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THE PLASMA MEMBRANE OF *SACCHAROMYCES CEREVISIAE*

ISOLATION AND SOME PROPERTIES

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Summary

The isolation of *Saccharomyces cerevisiae* plasma membrane was carried out after hypotonic lysis of yeast protoplasts treated with concanavalin A by two independent methods: a, at low speed centrifugation and b, at high speed centrifugation in a density gradient. Several techniques (electron microscopic, enzymic, tagging, etc.) were used to ascertain the degree of purification of the plasma membranes obtained. The low speed centrifugation technique as compared with the other method gave a higher yield of plasma membranes with a similar degree of purification.

Analysis of the yeast plasma membrane of normally growing cells by sodium dodecyl sulphate polyacrylamide gel electrophoresis showed at least 25 polypeptide bands. Twelve glycoprotein bands were also found, and their apparent molecular weights were determined. Treatment of the protoplasts with cycloheximide resulted in a significant decrease in the carbohydrate and protein content of the plasma membrane. The electrophoretic pattern of the plasma membrane of cycloheximide-treated cells showed a redistribution of the relative amounts of each protein band and a drastic reduction in the number of Schiff-positive bands. The isoelectric point of the most abundant proteins was low (p*I* 4) or lower than expected from previous data. A large part of the mannosyl transferase activity found in the cell (80%) was associated with the internal membranes, the remaining activity (20%) was located in the plasma membrane preparation. Part of the mannosyl transferase activity of the cells is located at the plasma membrane surface. Invertase (an external mannoprotein) is found in both the plasma and internal membranes, and as the specific activity dropped significantly following cycloheximide treatment of the cells, it is suggested that these membranes systems are the structures for the glycosylation of a precursor invertase and its subsequent release into the periplasmic space. Other transferase found in the plasma membrane preparation transfers glucose residues from UDPglucose to a poly($\alpha(1 \rightarrow 4)$ polymer identified as glycogen.

Introduction

Biosynthesis of the polymers involved in wall formation has been studied by several research groups using particulate membranous preparations. Crude [1,2] and modified [3] membrane preparations have been used as a source of mannosyl transferases and Nakajima and Ballou [3] have suggested that at least ten different enzymes are involved in mannan formation. However, little is known about their cellular location.

Mannan has been found on the surface of yeast protoplasts [4] but evidence that the plasma membrane is involved in the biosynthesis of mannan has not been obtained conclusively [5,6].

One unsolved problem concerns the possible location of the enzymes and the lipid carrier(s) which are directly involved in the synthesis of mannan glycoproteins. The unmasking of their location in the yeast cellular topography may indicate the place(s) where glycoproteins are synthesized and serve as a model for the glycoprotein pathway in eukaryotic cells.

Several methods have been described to isolate yeast plasma membrane [7—17] but the actual origin of the preparations obtained has not been definitively established as the criteria used to characterize them might be questioned. According to Kidby [14] the significance of yeast membrane characterization by chemical composition analysis, enzyme marker assay and electron microscopy, is uncertain. Surface labelling seems to be the most dependable method for identifying the plasma membrane in any isolation procedure involving free-living cells.

Scarborough [18] has described the isolation of *Neurospora crassa* plasma membrane by treatment of protoplasts with concanavalin A. This treatment reinforces the plasma membrane avoiding fragmentation after lysis of the protoplasts. A modification of this technique based on the use of a density gradient centrifugation has recently been reported in the isolation of yeast plasma membrane [19].

In this work we describe the isolation of the *Saccharomyces cerevisiae* plasma membrane using other adaptation of Scarborough's method. Low velocity centrifugation allows rapid and accurate separation of large amounts of yeast plasma membranes, a goal that cannot be achieved by other techniques. This method has the advantage that the plasma membranes are not exposed to gradient media of non-physiological density.

Materials and Methods

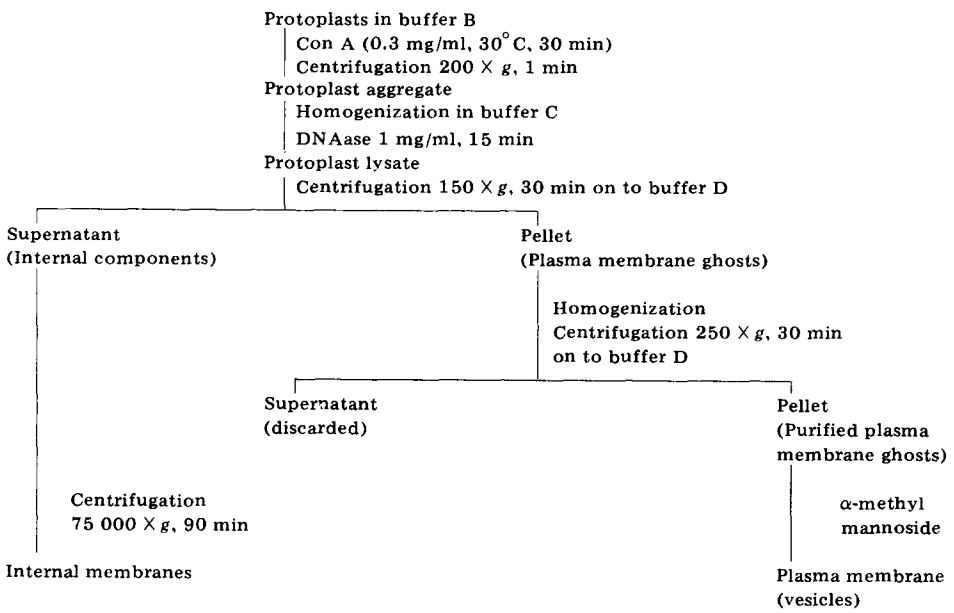
Chemicals

Lyophilized concanavalin A, α-methyl-D-mannoside, deoxyribonuclease (DN25), cytochrome *C*, alcohol dehydrogenase, phosphoglyceraldehyde dehydrogenase, bovine serum albumin, catalase and Coomassie Brilliant Blue R were purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). Lactoperoxidase was from Calbiochem; lysozyme and trypsin were obtained from Schuchardt. Human transferrin was from Boehringer. Sodium dodecyl sulphate and mercaptoethanol were from Merck (Darmstadt, Germany). [*ring* C-^{14}C]colchicine (spec. act. 15 mCi/mmol), UDP[U-^{14}C]glucose (spec. act. 260 mCi/mmol),

GDP[U-^{14}C]mannose (spec. act. 154 mCi/mmol), [1-^{3}H]glucose (spec. act. 5.3 Ci/mmol) and ^{125}I carrier free were obtained through the Radiochemical Centre (Amersham, U.K.). Ampholites were from LKB Instruments (Bromma, Sweden). Acrylamide, *N,N'*-methylenbisacrylamide, *N,N,N',N'*-tetramethylethylenediamine and ammonium persulphate were purchased from Serva Feinbiochemica. All other reagents were of analytical grade.

Organism

S. cerevisiae LK2G12, a diploid strain obtained from Dr. Halvorson, was maintained on slants of YPD medium (yeast extract, 1 g/peptone, 2 g/dextrose, 2 g/water, 1 l) solidified with 1.5% agar [20] and subcultured periodically.



SCHEME I

SCHEME OF MEMBRANE PREPARATION

Growth conditions. Cells were grown in YPD liquid media, in a rotary shaker at 250 rev./min. Cells (600 μg dry weight) were used to inoculate a 1000-ml Erlenmeyer flask with 300 ml medium and the crop was collected after about 14 h of incubation at 28°C (early exponential phase).

In some experiments a synthetic defined medium was also used. The composition per l was: 6.7 g yeast nitrogen base, 10 g succinic acid, 6 g sodium hydroxide and 20 g glucose.

Preparation of protoplasts

The protoplasts were prepared with either Glusulase or Helicase. Glusulase (Endo Laboratories, Inc.) was about four times more active than Helicase (L'Industrie Biologique Francaise). The activity of both enzymic preparations was dependent on the temperature and on pretreatment with mercaptoethanol. Cells (50 mg dry weight) were collected in the early exponential phase, washed

twice in 50 mM Tris · HCl (pH 7.4), containing 0.6 M KCl and 10 mM $MgSO_4$ (buffer A) and resuspended in 5 ml of the same buffer containing 20 mM mercaptoethanol. Glusulase (200 μl) was added to the suspension and protoplast formation was followed with a phase contrast microscope. Cells were converted into protoplasts within 20—30 min and after centrifugation at 2000 $\times g$ for 1 min they were washed twice with 50 mM Tris · HCl (pH 7.4), containing 0.9 M sorbitol and 10 mM $MgSO_4$ (buffer B).

Analytical determinations

Proteins were solubilized in sodium deoxycholate and measured by a modified Lowry method [21] using bovine serum albumin as a standard. Total carbohydrate was determined by the phenol-sulfuric acid method of Dubois [22].

The uptake of O_2 was measured with a Clark type electrode in a closed chamber at 30°C. The reaction mixture contained 10 mM sodium succinate and 1 mg/ml membrane protein in a final volume of 2 ml of 50 mM Tris · HCl buffer (pH 7.4).

Inorganic phosphate was determined as described [23], and the phospholipid phosphorus content of the lipid extracts was determined by the method of Barlett [24].

Radioactivity determinations. Whenever a β-emitter isotope was used, the radioactivity was measured in a Tri-Carb liquid scintillation spectrophotometer (Packard, Model 3320) using Bray's solution [25] with aqueous samples and a toluene-based scintillation mixture in non-aqueous samples [26].

The radioactivity due to [^{125}I]iodide was measured directly in a Intertechnic CG30 gamma counter.

Enzyme assays. Mannan synthetase activity. The enzymic preparations were incubated in 50 mM Tris/maleate buffer (pH 6.8)/5 mM $MnCl_2$/1 mM mercaptoethanol, at 30°C for 30 min with 0.1 μCi GDP[U-^{14}C]mannose (spec. act. 154 mCi/mmol), in a final volume of 0.5 ml. The reaction mixture was extracted twice with 20 vols. of chloroform/methanol (2 : 1, v/v) and the extracts washed according to Folch et al. [27]. The organic solvent-insoluble material was recovered on glass fibre filters and the radioactivity was counted [26].

Glucosyl transferase activity. The preparations were incubated in 50 mM Tris/maleate buffer (pH 6.8), 5 mM $MgCl_2$ at 30°C for 30 min in the presence of 1 μCi UDP[U-^{14}C]glucose (spec. act. 260 mCi/mmol), in a final volume of 1 ml. Samples were extracted with chloroform/methanol (2 : 1) and washed according to Folch et al. [27]. The organic solvent insoluble material was recovered in glass fibre filters Whatman GF/C and the radioactivity was determined.

Invertase activity was determined according to Gascón and Lampen [28] with 100—150 μg membrane protein in the reaction mixture.

Isolation of the plasmalemma

All operations were carried out in a cold room and in an ice-cooled water bath. The isolation of plasma membrane was carried out by two different procedures.

A. Low speed centrifugation. Yeast protoplasts obtained from 500 mg (dry wt.) cells were resuspended in 30 ml buffer B supplemented with 0.3 mg/ml

concanavalin A. The mixture was maintained at 30°C for 20—30 min until the protoplasts floculated. The protoplasts were washed twice with buffer B without concanavalin A and collected by centrifugation in a swinging bucket centrifuge at 200 g for 1 min. The pellet was then resuspended in 50 ml of 10 mM Tris · HCl buffer (pH 7.4) containing 5 mM $MgSO_4$ (buffer C) supplemented with 1 mg/ml DNAase. The protoplast suspension was homogenized in a Sorvall Omnimixer at the lowest speed possible for 5—10 min at 0°C and the lysate obtained was incubated a 30°C for 15 min in order to degrade the nuclear structure.

Aliquots of the lysate were layered over 3 vols. of 0.1 M Tris · HCl buffer (pH 7.4), 0.5 M mannitol (buffer D) and centrifuged at 150 $\times g$ for 30 min in a swinging bucket centrifuge. The supernatant containing the intracellular content was removed and the pellet (the plasma membranes) was resuspended in 100 ml of ice-cooled buffer C. The pellet was homogenized in a teflon homogenizer (20 strokes) and layered into 3.5 vols. of buffer C and centrifuged again at 250 $\times g$ for 30 min. The supernatant was removed and the pellet containing the plasma membrane ghosts resuspended in 20 ml of 0.01 M Tris · HCl buffer (pH 7.4)/1 M α-methylmannoside in order to eliminate concanavalin A. The suspension was incubated for 30 min, centrifuged and the pellet was resuspended in the appropriate buffer.

B. Density gradient centrifugation in Urografin. A cellular lysate (8 ml, 150 mg dry wt.) obtained by method A was layered onto a 55 ml gradient of Urografin ranging from 5 to 50% concentration [9]. The tubes were centrifuged at 75000 $\times g$ for 80 min in the SW-25-2 rotor of a L2-65B Beckman Ultracentrifuge. The gradient was recovered from the bottom upwards by means of a peristaltic pump.

Reaction with colchicine

Binding of colchicine to membrane preparations was measured by incubating the suspension of the membranes (0.65 ml in 50 mM Tris/maleate buffer (pH 6.5)/5 mM $MgCl_2$ in the presence of 0.1 μCi [*ring* C-^{14}C]colchicine (spec. act. 15 mCi/mmol) at 38°C for 20 min. The membranes were recovered on glass fibre discs, washed and the radioactivity was determined in the discs.

Iodination of the protoplasts

Protoplasts obtained from 250 mg of yeast cells resuspended in 3 ml of 0.1 M phosphate buffer (pH 7.5), containing 0.8 M sorbitol. Lactoperoxidase (30 μl), solution of 1 mg/ml and 10 μl of carrier-free [^{125}I]iodide (4 μCi/ml) were added to the suspension. The reaction was started by adding 10 μl of 0.03% H_2O_2 and after 10 min H_2O_2 together with [^{125}I]iodide were added again [29]. The reaction was stopped by centrifugation and the resulting pellet was washed twice in the initial buffer.

Analytical polyacrylamide gel electrophoresis

Analytical gel electrophoresis in sodium dodecyl sulphate was performed on 5%, 8% and 10% acrylamide gels by the system described by Zahler [30]. The gel rods were 0.6 × 10 cm. Samples containing approx. 200 μg protein were treated with an equal volume of a solution of 2% sodium dodecyl sulphate, 8 M

urea and 2% mercaptoethanol. Bromophenol blue was used as a tracking dye. Proteins were detected as described by Fairbanks [31] and glycoproteins by the method of Zacharius et al. [32].

The dyed gels were scanned at 575 nm (for Coomassie stain) and at 560 nm (for carbohydrate stain) in a Pye-Unicam SP-1700 spectrophotometer equipped with a densitometer, model Unicam SP-1809.

A plot relating the logarithm of molecular weight versus relative mobility was charted for 5% and 8% acrylamide gels by using proteins of known molecular weight.

Electrofocusing

Electrofocusing of samples was carried out after membrane samples (100 μg protein) had been dissolved in a small volume of a mixture of 10 M urea, 10% Triton X-100, 2% 2-mercaptoethanol, 1.6 mM EDTA, 20 mM Tris · HCl (pH 7.0) and placed on a 6% acrylamide supplemented with 1.5% Ampholytes rod gel, in a final volume of 150 μl. After a pre-electrofocusing process for 30 min at 0.3 mA per gel, the electrofocusing was developed at high voltage (200—400 V) for 24 h. When the run was over, pH was measured on 2-mm gel slices placed on 1.5 ml boiled distilled water for approx. 2 h. After fixing the gel with 12.5% trichloroacetic acid the protein bands were stained with 0.25% Coomassie Brilliant Blue R [33].

Electron microscopy

Washed membranes were fixed in 4% glutaraldehyde in 1 M phosphate buffer (pH 6.8), containing 10 mM $CaCl_2$ at 4°C for 90 min. After four rinses in the same buffer, the membranes were postfixed in 1% OsO_4 in 50 mM phosphate buffer (pH 6.8) for 1 h in the dark. The membranes were then washed several times to eliminate the residual fixative.

Protoplasts were fixed in 4% glutaraldehyde in buffer B supplemented with 10 mM $CaCl_2$ at 4°C for 90 min. Postfixation was carried out in the same conditions as for the membranes.

Dehydration was done in a graded series of acetone and Spurr [34] was used as the embedding medium. The sections were obtained with an LKB ultramicrotome (Ultratome III, LKB Produkter AB, Bromma, Sweden) fitted with glass knives prepared in the LKB 7800 B knifemaker device. After staining with uranyl acetate and lead citrate the sections were viewed and photographed in a Philips EM 300 electron microscope.

The electron-dense concanavalin A · ferritine complex was prepared as described by Stobo and Rosenthal [35].

Results and Discussion

Isolation of plasma membrane by method A

The behaviour of the membranes during the isolation procedures was determined by labeling the proteins accessible from the external part of the yeast plasma membranes by iodination in the following way. Cells of *S. cerevisiae* were grown in [1-^{3}H]glucose until the early exponential phase, then converted to protoplasts and iodinated and the plasma membrane isolated as described in

Materials and Methods. The isolation was carried out by method A and pelleting of the membranes was avoided by adding a cushion of 80% (v/v) sucrose to the centrifugation tubes during the first spinning. Almost all the ^{125}I was found at the interphase, whereas the largest amount of ^{3}H was found in the rest of the tube (Fig. 1). The material found at the interphase was centrifuged again and a similar distribution of radioactivity was obtained. As seen in Fig. 1, a minor second double-labelled peak was also found that may correspond either to aggregates of smaller number of plasma membranes or to a population of smaller size membranes.

Isolation of plasma membrane by density gradient centrifugation (method B)

When a protoplast lysate was spun in a continuous or discontinuous density gradient in Urografin four bands were found which were isolated with the help of a peristaltic pump. Better separations were obtained when the spinning was carried out in a continuous gradient as described in Materials and Methods. The band found at the top of the tube (Band I) did not enter the gradient, and might consist of lipoproteins together with soluble materials. Three other bands appeared in the gradient; they corresponded to vesicles of different sizes and shapes. Band II showed the presence of small vesicles, most of them of a mitochondrial morphology. The particulate materials present in the third and fourth bands were similar in size to that of intact protoplasts. These bands were formed by smaller (Band III) or larger (Band IV) aggregates of membrane clamps.

These results are different from those previously reported [19]; Band I did not enter the gradient and Band IV was formed neither by whole cells nor by wall residues and cellular debris. Phase-contrast microscopic observations indicated that the difference between bands III and IV lay in the size of the membrane aggregates.

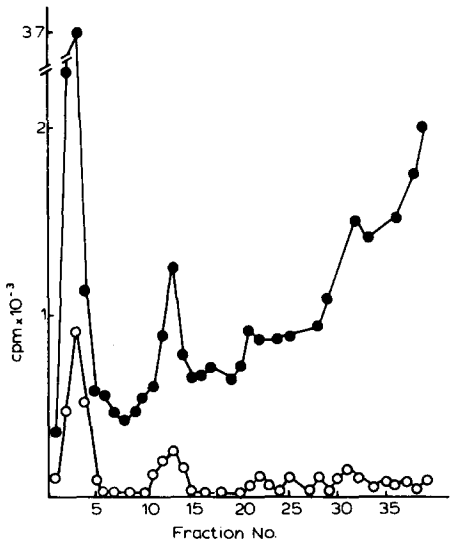


Fig. 1. Identification of yeast plasma membrane by surface labeling with ^{125}I. Radioactivity: ^{3}H, ●——●; ^{125}I, ○——○.

The isolation of plasma membranes by density gradient centrifugation is a good but rather time consuming technique; thus, in the present work the low speed centrifugation method was used routinely because of the higher yields of membranes obtained.

Microscopy

Fig. 2 shows phase-contrast and electron photomicrographs of samples taken at different steps of the isolation procedure. The morphology of protoplasts observed by phase contrast microscopy can be seen in Fig. 2A inset. Thin sections of the preparation were also studied with a Philips electron microscope and a whole complement of cytoplasmic organelles was visible over a background of ribosomes (Fig. 2A). The images show a higher resolution than those obtained from whole cells. The absence of a wall in protoplasts may facilitate penetration of fixatives thereby avoiding artifacts. The plasma membrane appeared as the outer most layer of the protoplast. It seemed morphologically unaffected by treatment with wall-dissolving enzymes because it kept its integrity around the cytoplasm. The protoplasts showed several inlets (Fig. 2A) and electron transparent and non transparent vesicles which are apparently leaving the cell (Fig. 2B). The existence of inlets and superficial vesicles results in a non-spherical morphology suggesting, that there are either wall remains or other supporting structures (e.g., microtubules) responsible for the non-spherical morphology in an isotonic medium.

The binding of concanavalin A to the membranes was studied by treating the protoplasts either directly with concanavalin A or conjugated with ferritin, a complex which permits direct observation of concanavalin A molecules as electron dense particles (Fig. 2C).

Spacing between plasma membraned of aggregated protoplasts was wider than reported for plant cell protoplasts [36]. No fusion of paired membranes could be seen in the preparations observed (Fig. 2D). Distribution of concanavalin A · ferritin molecules appeared to be all over the surface of the plasma membrane (Fig. 2C). These results suggest that under the experimental conditions mobile concanavalin A receptors are not detected in the yeast plasma membrane. These receptors have been described in mammalian cells to result in linear arrays [37] or a patchy distribution (capping) [38] upon binding of concanavalin A to the membranes. It was observed from the treatment of the protoplasts with concanavalin A · ferritin that the phytohaemagglutinin molecules were not closely bound to the plasma membrane surface, due to the fact that many of the electron dense granules were dispersed in a surrounding peripheral area outside the membrane (Fig. 2C, inset). These results suggest a marked difference between yeast plasma membranes and plasma membrane of higher cells. Yeast plasma membranes may present large amounts of glycoproteins forming dense sugar "hairs" all around, giving rise to an intermediate structure between the membrane itself and the cell wall. The high carbohydrate/protein ratio found in the analytical determinations supports this interpretation. The "hairs" may represent macromolecules that are being actively secreted (see below).

The existence of vesicles in continuity with the protoplasts (Fig. 2B) and externally to them (Fig. 2C, inset) might suggest a secretory role for these

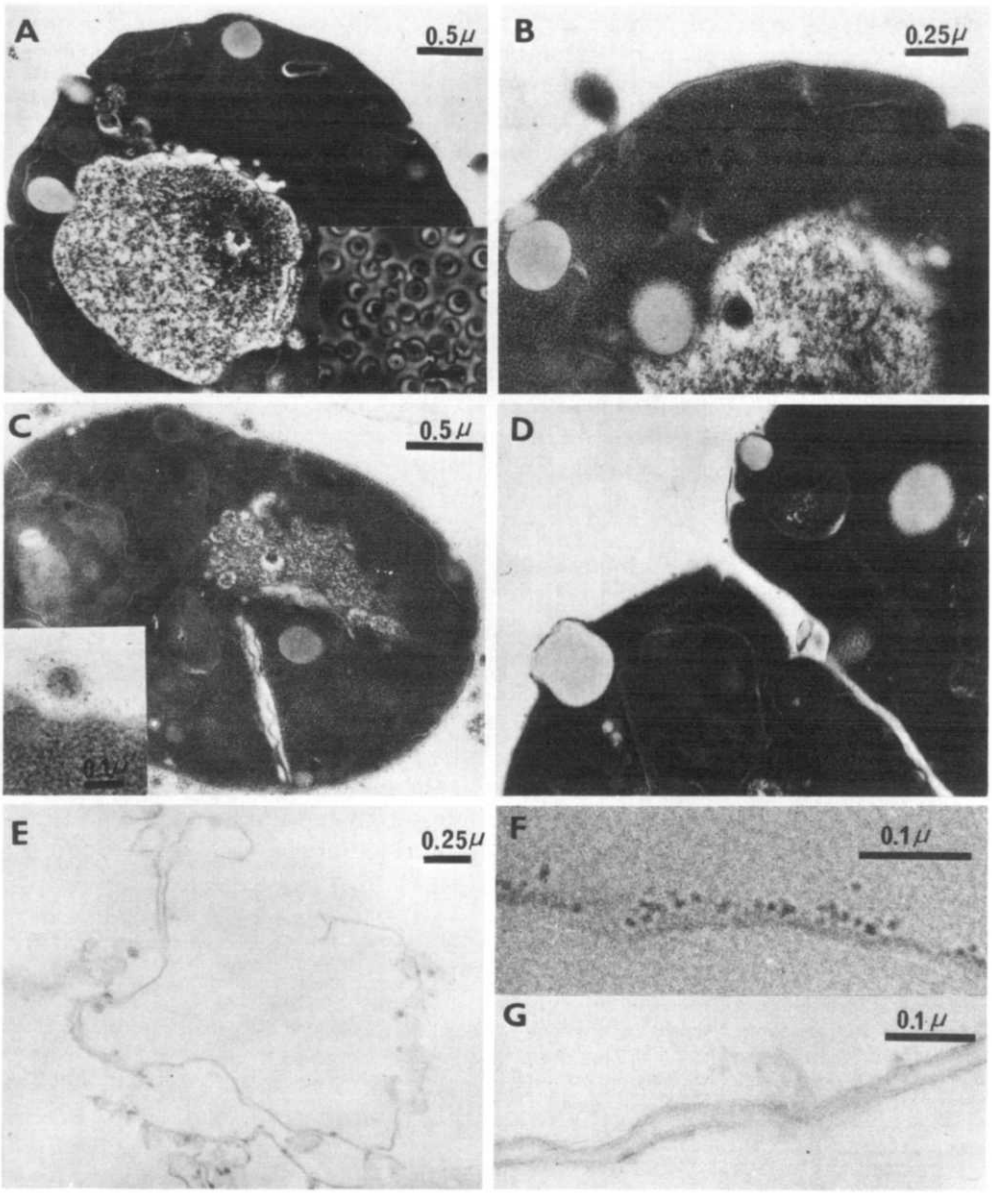


Fig. 2. Optical (A, inset) and electron photomicrographs of protoplasts and plasma membrane ghosts. See text for details.

organelles, but their actual physiological significance is unknown. Burgess and Lindstead [36] have described labelling of amorphous material, located externally to the plant plasma membrane by means of concanavalin A · ferritin. A secretory role of these vesicles in yeast did not seem to be appropriate since the labelled structures seem to be non-amorphous, and ribosome-filled.

Concanavalin A treated membranes appeared as large, open sheets, with the expected size of a protoplast (Fig. 2E). Other isolation procedures result in the breaking open of the plasma membrane following formation of small vesicles.

Concanavalin A was only visible on the external surface of the plasma membrane (Fig. 2F) and some paired membranes were also observed with the phytohaemagglutinin acting as a bridge between them (Fig. 2G). Small vesicles, probably attached to the membrane, were also frequently observed.

Chemical composition

A summary of some structural parameters of the isolated plasma membranes is shown in Table I. A high carbohydrate-to-protein ratio was found in the plasma membranes. The high amounts of carbohydrate found are probably due to the carbohydrate of structural components but also to the carbohydrate of the wall glycoproteins and polysaccharides which are transported across the plasma membrane. This is apparently so, since the carbohydrate concentration was drastically reduced when protein synthesis was halted by cycloheximide. This antibiotic inhibits synthesis of wall mannan glycoproteins at the level of peptide formation whereas glycosylation and secretion of those peptides already formed does take place normally [26,39]. A decrease was also observed following glucose starvation of the cells. This treatment results in a desintegration of polysomes with the concomitant protein synthesis blockage. The carbohydrate/protein ratio of glucose starved cells was higher than in cycloheximide treated cells, since this procedure does not totally inhibit protein synthesis [40,41].

Colchicine binding activity was found both in plasma and internal membrane suggesting the existence of tubulin-like proteins.

Despite recent reports on the occurrence of tubulin in plasma membranes of brain and thyroid cells [42,43] little is known about its function. Detection of tubulin in yeast homogenates [44] and colchicine binding proteins in yeast plasma membrane suggests that microtubules might be implicated in the maintenance of the bizarre protoplast shape described above (Fig. 2) and perhaps

TABLE I

STRUCTURAL PARAMETERS OF YEAST MEMBRANES

Analytical determinations were carried out under standard conditions as described in Materials and Methods. Values are referred to control (normally growing cells), cycloheximide treated (cells grown in the presence of 100 μg/ml cycloheximide) and glucose starved (cells grown in synthetic defined medium in the absence of glucose for 2 h). 'Internal membranes' in the present context refer to all yeast membrane systems with the exception of the plasma membrane.

	Plasma membrane	Internal membranes
Carbohydrate/protein ratio		
Control	15.29	0.79
Cycloheximide treated	1.07	n.d.
Glucose starved	3.39	n.d.
Inorganic phosphate/protein ratio (μg phosphate/mg protein)	4.47	8.54
Phospholipid/protein ratio (μmol phosphate/mg protein)	0.48	n.d.
DNA (μg/mg protein)	9.80	n.d.
RNA (μg/mg protein)	50.00	n.d.
Colchicine binding (μmol/mg protein)		
Control	0.21	0.29
Cycloheximide treated	0.12	n.d.
Glucose starved	0.57	n.d.

n.d., not determined.

in the secretion process too. This indicates that the colchicine binding proteins may well form a cytoskeleton responsible for the sorting out of transmembrane and secretion proteins from the non-interacting ones [45]. There was little contamination by nucleic acids. The small amount of RNA could be reduced still further by treatment with RNAase.

Structure of the membrane

Distribution of proteins and glycoproteins in plasma membrane was studied by sodium dodecyl sulphate gel electrophoresis and by electrofocusing. The electrophoretic profiles of Coomassie and Schiff-positive components of the membrane preparations are shown in Fig. 3. Plasma membrane vesicles after detachment of concanavalin A from plasma membrane concanavalin A ghosts were solubilized by treatment with 2% sodium dodecyl sulphate containing 2% mercaptoethanol and 8 M urea at 100°C for 5—10 min. When 2-mercaptoethanol was omitted, no marked differences could be observed either in the protein or in the glycoprotein electrophoretic patterns, suggesting that there are no disulphide groups with a structural role in the yeast plasma membrane. The apparent molecular weight of the components was estimated by plotting their relative mobilities in a graph obtained by running proteins of knonw molecular weights in 5% and 8% polyacrylamide gels. In plasma membranes of normally growing cells about 25—30 polypeptide bands could be detected, with a predominance of those of a high molecular weight. A band with an apparent molecular weight of 30000 was calculated to be a major component of the plasma membrane. At least two fast-moving bands were observed in 8% gels (Fig. 3B); they correspond to low molecular weight glycoproteins, since they also reacted with Schiff stain. Four Coomassie-positive bands appeared in 5% gels which did not enter 8% polyacrylamide gels due to their high molecular weight. The apparent molecular weights of all of the polypeptide bands ranged from 10000 to 300000, with a predominance of proteins with higher molecular weights. Perhaps some bands represent mannan subunits, whilst the 30000 band may well be the main structural component of yeast plasma membrane.

Up to six different well-defined glycoprotein bands were detected in 5% polyacrylamide gels (Fig. 3A) and at least 11 different bands could be found in 8% gels. (Fig. 3B). Two fast-moving bands appeared in 8% gels, and have already been mentioned. The estimated apparent molecular weight for the glycoprotein bands ranged from 28 000 to 240 000, most of the Schiff-positive material corresponding to molecular weight higher than 50 000.

When exponentially growing cells were treated with 100 μg/ml cycloheximide in the growth medium before plasma membrane isolation, a drastic change in the relative amount of the polypeptide bands was observed (Fig. 3D). The main bands were found to be of lower molecular weight than in normally growing cells and a better separation between them was evident. Many Schiff-positive bands disappeared, leaving two main, high molecular weight ones and a third reduced one corresponding to the polypeptide band of about 30000. The polypeptide bands found after cycloheximide treatment might represent the real structural components of the yeast plasma membrane, the additional bands found on normally growing cells might then represent the non-structural material which is actively secreted across the plasma membrane.

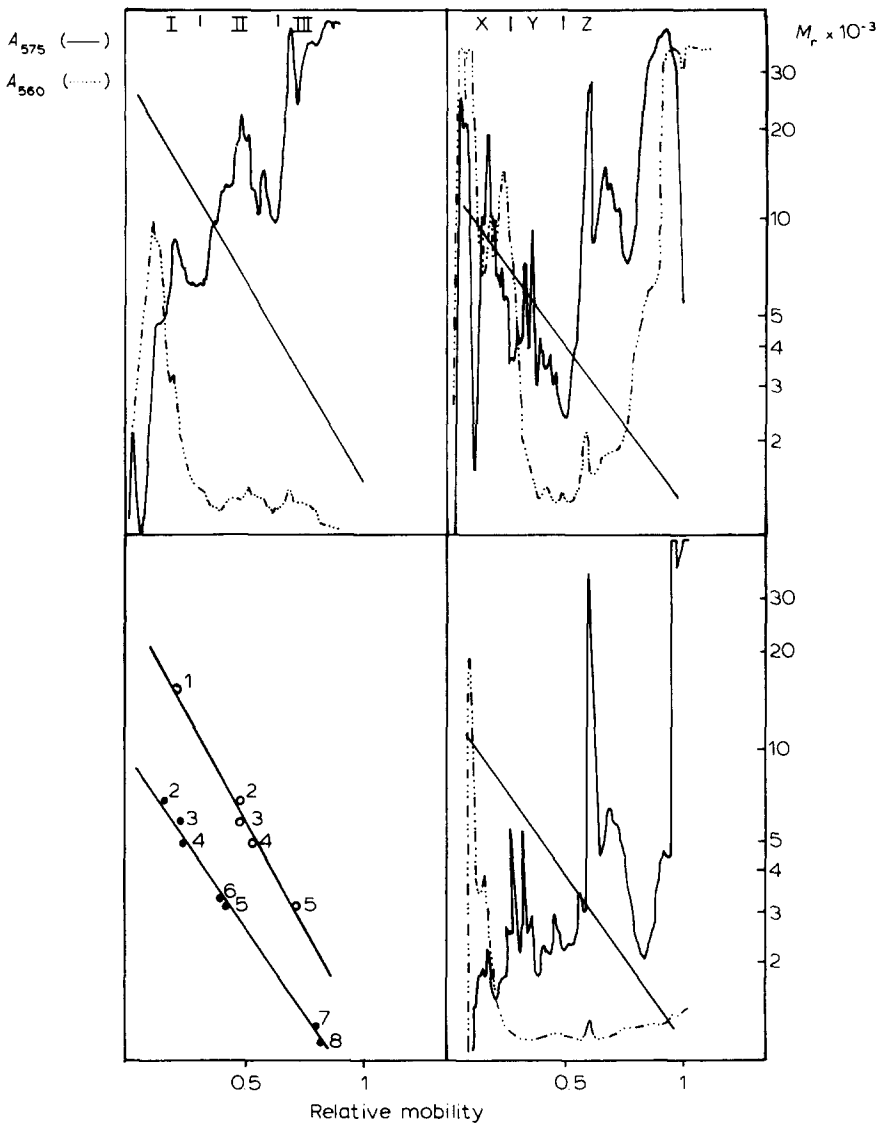


Fig. 3. Densitometer tracings of sodium dodecyl sulphate polyacrylamide gels showing the polypeptide (continuous line) and carbohydrate (broken line) electrophoretic pattern of the components of the yeast plasma membrane of normally grown (A and B) and cycloheximide treated cells (D). Graph C is a plot relating the logarithm of molecular weight (ordinates) versus relative mobility (abscissa) in 5% (○) and 8% (●) polyacrylamide gels (1, γ-globulin; 2, human transferrin; 3, bovine serum albumin; 4, catalase; 5, phosphoglyceraldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, lysozyme; 8, cytochrome *c*).
Three main groups of polypeptide bands were observed in 5% gels; group I (110000—300000 apparent molecular weight) represents high molecular weight components which did not enter 8% gels; groups II (45000—110000 apparent M_r) and III (25000—45000 apparent M_r) were more finely defined in 8% gels, giving rise also to three differentiated groups of bands (X, Y and Z). When the cells were treated with cycloheximide, bands belonging to groups X (60000—90000 apparent M_r) and Y (32500—60000 apparent M_r) showed a minor overlapping and an opposite distribution in the relative amount of each band, with an increased relative quantity of those bands of lower molecular weight and a decrease relative quantity of those bands with higher molecular weight. Group Z (10000—32500 apparent M_r) showed a similar pattern in normally grown and in cycloheximide treated cells.

The pattern of electrofocused proteins of yeast plasma membrane showed the presence of at least seven clearly defined protein groups, the majority of them showing low isoelectric points (p*I* 3.1, 3.4, 3.7, 4.2, 5.2 and 6.7) (Fig. 4).

Enzyme activities

A summary of the enzyme activities studied so far in plasma and internal membranes is shown in Table II.

Invertase was found in both plasma and internal membranes, and, though the activity detected was low (about 4% of the total invertase of the protoplasts), it seems close to the point of being secreted, since cycloheximide significantly reduced its specific activity (Table II). Both membrane types showed also mannosyl transferase activity but the amount of sugar transferred into glycoproteins by the latter group of membranes was four times as high as that of the plasma membranes. Transfer of mannose into dolichol derivatives [46,47] was about eight-fold (Table II).

Preferential location of the transferases in the internal membranes lead to the idea that they might be the main places of protein glycosylation or that they are synthesized intracellularly and then transported to the plasma membrane. This latter hypothesis is suggested by the high ratio of carbohydrate to protein found in the plasma membrane (Table I).

The presence of lipid carrier in the plasma membrane may be due to the fusion of internal vesicles with it during the secretion process of wall materials. This granulocrine mode of secretion has been proposed [48] and has been proved to occur by Moor [49] and Sentandreu and Northcote [50] during yeast cell division. But an eccrine mechanism has also been postulated and the important decrease in carbohydrate found at the plasma membrane level as a result of cycloheximide treatment might have a role in this process. The electron micrographs show the presence of concanavalin A · ferritin binding molecules all around the plasma membrane indicating that secretion of mannan by protoplasts is not a localized procedure. These considerations and the fact that

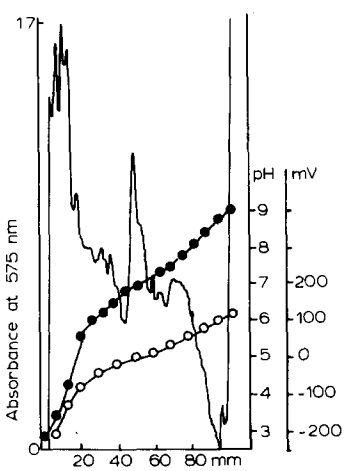


Fig. 4. Densitometer profile of polyacrylamide gel electrofocusing of yeast plasma membrane proteins. pH, ●———●; mV, ○———○; absorbance, continuous line.

TABLE II

ENZYMIC PARAMETERS OF YEAST MEMBRANE SYSTEMS

Conditions of assay have been described in Materials and Methods and a value of 100% was given to the total invertase activity detected. Cycloheximide treatment was as described in Table I. Respiration is given as nmol O_2 taken up/mg protein per min.

	Invertase activity (units)				Mannosyl transferases (mannose transferred)				Respiration (units)
	Control		Cycloheximide treated		Glycoprotein		Glycolipids		
	Spec. act.	Percent	Spec. act.	Percent of control	pg/mg protein	Percent	pg/mg protein	Percent	
Plasma membrane	525	100	193	36.8	4.03	18	0.46	2	0.26
Internal membranes	1312	100	n.d.	—	14.57	64	3.75	16	1.80

n.d., not determined.

invertase is present in both the internal and plasma membranes and that the addition of cycloheximide to cells results in a plasma membrane with reduced carbohydrate concentration raises the interesting possibility that both mechanisms might function in the secretion of wall materials by *S. cerevisiae*. Firstly, when large amounts of wall materials are needed, such as during the budding process, an orientated flow of vesicles would produce an accumulation of the needed materials at the bud region. The wall materials would then be passed across the plasma membrane by reverse pinocytosis. Secondly an eccrine mechanism of secretion would take place around the whole cell when the cellular requirements of wall materials were reduced, as in non-budding cells, thus resulting in a isodiametric thickening of the wall. This phenomenon is particularly evident in aged cells.

The mannosyl transferases found in the outer surface of the plasma membrane appear to be functional, as is suggested by the decrease in mannose incorporated into glycoproteins (15%) when treated with concanavalin A (results to be described elsewhere). The phytohaemagglutinin may block terminal mannosyl residues of the growing chains of the glycoproteins. This phenomenon is coupled with an increase in the sugar incorporated into lipid carrier(s) (100%).

Studies on the glucosyl transferase activity revealed that glucose was incorporated almost exclusively into polysaccharides (99%), and specifically in glycogen. About 1% of the glucose incorporated turned out to be present in the form of glycolipids because they were extracted with chloroform/methanol (2 : 1, v/v) and eluted from a silicic acid column with acetone [51] (results to be described elsewhere).

Plasma membranes have small oxygen uptake when supplemented with succinate as an electron source. The activity was about ten times lower then that found in the internal membranes. As this activity appears to be carried out by mitochondria and can be employed as a marker enzyme for such an organelle it seems that the preparation of plasma membranes obtained is devoid of contamination by mitochondria (Table II).

From the results obtained with the iodide peroxidase treatment of protoplasts and the morphological observations and enzymic determinations carried out with a membrane preparation, we are confident that we have isolated the yeast plasma membrane. Its isolation can be carried out by low speed centrifugation of protoplast lysates previously treated with concanavalin A.

The preferential location of mannosyl transferase(s) in the internal membranes aggrees with the results obtained in higher cells [52—54], suggesting that the glycosyl transferases involved in the formation of the core region of glycoproteins are localized in the endoplasmic reticulum. We also present evidence of the presence of ectomannosyl transferase(s) on the surface of yeast protoplasts as in higher cells [55]. Glycosylation of secretory proteins may require the cooperation of both membrane systems. While the rough endoplasmic reticulum and other internal systems probably play a dominatn role in the formation of the core region, the plasma membrane may serve to complete the carbohydrate side chains. The significance of the ectomannosyl transferase(s) in terminating the carbohydrate chains of the secretory glycoproteins remains to be assessed. Finally, from the distribution of mannosyl transferase(s) and invertase before and after cycloheximide treatment it would appear that the secretion

process of yeast wall glycoproteins could possibly be carried out by both a vesicular system (granulocrine mode) as well as directly through the plasma membrane (eccrine mode).

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