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ROLE OF STEROL STRUCTURE IN THE THERMOTROPIC BEHAVIOR OF PLASMA MEMBRANES OF *SACCHAROMYCES CEREVISIAE*

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Plasma membranes from *Saccharomyces cerevisiae* were prepared by a new procedure involving lyticase treatment of the yeast cells. The plasma membranes were right-side-out, closed vesicles of uniform appearance with a sterol to phospholipid molar ratio of 0.365. The thermotropic behavior of these plasma membranes from wild-type yeast and from sterol mutants was examined by differential scanning calorimetry, fluorescence anisotropy and Arrhenius kinetics of plasma membrane enzymes. While differential scanning calorimetry failed to demonstrate any lipid transition, fluorescence anisotropy data indicated that lipid transitions were occurring in the plasma membranes of the yeast sterol mutants but not the sterol wild-type. The temperature dependence of the plasma membrane enzymes, chitin synthase and Mg^{2+}-ATPase, was also investigated. The Arrhenius kinetics of chitin synthase did not reveal any transitions in either the sterol mutant or wild-type plasma membranes, yet the Arrhenius kinetics of the Mg^{2+}-ATPase suggested that lipid transitions were occurring in both cases.

Introduction

The role of sterols in the maintenance of lipid bilayer integrity and fluidity has been studied extensively in membrane physiology [1–4]. The influence of sterol structure on this role, however, is only beginning to be investigated. One of the easiest systems for such studies is the isolation of membranes from *Saccharomyces cerevisiae*. It is possible to obtain yeast sterol mutants which no longer accumulate ergosterol, the normal end-product of sterol biosynthesis in yeast, but which instead accumulate intermediates in the sterol biosynthetic pathway [5]. Thus, the altered sterol composition of yeast sterol mutants' membranes allows for comparative studies of the effect of sterol structure on the thermotropic behavior of membranes. In one such study, Lees et al. [6] determined the order of membrane rigidity based on the type(s) of sterol present in the mutants. Using mitochondrial membranes, we have previously [7,8] observed differences between the lipid phase transitions of these mutants and the mitochondrial membranes of wild-type strains.

Because the mitochondrial membranes are relatively low in sterol [9] and because some mutants of interest lack respiratorially competent mitochondria [10], we have developed a new plasma membrane system for the continuation of these studies. Using this plasma membrane system we have demonstrated the existence of transitions in the sterol mutant membranes not present in wild-type membranes.

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Methods and Materials

Growth of strains

Yeast strain S288C, and two mutant strains derived from S288C, JR 1 and JR 5 (Table I) were grown overnight at 27°C on 1% tryptone, 0.5% yeast extract and 2% ethanol to early log phase. Final growth yield was never greater than 2 g/l wet weight.

Isolation of plasma membranes

Method 1: Lyticase digestion. Yeast cells were harvested by centrifugation at $4000 \times g$, pretreated with 0.5 M β-mercaptoethanol/0.1 M Tris buffer (pH 9.3) for 5 min at 30°C, and washed twice in lyticase buffer of 1.1 M sorbitol/10 mM phosphate/1 mM EDTA (pH 7.5). The cell walls were digested to form spheroplasts by incubation in lyticase for 1 h at 30°C with gentle shaking (1 g wet wt. cells/3 ml lyticase). Lyticase was prepared from *Oerskovia xanthineolytica* grown on modified Sistrom's medium [11] containing 1.5% washed, autoclaved, lyophilized yeast cells, for 72 h. The *O. xanthineolytica* was pelleted by centrifugation of the medium at $14500 \times g$ for 20 min, and the supernatant, containing the extracellular digestive enzymes, was dialyzed against the above buffer extensively. This dialysate (designated here as lyticase) was stored at 4°C and could be used for cell-wall digestion up to 6 weeks after preparation.

The spheroplasts were washed three times in 0.9 M sorbitol/10 mM Tris/1 mM EDTA buffer (pH 7.4) and then osmotically lysed by vortexing for 1 min in lysis buffer (10 mM Tris/1 mM EDTA buffer (pH 7.4) or 50 mM imidazole/2 mM $MgSO_4$ buffer (pH 6.5)) at 1 ml/g wet wt. spheroplasts. After incubation on ice for 10 min, another 1 ml of buffer per g wet wt. spheroplasts was added to the suspension and vortexed for 1 min. Following incubation on ice for another 10 min, 2 ml of lysate was loaded on 10-20-30-40-50-60-70% discontinuous sucrose gradients (2:2:2:2:2:2:1 ml) containing 10 mM Tris/1 mM EDTA (pH 7.5) and covered with 1 ml of lysis buffer. The gradients were centrifuged at $27000 \times g$ for 30 min and fractionated from the top in either 0.5 ml or 1 ml aliquots.

Method 2: Glusulase digestion. Yeast cells were harvested by centrifugation at $4000 \times g$ for 1 min, and the cell walls digested by glusulase as described by McLean-Bowen and Parks [7]. The spheroplasts were washed and lysed, and the plasma membranes were isolated as above (see Method 1).

Method 3: Concanavalin A conjugation. Yeast cells were treated with lyticase as described under Method 1. The spheroplasts were washed in 0.9 M sorbital/10 mM Tris/10 mM $MgSO_4$ buffer (pH 7.5) three times and incubated for 30 min at 27°C with gentle shaking in the same buffer containing fluorescein isothiocyanate-Concanavalin A (FITC-Concanavalin A) or unlabelled concanavalin A at 1 mg concanavalin A/g wet wt. spheroplasts per 0.5 ml buffer. After incubation with the fluorescent concanavalin A, the spheroplasts were washed three times with the same buffer (sans Con A) and lysed as described above in Method 1. The fluorescence of the concanavalin A-labelled bands from the discontinuous sucrose gradients was determined on an Aminco-Bowman spectrofluorophotometer.

Radioactive labelling of the plasma membranes

The spheroplasts formed from the glusulase

TABLE I

YEAST STRAINS

nys = nystatin: resistant concentrations in units nystatin/ml medium.

Strain	Phenotype	Sterol(s) accumulated	Defect in sterol biosynthesis
S288C	wild-type, nys^s (3 units/ml)	ergosterol	–
JR1	nys^r (8 units/ml)	ergosta-7,22-dienol	Δ^5desaturase
JR 5	nys^r (14 units/ml)	zymosterol cholesta-5,7,22,24-tetraenol	Δ^{24}methyltransferase

and lyticase digestions were each labelled with *N*-[^{3}H]ethylmaleimide as described by Schibeci et al. [12]. The spheroplasts were lysed and the plasma membranes isolated as described above (see Method 1). The radioactivity in the sucrose gradient bands was determined by counting 100-μl aliquots diluted in Aquasol II scintillation fluid in a Beckman LS8000 scintillation counter.

Quantitation of membrane components

Lipids were extracted from the sucrose gradient bands by the procedure of Bligh and Dyer [13] and separated by the thin-layer chromatography system of Skipski and Barclay [14]. Sterols were quantitated by gas-liquid chromatography with cholesterol and cholestane as internal standards as previously described [9,15]. Phospholipids were quantitated by the method of Ames [16], and protein was quantitated by the method of Lowry et al. [17] using bovine serum albumin as the protein standard.

Transmission electron microscopy

The bands from the sucrose gradients were fractionated as described above and pelleted in 10 mM Tris/1 mM EDTA buffer (pH 7.4) by centrifugation at 48 000 × *g* for 30 min. The pellets were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0), solidified in 2% ionagar, and stained with 2% OsO_4/cacodylate buffer (1 : 1, v/v). After successive washing in 30% acetone, 50% acetone, 70% acetone with uranyl acetate, and 100% acetone, the samples were infiltrated with Spurrs *. The thin sections were treated with Reynold's lead nitrate, sodium acetate stain prior to examination on a Philips EM 300 transmission electron microscope.

Enzyme assays

Chitin synthase activity was assayed by the method of Cabib [18] with the exception that the *N*-acetylglucosamine particles were isolated by filtration on to 0.45 μm Millipore filters instead of by centrifugation techniques. Mg^{2+}-ATPase activity was assayed according to the procedure of Cross and Kohlbremmer [19]. α-Mannosidase was assayed by the method of Van der Wilden et al. [20]. Cytochrome oxidase activity was measured as described by Wharton and Tzagoloff [21] and NADPH-cytochrome *c* reductase was measured as described by Sottocasa et al. [22]. DNA content was determined by the procedure of Burton [23].

In the enzyme inhibition studies, nystatin was incubated with the plasma membranes for 30 min prior to the start of the reactions. Nystatin was solubilized in dimethylformamide and diluted with the appropriate buffer to make stock solutions such that the final concentration of dimethylformamide in the enzyme reactions was less than 0.1%. This concentration of dimethylformamide did not affect enzyme activity.

The sidedness of the plasma membrane vesicles was determined by the inhibition of chitin synthase by glutaraldehyde using the procedure of Duran et al. [24]. The final concentration of glutaraldehyde was 0.25% in all cases. Spheroplasts treated with glutaraldehyde before lysis were washed prior to disruption as previously described [24]. Plasma membranes isolated from the lysate containing glutaraldehyde were washed after isolation from the sucrose gradients. Plasma membranes isolated from the sucrose gradients and then treated with glutaraldehyde were also washed as above.

Physical studies

Fluorescence anisotropy was measured on a computerized fluorescence anisotropy spectrometer [25] using 1,6-diphenyl-1,3,5-hexatriene as the probe [7]. The absorbance at 460 nm was 0.3 for all preparations and the concentration of diphenylhexatriene was 1 μM in 10 mM Tris/1 mM EDTA (pH 7.4) buffer.

High-sensitivity differential scanning calorimetry (DSC) was performed on a Micro-Cal I as previously described [26] using 10 mg protein/ml of 10 mM Tris/1 mM EDTA buffer (pH 7.4) as the sample concentration. Plasma membranes were scanned at a rate of 20 K/h from 2.5°C to 75.0°C. Proteolysis of the membranes was performed by adding 1 mg pronase/ml of plasma membrane suspension and incubating the suspension at 25°C for 1 h prior to scanning.

Materials

Snail gut enzyme preparation Glusulase was

* Spurrs embedding plastic.

purchased from Endo Laboratories, Inc. Concanavalin A, concanavalin A fluorescein isothiocyanate (FITC-Con A), bovine serum albumin, cholesterol, nystatin, pronase, 1,6-diphenyl-1,3,5-hexatriene (DPH), and enzyme reagents were from Sigma Chemical Co. Aquasol II and *N*-[*ethyl*-2-^{3}H]ethylmaleimide were purchased from New England Nuclear. Uridine diphospho-*N*-[*acetyl*-1-^{14}C]acetylglucosamine was from International Chemical and Nuclear Corp. Dipalmitoylphosphatidylcholine (DPPC) used in the DSC analysis was purchased from Calbiochem-Behring. All other chemicals and solvents employed were reagent grade, and the solvents were redistilled prior to use.

Instrumentation

A Sorvall RC-2B centrifuge was used for the centrifugations involved in plasma membrane isolation. Spectrophotometric assays for enzyme activities and other analyses were conducted on a Beckman DU-8 spectrophotometer. GLC quantitation of sterols was performed on a Supelco SE-30 column in a Varian Series 2700 gas chromatograph equipped with a CDS-111 data processor. Densities of gradient fractions were determined by a ABBE-3L Bausch & Lomb refractometer. Radioactivity was measured in a Beckman LS8000 scintillation counter.

Results

Identification of plasma membranes

Using the procedure of lyticase digestion of the yeast cell walls (Method 1), the top band ($\rho = 1.03$) of the sucrose gradients contained the bulk of the plasma membranes as demonstrated by the distribution of the yeast plasma membrane markers chitin synthase and Mg^{2+}-ATPase (Fig. 1, Table II). About 90% of these enzyme activities was located in this band. These results were confirmed by radioactive labelling experiments of the plasma membrane proteins with *N*-ethylmaleimide (Fig. 2). Again, in these experiments 90% of the plasma membrane was shown to fractionate in the top band.

The purity of this plasma membrane fraction was examined by the use of other markers enzymes (Fig. 3, Table II). Low levels of cytochrome

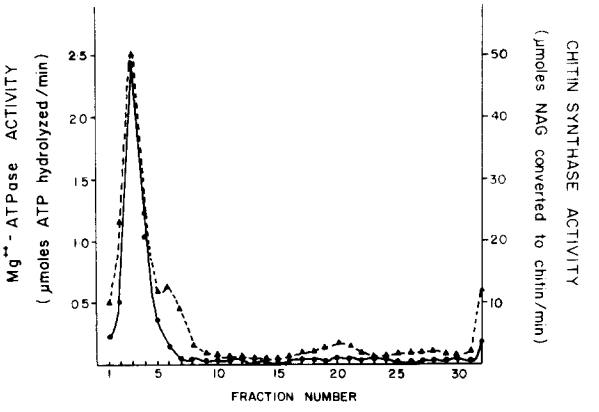


Fig. 1. Enzyme activities of Mg^{2+}-ATPase and chitin synthase in sucrose gradients of lysate from lyticase-prepared spheroplasts (Method 1). Lysate and gradient fractions were prepared as described in Methods. Mg^{2+}-ATPase activity (▲———▲) and chitin synthase activity (●———●) were measured in 0.5 ml fractions of the discontinuous sucrose gradients as described in Methods and Materials where fraction 1 represents the top of the gradient. NAG, *N*-acetylglucosamine.

c oxidase activity in this band illustrated little mitochondrial contamination, the major mitochondrial band being much lower in the sucrose gradients (Fig. 3A). Some contamination by microsomes (NADPH-cytochrome *c* reductase) was evident (Fig. 3A). However, this contamination was always less than 10% of the total enzyme activities in the gradients and could be minimized by layering lysis buffer over the lysate prior to centrifugation of the sucrose gradients. Other markers for vacuoles (α-mannosidase) and nuclei (DNA content) demonstrated little or no contamination of the plasma membrane fraction with these organelles (Fig. 3B). The purity of the plasma membranes was thus established, with contamination by other fractions being less than 10%.

As these results appeared to be inconsistent with previous work [12,24,27–35], which reported plasma membrane fractions of much greater densities, two other techniques for isolation of plasma membranes were utilized. The first (Method 3) involved the use of the lectin concanavalin A to reinforce the plasma membrane prior to lysis of the spheroplasts (see Methods and Materials). Indeed, in these experiments, enzyme markers revealed a plasma membrane fraction of much greater density (Fig. 4). These results were con-

TABLE II

SPECIFIC ACTIVITIES OF MARKER ENZYMES AND LIPID DISTRIBUTION IN GRADIENTS

Cells were digested with lyticase, the cellular lysate run on sucrose gradients, and the enzyme activities and lipid distribution quantitated per mg protein as described in Methods and Materials.

Fraction [a]	Mg^{2+}-ATPase [b] (μmol·min^{-1}·mg^{-1})	Chitin synthase (μmol·min^{-1}·mg^{-1})	NADPH-cyt. *c* reductase (μmol·min^{-1}·mg^{-1})	Cytochrome *c* oxidase (μmol·min^{-1}·mg^{-1})	α-Mannosidase (nmol·min^{-1}·mg^{-1})	DNA (μg·mg^{-1})	Phospholipid (μmol·mg^{-1})	Sterol (μmol·mg^{-1})
1	0.008	0.34	0.0054	0.0010	0.27	0.07	0.013	0.0089
2	0.037	0.92	0.0744	0.0014	0.50	0.19	0.045	0.0036
3	0.222	4.63	0.0088	0.0083	0.83	0.20	0.104	0.0372
4	0.192	4.22	0.0039	0.0078	1.37	0.29	0.086	0.0336
5	0.129	4.29	0.0089	0.0054	7.14	0.57	0.112	0.0311
6	0.080	1.57	0.0075	0.0025	14.50	1.01	0.125	0.0109
7	0.027	0.63	0.0033	0.0100	9.33	0.80	0.083	0.0260
8	0.007	0.74	0.0074	0.0259	7.04	0.67	0.062	0.0117
9	0.006	0.20	0.0020	0.0530	1.00	0.60	0.300	0.0056
10	0.004	0.45	0.0051	0.0538	0.77	0.64	0.336	0.0123
11	0.005	0.45	0.0034	0.0716	0.80	0.95	0.321	0.0091
12	0.005	0.75	0.0050	0.0675	1.75	1.30	0.250	0.0059
13	0.000	0.53	0.0052	0.0029	1.05	1.68	0.044	0.0033
14	0.000	0.50	0.0100	0.0027	3.33	2.33	0.028	0.0025
15	0.003	0.33	0.0033	0.0050	1.80	1.00	0.042	0.0020
16	0.026	2.02	0.0081	0.0113	2.10	0.65	0.141	0.0323

[a] Fractions were collected in 1 ml aliquots from the top of the gradients; fraction 1 represents the top of the gradient.
[b] Mg^{2+}-ATPase activity measured was oligomycin-insensitive as described in Methods and Materials.

firmed by conjugating the spheroplasts with fluorescein-concanavalin A before lysis and then measuring the fluorescence intensity of each fraction to locate the labelled plasma membrane. In these experiments (Fig. 4) the bulk of the plasma membrane was again located at much greater density ($\rho = 1.22$) than was seen without concanavalin A conjugation. There was, however, also a small peak of intensity at the top of the gradients ($\rho = 1.03$), which appeared consistently and represented some 20% of the total fluorescein-concanavalin A label in the gradients.

The other method of preparing a plasma membrane fraction employed the snail gut preparation Glusulase (Method 2) for digestion of the cell walls instead of lyticase (see Methods and Materials). The pattern of enzyme marker distribution in the gradients was similar to the experiments involving the concanavalin A conjugation, and labelling with *N*-[^{3}H]ethylmaleimide also confirmed this pattern of distribution (Fig. 2). A large band of high density ($\rho = 1.22$) representing the majority of the plasma membrane was located near the bottom of the gradients with a smaller band ($\rho = 1.03$) located near the top of the gradients.

The difference between these two types of plasma membrane fraction appears to be the size of the vesicles and the amount of internal components associated with the vesicles. The plasma membrane vesicle size would be anticipated to be larger with concanavalin A reinforcement, which would prevent the plasma membrane from fragmenting into smaller vesicles. The amount of cell wall digestion could also be a factor in the fragmentation of the plasma membranes, as any remaining cell wall could stabilize the membranes [36]. Therefore, if Glusulase was less efficient in cell wall digestion than the lyticase, the Glusulase-

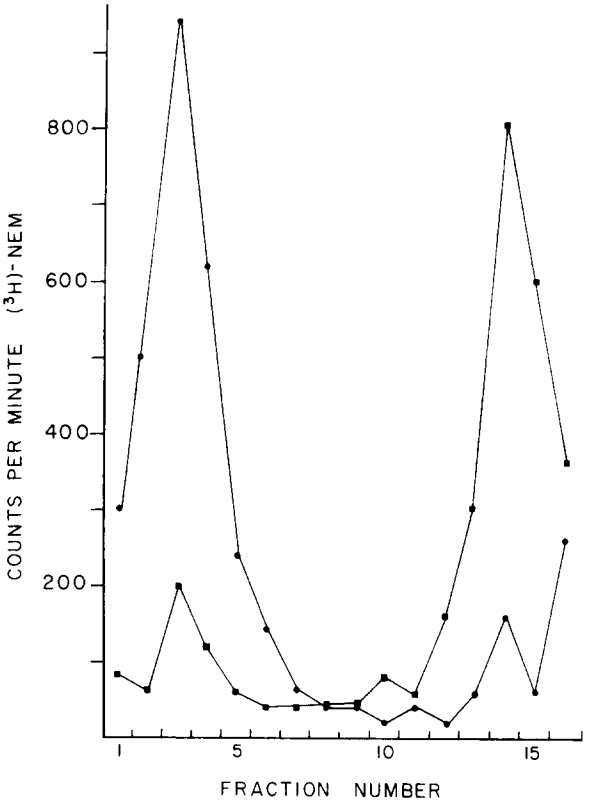


Fig. 2. Comparison of *N*-[^{3}H]ethylmaleimide (NEM) labelling of spheroplasts from lyticase digestion (Method 1) and Glusulase digestion (Method 2). Spheroplasts were labelled with [^{3}H]ethylmaleimide and lysed, and the lysate was run on discontinuous sucrose gradients as described in Methods and Materials. Gradients were fractionated from the top (fraction 1 represents the top of the gradient) in 1 ml aliquots. Radioactivity in fractions prepared by Method 1 (●———●) and by Method 2 (■———■) was determined as described in Methods and Materials.

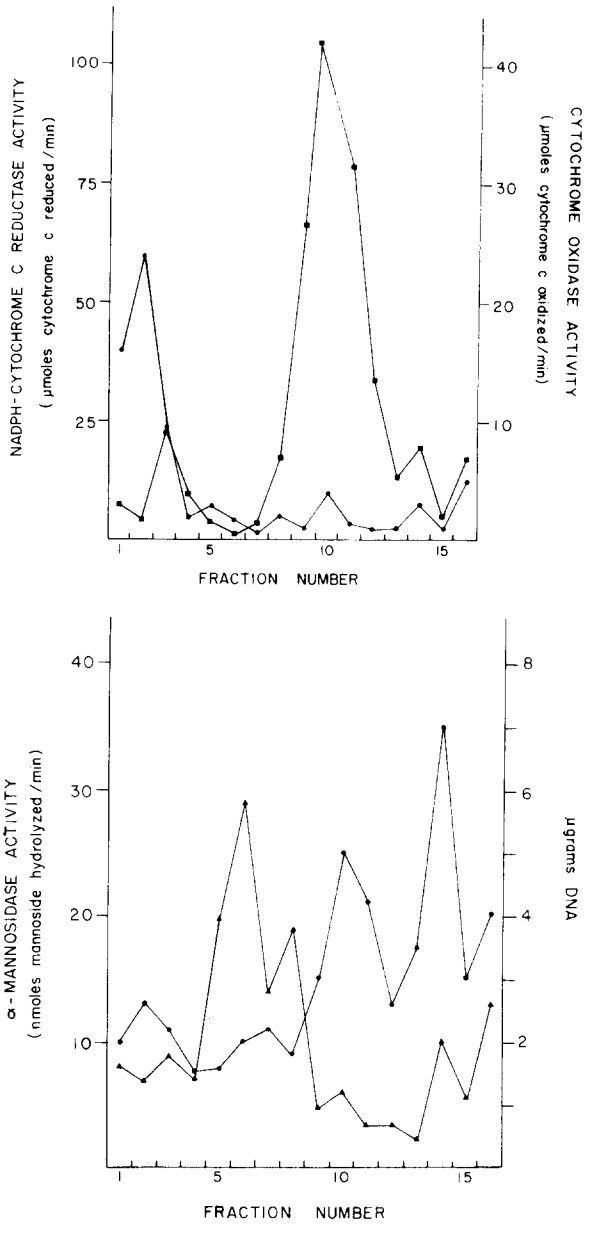


Fig. 3. Marker enzyme activities in sucrose gradients of lysate from lyticase-digested spheroplasts (Method 1). The lysate and discontinuous sucrose gradients (Method 1) were prepared and the enzyme activities determined for 1 ml fractions (fraction 1 is top of the gradient) as described in Methods and Materials. (A) Cytochrome oxidase (■———■), NADPH-cytochrome *C* reductase (●———●). (B) α-Mannosidase (▲———▲), DNA content (●———●).

prepared plasma membranes would contain larger vesicles with more cell wall and internal components present. We have observed using the lyticase digestion procedure that any factor which influences the efficiency of digestion affects the yield and distribution of the plasma membrane fraction. Thus, the age of the yeast culture, the particular yeast strain, the temperature, pH and length of digestion, and the age of the lyticase preparation are all critical factors. It is of interest to note that others have often reported a small band of plasma membrane of density similar to that of our plasma membrane fraction in their gradients [12,24,30–33,35], as well as a larger plasma membrane fraction of greater density.

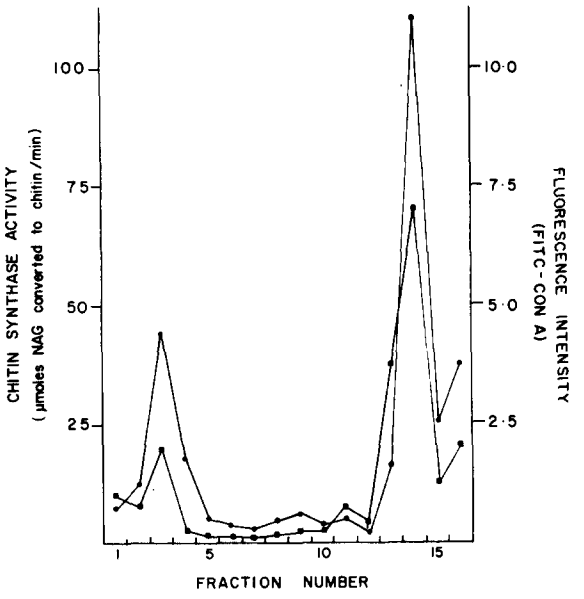


Fig. 4. Effects of concanavalin A conjugation (Method 3). FITC-Con A was used to reinforce lyticase-digested spheroplasts (Method 3), lysed, and fractionated on discontinuous sucrose gradients as described in Methods and Materials. Fluorescence intensity of FITC-Con A (●———●) and chitin synthase enzyme activity (■———■) were measured for 1 ml fractions (fraction 1 being top of the gradient) as described in Methods and Materials.

Characterization of plasma membrane vesicles

The sidedness of the plasma membrane vesicles was determined by inhibition of chitin synthase by glutaraldehyde. Using this technique, Duran et al. [24] have demonstrated that chitin synthase is located on the inner surface of the yeast plasma membrane. In similar experiments, we treated the spheroplasts before, during and after lysis. Our results (Table III) indicate that when the inner surface of the plasma membrane was exposed to glutaraldehyde, the chitin synthase was inhibited (i.e., when glutaraldehyde was present during lysis). If glutaraldehyde was added after lysis, then the chitin synthase was not inhibited, indicating that the plasma membrane vesicles are right-side-out (Table III).

The plasma membranes were further characterized by examination of the fraction with transmission electron microscopy. Using the lyticase digestion procedure (Method 1), the plasma membrane preparation ($\rho = 1.03$) was seen as many, small,

TABLE III

GLUTARALDEHYDE INHIBITION OF CHITIN SYNTHASE IN DETERMINING SIDEDNESS OF PLASMA MEMBRANE VESICLES

Plasma membrane vesicles were isolated from lyticase-treated spheroplasts in the absence and presence of glutaraldehyde and the chitin synthase activity was determined as described in Methods. NAG, *N*-acetylgluosamine.

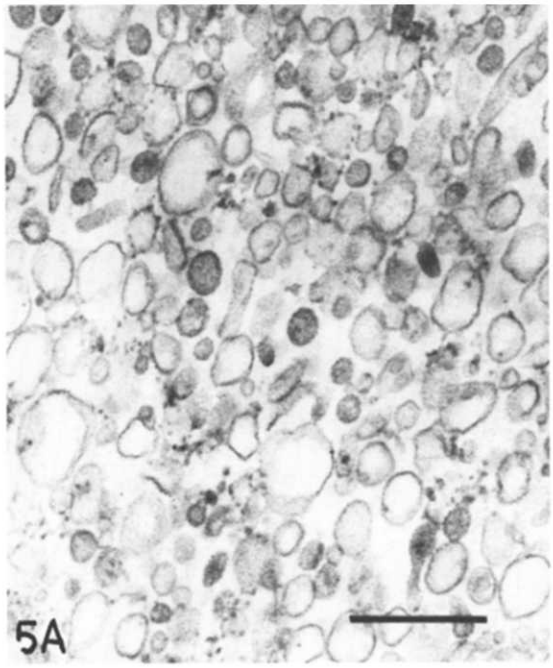
Addition of glutaraldehyde	Chitin synthase activity (μmol NAG converted to chitin/min per mg protein)
None	124.9
Before lysis	109.5
During lysis	20.0
After lysis	102.2

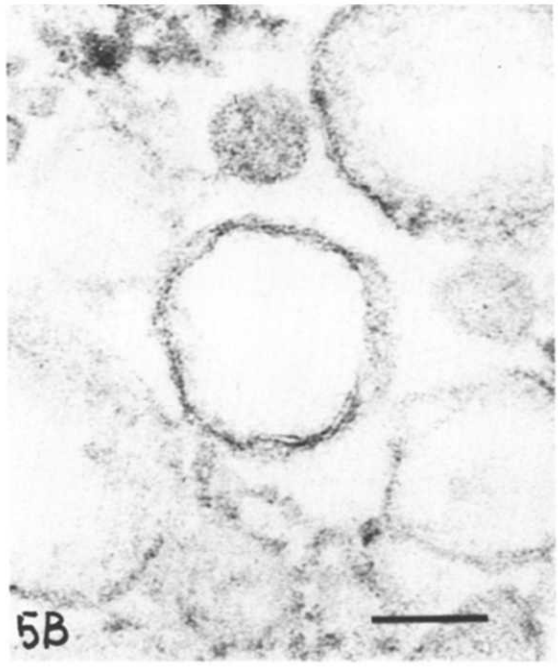
uniform, closed, bilayer vesicles (Fig. 5). These vesicles did not appear to contain any internal contamination. The small, lower band located where the bulk of the plasma membrane appeared using the other procedures ($\rho = 1.22$) was also examined. The band was composed of cellular material loosely enclosed in large vesicles, extraneous cellular components, and non-vesicularized membranes (Fig. 6). The asymmetric appearance of the vesicles suggested that little cell wall remained.

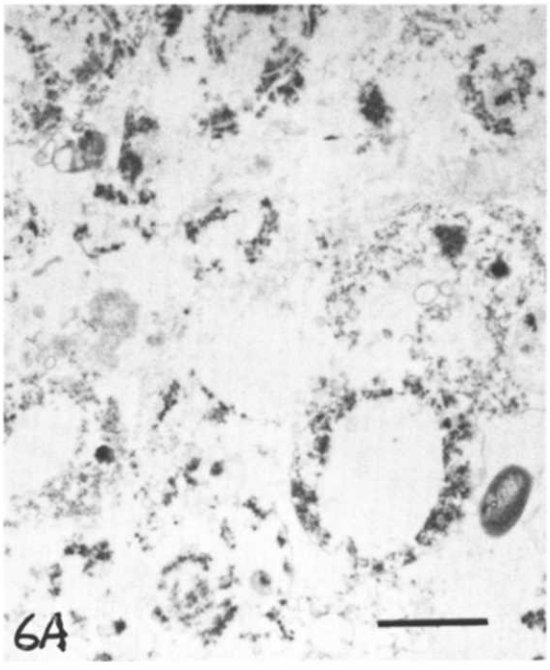
As the plasma membrane fraction from the lyticase lysis procedure (Method 1) had the least contamination of other cellular components, the largest yield, and the most uniform non-contaminated vesicles, this was the procedure of choice for subsequent experiments. The lyticase digestion procedure may be of great use in those cases where low contamination of the plasma membrane fraction by cell wall and other components is essential or where uniform closed vesicles are desired. Using the procedure, Louma and Ferro (personal communication) have identified the same plasma membrane band by chitin synthase and radioactive labelling with dansyl chloride, and found this fraction suitable for *S*-adenosylmethionine permease studies.

Sterol quantitation of plasma membranes

The sterol composition of the yeast plasma membrane isolated by lyticase digestion (Method 1) was quantitated on the basis of phospholipid







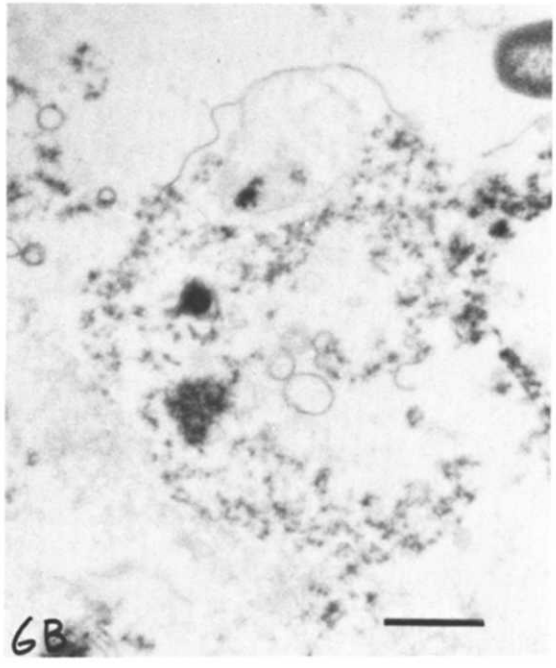


Fig. 5. Electron micrographs of plasma membranes (low density) from lyticase-digested spheroplasts (Method 1). (A) Bar = 0.5 μm; 36000×. (B) Bar = 0.1 μm; 148800×.

Fig. 6. Electron micrographs of high-density plasma membrane fraction from lyticase-digested spheroplasts (Method 1). Preparation of fractions and microscopy are described in Methods and Materials. (A) Bar = 2 μm; 6800×. (B) Bar = 1 μm; 12800×.

and protein content (Tables II and IV). The ergosterol-to-phospholipid molar ratio of the plasma membrane was determined to be 0.365 compared to a 0.321 ergosterol-to-phospholipid molar ratio of whole yeast cells. The ergosterol-to-protein ratio was calculated to be 0.0372 μmol per mg for these plasma membranes and 0.0225 μmol per mg for whole cells. The plasma membranes are thus enriched in sterol over the whole cell and other cellular components such as nuclei and mitochondria (Table IV).

Physical studies of the plasma membranes

The thermotropic behavior of yeast plama membranes was first investigated by high-sensitivity differential scanning calorimetry (DSC). The differential scanning calorimetry of the plasma membranes from wild-type yeast gave a large protein transition at 50°C which could be eradicated by treatment with pronase (Fig. 7). This peak was superimposed over two other broad transitions occurring from 5°C to 45°C and from 45°C to 100°C. The significance of these broad transitions is not clear. They are unaffected by pronase and cannot be observed if the same sample is scanned a second time (Fig. 7). The possibility that this phenomenon represents a bulk lipid transition was investigated by DSC scans of lipid extracted from wild-type yeast plasma membranes (Fig. 8). The total extracted lipid and the extracted phospholipid both failed to show any such transitions.

The plasma membranes isolated from the yeast sterol mutants gave DSC scans identical to those of the wild-type yeast plasma membranes, despite the differences in the sterol structures of those

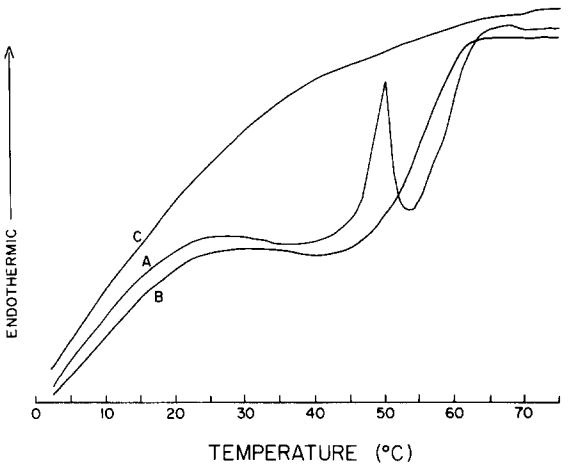


Fig. 7. High-sensitivity differential scanning calorimetry heating scans of plasma membranes (10 mg protein/ml). (A) First scan without pronase treatment. (B) First scan with pronase treatment. (C) Second scan of (A). Plasma membrane preparation for DSC measurements is described in Methods and Materials.

membranes. A protein transition at 50°C (which could be eliminated with pronase) overlapped with other broad transitions starting at approx. 5°C and 45°C. The total extracted lipid from the plasma membranes of the sterol mutants again failed to show any transitions (Fig. 8).

Further physical studies on the fluidity of the yeast plasma membranes involved fluorescence anisotropy measurements of the hydrophobic probe diphenylhexatriene. In these experiments, transitions were observed in the plasma membranes from the sterol mutants (Fig. 9). These transitions occur between 22 and 25°C, varying with the type

TABLE IV

STEROL COMPOSITION IN *S. CEREVISIAE*

	Sterol : protein (μmol/mg)	Phospholipid : protein (μmol/mg)	Sterol : phospholipid (μmol/μmol)	Ref.
Whole cells	0.0225	0.070	0.321	this paper
Mitochondria	0.0094	0.313	0.030	9
Plasma membrane [a]	0.0372	0.102	0.365	this paper
Nuclear membranes	–	–	0.273	50
Vacuoles	–	–	0.298	51

[a] Plasma membrane as isolated after lyticase digestion (Method 1). Lipids were extracted and quantitated as described in Methods and Materials.

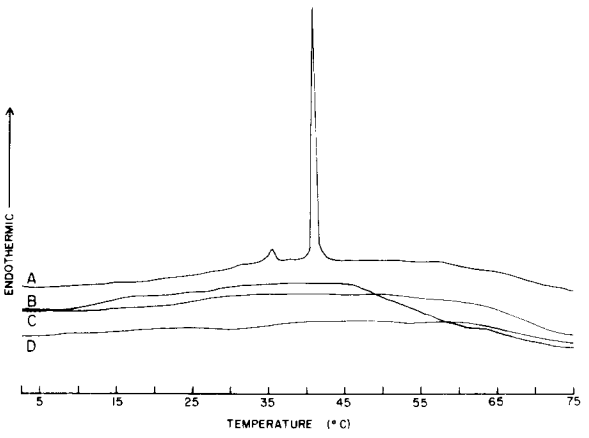


Fig. 8. High-sensitivity differential scanning calorimetry heating scans of lipid extracted from plasma membranes. (A) DPPC (0.5 mg/ml). (B) JR 1 lipids (5 mg/ml). (C) JR 5 lipids (5 mg/ml). (D) S288C lipids (5 mg/ml). Preparation of plasma membranes and extraction of lipids are described in Methods and Materials.

of sterol present. Such transitions were not observed in plasma membranes isolated from wild-type yeast containing ergosterol (Fig. 9).

Enzymatic studies of plasma membranes

Arrhenius kinetics of the plasma membrane enzymes chitin synthase and MG^{2+}-ATPase yielded results very different from the physical studies. Contrary to the fluorescence anistropy experiments, the Arrhenius kinetics of the chitin synthase did not demonstrate transitions in either plasma membranes isolated from wild-type yeast or from the sterol mutants (Fig. 10). No change in the activation energy of chitin synthase in any plasma membrane preparation was observed between 15°C and 40°C.

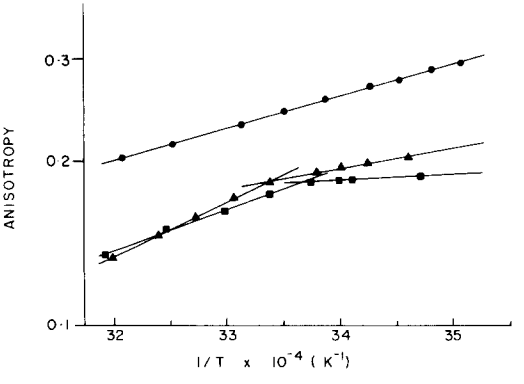


Fig. 9. Arrhenius curves of fluorescence anisotropy of plasma membranes. Fluorescence anisotropy of diphenylhexatrieme in plasma membranes from JR 1 (▲———▲), from JR 5 (■———■), and from S288C (●———●) was measured as described in Methods and Materials from 15 to 40°C.

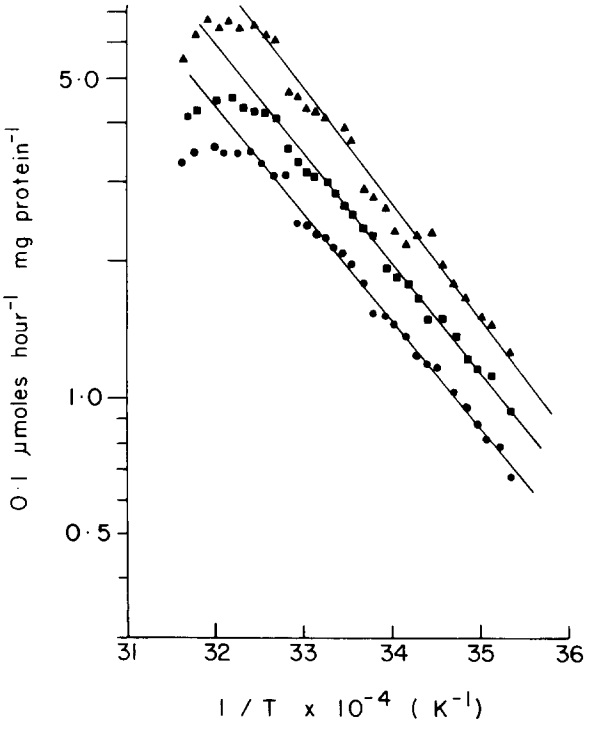


Fig. 10. Arrhenius curves of chitin synthase activity. Chitin synthase activity of plasma membranes from JR 1 (▲———▲), from JR 5 (■———■), and from S288C (●———●) was measured as described in Methods and Materials from 15 to 40°C.

The Arrhenius kinetics of the Mg^{2+}-ATPase, on the other hand, showed transitions both in plasma membranes from the yeast sterol mutants and from the wild-type yeast (Fig. 11). The temperatures at which these transitions occurred varied between 20 and 25°C, similar to those of the fluorescence anisotropy experiments. As these yeast strains are isogenic with the exception of the sterol produced, the actual temperature of transition appears to be dependent on the structure of the sterol(s) accumulated by the organism.

Because of the anomalous behavior of these two plasma membranes enzymes with respect to their activities over the physiological temperature range,

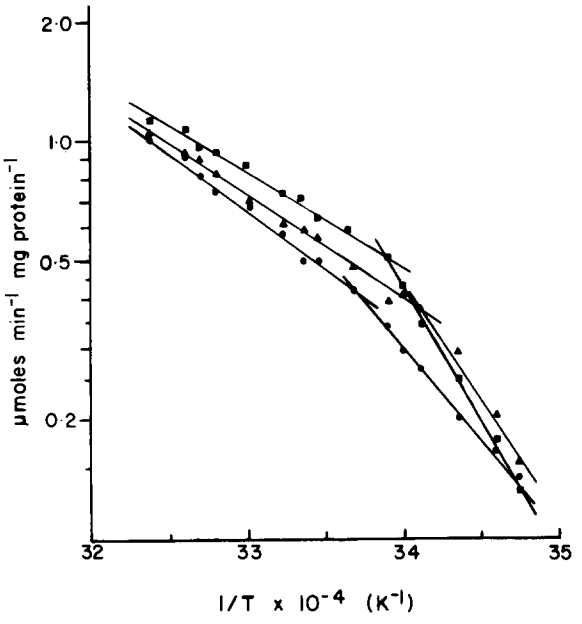


Fig. 11. Arrhenius curves of plasma membrane Mg^{2+}-ATPase activity. Mg^{2+}-ATPase activity of plasma membranes from JR 1 (●———●), from JR 5 (■———■), and from S288C (▲———▲) was measured as described in Methods and Materials from 15 to 40°C.

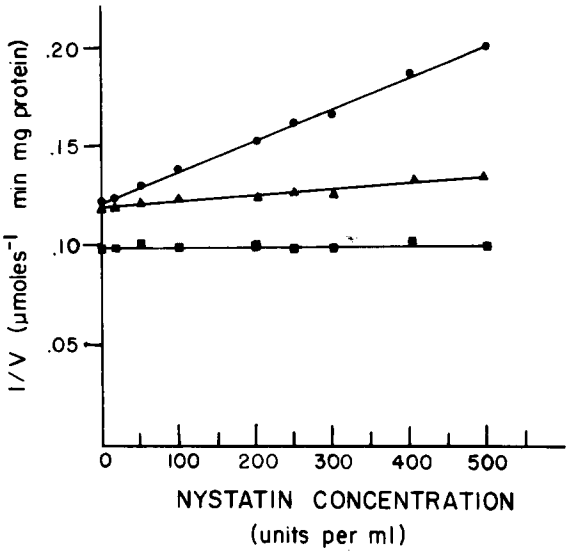


Fig. 12. Dixon curve of inhibition of nystatin on chitin synthase activity. Chitin synthase activity of plasma membranes from JR 1 (▲———▲), from JR 5 (■———■), and from S288C (●———●) was measured with increasing concentrations of nystatin (units/10 mg protein per ml) as described in Methods and Materials.

their membrane characteristics were investigated further by nystatin inhibition studies. Nystatin is a polyene antimycotic agent which disrupts fungal cellular membranes by complexing with sterols [37]. The sterol mutants used in this study were isolated on the basis of their resistance to nystatin (Table I). Thus it was of interest to determine if nystatin would interfere in the plasma membrane enzyme activities of the nystatin-sensitive, wild-type yeast and the nystatin-resistant, sterol mutants.

Addition of nystatin did not affect the enzyme activity of the Mg^{2+}-ATPase from either wild-type or sterol mutant plasma membranes. Even at concentrations of 1000 units nystatin/ml per 10 mg protein, no inhibition of the Mg^{2+}-ATPase was observed in the wild-type yeast whose growth inhibition concentration is 3 units nystatin/ml (Table I) (McCammon, M.T. and Parks, L.W., unpublished data).

Nystatin did, however, affect the activity of chitin synthase (Fig. 12). In the wild-type yeast plasma membranes, chitin synthase enzyme activity was decreased 10-fold by 10 units nystatin/ml per 10 mg protein. The chitin synthase from the sterol mutants' plasma membranes did not show such dramatic inhibition of enzyme activity by nystatin. On the basis of the resistance of these sterol mutants to nystatin, this result is not unexpected. Even at 100 units nystatin/ml per 10 mg protein, chitin synthase enzyme activity in the plasma membranes from the sterol mutants was reduced only 10%. Thus, the nystatin inhibition studies corroborated the differences in membrane behavior between these two plasma membrane enzymes.

Discussion

The fluidizing and ordering effects of sterols on membrane bilayers have been well documented [1,2,4,38]. In model membrane systems, sterols have been shown to eliminate phospholipid phase transitions [1,2,38]. The ability to eliminate these phase transitions has been linked to the structure of the sterol [38,39]. It was of interest to consider effects of sterol structure on the thermotropic behavior of an in vitro membrane system using both physical

techniques and enzymatic analyses.

The physical studies of the yeast plasma membranes gave conflicting results. Analysis by high-sensitivity differential scanning calorimetry did not reveal any lipid transitions in the plasma membranes isolated from either the wild-type yeast or the sterol mutants. The fluorescence anisotropy data, however, indicated that transitions were occurring in the plasma membrane of the sterol mutants which were not occurring in the wild-type plasma membranes. It is possible that any lipid transition which is occurring in the sterol mutants' plasma membrane is undetected by DSC because of the large protein transitions occurring. This seems unlikely, however, in view of the fact that the extracted lipids do not reveal transitions, either. We have observed a similar discrepancy in our results between differential scanning calorimetry and fluorescence anisotropy of mitochondrial membranes isolated from wild-type yeast and from sterol mutants [26]. We have argued that the fluorescence anisotropy measurements are more sensitive to lipid transitions in small regions. Such transitions would be unobserved by differential scanning calorimetry which can only detect bulk lipid transitions.

This picture is complicated by the enzymatic analysis of the yeast plasma membranes. Changes in activation energy were observed by the Arrhenius kinetics of the plasma membrane Mg^{2+}-ATPase in both wild-type and mutants. The Arrhenius kinetics of the chitin synthase, on the other hand, did not show any clear transition. No distinguishable break could be discerned in the Arrhenius plots in numerous experiments using either type of plasma membrane. It is of interest that the kinetics of the wild-type plasma membrane Mg^{2+}-ATPase indicated a transition not detected by fluorescence anisotropy measurements. This suggests that the two enzymes, Mg^{2+}-ATPase and chitin synthase, exist in two different environments.

This was further investigated by the addition of nystatin in the enzyme assays of the plasma membrane preparations. Nystatin is an antimycotic agent which binds ergosterol and disrupts the membranes of wild-type yeast. Nystatin's effectiveness in complexing the sterol intermediates accumulated by our yeast sterol mutants is greatly diminished, however, and their membranes remain intact conferring resistance to nystatin. The addition of nystatin to the plasma membrane preparations had no effect on the activity of the Mg^{2+}-ATPase in either the wild-type or sterol mutants. The chitin synthase activity was affected by the inclusion of nystatin as previously demonstrated [40], the plasma membranes from wild-type yeast showing a marked inhibition. As expected, the phase membranes from the sterol mutants showed much less inhibition of chitin synthase by nystatin. These nystatin inhibition studies of the two plasma membrane enzymes support the supposition for different lipid environments.

As one possible explanation for the conflicting results, we propose that there are at least two different domains in the yeast plasma membranes, one being sterol-rich and the other being sterol-poor. In this model, the chitin synthase is preferentially localized in a sterol-rich region. Thus, no transition is seen in the Arrhenius kinetics of the chitin synthase in the sterol-rich domains. Such transitions are prevented, even if the sterol structure is defective as in the case of sterol mutants, because the total sterol concentration is so high. Moreover, nystatin will inhibit the wild-type chitin synthase (and to a much lesser extent, the sterol mutants' chitin synthase) by binding the sterol present in the sterol-rich domains and disturbing the local environment.

The Mg^{2+}-ATPase in this model, however, would exist in a sterol-poor domain. Therefore, transitions are seen in both wild-type and sterol mutant Arrhenius kinetics of Mg^{2+}-ATPase because insufficient sterol is present in the local environment to prevent such transitions. Nystatin, on the other hand, will have little or no effect on the Mg^{2+}-ATPase as there is little sterol present in this domain to bind and thus disrupt enzyme activity. It has been shown for sarcoplasmic reticulum Ca^{2+}-ATPase that sterol is probably excluded from the boundary lipid layer surrounding the protein [41] and that sterols may even inhibit the Ca^{2+}-ATPase activity [42].

The idea of different lipid domains within membranes has been proposed previously [43,44]. Regions of low cholesterol concentration have been identified in such diverse membranes as tight junctions of oviduct cells [45], in frog postsynaptic

membranes at the neuromuscular junctions [46], and in rat pancreatic Golgi cisternae membranes [47]. In model membrane systems, it has been established that cholesterol has more affinity for negatively charged phospholipids or phospholipids of low transition temperature [48,49], giving rise to domains of low and high cholesterol concentration within the model membrane bilayers of mixed phospholipid content. However, the localization of different membrane enzymes within those domains has never been clearly illustrated in an in vitro membrane system.

As this model is obviously only one of many that could satisfactorily explain the observed phenomena, research is presently proceeding to verify the existence of these sterol-determined domains. The size, nature and transiency of any such domains also remains to be investigated.

Acknowledgements

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