JOURNAL OF BACTERIOLOGY, May 1991, p. 3101–3108
0021-9193/91/103101-08$02.00/0

Vol. 173, No. 10

The Phosphoinositol Sphingolipids of *Saccharomyces cerevisiae* Are Highly Localized in the Plasma Membrane

JANA L. PATTON AND ROBERT L. LESTER*

Department of Biochemistry, University of Kentucky, Lexington, Kentucky 40536

Received 11 January 1991/Accepted 12 March 1991

To investigate the vital function(s) of the phosphoinositol-containing sphingolipids of *Saccharomyces cerevisiae*, we measured their intracellular distribution and found these lipids to be highly localized in the plasma membrane. Sphingolipids were assayed in organelles which had been uniformly labeled with [^{3}H]inositol or ^{32}P and by chemical measurements of alkali-stable lipid P, of long chain bases, and of very long chain fatty acids. We have developed an improved method for the preparation of plasma membranes which is based on the procedure of Duran et al. (Proc. Natl. Acad. Sci. USA 72:3952–3955, 1975). On the basis of marker enzyme and DNA assays carried out with a number of preparations, the plasma membranes contained less than 10% vacuolar membranes (α-mannosidase) and nuclei (DNA); the contamination by the endoplasmic reticulum (NADPH-cytochrome *c* reductase) varied from 0 to 20%. The plasma membrane preparations showed a 13-fold increase in the specific activity of vanadate-sensitive ATPase, compared with that in the homogenate, with a yield ranging from 50 to 80%. A comparison of the distribution of the ATPase with that of sphingolipids assayed by a variety of methods showed that 80 to 100% of the sphingolipids are localized in the plasma membrane; the sphingolipids constitute about 30% of the total phospholipid content of the plasma membrane. Minor amounts of sphingolipids that were found in isolated mitochondria and nuclei can be attributed to the presence of small amounts of plasma membrane in these fractions. These results suggest that one or more essential functions of these lipids is in the plasma membrane. Furthermore, sphingolipids may be useful chemical markers of the plasma membrane of *S. cerevisiae*.

Sphingolipids are ubiquitous eukaryotic membrane constituents. Their study began with their discovery in the human brain by Thudichum (48) and is currently focused on their possible role as mediators of membrane signal transduction (12, 14–16). We have chosen to study the role of sphingolipids in *Saccharomyces cerevisiae*, an organism in which molecular genetic techniques can be exploited and which is useful for studies of phospholipid biosynthesis and regulation (6) and of membrane biogenesis, including the intracellular transport and trafficking of lipids and membrane proteins (9, 38).

The fact that hundreds of sphingolipids have been chemically characterized in animals (13) to some extent complicates the task of assigning specific roles to individual sphingolipids. *S. cerevisiae* has the advantage of containing only three major sphingolipids: inositol-P-ceramide (IPC), mannose-inositol-P-ceramide (MIPC), and mannose(inositol-P)$_2$-ceramide [M(IP)$_2$C]. The ceramide is composed of the long chain base phytosphingosine which is N-acylated with an hydroxy C_{26} fatty acid (44, 46). These lipids represent approximately 40% of the total inositol-containing lipids in *S. cerevisiae*. The clearest evidence for an essential role for sphingolipids was the isolation of the first sphingolipid-defective mutant described for any organism: a strain of *S. cerevisiae* with an obligate requirement for a sphingolipid long chain base, such as phytosphingosine, for growth (51) and viability. This mutant lacks serine palmitoyltransferase (33), the first enzyme in sphingolipid long chain base synthesis.

Here we have studied the subcellular distribution of yeast sphingolipids in order to help elucidate their role in the cell. We have examined plasma membranes, mitochondria, and nuclei for the presence of sphingolipids and have found that most, if not all, of these lipids are found in the plasma membrane. We have also developed an improved and simple method for the preparation of yeast plasma membranes. These data indicate that a major function(s) of sphingolipids is likely to be related to the plasma membrane and also suggest that sphingolipid content may be a useful chemical marker for the yeast plasma membrane. Abstracts of this work have appeared previously (29, 30).

MATERIALS AND METHODS

Materials. The sources of materials are as follows: [2-^{3}H]*myo*-inositol and [^{3}H]mannose, American Radiochemicals, Inc.; $^{32}P_i$, ICN Biomedicals Inc.; [^{35}S]methionine and [1-^{14}C]leucine, New England Nuclear; Cellufluor, Polysciences, Inc.; 4-biphenylcarbonyl chloride, Sigma Chemical Co.; Zymolyase 100T, Seikagaku Kogyo Co.; peptone and yeast extract, Difco Laboratories.

Yeast strain and culture conditions. *S. cerevisiae* W303-1B (*MATa ade2-1 can1-100 ura3-1 his3-11,15 trp1-1 leu2-3,112*; obtained from R. J. Rothstein, Columbia University [32]) was grown aerobically at 30°C with shaking. Turbidity was monitored by measurement of A_{650} with a Zeiss PMQ-Z spectrophotometer, and cells were harvested in the logarithmic phase, at absorbances of 5 to 8. For isolation of mitochondria, the culture medium consisted of 1% yeast extract, 1% Bacto-Peptone, 0.5% KH_2PO_4, and 2% ethanol. For isolation of plasma membranes and nuclei, the medium consisted of 1% Bacto-Peptone, 1% yeast extract, 50 mM Na succinate (pH 5.0), 0.001% *myo*-inositol, 0.05% KH_2PO_4, and 4% glucose. In some experiments the medium was supplemented with [2-^{3}H]*myo*-inositol or $H_3{}^{32}PO_4$ for measurement of sphingolipids. Cells grown for plasma membrane isolation were supplemented with [1-^{14}C]leucine or

* Corresponding author.

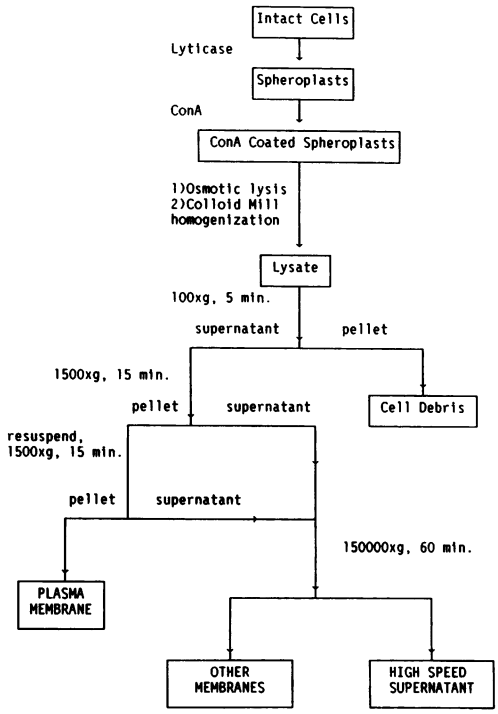

FIG. 1. Scheme for plasma membrane isolation. Details of the procedure are given in Materials and Methods.

[^{35}S]methionine for measurement of protein. For labeling the membranes with mannose, the culture medium was supplemented with 0.4% mannose and [^{3}H]mannose (0.2 μCi/ml).

Preparation of lyticase. Plasma membranes and nuclei were prepared from spheroplasts made with lyticase, an enzyme preparation derived from the culture filtrate of *Oerskovia xanthineolytica* (strain provided by William T. Garrard, University of Texas Southwestern Medical Center) and prepared by the method of Scott and Schekman (41).

Preparation of plasma membranes. The method is outlined in Fig. 1. Spheroplast formation was carried out essentially by the procedure of Szent-Gyorgyi and Isenberg (47). Cells were harvested at 3,000 × *g* for 5 min, washed with S buffer (1.2 M sorbitol, 40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.5], 0.5 mM $MgCl_2$), and suspended in S buffer to 4 ml/g (wet weight). Based upon the weight of the cell pellet, an empirically determined amount of lyticase (the volume required to convert a given weight of cells to spheroplasts in approximately 60 min) was diluted with an equal volume of 2× S buffer, readjusted to pH 7.5, and then added to the cell suspension. This cell suspension containing lyticase was then made 0.5% with respect to β-mercaptoethanol. Spheroplasting was carried out in a shallow layer (~1 to 2 cm) in a gently shaking flask at 30°C. Conversion to spheroplasts was monitored by using Cellufluor, a fluorescent dye which stains carbohydrates of the cell wall (18). The spheroplasts were then diluted two to three times with S buffer and centrifuged at 2,000 × *g* for 5 min. Spheroplasts were washed once in S buffer.

Plasma membranes were prepared from the spheroplasts by a modification of the method of Duran et al. (11). Spheroplasts were washed once with concanavalin A (ConA) buffer (1.2 M sorbitol, 0.05 M Tris-Cl [pH 7.5], 1 mM magnesium acetate, 1 mM $CaCl_2$, 1 mM $MnCl_2$) and then suspended in ConA buffer to a volume of 20 ml/g (wet weight) of cells. An equal volume of ConA buffer containing 3.5 mg of ConA per g (wet weight) of cells was added to this suspension by dropwise addition while gently stirring at room temperature. After the addition of ConA, gentle stirring was continued at room temperature for 10 min. The ConA-coated spheroplasts were centrifuged at 1,000 × *g* for 5 min, washed 2 times with ConA buffer, suspended in lysis buffer [5 mM piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (PIPES), 2 mM EDTA, pH 7.5] in a volume of approximately 50 ml per 3 g (wet weight) of cells, and homogenized in a colloid mill (Gifford-Wood Mini Mill) set at 60% maximal speed and with a rotor-stator distance of 5 to 10 thousandths of an inch. Homogenization was carried out in an ice water bath for three to five 30-s periods or until the plasma membrane ghosts looked relatively free of particulate contamination, as assessed by light microscopy. On the few occasions when the homogenization was incomplete, the homogenate was centrifuged at 100 × *g* for 5 min in 15-ml conical tubes in order to sediment a low-speed pellet consisting of unlysed spheroplasts, cell wall debris, and any aggregates which may have formed. Although the plasma membranes will begin to sediment at that low speed, only the material which actually formed a pellet and did not pour off was considered to be cell debris. The homogenate was then centrifuged at 1,500 × *g* for 15 min at 4°C to sediment crude plasma membranes. The supernatant (S1) was saved. The plasma membranes were washed at least twice by suspending the membrane pellet in lysis buffer and centrifuging at 1,500 × *g* for 15 min at 4°C in 15-ml conical tubes. The supernatants from the washes were combined with the supernatant of the first centrifugation step, S1, to constitute all other sedimentable membranes. All prior steps of centrifugation were done in a swinging bucket rotor. The other membranes were sedimented at 150,000 × *g* for 1 h at 4°C. Both plasma membranes and the other membranes were suspended in a mixture containing 20% glycerol, 10 mM Tris-Cl (pH 7.5), and 0.1 mM EDTA and stored at −80°C.

Preparation of mitochondria. Spheroplast formation was carried out as described by Daum et al. (8) except that Zymolyase 100T was used at a final concentration of 100 U/g (wet weight) of cells. Isolation of mitochondria was carried out essentially as described previously (8) by differential centrifugation of the homogenized spheroplasts. The homogenization buffer was 0.6 M sorbitol and 10 mM Tris-Cl, pH 7.5. The mitochondrial supernatant was centrifuged at 200,000 × *g* at 4°C for 30 min to sediment the other membranes. The other membranes and mitochondrial pellets were resuspended in homogenization buffer with a Dounce homogenizer and stored at −80°C.

Preparation of nuclei. Spheroplasts were prepared as described above for the preparation of plasma membranes. Nuclei were isolated by the method of Szent-Gyorgyi and Isenberg (47). The upper and lower layers from the first centrifugation step were separately diluted with 20 mM PIPES (pH 7.5)–2 mM EDTA and centrifuged at 200,000 × *g* for 1 h at 4°C; these pellets and the high-speed supernatant were saved for analysis. Nuclei were suspended in Ficoll buffer (47) and stored at −80°C.

Enzyme and protein assays. Published procedures were used to assay cytochrome *c* oxidase (27), vanadate-sensitive ATPase (21), α-mannosidase (55), NADPH-cytochrome *c* reductase (37), and chitin synthetase (20). Protein concentrations were assayed by the method of Lowry et al. (25) or by the bicinchoninic acid method (43) with bovine serum albumin as the standard. For the estimation of protein in plasma membrane preparations, which contained high levels

of ConA, cells were labeled with either [1-^{14}C]leucine or [^{35}S]methionine (0.025 to 0.5 μCi/ml of culture medium). The radioactivity observed in the isolated organelles was then converted to protein by correlation to the actual protein content (as measured by the Lowry or bicinchoninic acid method) of lysed, labeled spheroplasts not coated with ConA.

Isolation and estimation of DNA. The isolation of DNA from various cellular particles was carried out by the method of Kumari et al. (22). DNA estimation was performed by the method of Abraham et al. (1), except that amyl acetate was not added to extract the colored product of the diphenylamine-deoxypentose sugar reaction. To 2 ml of perchloric acid-hydrolyzed DNA was added 2 ml of 4% diphenylamine in glacial acetic acid, which was followed by mixing with 0.1 ml of aqueous acetaldehyde (1.6 mg/ml). Calf thymus DNA standards (1 to 25 μg/ml) in 1 M perchloric acid were treated with diphenylamine and acetaldehyde as described above. Samples and standards were then incubated for 1 h at 56°C, and the A_{595} was measured.

Sphingolipid analysis. Cells were grown in culture media supplemented with [^{3}H]inositol (0.2 to 0.8 μCi/ml) or $H_3{}^{32}PO_4$ (0.8 to 1.2 μCi/ml) to label sphingolipids. The cells were fractionated as described above, and the membrane suspensions were extracted with 1.4 times their volume in ethanol-diethyl ether-pyridine (15:5:1) containing 100 μl of concentrated NH_4OH per 100 ml. The lipid extract was subjected to mild alkaline methanolysis, separated from nonlipids to produce a sphingolipid fraction (3), and resolved by high-pressure liquid chromatography (HPLC) (51). In some cases sphingolipids were assayed by measuring the alkali-stable lipid P content; samples were extracted, deacylated, and separated from nonlipids as described above, and the sphingolipid fraction was assayed for lipid P (3).

Long chain base analysis. A lipid extract of membranes (0.5 to 3 mg of protein) prepared as described above was dried under nitrogen and hydrolyzed in methanolic HCl, and the long chain bases converted to UV-absorbing biphenyl-carbonyl derivatives were resolved by HPLC as previously described (10, 19).

Fatty acid analysis. Membranes (0.5 to 3 mg of protein) were extracted as for long chain base analysis, the extracts were dried under nitrogen and saponified, and the fatty acids were converted to UV-absorbing phenacyl derivatives (54), which were resolved by using reverse-phase HPLC as described previously (10).

Sterol analysis. Total lipids were extracted from membranes as described previously (24). Five volumes of ethanol-diethyl ether (3:1) were added to an aqueous membrane suspension. After 20 min, 3 volumes of petroleum ether were added and the samples were mixed. Six volumes of 1 M KCl were added, the tubes were mixed vigorously and centrifuged briefly at low speed to separate the phases, and the upper phase was saved. The lower phase was extracted with 3 volumes of petroleum ether, and the combined upper phases were evaporated to dryness under a stream of nitrogen. Sterols were estimated essentially as described by Courchaine et al. (7) with ergosterol as a standard.

Lipid P assay. Samples containing up to 0.15 μmol of P were evaporated to dryness. Organic P was converted to P_i by refluxing for 1 h with 0.4 ml of 70 to 72% perchloric acid; P_i was determined as described by Bartlett (2).

Assay of glycerophospholipids. Membranes from cells grown in the presence of $H_3{}^{32}PO_4$ were extracted as described above for the analysis of sterols (24). The dried lipid extracts of plasma membrane and other membrane samples corresponding to 3 and 11 mg of membrane protein, respectively, were resuspended in 2 ml of CH_3OH-toluene (1:1). After treatment for 1 h at room temperature with 2 ml of 0.2 N KOH in CH_3OH, 0.4 ml of 1 N acetic acid and 4 ml (each) of $CHCl_3$ and H_2O were added. The samples were mixed thoroughly and briefly centrifuged to separate the phases, and the upper phases containing the glycerophosphoryl esters derived from the deacylated lipids were removed and dried under N_2. Anion-exchange chromatography of the glycerophosphoryl esters was carried out with an ammonium formate-borate gradient, as described in reference 24, which was followed by Cerenkov counting.

Determination of the effect of spheroplasting enzymes on sphingolipids. To test the possibility that both the Zymolyase and lyticase preparations used in the present study could cause sphingolipid degradation, the following experiments were carried out. An alkali-stable sphingolipid fraction was prepared (51) from [^{3}H]inositol-labeled cells and from the lyticase-derived spheroplasts. An amount of each sample equivalent to about 1 absorbance unit was chromatographed on EDTA-treated SG-81 paper developed with $CHCl_3$–CH_3OH–4.2 N NH_4OH (9:7:2, vol/vol) in order to separate the sphingolipids (45). Each lane was cut into 1-cm pieces, and the radioactivity was measured by scintillation counting.

To test the effect of Zymolyase on sphingolipids, purified, radiolabeled lipids were incubated with Zymolyase at concentrations of approximately 10 U/nmol of sphingolipid (a much higher concentration than would occur during normal spheroplasting conditions). After 2 h, the solutions were directly spotted onto EDTA-treated SG-81 paper and chromatographed and counted as described for the lyticase experiments.

RESULTS

Plasma membrane isolation. The method described here for plasma membrane preparation is outlined in Fig. 1, and while it is based on the procedure of Duran et al. (11), it differs in a few important respects. Lyticase, prepared in this laboratory, was used instead of Glusulase for the preparation of spheroplasts. It was found that both Glusulase and a commercial sample of lyticase (Zymolyase 100T) formed spheroplasts that were both less complete (more cell wall remaining) and more unstable than those which could be formed using lyticase prepared in this laboratory. This was an important finding, since stable, completely spheroplasted cells resulted in stable ConA-coated spheroplasts which could subsequently be lysed in a controlled manner. Premature lysis yielded aggregates of cellular debris which contaminated the final plasma membrane preparation. The procedure for the formation of ConA-coated spheroplasts was also altered in that the spheroplasts were suspended at a lower concentration in a modified buffer and the ConA reagent was more dilute and was added in a dropwise manner, resulting in a far more uniform coating of the spheroplasts with little or no visible flocculation (unlike the previously described method). Finally, of the several procedures employed for the homogenization of the ConA-coated spheroplasts (e.g., mild sonic treatment, Potter-Elvehjem), the best was the use of a colloid mill, which forced the spheroplasts through a reproducibly restricted gap between the rotor and stator; these conditions not only disrupted the spheroplasts but gave a dispersion of the plasma membrane ghosts free of other cellular contaminants, which remained attached with alternate methods of homogenization. This homogenization procedure allows the subsequent isolation

TABLE 1. Composition of isolated plasma membrane fractions[a]

Membrane component	Recovery[b]		Amt of component[c]	Relative enrichment[d]
	Mean ± SD (n)	Range		
Protein	5.2 ± 2.3 (7)	3.3–10.7		
Vanadate-sensitive ATPase	64.8 ± 15.1 (7)	47–81.8	1,200	13.2
α-Mannosidase	6.3 ± 5.0 (7)	0.7–16.7	0.082	0.9
NADPH-cyt *c* reductase	16.2 ± 9.6 (6)	0.0–21.1	34	3.5
DNA	4.2 ± 2.7 (2)	1.5–6.8	4.4	1.2

[a] Protein, DNA, and enzymes were assayed and plasma membranes were isolated as described in Materials and Methods. cyt *c*, cytochrome *c*.
[b] Values are expressed as percentage of the homogenate.
[c] Values for enzymes are expressed in nanomoles per minute per milligram of protein; the value for DNA is expressed in micrograms per milligram of protein. Values shown represent a typical preparation.
[d] Total homogenate = 1.0. Values shown represent a typical preparation.

of plasma membranes of high yield and purity by low-speed centrifugation followed by low-speed washes.

Evaluation of the purity of the isolated plasma membrane fraction. Purity of the plasma membrane fraction was evaluated by measuring various enzymes and DNA as markers for contaminating organelles. It is clear from Table 1 that ~50 to 80% yields of the plasma membrane were obtained, as determined on the basis of the recovery of the vanadate-sensitive ATPase, a plasma membrane marker. On average, approximately 90% of the ATPase activity found in the plasma membrane fractions was vanadate sensitive. Generally, these preparations contained less than 10% of the homogenate protein, the vacuolar α-mannosidase activity, and the DNA. The contamination of the plasma membrane fraction by the endoplasmic reticulum was variable, as judged by the marker NADPH-cytochrome *c* reductase, which showed a mean recovery of 16.2% with a range of 0 to 21.1%. A typical preparation (Table 1) showed that the specific activity of the ATPase was enriched 13.2-fold over that of the homogenate. While the specific activity of the NADPH-cytochrome *c* reductase and the DNA content were also slightly enriched over those of the homogenate, the overall plasma membrane contamination determined by these organelle markers was quite low.

TABLE 2. Lipid composition of typical plasma membrane preparations

Expt and components[a]	Content[b]	% of homogenate	Relative enrichment[c]	Phospholipid content (mol%)
I				
Vanadate-sensitive ATPase	1,200	72.6	13.2	
Sphingolipid P	207	57.4	10.4	
Long chain base	207	64.7	11.8	
Very long chain fatty acid	250	67.4	12.3	
II				
Vanadate-sensitive ATPase		81.8		
Sphingolipids	159.1	75.2		30.7
Phosphatidylcholine	88.3	42.4		17.0
Phosphatidylethanolamine	72.5	47.5		14.0
Phosphatidylinositol	143.3	48.3		27.7
Phosphatidic acid	13	62		2.5
Phosphatidylserine	19.8	45		3.8
Cardiolipin	21.9	48		4.2
Sterol	486	59		

[a] In experiment I, sphingolipids were assayed by measuring the amount of alkali-stable lipid P and the amount of $M(IP)_2C$ relative to the amounts of IPC and MIPC, estimated by separation of the lipids by HPLC. In experiment II, sphingolipids were estimated by measuring the amount of alkali-stable lipid P and assuming that 57% of the sphingolipids were $M(IP)_2C$. Preparation II contained 10% of the total α-mannosidase activity and 29% of the total NADPH-cytochrome *c* reductase activity. Long chain bases are composed of 53% C_{18} and 47% C_{20} phytosphingosine, and very long chain fatty acids are composed of 23% C_{26} and 77% OH C_{26} fatty acids.
[b] ATPase values are expressed in nanomoles per minute per milligram of protein and lipid values are expressed in nanomoles per milligram of protein.
[c] Total homogenate = 1.

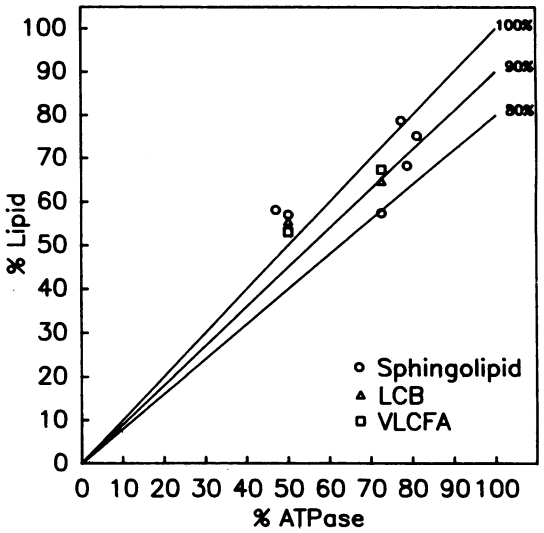

FIG. 2. Sphingolipid and vanadate-sensitive ATPase recovery in several plasma membrane preparations. For several plasma membrane preparations, the recovery of vanadate-sensitive ATPase is plotted against the recovery of sphingolipid measured as alkali-stable P (○), long chain bases (△), and very long chain fatty acids (□). Theoretical lines are drawn to indicate 100, 90, and 80% localization of the homogenate sphingolipids in the plasma membrane, assuming 100% of the ATPase is in the plasma membrane.

Measurement of sphingolipid components in isolated plasma membranes. Several parameters which are diagnostic for sphingolipids were measured and compared with the recovery of vanadate-sensitive ATPase in plasma membranes. We measured the total amount of sphingolipid long chain bases present in the lipid extract that could be liberated by acid hydrolysis; as shown earlier these were C_{18} and C_{20} phytosphingosines (44). Also measured in the lipid extract were very long chain fatty acids, primarily C_{26}, characteristic of yeast sphingolipids (44). Results from a typical preparation are summarized in Table 2 (experiment I) along with a measurement of the base-stable lipid P. Depending upon the parameter measured, the sphingolipid content (in nanomoles per milligram of protein) was found to vary as follows: plasma membrane (207 to 250), total sedimentable membranes (46 to 53), and homogenate (18 to 20). It can be seen that all three sphingolipid markers, long chain base, very long chain fatty acid, and alkali-stable lipid P gave equivalent results.

Correlation of sphingolipid content and vanadate-sensitive ATPase activity in various plasma membrane fractions. In Fig. 2 we have compared the percent recovery of vanadate-sensitive ATPase activities in six plasma membrane preparations with the percent recovery of sphingolipids, measured

TABLE 3. Composition of the isolated mitochondrial fraction[a]

Mitochondrial component	% of total membranes	Relative enrichment[b]
Protein	21.7	
Cytochrome *c* oxidase[c]	87	4.3
Chitin synthase[d]	1.6	0.08
[^{3}H]mannose	13	0.68
Total sphingolipids	7.4	0.34

[a] Protein, enzymes, and [^{3}H]mannose were assayed and mitochondria were isolated as described in Materials and Methods. Sedimentable [^{3}H]mannose was measured in a separate experiment in which cells were grown with [^{3}H]mannose. Cells were labeled with [^{3}H]inositol for the measurement of sphingolipids as described in Materials and Methods.
[b] Total membrane = 1.0.
[c] Specific activity, 3.0×10^{-3} min^{-1} mg of $protein^{-1}$.
[d] Specific activity, 0.25 nmol/h/mg of protein.

TABLE 4. Composition of isolated nuclear fraction[a]

Component	% of membrane	Relative enrichment[b]
Protein	3.3	
Vanadate-sensitive ATPase	13.4	0.54
NADPH-cyt *c* reductase	33.3	1.36
DNA	54	2.18
Total sphingolipid P	11.9	0.46

[a] Enzymes, protein, and DNA were assayed and nuclei were isolated as described in Materials and Methods. Sphingolipids were assayed by measuring alkali-stable lipid P as described in Materials and Methods. The specific activities are as follows: ATPase, 187 nmol/min/mg of protein; NADPH-cytochrome *c* (cyt *c*) reductase, 110 nmol/min/mg of protein; DNA, 11.6 μg/mg of protein; sphingolipid P, 8.5 nmol/mg of protein.
[b] Total membranes = 1.0.

as alkali-stable P (all six preparations) and as long chain bases and very long chain fatty acids (two preparations). Lines are drawn to indicate where points would fall if 100, 90, or 80% of the homogenate sphingolipids were localized in the plasma membrane. As can be seen, the majority of points lie within the 80 to 100% range. There are a few points which lie above the 100% line, which is, of course, theoretically impossible, since it would mean that more than 100% of the sphingolipids were found in the plasma membrane. Those points, along with the 80 to 100% variance, are more likely attributable to the error inherent in the ATPase assay than to the sphingolipid measurements, which have been more reliable in our experience. Thus, within the error limits of the various measurements, we can conclude that 90% or more of the yeast sphingolipid is localized in the plasma membrane. Direct measurements of the sphingolipid content of other isolated organelles, i.e., mitochondria and nuclei, are consistent with this conclusion.

Composition of isolated mitochondria. The mitochondrial preparation was obtained from cells cultured either with [^{3}H]inositol to label the sphingolipids or with [^{3}H]mannose, which should label plasma membrane glycoproteins. Because of the high levels of ATPase in the mitochondria, it was difficult to measure the vanadate-sensitive plasma membrane ATPase that might be present as a contaminant. As shown in Table 3, the mitochondrial fraction contains 87% of the sedimentable cytochrome *c* oxidase and 21.7% of the sedimentable total membrane protein. A small contamination with other organelles is indicated by the presence of 13% of the sedimentable mannose and 1.6% of the putative (23, 35) plasma membrane marker chitin synthase. The 7.4% of the total sphingolipids found in the mitochondrial preparation could thus be accounted for by contamination with plasma membranes.

Composition of isolated nuclei. The isolated nuclei and other sedimentable membranes were assayed for protein, marker enzymes, DNA, and alkali-stable lipid P corresponding to sphingolipids. As shown in Table 4, the nuclear preparation obtained in about a 54% yield, as judged by DNA recovery, was contaminated with 13.4% of the plasma membrane and 33.3% of the endoplasmic reticulum, as judged by vanadate-sensitive ATPase and NADPH-cytochrome *c* reductase assays, respectively. The sphingolipid P found in this fraction, 11.9% of the total, could thus be accounted for by contamination with plasma membranes.

Phospholipid composition of the plasma membrane fraction. Table 2 (experiment II) gives the phospholipid composition of the plasma membrane fraction obtained for one preparation. In this case, 81.8% of the plasma membrane ATPase was recovered along with 75.2% of the sphingolipids. The molar sphingolipid content was estimated by assuming that 57% of the alkali-stable lipid P is composed of $M(IP)_2C$, with the balance being IPC and MIPC (a typical percentage found in those preparations in which the individual sphingolipids were identified). In contrast, only 42 to 48% of the glycerophospholipids cardiolipin, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine were found in this fraction, indicating that those lipids are not uniquely localized in the plasma membrane. The amount of phosphatidic acid found in the plasma membrane may be spuriously high because of breakdown of the other phospholipids, possibly by a phospholipase D. Sphingolipids, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine are the most abundant phospholipids in the plasma membrane fraction; phosphoinositol-containing lipids constitute over 50 mol% of the phospholipid. Notably, 48% of the homogenate cardiolipin is found in the plasma membrane fraction, suggesting that this lipid may not be totally confined to the mitochondrial compartment. Although this number may be somewhat high because of contamination with other organelles, none of these preparations has ever had contamination of such a magnitude as to account for the entire 48% found here. Because these cells were grown on glucose in order to assay for the vanadate-sensitive ATPase without interference from the mitochondrial ATPase, we were unable to assay for mitochondrial membrane enzymes. However, regarding other membrane markers for this particular preparation, there was approximately 29% of the homogenate endoplasmic reticulum marker NADPH-cytochrome *c* reductase and there was 10% of the vacuolar α-mannosidase activity of the homogenate. Thus, the contamination with other organelles cannot fully account for the cardiolipin observed and a significant percentage of the cellular cardiolipin may be found in the plasma membrane.

Analysis of long chain bases in high-speed supernatant. To confirm the fact that all sphingolipids are membrane bound and remain so during the course of plasma membrane isolation, the high-speed supernatant was analyzed for the presence of long chain bases. Less than 1% of the total cellular long chain bases was detected, indicating that sphingolipids are not present in the soluble fraction of the cell.

Effect of spheroplasting enzymes on yeast sphingolipids. The cell wall-digesting preparation Glusulase used by others to prepare yeast spheroplasts (11) had been shown (unpublished observation) to degrade yeast sphingolipids. Accordingly, Zymolyase and lyticase preparations were examined

as possibly causing sphingolipid degradation (see Materials and Methods). With either lyticase or Zymolyase there was no significant qualitative or quantitative difference between the sphingolipid chromatographic profiles obtained for whole cells and those obtained for spheroplasts.

DISCUSSION

Our work shows that the sphingolipids of *S. cerevisiae* are highly localized in the plasma membrane. This result is based on an improved method for plasma membrane preparation, on the comparable enrichment of sphingolipids and the plasma membrane ATPase, and on the quantitative evaluation of its level of contamination with other organelles. In preparations yielding 50 to 80% of the plasma membrane vanadate-sensitive ATPase, the mean contamination with vacuolar membrane α-mannosidase was 6.3% and with DNA was 4.2%. The most significant contamination appeared to be that of the endoplasmic reticulum, as judged by a highly variable contamination with NADPH-cytochrome *c* reductase (mean recovery, 16.2%). The assumption that NADPH-cytochrome *c* reductase is an endoplasmic reticulum marker in *S. cerevisiae* is based on studies by Schatz and Klima (37). In contrast, Serrano (42) found that most of the reductase was associated with particles containing mitochondrial cytochrome *c* oxidase and therefore judged it not to be a good microsomal marker. In addition, we found a large portion (≈40%) of the activity to be soluble, as did Serrano (42). Thus, the quantitative significance of the contamination with NADPH-cytochrome *c* reductase is not easily interpretable. Nevertheless, the distribution of the reductase does not parallel that of sphingolipids since the nuclear preparation (Table 4), which had 33.3% of the NADPH-cytochrome *c* reductase activity, contained only 11.9% of the sphingolipids. Likewise, it is clear from both the nuclear and mitochondrial data that sphingolipids are not enriched in either of those organelles. In fact, the amounts of sphingolipid present in the nuclei and mitochondria are in close correspondence with the amount of contamination present from the plasma membrane markers vanadate-sensitive ATPase and sedimentable [^{3}H]mannose. Given all the uncertainties of marker assays, it is clear that sphingolipids are highly localized in the plasma membrane fraction and that their presence therein cannot be accounted for by contaminating organelles. The level of sphingolipids found, 30% of the plasma membrane phospholipids, might be overestimated given the existence of glycerophospholipases in *S. cerevisiae* (for references, see Witt et al. [53]).

Nurminen et al. (28) have described cell envelope fractions containing fragments of the cell wall and the plasma membrane of *S. cerevisiae* in which sphingolipids were found (50). No quantitative data was provided to permit conclusions about the overall subcellular distribution or content of sphingolipids.

Three main strategies have been employed by others to isolate plasma membranes from *S. cerevisiae*; however, of the many procedures described, few address the issues of yield, enrichment, and purity, making it difficult to compare those works to our own. The first method involves mechanical disruption of the cells, which is then followed by differential or density gradient centrifugation (23, 31, 42, 52). To disrupt the cell wall, relatively harsh mechanical treatments are required; this can result in breakage of organelles and extensive cross-contamination of membrane fractions. Although this approach may be useful, no one has yet documented a preparation of high yield, enrichment, and purity. Because there is a need for density gradient centrifugation in many instances, this procedure is relatively more time-consuming than our method.

A second strategy of plasma membrane preparation involves isolation of a cell wall fraction with the attached plasma membrane after mechanical disruption by glass beads and subsequent enzymatic (snail gut enzyme) removal of the cell wall (28). The yield, the enrichment of some plasma membrane marker over the homogenate, and the possible contamination by other organelles were not addressed in the description of this procedure.

In the third, most commonly used method, spheroplasts are enzymatically prepared and lysed and then plasma membranes are isolated by differential or density gradient centrifugation. As stated earlier, even though some of these methods (4, 17, 26, 39) probably result in quite clean plasma membranes, many authors do not report quantitative parameters such as yield, enrichment, or purity. An exception is the procedure of Bottema et al. (4), who obtained approximately 90% of the ATPase activity with little contamination by other membrane markers. Perhaps because of the yeast strain used, in our hands (unpublished data) this procedure resulted in plasma membranes with substantial contamination with other organelles.

Spheroplasts have been treated with cationic microbeads (40) and ConA in order to stabilize the plasma membrane and alter its density for ease of separation during isolation steps. First used by Scarborough with *Neurospora crassa* (36) and subsequently by others with *S. cerevisiae* (5, 11, 34, 49), several procedures have been described which use spheroplasts coated with ConA to give preparations of ghosts and large plasma membrane sheets. Some workers (11, 49) use density gradient centrifugation and various other manipulations such as α-methyl mannoside treatment, centrifugation through 20% sucrose, and sonication in high salt concentrations. Our approach is simple in that ConA-coated plasma membranes are isolated by low-speed centrifugation following a controlled homogenization. Santos et al. (34) and Bussey et al. (5) described a procedure similar to ours in that ConA-coated spheroplasts were lysed and their plasma membranes were purified by low-speed centrifugation. However, thorough marker enzyme analysis to assess contamination by other organelles was not carried out. Both (5, 34) reported a high yield of plasma membranes and Bussey et al. (5) obtained an 8- to 10-fold purification of plasma membranes with respect to the homogenate. The method described here for plasma membrane isolation is fast and efficient and results in relatively pure plasma membranes which have not been subjected to harsh mechanical treatment or to solutions of nonphysiological density.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grants AI20600 and GM41302. J.L.P. was supported in part by a Lucille Parker Markey Fellowship.

We acknowledge the excellent technical assistance of Gerald B. Wells. We thank Charles J. Waechter for a critical reading of the manuscript.

REFERENCES

1. **Abraham, G. N., C. Scalletta, and J. H. Vaughan.** 1972. Modified diphenylamine reaction for increased sensitivity. Anal. Biochem. **49**:547–549.
2. **Bartlett, G. R.** 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
3. **Becker, G. W., and R. L. Lester.** 1980. Biosynthesis of phosphoinositol-containing sphingolipids from phosphatidylinositol

by a membrane preparation from *Saccharomyces cerevisiae*. J. Bacteriol. **142**:747–754.
4. **Bottema, C. D. K., C. A. McLean-Bowen, and L. W. Parks.** 1983. Role for sterol structure in the thermotropic behavior of plasma membranes of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **734**:235–248.
5. **Bussey, H., D. Saville, M. R. Chevallier, and G. H. Rank.** 1979. Yeast plasma membrane ghosts: an analysis of proteins by two-dimensional gel electrophoresis. Biochim. Biophys. Acta **553**:185–196.
6. **Carman, G. M., and S. A. Henry.** 1989. Phospholipid biosynthesis in yeast. Annu. Rev. Biochem. **58**:635–639.
7. **Courchaine, A. J., W. H. Miller, and D. B. Stein.** 1959. Rapid semimicro procedure for estimating free and total cholesterol. Clin. Chem. **5**:609–614.
8. **Daum, G., P. C. Bohni, and G. Schatz.** 1982. Import of proteins into mitochondria. J. Biol. Chem. **257**:13028–13033.
9. **Daum, G., and F. Paltauf.** 1990. Lipid transport in microorganisms. Experientia **46**:586–592.
10. **Dickson, R. C., G. B. Wells, A. Schmidt, and R. L. Lester.** 1990. Isolation of mutant *Saccharomyces cerevisiae* strains that survive without sphingolipid. Mol. Cell. Biol. **10**:2176–2181.
11. **Duran, A., B. Bowers, and E. Cabib.** 1975. Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Natl. Acad. Sci. USA **72**:3952–3955.
12. **Ghosh, T. K., J. Bian, and D. L. Gill.** 1990. Intracellular calcium release mediated by sphingosine derivatives generated in cells. Science **248**:1653–1656.
13. **Hakomori, S., and J. N. Kanfer (ed.).** 1983. Chemistry of glycosphingolipids in sphingolipid biochemistry, p. 1–150. Plenum Publishing Corp., New York.
14. **Hakomori, S.** 1990. Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. J. Biol. Chem. **265**:18713–18716.
15. **Hannun, Y. A., and R. M. Bell.** 1989. Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. Science **243**:500–507.
16. **Hannun, Y. A., C. R. Loomis, A. H. Merrill, and R. M. Bell.** 1986. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding *in vitro* and in human platelets. J. Biol. Chem. **261**:12604–12609.
17. **Henschke, P. A., D. S. Thomas, A. H. Rose, and F. J. Veazy.** 1983. Association of intracellular low-density vesicles with plasma membranes from *Saccharomyces cerevisiae* NCYC 366. J. Gen. Microbiol. **129**:2927–2938.
18. **Hughes, J., and M. E. McCully.** 1975. The use of an optical brightener in the study of plant structure. Stain Technol. **50**: 319–329.
19. **Jungalwala, F. B., J. E. Evans, E. Bremer, and R. H. McCluer.** 1983. Analysis of sphingoid bases by reversed-phase high performance liquid chromatography. J. Lipid Res. **24**:1380–1388.
20. **Kang, M. S., N. Elango, E. Mattia, J. Au-Young, P. W. Robbins, and E. Cabib.** 1984. Isolation of chitin synthetase from *Saccharomyces cerevisiae*. J. Biol. Chem. **259**:14966–14972.
21. **Koland, J. G., and G. G. Hammes.** 1986. Steady state kinetic studies of purified yeast plasma membrane proton-translocating ATPase. J. Biol. Chem. **261**:5936–5942.
22. **Kumari, L., J. R. Decallonne, and J. A. Meyer.** 1975. Deoxyribonucleic acid metabolism and nuclear division during spore germination in *Fusarium oxysporum*. J. Gen. Microbiol. **88**:245–252.
23. **Leal-Morales, G. A., C. E. Bracker, and S. Bartnicki-Garcia.** 1988. Localization of chitin synthetase in cell-free homogenates of *Saccharomyces cerevisiae*: chitosomes and plasma membrane. Proc. Natl. Acad. Sci. USA **85**:8516–8520.
24. **Lester, R. L., and M. R. Steiner.** 1968. The occurrence of diphosphoinositide and triphosphoinositide in *Saccharomyces cerevisiae*. J. Biol. Chem. **243**:4889–4893.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Martinez, A. F., and J. Schwenke.** 1988. Chitin synthetase activity is bound to chitosomes and to the plasma membrane in protoplasts of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **946**:328–336.
27. **Mason, T. L., R. O. Poyton, D. C. Wharton, and G. Schatz.** 1973. Cytochrome *c* oxidase from baker's yeast. J. Biol. Chem. **248**:1346–1354.
28. **Nurminen, T., E. Oura, and H. Suomalainen.** 1970. The enzymic composition of the isolated cell wall and plasma membrane of baker's yeast. Biochem. J. **116**:61–69.
29. **Patton, J. L., G. B. Wells, and R. L. Lester.** 1988. Intracellular distribution of inositol sphingolipid in yeast. J. Cell Biol. **107**: 362a.
30. **Patton, J. L., G. B. Wells, and R. L. Lester.** 1990. Inositol sphingolipid in yeast is enriched in the plasma membrane. FASEB J. **4**:A1805.
31. **Peters, P. H. J., and G. W. F. H. Borst-Pauwels.** 1979. Properties of plasma membrane ATPase and mitochondrial ATPase of *Saccharomyces cerevisiae*. Physiol. Plant. **46**:330–337.
32. **Petko, L., and S. Lindquist.** 1986. Hsp 26 is not required for growth at high temperatures, nor for thermotolerance, spore development, or germination. Cell **45**:885–894.
33. **Pinto, W. J., G. B. Wells, A. C. Williams, K. A. Anderson, E. C. Teater, and R. L. Lester.** 1986. Characterization of a *Saccharomyces cerevisiae* mutant defective in inositol sphingolipid synthesis. Fed. Proc. **45**:1826.
34. **Santos, E., J. R. Villaneuva, and R. Sentandreu.** 1978. The plasma membrane of *Saccharomyces cerevisiae*. Isolation and some properties. Biochim. Biophys. Acta **508**:39–54.
35. **Sburlati, A., and E. Cabib.** 1986. Chitin synthetase 2, a presumptive participant in septum formation in *Saccharomyces cerevisiae*. J. Biol. Chem. **261**:15147–15152.
36. **Scarborough, G. A.** 1975. Isolation and characterization of *Neurospora crassa* plasma membranes. J. Biol. Chem. **250**: 1106–1111.
37. **Schatz, G., and J. Klima.** 1964. Triphosphopyridine nucleotide: cytochrome c reductase of *Saccharomyces cerevisiae*: a "microsomal" enzyme. Biochim. Biophys. Acta **81**:448–461.
38. **Schekman, R.** 1985. Protein localization and membrane traffic in yeast. Annu. Rev. Cell Biol. **1**:115–143.
39. **Schibeci, A., J. B. M. Rattray, and D. K. Kidby.** 1973. Isolation and identification of yeast plasma membrane. Biochim. Biophys. Acta **311**:15–25.
40. **Schmidt, R., R. Ackermann, Z. Kratky, B. Wasserman, and B. Jacobson.** 1983. Fast and efficient purification of yeast plasma membranes using cationic silica microbeads. Biochim. Biophys. Acta **732**:421–427.
41. **Scott, J. H., and R. Schekman.** 1980. Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. J. Bacteriol. **142**:414–423.
42. **Serrano, R.** 1978. Characterization of the plasma membrane ATPase of *Saccharomyces cerevisiae*. Mol. Cell. Biochem. **22**:51–63.
43. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
44. **Smith, S. W., and R. L. Lester.** 1974. Inositolphosphorylceramide, a novel substance and the chief member of a major group of yeast sphingolipids containing a single inositolphosphate. J. Biol. Chem. **249**:3395–3405.
45. **Steiner, S., and R. L. Lester.** 1972. Studies on the diversity of inositol-containing yeast phospholipids: incorporation of 2-deoxyglucose into lipid. J. Bacteriol. **109**:81–88.
46. **Steiner, S., S. Smith, C. J. Waechter, and R. L. Lester.** 1969. Isolation and partial characterization of a major inositol-containing lipid in baker's yeast: mannosyl-diinositol, diphosphoryl-ceramide. Proc. Natl. Acad. Sci. USA **64**:1042–1048.
47. **Szent-Gyorgyi, C., and I. Isenberg.** 1983. The organization of oligonucleosomes in yeast. Nucleic Acids Res. **11**:3717–3736.
48. **Thudichum, J. L. W.** 1884. A treatise on the chemical constitution of brain. Balliere, Tindall, and Cox, London.
49. **Tschopp, J., and R. Schekman.** 1983. Two distinct subfractions in isolated *Saccharomyces cerevisiae* plasma membranes. J. Bacteriol. **156**:222–229.

50. **Tyorinoja, K., T. Nurminen, and H. Suomalainen.** 1974. The cell-envelope glycolipids of baker's yeast. Biochem. J. **141**:133–139.
51. **Wells, G. B., and R. L. Lester.** 1983. The isolation and characterization of a mutant strain of *Saccharomyces cerevisiae* that requires a long chain base for growth and for synthesis of phosphosphingolipids. J. Biol. Chem. **258**:10200–10203.
52. **Welten-Verstegen, G. W., P. Boer, and E. P. Steyn-Parve.** 1980. Lipid-mediated glycosylation of endogenous proteins in isolated plasma membrane of *Saccharomyces cerevisiae*. J. Bacteriol. **141**:342–349.
53. **Witt, W., H.-J. Bruller, G. Falker, and G. F. Fuhrmann.** 1982. Purification and properties of a phospholipid acyl hydrolase from plasma membranes of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **711**:403–410.
54. **Wood, R., and T. Lee.** 1983. High-performance liquid chromatography of fatty acids: quantitative analysis of saturated, monoenoic, polyenoic and geometrical isomers. J. Chromatogr. **254**:237–246.
55. **Yoshihisa, T., Y. Ohsumi, and Y. Anraku.** 1988. Solubilization and purification of α-mannosidase, a marker enzyme of vacuolar membranes in *Saccharomyces cerevisiae*. J. Biol. Chem. **263**: 5158–5163.