), the respiratory mutants were isogenic with the wild type strains in all characters other than their respiratory status. We have analyzed the phospholipids of neutral and suppressive cytoplasmic mitochondrial mutants in view of the possibility that the basic defect in each of these two mutants might be different. This was considered especially important in the light of the observed differences in buoyant density of mitochondrial DNA in mutants of this type (Mounolou, Jakob, and Slonimski, 1966). The strains were cultured under growth conditions designed to induce variations in the complement of mitochondrial membranes.

MATERIALS AND METHODS

Strains of Yeast

All strains of yeast employed in these studies were provided from the collection of Dr. P. P. Slonimski. Three sets of isogenic yeast strains were studied. The phospholipid composition of the first two of these sets was determined, while the third set was used for genetic testing. The characteristics of these strains are tabulated in Table I. An explanation of the significance of the rho factor (ρ) was given elsewhere (Getz et al., 1970).

The particular set of haploid strains was chosen because it contains a large amount of mitochondrial DNA in comparison to the diploid yeast 237 (Mounolou, 1968). Several chromosomal loci controlling mitochondrial function have been identified (Sherman and Slonimski, 1964). This set of strains varies in the locus designated P_7 (respiratory-competent wild type) or p_7 (respiratory-deficient mutant).

Culture Media

Cultures were grown aerobically on enriched media as described by Getz et al. (1970). In some experiments 2% galactose or 2% Na lactate replaced glucose as the carbon source in the medium. For cell fractionation, diploid yeast, wild type ($YF\rho^+$) and "petite" ($YFA\rho^-$), were grown to midexponential phase in 2% galactose. Strains of the haploid set ($scl_1 p_7 \rho^+$; $R_1 P_7 \rho^+$; $g p_7 \rho^-$; $116 p_7 \rho^-$) were

TABLE I
Yeast Strains

Strain	Cytoplasmic petite	Chromo- somo- mal petite	Mat- ing type	Auxotrophy	Abbreviations used in text	Comment
Diploid set						
Yeast foam 237	—	—	—	—	YF ρ^+	
Yeast foam 237 A	+	—	—	—	YFA ρ^-	
Haploid set						
D243-2B-Scl ₁	—	+	α	ad ₁ lys ₂ *	scl ₁ p ₇ ρ^+	Reversion rate 1 in 10 ⁴
D243-2B-R ₁	—	—	α	ad ₁	R ₁ P ₇ ρ^+	Revertant with respect to respiratory deficiency
D243-2B-g	+ (neutral)	+	α	ad ₁ lys ₂	g ⁻ p ₇ ρ^-	17% suppressive‡
D243-2B-116	+ (suppressive)	+	α	ad ₁ lys ₂	116 p ₇ ρ^-	92% suppressive‡
Test set (haploid)						
C982-19D	—	—	a	hi ₁ tr ⁻	19 d ρ^+	
C982-19dA	+	—	a	hi ₁ tr ⁻	19 d A ρ^-	

* Note that in reversion with respect to respiratory deficiency, auxotrophy for lysine is lost.

‡ The degree of suppressiveness was obtained by measuring the percentage of diploid petite colonies obtained after crossing with grande test strain 19d ρ^+ and plating the zygotes on minimal medium with a low glucose content. The crosses were made with test set yeast according to standard procedures (Ephrussi and Grandchamp, 1965; Jakob, 1966). The plates were stained using the tetrazolium overlay technique (Ogur and Rosen, 1950) to determine the number of respiratory-sufficient colonies (which stain red).

grown in the chemostat as described by Mounolou, Jakob, and Slonimski (1966). The medium used for chemostat growth (3-S) contained in 1 liter: 10 g yeast extract, 1 g KH₂PO₄, 1.2 g (NH₄)₂SO₄, and 50 g glucose. 15 ml of Antifoam A (Dow Corning Corp., Midland, Mich.), 1 g streptomycin, and 5,000,000 units penicillin were added to 8 liters of culture medium. Precultures were grown in 3S medium containing only 0.5% glucose and were collected in the exponential phase of growth in order to introduce a low glucose concentration at the beginning of the culture in the chemostat. The 500 ml chemostat flasks contained 88–136 ml of medium. The flow rate was about 18.0 ml/hr and generation times were between 4.2 and 5.4 hr. The collection of the first 48 hr was discarded. The optical density of the cultures was constant during the next 3 days of collection and was indicative of stationary growth phase. Glucose was not detectable in the medium during the steady state growth. Yeast cells were collected for phospholipid analyses on the 3rd–6th day. Cultures were tested daily for growth on different media to ensure that no respiratory sufficient revertants had appeared.

Strains of the haploid set were also grown in shaker flasks to the exponential phase of growth in 3S medium

containing 5% glucose. In some cases, the respiratory-sufficient haploid strain R₁ P₇ ρ^+ was also grown to late stationary phase in 3S medium, and to late stationary and midexponential phases in enriched medium containing 2% glucose.

Yeast Collection and Breakage

The yeast cells were harvested by centrifugation at appropriate stages of the growth cycle, washed twice with ice-cold water, and either broken or frozen immediately thereafter. When phospholipid analysis was to be of the whole yeast only, cell breakage was performed at 0–5°C in the Ribi cell fractionator (Ivan Sorvall Inc., Norwalk, Conn.) as described by Getz et al. (1970). In most cases cells were broken within a week of collection. On the other hand, the cells of the haploid set (R₁ P₇ ρ^+ and mutant cells) grown in the chemostat, and log phase cultures of these same yeast strains grown in 3S medium, but not in the chemostat (grown in Gif-sur-Yvette, France), were stored as a yeast cake at –20°C for 6 months before mechanical breakage and lipid extraction. Small samples of the broken yeast cell suspension were

saved for protein determination according to Lowry et al. (1966).

Cell Fractionation

Cell fractions used to study phospholipid distribution (Table II) were isolated from protoplasts of diploid yeast. Protoplasts were prepared with glucylase (Endo Laboratories, Inc., Garden City, N. Y.) as described by Rabinowitz et al. (1969). They were disrupted as previously described (Rabinowitz et al., 1969) except that the concentration of ethylenediaminetetraacetate (EDTA) in breaking solution was increased from 1×10^{-3} M to 5×10^{-3} M in the hope of minimizing phospholipolytic activity.

A portion of the broken protoplast homogenate was saved for lipid and protein analysis and for cytochrome oxidase assay. The remainder of the suspension was centrifuged in the Servall RC2B refrigerated centrifuge at 1500 *g* for 10 min. The pellet constituted the debris fraction. The supernatant was centrifuged again at 1500 *g* for 10 min and the pellet was discarded. This last step was repeated once more, to reduce contamination of mitochondria by whole protoplasts. The supernatant of the last 1500 *g* centrifugation was centrifuged at 17,300 *g* for 10 min. The pellet was resuspended in protoplast breaking solution and re-centrifuged at 17,300 *g* for 10 min. This last washing procedure was repeated twice more, the final three times washed pellet constituting the mitochondrial fraction. The supernatant from the sedimentation of the mitochondrial fraction was centrifuged at 48,000 *g* for 30 min. The pellet from this centrifugation is designated the intermediate fraction, and the supernatant was sedimented in the Spinco L2-65 centrifuge in rotor No. 60 at 60,000 rpm for 60 min to yield the microsomal pellet and a supernatant that was discarded. The debris, mitochondrial, intermediate, and microsomal fractions were saved for lipid analysis, protein determination, and cytochrome oxidase assay. The same fractionation procedure was employed for diploid petite yeast (YFA ρ^-) except that the mitochondrial fraction was obtained and washed at 27,000 *g* for 10 min. The mitochondrial fractions of both wild type (YF ρ^+) and petite (YFA ρ^-) yeasts were examined by electron microscopy.

Electron Microscopy

Mitochondrial pellets for electron microscopy were prepared from a small sample of the mitochondrial suspension removed prior to the final centrifugation for the third wash of the mitochondrial fraction. The entire pellet from this sample was fixed for electron microscopy in an attempt to ensure random sampling of the mitochondrial fraction. The mitochondrial pellets were fixed for 1 hr at 4°C in 4% glutaralde-

hyde (Polysciences, Inc., Rydal, Pa.) in 0.1 M phosphate buffer and 1% sorbitol. Shortly after introduction into the fixation medium, pellets were divided into pieces less than 1 mm across. These pieces were washed in phosphate-buffered sorbitol, postfixed in 1% osmium tetroxide in phosphate-buffered sorbitol, and embedded in Epon. Thin sections were stained with uranyl acetate and Reynolds' lead citrate.

*Cytochrome *c* Oxidase Assays*

Cytochrome *c* oxidase was measured as described by Rabinowitz et al. (1969). In the case of the cell fractionation experiments, results are expressed as the first order rate constant K/min per mg protein calculated as described by Cooperstein and Lazarow (1951). In the case of yeast grown under various different conditions for lipid analysis of whole yeast, enzyme activities were determined on a mechanically broken yeast suspension and results are expressed as μ moles cytochrome *c* oxidized/min per mg protein. Cytochrome *c* oxidase activity was largely inactivated by breakage of yeast in the Ribi cell fractionator at 40,000 pounds per square inch, the breakage method employed for whole yeast to be subjected to lipid analysis. Therefore, for enzyme assays on whole yeast, samples of yeast, grown under the same conditions as the samples employed for lipid analysis, were broken mechanically in the Braun shaker (W. Braun, Co., Chicago, Ill.) as described by Henson, Weber, and Mahler (1968). The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant was used for oxidase assay. (We are grateful to Dr. B. Levin for help with these experiments.) Repeated assays for cytochrome *c* oxidase in petite yeast revealed no demonstrable activity.

Lipid Extraction and Chromatographic Analysis

Procedures for the extraction, chromatographic quantitative determination of lipid, and expression of results have been described by Getz et al. (1970). The only variation was that samples prepared in the cell fractionation experiments were initially extracted with 4 vol of absolute ethanol (final concentration of ethanol 80%), held for 15 min at 80°C, and shaken at room temperature overnight. This substituted for the overnight room temperature extraction with 19 vol of ethanol (final concentration of ethanol 95%) used with all other yeast samples. This variation was an attempt to limit the phospholipolytic activity in the fractions prior to analysis, as suggested by Letters (1967). Evidence for the quantitative nature of this extraction procedure has been provided by Letters (1966, 1967) and Harrison and Trevelyan (1963). Although peroxides were not measured directly in the lipid extract, major deterioration of lipids did not ap-

TABLE II
Distribution of Phospholipids and Cardiolipin in Subcellular Fractions of Wild Type Yeast (*YFp⁺*)

Fraction	Homogenate (% PLP)*	Debris (% PLP)	Mitochondria (% PLP)	Intermediate (% PLP)	Microsomes (% PLP)
DPG‡	6.5	9.8	15.6	5.0	1.6
PE	14.1	17.2	22.0	16.0	12.4
PC	28.6	31.6	32.5	29.4	40.5
DMPE	1.7	0.8	0.9	1.7	1.4
PG	2.7	1.4	2.0	1.5	
PA	1.5	2.6	1.0	2.6	0.7
PS	4.6	5.4	3.3	8.6	7.3
LPE	7.9	1.7	3.6	4.5	2.0
PI	12.7	22.1	10.7	16.6	27.8
LPC	10.8	2.5	4.2	5.7	2.7
?	1.8	0.5		0.7	
?	6.7	4.8	4.2	7.8	3.7
PLP $\mu\text{g/g}\S$ protein	3285	2050	8030	7920	5235
Cytochrome oxidase K/min per mg protein	21.9	29.9	160.9	37.0	6.5
DPG phosphorus $\mu\text{g/g}$ protein	214	201	1252	396	83.7
% of whole yeast DPG in fraction	100	15.4	64.0	16.1	4.4
% of whole yeast DPG in frac- tion corrected for mitochon- drial contamination		0		4.4	1.7
% of whole yeast PLP in frac- tion	100	13.5	35.2	27.7	23.6
% of whole yeast protein in fraction	100	34.7	23.0	18.4	23.7

* % PLP refers to the percentage of phospholipid phosphorus recovered from the respective thin layer chromatograms.

‡ Abbreviations: DPG, diphosphatidyl glycerol or cardiolipin; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline or lecithin; DMPE, dimethylphosphatidyl ethanolamine; PG, phosphatidyl glycerol; PA, phosphatidic acid; PS, phosphatidyl serine; LPE, lysophosphatidyl ethanolamine; PI, phosphatidyl inositol; LPC, lysophosphatidyl choline or lysolecithin; PLP, phospholipid phosphorus.

§ Total phospholipid phosphorus recovered in the whole extract for each gram of whole yeast protein or fraction protein extracted.

|| The calculations of these percentages were all based upon setting the total cardiolipin, phospholipid, or protein recovered in the sum of the fractions at 100%.

pear to take place as judged by the very pale color of the extract and the absence of any significant material remaining at the origin of thin-layer chromatograms. Sterol analysis was made by the Liebermann-Burchard reaction (Huang et al., 1961) performed on the whole purified lipid extracts. Ergosterol was employed as standard for these analyses.

RESULTS

Lipid Composition of Subcellular Fractions of Wild Type Yeast (*YFp⁺*)

Only by the preliminary formation of protoplasts with glucylase can one expect to obtain a

good yield of intact mitochondria. Mechanical breakage of whole yeast generally results in a low yield of mitochondria, probably due to the high rate of disruption. Disruption of mitochondria will militate against a satisfactory separation of mitochondrial membranes from other subcellular fractions. Since protoplasts are more readily formed from log phase than from stationary cells, galactose-grown cells, harvested in log phase, were employed for yeast cell fractionation. Galactose was chosen as carbon source in order to obtain yeast with a large mitochondrial complement, and this carbon source could also be employed for petite ($YFA\rho^-$) yeast. Either because of the presence of lipolytic enzymes in snail juice or because of activation of endogenous yeast phospholipases during the preparation of yeast cell fractions, considerable difficulty was experienced in obtaining fractions which did not contain large quantities of lysophospholipids, such as lysophosphatidyl ethanolamine or lysophosphatidyl choline. That the extraction pro-

cedure per se was not responsible for the phospholipolysis is suggested by the relatively low amounts of lysophospholipids found in extracts made according to the same procedure from yeast broken mechanically in the Ribi fractionator and extracted immediately. In attempts to minimize such artifactual degradation of phospholipids, the concentration of EDTA in the breakage solution was increased from 0.001 M to 0.005 M, and, immediately after the fraction was obtained, extraction of lipids was initiated with ethanol (80%) for 15 min at 80°C. Despite this, however, substantial amounts of lysophospholipids were found in the yeast cell fractions, the phospholipid composition of which is presented in Table II. Continuing phospholipolysis during the course of fractionation also probably accounts for the fact that all fractions of the petite ($YFA\rho^-$) yeast contained less phosphatidyl inositol and more of an unknown phospholipid, probably lysophosphatidyl inositol, than the starting homogenate (see Table III). Comparison of the

TABLE III
Distribution of Phospholipids and Cardiolipin between Subcellular Fractions of Petite Yeast ($YFA\rho^-$)

Fraction	Homogenate (% PLP)	Debris (% PLP)	Mitochondria (% PLP)	Intermediate (% PLP)	Microsomes (% PLP)
DPG*	4.9	5.7	9.1	2.7	1.2
PE	14.7	15.7	20.5	13.0	13.7
PC	37.6	41.6	35.8	36.1	38.1
DMPE	1.1	Trace	Trace	Trace	0.8
PG	Trace	0.5	0.8	0.6	0.6
PA	2.2	2.4	1.5	2.9	1.3
PS	9.7	9.2	7.4	5.7	3.9
LPE	Trace	0.5	1.9	8.3	5.4
PI	23.9	19.9	16.7	8.9	7.2
LPC	1.6	Trace	1.9	16.0	14.2
?	4.3	4.4	4.7	5.7	13.8
PLP $\mu\text{g/g}$ protein	3316	4500	14340	4640	3109
DPG phosphorus $\mu\text{g/g}$ protein	162.5	256.4	1224	125	37.3
% of whole yeast DPG in fraction	100	40.8	45.0	9.4	4.9
% of whole yeast PLP in fraction	100	36.6	25.2	17.7	20.7
% of whole yeast protein in fraction	100	39.8	8.6	18.8	32.7

* For abbreviations and mode of expression and calculation of data see footnotes to Table II.

results for the homogenate, shown in Table II, with the results for galactose-grown cells disrupted mechanically prior to lipid analysis, suggests strongly that the presence of lysophospholipids in the glucosylase-broken homogenate of yeast is an artifact of the preparative procedure. The level of phospholipid per gram of yeast protein suggests that the lipolysis of phospholipid did not proceed beyond the stage of lysophospholipid. Most important, for the purposes of this paper, the lipolysis does not appear to have affected the cardiolipin.

Bearing in mind the limitations of this analysis, several features of the composition of cell fraction phospholipids are evident. The mitochondrial fraction is characterized by its high cytochrome *c* oxidase activity and its appearance on electron microscopic examination of a representative mitochondrial pellet (Fig. 1). The distinctive feature of the mitochondrial phospholipid is the large proportion of cardiolipin (15.6%) contained in this fraction. Mitochondria also contain a higher ratio of phosphatidyl ethanolamine to phosphatidyl choline than any other cell fraction. They contain a lower proportion of phosphatidyl serine and phosphatidyl inositol than is found in the other cell fractions, while the microsomal fraction contains large amounts of phosphatidyl choline and phosphatidyl inositol and relatively little cardiolipin and phosphatidyl ethanolamine. Similar characteristics were observed in the phospholipid composition of a mitochondrial fraction and a microsomal fraction prepared in an equivalent fashion from glucose-grown aerobic stationary wild type diploid yeast ($YF\rho^+$). The relative cardiolipin content of a mitochondrial fraction of anaerobic wild type diploid yeast ($YF\rho^+$) was also twice that of the whole yeast. The mitochondrial fraction had the highest ratio of phospholipid to protein of all cell fractions. At least 64% of the whole yeast cardiolipin was recovered in the mitochondrial fraction although it contained only 35.2% of the whole yeast phospholipid and 23.0% of the yeast protein. When corrections are made for the contamination of the debris, intermediate and microsomal fractions by mitochondrial membranes on the basis of their specific activity of cytochrome *c* oxidase, it is seen that all but a small proportion of the cardiolipin found in the intermediate and microsomal fractions (total

$\pm 6.0\%$) can be attributed to the contaminating mitochondria. In other words, in excess of 90% of the whole yeast cardiolipin appears to be associated with mitochondrial membranes.

Lipid Composition of Subcellular Fractions of Respiratory-Deficient Yeast ($YFA\rho^-$)

Respiratory-deficient diploid yeast grown to log phase in galactose was subjected to subcellular fractionation in the same fashion as was wild type yeast. However, to ensure a reasonable recovery of mitochondrial membranes, the mitochondrial fraction was sedimented at 27,000 *g* for 10 min rather than 17,300 *g* used for the sedimentation of the wild type mitochondrial fraction. The characterization of the mitochondrial fraction from the respiratory-deficient yeast rests upon the electron microscopic appearance shown in Fig. 2. The micrograph shown is from a representative pellet of the mitochondrial fraction. In general the distinction between phospholipid composition of the mitochondrial fraction and that of the other cell fractions was similar to that obtained for the wild type yeast cell fractions. The data are presented in detail in Table III. The mitochondrial fraction had a high cardiolipin and relatively high phosphatidyl ethanolamine content. The mitochondrial fraction had the highest ratio of phospholipid to protein of all the cell fractions. The cardiolipin content per gram of protein in the petite mitochondrial fraction was strikingly similar (1224 vs. 1252) to that found in the corresponding mitochondrial fraction of the wild type yeast. Much of the whole cell cardiolipin (45%) was recovered in the mitochondrial fraction, which contained 25.2% of the yeast phospholipids and only 8.6% of the yeast protein. A comparison of the subcellular distribution of cardiolipin with that of either cytochrome *c* or mitochondrial adenosine triphosphatase (ATPase), both of which are present in petite mitochondria, would establish whether in the respiratory-deficient yeast, too, cardiolipin is an exclusive marker of mitochondrial membranes. Clearly, however, petite mitochondria do contain large quantities of cardiolipin, and it is likely that most or all of this phospholipid is also associated with mitochondrial membranes.

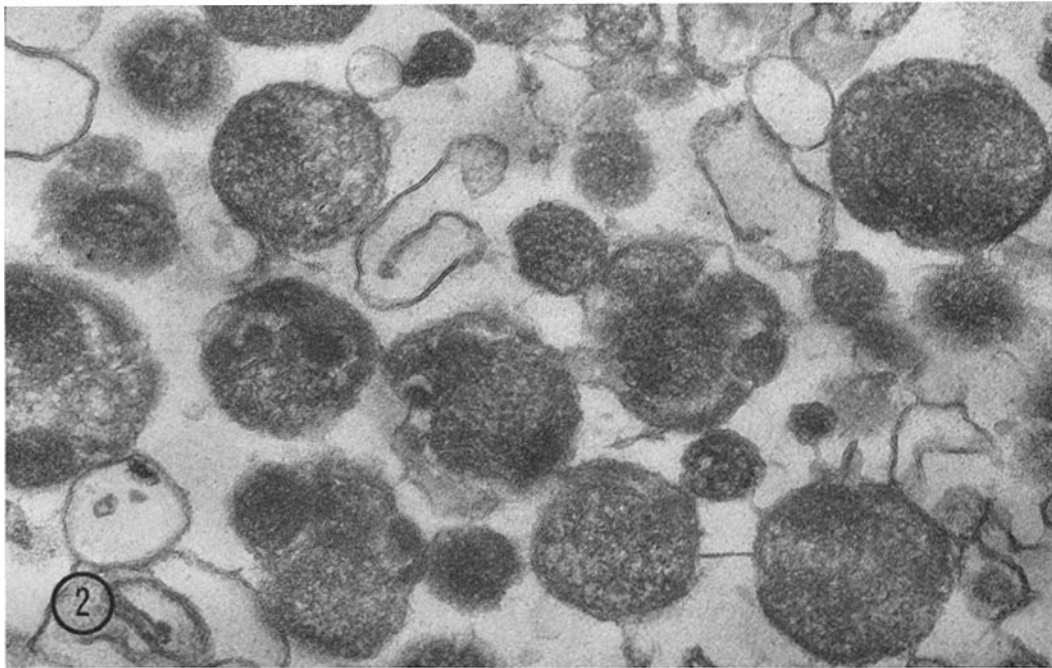
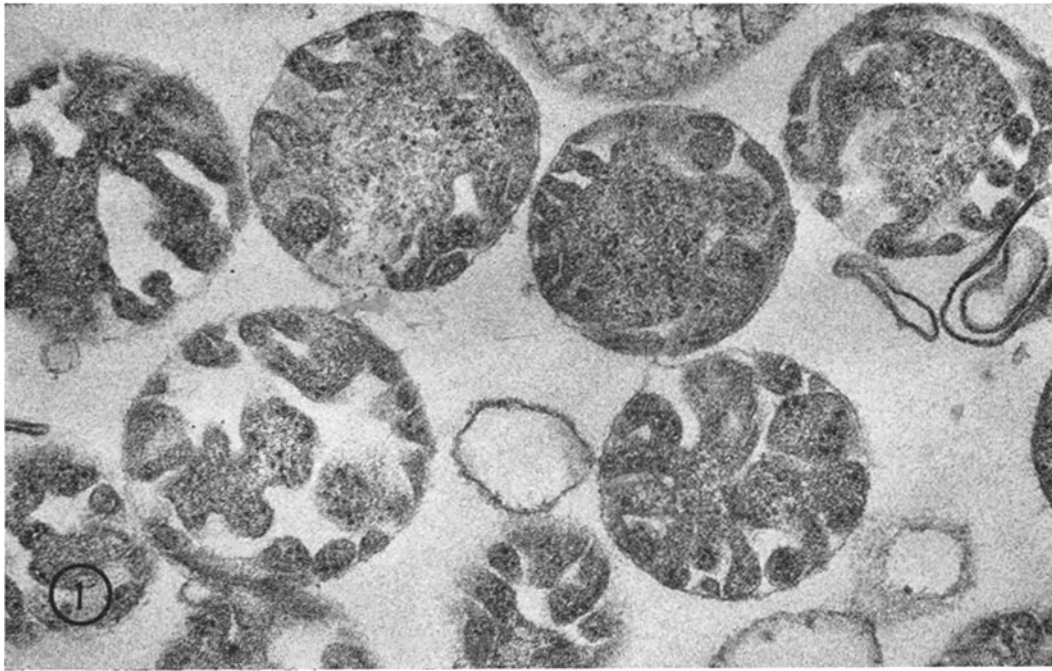


FIGURE 1 Portion of mitochondrial pellet obtained from wild type (YFp⁺) cells. $\times 46,000$.

FIGURE 2 Portion of mitochondrial pellet obtained from petite (YFAp⁻) cells. $\times 59,000$.

TABLE IV
Effect of Carbon Source on Phospholipid Composition of Log Phase Wild Type ($YF\rho^+$) and Petite ($YFA\rho^-$) Yeast*

Yeast strain Carbon source	$YF\rho^+$			$YFA\rho^-$	
	Glucose (3) †, § (% PLP) ‖	Galactose (1) (% PLP)	Lactate (1) (% PLP)	Glucose (2) § (% PLP)	Galactose (1) (% PLP)
DPG ‖	3.3	7.1	6.6	1.3	3.8
PE	21.6	20.5	17.9	21.8	15.9
PC	41.6	39.6	47.3	39.8	43.4
DMPE	1.4	0.7		1.0	
PG	0.3	0.5	1.2	0.6	0.9
PA	0.8	0.5	2.1	1.8	2.3
PS	7.5	5.2	7.0	7.7	4.7
LPE	1.0	0.2		0.2	1.3
PI	20.3	24.6	16.0	21.8	26.1
LPC	0.3	1.3	3.0	2.2	1.5
?	0.4		1.4	0.7	
?	0.9			1.7	0.1
PLP μ g/g protein	2800		2990	2840	3200

* All cultures were grown in enriched medium in which glucose was replaced by the designated substrate at the 2% level. All samples of yeast were mechanically disrupted.

† Numbers in parentheses refer to the numbers of independent cultures analyzed.

§ The data in these columns are from Getz et al. (1970).

‖ For abbreviations and explanation of mode of expression of data see footnote to Table II.

Effect of Carbon Source on Phospholipid Composition of Exponential Phase Diploid Yeast

Since glucose is known to repress the formation of mitochondria, the effects of galactose and lactate replacement of glucose as the major carbon source in the enriched medium were investigated. The results of these experiments are presented in Table IV. The total content of phospholipid and the composition with respect to the major phospholipids were strikingly similar in both wild type ($YF\rho^+$) and petite ($YFA\rho^-$) log phase yeast irrespective of carbon source. The cardiolipin content, however, varied significantly with carbon source and presumably with state of repression of mitochondrial function.

Effect of Growth Conditions on the Cardiolipin Content of Diploid Yeasts

The effects of variations of growth conditions on the cardiolipin content of wild type ($YF\rho^+$) and petite ($YFA\rho^-$) yeast are presented in summary form in Table V. The degree of repression of respiratory function, as represented by cyto-

chrome *c* oxidase activity, and of cardiolipin formation were not similarly affected in a quantitative sense by either glucose or anaerobiosis. Thus, while respiratory activity of nonrepressed or incompletely repressed yeast was four- to sevenfold greater than that of glucose-repressed yeast and 16- to 30-fold greater than that of anaerobic yeast, the differences in cardiolipin content varied only over a two- to fivefold range. Cytoplasmic mutant yeast (ρ^-) had no demonstrable cytochrome oxidase activity, but contained one-third to two-thirds as much cardiolipin as wild type yeast (ρ^+) under the corresponding conditions of growth.

Effect of Growth Conditions and of Mutations Affecting Mitochondrial Function on Cardiolipin Content of Haploid Yeast

As in the case of diploid yeast, high glucose repressed the respiratory activity (in the respiratory competent $R_1 P_7 \rho^+$) and cardiolipin formation in both normal and mutant haploid yeast.

During the growth, harvesting, or storage of the haploid yeast and its derived mutants grown under chemostat conditions, there was activation

TABLE V
Lipid and Cardiolipin Content of Diploid Strains under Various Growth Conditions

Yeast	Gas phase	Carbon source	PLP $\mu\text{g}^*/\text{g}$ protein	Cardiolipin (DPG)* (% Total PLP)	Cardiolipin phosphorus ($\mu\text{g}/\text{g}$ protein)	Cytochrome oxidase ($\text{m}\mu\text{moles}/\text{min}$ per mg protein)
A. Stationary growth						
YF ρ^+	O ₂	Glucose	2780(2)‡	7.5	216.0	136
YFA ρ^-	O ₂	Glucose	1830(3)	3.7	71.0	
YF ρ^+	N ₂	Glucose	1050(2)	4.7	49.1	<5
YFA ρ^-	N ₂	Glucose	2260(1)	0.4	9.0	
B. Exponential growth						
YF ρ^+	O ₂	Glucose	2800(3)	3.3	92.4	20
YFA ρ^-	O ₂	Glucose	2840(2)	1.3	36.9	
YF ρ^+	O ₂	Galactose	3110(2)	6.8	211.5	152
YFA ρ^-	O ₂	Galactose	3260(2)	4.4	143.5	
YF ρ^+	O ₂	Lactate	2990(1)	6.6	197.7	82
YF ρ^+	N ₂	Glucose	1790(2)	2.5	44.8	<5
YFA ρ^-	N ₂	Glucose	2210(2)	1.5	33.1	

* Expression of these results is as indicated in the footnote to Table II.

‡ Figures in parentheses refer to the number of independent cultures analyzed.

of the phospholipolytic enzymes. This was evident from the relatively large amounts of lysolecithin, lysophosphatidyl ethanolamine, and lysophosphatidyl inositol found in extracts of these yeasts. The major objective of this study, however, was to ascertain whether the mutation was associated with a loss of mitochondrial lipids, especially cardiolipin, and whether there was a relationship between the cardiolipin content and the nature of the mutation affecting mitochondrial structure and function. This was why we chose to study these mutants grown under chemostat conditions, i.e., conditions that would permit the maximal development of mitochondrial membranes. It can be seen from Table VI B that in each class of mutant the chemostat-grown cells contained more cardiolipin than the log phase cells. The chromosomal mutant contained the same amount of cardiolipin as its corresponding wild type yeast. On the other hand, as has been observed with the diploid strains, the cytoplasmic petites had significantly less cardiolipin than the wild type R₁ P₇ ρ^+ . In this respect there was little difference between the neutral and suppressive cytoplasmic petites. In the case of log phase-grown cells, respiratory competent and incompetent cells contained similar amounts of cardiolipin.

DISCUSSION

The phospholipid composition of the mitochondria of wild type diploid yeast (YF ρ^+) is strikingly similar to that reported for mitochondria of several mammalian tissues (Fleischer et al., 1967; Getz et al., 1968) and, more recently, of yeast (Letters, 1966; Paltauf and Schatz, 1969). The phospholipid composition of mitochondria in all of these cell types is characterized by a high proportion of cardiolipin and a higher ratio of phosphatidyl ethanolamine to lecithin than is found in other cellular fractions. In few previous reports have the available data permitted an assessment of the proportion of total cellular cardiolipin which is located in the mitochondria. In this study, it is evident that more than 90% of the total yeast cardiolipin is confined to the mitochondrial membranes, presumably the inner mitochondrial membrane. This is also true for fetal and adult rat liver (Jakovic et al., 1971) and probably for rat brain as well (Eichberg, Whittaker, and Dawson, 1964).

The information on the localization of cardiolipin to mitochondrial membranes suggests that this phospholipid may be used as a suitable structural marker of these membranes. Such a marker may be particularly useful in view of the

TABLE VI
Lipid and Cardiolipin (DPG) Content of Haploid Strains (D243-B) (Normal and Different Mutants)

Growth condition and medium	PLP* $\mu\text{g/g}$ protein	Cardiolipin* (DPG) (% total phospholipids)	Cardiolipin (DPG) (phosphorus $\mu\text{g/g}$ protein)	Cytochrome oxidase ($\text{m}\mu\text{moles/min}$ per mg protein)	
<i>A. Normal cells ($R_1 P_7 \rho^+$) in various media</i>					
Late stationary aerobic enriched 2% glucose	1410	13.8	195.0	92.0	
Late stationary aerobic chemostat medium	1990	13.6	271.1		
Late stationary anaerobic enriched 2% glucose	1230	3.3	40.5	<5.0	
Exponential aerobic chemostat	3080	1.7	51.1	19.0	
<i>B. Normal and mutant cells grown in chemostat medium ‡</i>					
Yeast strain	Cells grown in chemostat			Log phase cells	
	PLP* $\mu\text{g/g}$ protein	Cardiolipin* (DPG) (% total phospholipids)	Cardiolipin (DPG) (phosphorus $\mu\text{g/g}$ protein)	Sterol mg/g protein	Cardiolipin (DPG) (phosphorus $\mu\text{g/g}$ protein)
$R_1 P_7 \rho^+ \S$	1870	9.9	185	20.2	68.0
$sc1_1 p_7 \rho^+ \P$ (Chromosomal mutant)	1600	12.1	194	23.4	61.0**
$g p_7 \rho^-$ (Neutral cytoplasmic petite)	1800	5.9	106	27.1	41.4
116 $p_7 \rho^-$ (Suppressive cytoplasmic petite)	1320	5.3	70	28.5	53.2

* Expression of these results is as indicated in the footnote to Table II.

‡ All cultures referred to in this section of this table were grown in chemostat medium (3-S). The log phase cells were grown in this medium but not in the chemostat.

§ 16 % of this culture had become petite (ρ^-).

|| At harvesting of this culture 4.3% of cells were petite (ρ^-).

¶ On the last day of the collection from the chemostat 29% of this strain had become double petite, i.e., $sc1_1 p_7 \rho^-$. This was checked by crossing these cells with test yeast 19 d $A\rho^-$.

** At harvesting of this culture 8.5% of diploid colonies growing from a cross with test yeast 19 d $A\rho^-$ were petite, i.e., $sc1_1 p_7 \rho^-$.

recent findings by Fukuhara (1969) that amounts of mitochondrial DNA do not fluctuate much with the physiological state of yeast. This situation has the particular advantage that it may be used to mark mitochondrial membranes, irrespective of their functional competence, as exemplified by the presence of significant concentrations of cardiolipin in anaerobic yeast, where

it is concentrated in promitochondria (Paltauf and Schatz, 1969), and in respiratory-deficient yeast (this study). That mitochondrial membrane biogenesis may be regulated independent of the development of respiratory capacity is clearly demonstrated by the aerobic development of mitochondria in anaerobically grown yeast even in the presence of cyanide, an inhibitor of

cytochrome oxidase (Slonimski, 1956). Our own studies reported herein suggest that fluctuations in cardiolipin concentration (tabulated in Tables V and VI) reflect alterations in the development of mitochondrial membranes under a wide variety of growth conditions and genetic controls. Furthermore, they support the notion that the mechanism responsible for the regulation of mitochondrial membranes by glucose and anaerobiosis, as measured by variations in yeast cardiolipin content, is still operative even in yeast that is genetically unable to support terminal respiratory activity. Evidence in favor of such a relationship may be briefly summarized as follows:

(a) Mitochondrial development and respiratory capacity occur more prominently in the stationary phase of aerobic yeast growth in glucose (Ephrussi et al., 1956; Utter, Duell, and Bernofsky, 1967). Cardiolipin content of stationary yeast was much higher than that of glucose-grown log phase cells of all strains examined, including the cytoplasmic mutants (see Table V). For the wild type yeast the cardiolipin content of the yeast in various phases of growth was related to the cytochrome oxidase activity (see Tables V and VI A).

(b) The synthesis of respiratory assemblies and the formation of mitochondrial membrane are markedly suppressed under anaerobic conditions (Slonimski, 1953; Yotsuyanagi, 1962; Wallace, Huang, and Linnane, 1968). Anaerobic yeast, at each phase of growth examined, invariably contains less cardiolipin than aerobic yeast, even when the content of the major phospholipid, lecithin is comparable. It is, however, noteworthy that cardiolipin was always present in measurable amounts in anaerobic yeast, even when grown in medium unsupplemented with Tween 80 and ergosterol (data not shown here). Assuming cardiolipin is exclusively localized in yeast mitochondrial inner membrane as appears to be the case in liver (Parsons et al., 1967; McMurray and Dawson, 1969), this may be taken to indicate that the mitochondrial membrane does not completely disappear from anaerobic yeast, even under conditions of minimal supplementation. Recent studies (Criddle and Schatz, 1969; Paltauf and Schatz, 1969; Plattner and Schatz, 1969) have demonstrated the presence of mitochondrial membranes in anaerobic yeast and have shown that cardiolipin is a component of the mitochondrial mem-

brane derived from anaerobic yeast. A similar situation apparently is encountered in the cytoplasmic mitochondrial mutants.

(c) Cytoplasmic mutants, both suppressive and neutral petite, have relatively poorly developed mitochondrial profiles (Yotsuyanagi, 1962) and virtually no respiratory capacity. These mutants also contain less cardiolipin than the corresponding wild type cells (Tables V and VI).

(d) Chromosomal mutation affecting mitochondrial function is usually associated with relatively normal mitochondrial morphology when the mutant is grown under conditions of maximal mitochondrial development (Yotsuyanagi, 1962) (i.e., stationary or chemostat growth) despite the absence of respiratory function. The cardiolipin content of such a mutant grown under nonrepressing conditions was essentially normal (Table VI B).

(e) Lactate does not repress the development of mitochondrial membranes or of respiratory capacity at all, and galactose does so less readily than glucose (Polakis, Bartley, and Meek, 1965; Wallace, Huang, and Linnane, 1968). Log phase yeast foam "grande" ($YF\rho^+$) and cytoplasmic petite ($YFA\rho^-$) grown on lactate and/or galactose had a cardiolipin content at least as high as that found in late stationary yeast grown on glucose, i.e., in derepressed yeast. In the case of the diploid cytoplasmic petite ($YFA\rho^-$) yeast grown to log phase in galactose, the cardiolipin content was twice that found in similar cells grown to late stationary phase in glucose, presumably because the latter yeast, being unable to utilize the products of glycolysis for anabolic energy requirements, may enter a stage of cytoplasmic turnover earlier than wild type yeast.

Cardiolipin was present in all respiratory mutants studied. The relatively high concentration of cardiolipin in the diploid cytoplasmic petite yeast ($YFA\rho^-$) grown to log phase in galactose (Table V) particularly suggests that there is no major defect in cardiolipin synthesis in this mutant. It is of interest that the chromosomal petite, which as far as can be ascertained has qualitatively normal mitochondrial DNA (Mounoulou, Jakob, and Slonimski, 1966), had a normal cardiolipin content when grown in the chemostat under maximally derepressed conditions. Thus, the genetic defect in this case probably does not

diminish the mass of inner mitochondrial membrane under appropriate conditions.

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