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COMPARATIVE STUDIES OF THE STRUCTURE AND COMPOSITION OF THE PLASMALEMMA AND THE TONOPLAST IN *SACCHAROMYCES CEREVISIAE*

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Summary

The two membranes, plasmalemma and tonoplast (*Saccharomyces cerevisiae* H 1022), are characterized ultrastructurally by their different texture in the corresponding freeze-fracture faces and their silver staining properties.

Biochemical characterization with regard to proteins and lipids indicated that the ratio of protein to lipid is significantly higher in the plasmalemma as compared to the tonoplast. Moreover, a pronounced difference appears to exist for both the amount and the composition of total lipids, phospholipids and sterols. The protein patterns of the plasmalemma and the tonoplast reveal only minor differences, as judged by sodium dodecyl sulphate gel electrophoresis.

Introduction

For a comparative study of membranes we have restricted ourselves to the investigation of two kinds of membranes, namely the plasmalemma (plasma membrane), and the tonoplast (vacuolar membrane) in *Saccharomyces cerevisiae* H 1022. In three recent papers we described preparation and identification of plasmalemma from *S. cerevisiae* DGI 251 [1,2,3]. The plasmalemma was isolated from mechanically-disrupted yeast cells without the use of enzymes. Tonoplasts were gained from lysed protoplasts [4,5]. The protoplasts were obtained, however, by digesting the yeast cell wall with snail gut juice. The membranes have been compared electron microscopically after freeze fracturing, and chemically by investigating the membrane proteins and lipids. Dif-

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ferences and similarities in the structure of the two kinds of membranes are discussed in relation to their biogenesis and function.

Materials and Methods

The following yeast cells have been used for the preparation of plasmalemma: *S. cerevisiae* H 1022 (ETH Zürich) and *S. cerevisiae* DGI 251 (Hefefabriken Hindelbank AG, Bern). For preparation of tonoplasts only *S. cerevisiae* H 1022 has been taken.

S. cerevisiae H 1022 was grown in air at 30° C on a synthetic glucose-limited medium [6]. In order to prepare protoplasts the yeast cells were harvested in their first exponential phase. For the preparation of plasmalemma, cells were harvested in the stationary phase. *S. cerevisiae* DGI 251 was purchased as a fresh pressed cake and starved under aeration for 15 h before use.

Isolation of tonoplast

For isolation of tonoplast the procedures of Wiemken [7,8] and Schaffner [9] were slightly modified. 1 vol. washed wet cells was supplemented by 3 vols. of 0.01 M citrate buffer (pH 6.5) containing cysteamine hydrochloride, 0.05 M EDTA and 0.85 M sorbitol. Under constant gentle shaking the cells were kept at 30° C for 30 min. After this, the cells were washed twice with citrate buffer containing 0.85 M sorbitol. The cell sediment was resuspended with an equal volume of the above solution to which 25 mg lyophylized snail gut juice ("Helicase", Industrie Biologique Francaise S.A. Gennevilliers, France) per ml sediment were added. Incubation was at 30° C for 2 h by slow motion shaking. After this time most of the protoplasts had lost their cell wall. The protoplasts were washed twice in the above medium without snail gut juice but with an addition of 1 mM phenylmethane sulfonylfluoride as a protease inhibitor. The cell sediment was weighed and mixed with a solution of 15% (w/v) Ficoll (M_r 400 000, Pharmacia Uppsala, Sweden) in citrate buffer containing 0.1 M sorbitol to give a final concentration of 10% Ficoll. The suspension of protoplasts was stirred slowly during 30 min at 4° C. By this treatment most of the protoplasts lyse and release vacuoles swollen because of the hypotonicity of the solution, but remain intact as vesicles. The resulting suspension was taken at the bottom of a discontinuous gradient in citrate buffer which was built up toward the top with 9% Ficoll in 0.1 M sorbitol, 7% Ficoll in 0.1 M sorbitol, 0.1 M sorbitol and on the top buffer only. Centrifugation for 90 min at 25 000 rev./min in a Beckman SW 27 rotor caused flotation of vacuoles and of lipid granules. The lipid granules were mainly found at the top, whereas the vacuoles form a double band at the interphase between 7% Ficoll and 0.1 M sorbitol citrate solution. The vacuoles form an upper homogeneous layer and a lower, rather flaky layer. In phase contrast microscopy the upper layer showed only vacuoles contaminated by lipid granules, whereas the lower layer contained in addition some plasmatic remnants. Purification of the vacuoles was done in another discontinuous gradient. The double layer was collected and mixed 1 : 1 with the above 7% Ficoll solution. The resulting suspension was gently mixed in a Potter-Elvehjem homogenizer to release lipid granules from vacuoles. The suspension was used as bottom layer followed by a layer of 0.1 M sorbitol in

citrate buffer and at the top citrate buffer only. Centrifugation as above leads to the formation of a homogeneous layer of vacuoles at the interphase between 7% Ficoll and sorbitol solution. This layer was still contaminated by some lipid granules. The layer was collected and mixed 1 : 4 with 1 M sorbitol in citrate buffer which induces the vacuoles to shrink. The resulting tonoplast membranes were sedimented at 20 000 rev./min for 60 min (Beckman, SW 40). The sediment was resuspended in the 1 M sorbitol citrate solution and centrifuged as before. No further lipid granules floated during this centrifugation. The ratio of protein to total lipid in this and the preceding sediment was constant within the range of the standard error as given in Table I. The tonoplast sediment was white and homogeneous. It was kept frozen at −20° C before analytical use.

Isolation of plasmalemma

The method of plasmalemma vesicles preparation has been described by Fuhrmann et al. [3] and the purity of the plasmalemma vesicles has been demonstrated [1,3]. To release cytosolic inclosures, the vesicles were lysed by transferring them from the preparation solution with an osmotic pressure of 760 mosM to a 10 mM Tris buffer solution, pH 7.0, with an osmotic pressure of approx. 20 mosM. After washing 3 times with this solution the inclosures were removed. There were no proteins detectable in the supernatant after a second lysis and the plasmalemma was devoid of the cytosolic enzyme ethanol dehydrogenase. For chemical analysis the vesicles were protected by the addition of 1 mM phenylmethane sulfonylfluoride to the buffer and stored deep frozen.

Analytical methods

Protein was determined before lipid analysis according to Lowry et al. [10] with bovine serum albumin as standard.

Extraction of lipids and determination of main lipid classes. Approximately 20 mg of lyophilised membranes were treated with 5 ml of chloroform/methanol (2 : 1, v/v) for 8 h at room temperature. The extraction was repeated twice for 4 h with fresh solvent. The combined extracts were washed according to Folch et al. [11]. The dry residue, after rotary evaporation of the solvents, was reextracted with chloroform for 1 h. Extraction was repeated twice for 30 min, the extracts were filtered, combined and dried under N_2 at 30° C. After in vacuo drying over P_2O_5 the dry weight of the extract was determined gravimetrically. Lipid phosphorus was measured with the method of Bartlett [12]. Sterols were determined by a modified Liebermann-Burchardt reaction [13] with ergosterol as standard. Identification of neutral lipids from tonoplast and plasmalemma was achieved by thin-layer chromatography on silica gel HR plates (E. Merck, G.F.R.), with petroleum ether (b.p. 40—60° C)/diethyl ether/acetic acid (75 : 25 : 1, v/v) as developing solvent. The chromatograms were sprayed with H_2SO_4 (5% ethanol solution) and heated at 180° C for 30 min. The stained lipids were identified by their R_F values in relation to lipid standards. The plasmalemma phospholipids were separated from non-phosphorus lipids by means of silicic acid column chromatography (Unisil, Clarkson Chemical Co.). Fractionation was accomplished by eluting successively non-phospholipids and phospholipids with chloroform and methanol, respectively. The solvents were removed and the residues dried in vacuo.

Separation of phospholipids from tonoplast and plasmalemma was accomplished by two-dimensional thin-layer chromatography as described by Broekhuyse [14]. For detection of phospholipids the chromatograms were sprayed with acid molybdate reagent. The identity of the individual phospholipids was determined by comparing their mobility to known substances. For quantitative evaluation the chromatograms were stained with iodine vapour and the individual phospholipid spots were scraped off the plates. The organic material was digested and the phosphorus content of each component determined according to Bartlett [12].

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis of tonoplast and plasmalemma was done according to the method of Fairbanks et al. [15]. The acrylamide/bisacrylamide concentration was 7.06% and approx. 100 μg protein was applied per gel.

Electron microscopy

Freeze etching was carried out according to Moor [16,17] in a Balzers freeze-etch device BA 500 M. Double replica of the membranes have been prepared by using the sandwich technique of Mühlethaler et al. [18—19]. Silver-staining was performed following the procedure of Picket-Heaps [20], but instead of block staining the different steps were performed in suspension. Blocking of sulfhydryl groups was achieved by treating aldehyde fixed protoplasts with 0.1 M iodoacetic acid titrated to pH 8 with NH_4OH for 75 min. Aldehyde groups were blocked by treatment with 2% sodium bisulfate at 60°C for 60 min. Fixation and embedding of cells was done as described by Kopp [21].

Results

In Fig. 1, a and à, the complementary fracture faces of the plasmalemma are shown. Plasmatic (PF) and exoplasmatic (EF) fracture faces of the plasmalemma exhibit the characteristic invaginations (iv) and areas with hexagonal patterns (encircled areas). In contrast, the corresponding fracture faces of the tonoplast (Fig. 1, b and b̀) show a uniform random distribution of particles. In comparison to plasmalemma the number of particles is less than half.

In Fig. 2 an ultrathin section of a silver-stained protoplast is pictured. The plasmalemma demonstrates a distinct deposition of silver granules, whereas the tonoplast does not. Sometimes silver-stained precipitates (double arrows) are found in the vacuole. The plasma shows some diffuse staining.

In order to compare the protein content of plasmalemma and tonoplast we used the method of Lowry et al. [10] with bovine serum albumin as standard and every effort was made to treat parallel samples identically. It is not possible to obtain reliable absolute values of the membrane protein content, because different standards and different methods give different results [22]. Based on our estimations, the ratio of protein to total lipid was the same in both *S. cerevisiae* species in their plasmalemma as can be seen in Table I, namely 2.1. ± 0.1. The tonoplast had a significantly lower amount of protein which can be deduced from the low ratio of protein to total lipid of 0.66 ± 0.067.

Differences in phospholipid, neutral lipid and ergosterol content were also

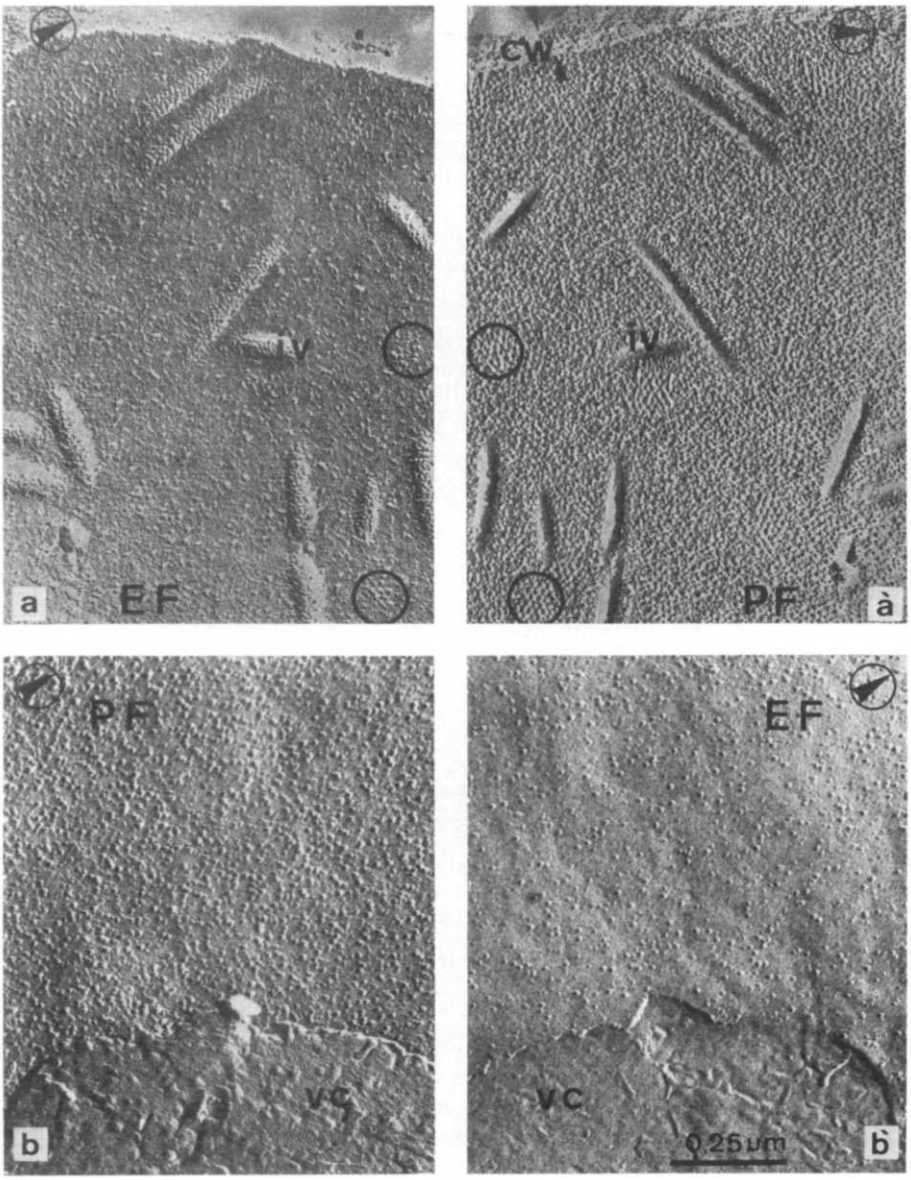

Fig. 1. Complementary replicas of yeast cell plasmalemma and tonoplast. The micrographs are mirror images to the perpendicular axis (dashed line). The encircled arrows indicate the direction of shadowing. Magnification, X60 000. a, EF (exoplasmatic) and à, PF (plasmatic-fracture face) of the plasmalemma at early stationary phase. Encircled areas show particles in hexagonal array. cw, Cell wall; iv, invagination. b, PF and b̀ EF of a tonoplast. vc, Vacuolar lumen. Except for the invaginations and the hexagonal arrays in a, particles in a and b appear to be distributed at random.

significant between plasmalemma and tonoplast as shown in Table I. When related to the percentage of total lipid the plasmalemma contained only between 5 and 6% of phospholipid against 40% of phospholipid in the tonoplast. The difference for neutral lipids was 94% in the plasmalemma compared

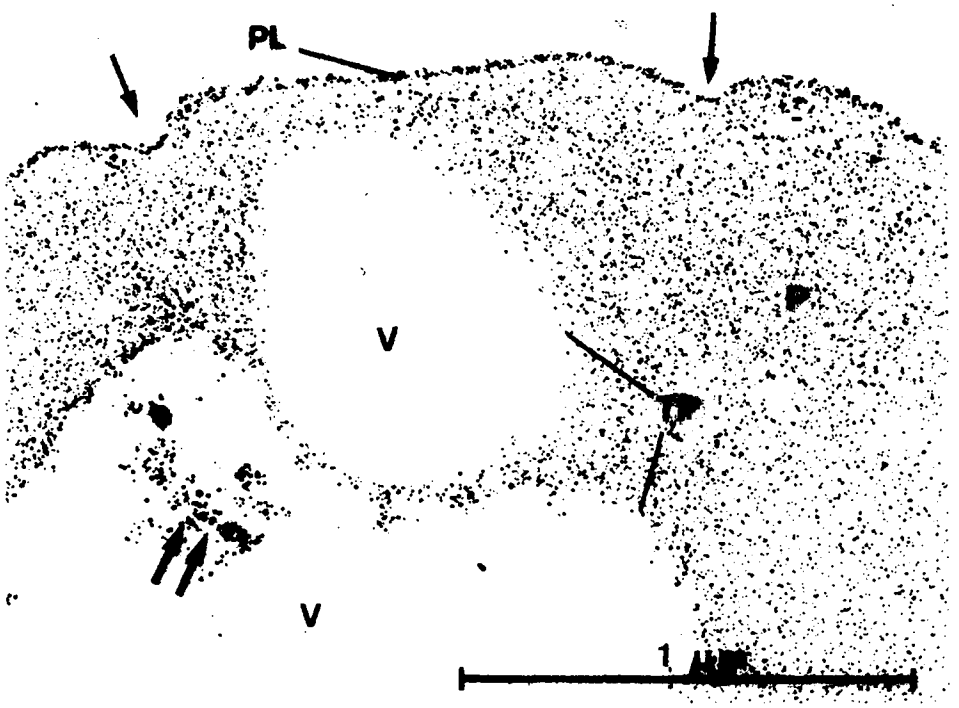

Fig. 2. Ultrathin section of a yeast protoplast stained with silver hexamethylene tetramine after periodic acid treatment. The plasmalemma (PL) shows a distinct stain, whereas the tonoplast membrane (TP) does not. The single arrows point to cross-sectioned membrane invaginations (cf. Fig. 1a and 1a). Inside the vacuole (V) some precipitated material of unknown composition was sometimes found (double arrows). Magnification, X60 000.

to 60% in tonoplast. Also the ergosterol content in plasmalemma was significantly higher with 25 to 26% in the neutral fraction compared to 6% in the corresponding fraction of the tonoplast.

The next figure (Fig. 3) shows a two-dimensional thin-layer chromatogram of the phospholipids of tonoplasts, and of plasmalemma from *S. cerevisiae* H 1022. The main difference in these two membranes is the absence of phosphatidic acid in tonoplasts. Lysophosphatidylcholine, on the other hand, is

TABLE I

	Plasmalemma (DGI 251 Hindelbank)	Plasmalemma (H 1022 ETH Zürich)	Tonoplast (H 1022 ETH Zürich)
Protein: total lipid	2.1 ± 0.11 (6)	2.1 ± 0.125 (5)	0.66 ± 0.067 (4)
Phospholipid (% of total lipid)	6.4 ± 1.00 (6)	5.4 ± 0.71 (5)	40.0 ± 4.87 (4)
Neutral lipid (% of total lipid)	93.5	94.6	60.0
Ergosterol (% in neutral lipid fraction)	26.2 ± 2.33 (6)	25.0 ± 0.61 (5)	6.05 ± 0.74 (4)
Sterol: phospholipid			
(by weight)	4.65 ± 0.82 (6)	4.6 ± 0.53 (5)	0.16 ± 0.015 (4)
(molar)	8.97	8.9	2.9

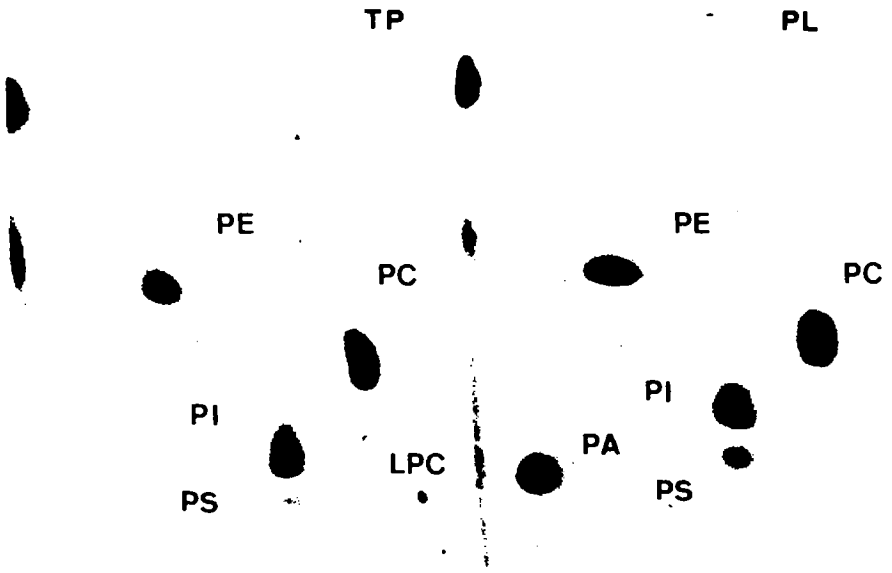

Fig. 3. Two-dimensional thin-layer chromatograms of yeast plasmalemma (PL) and tonoplast (TP) phospholipids. Phospholipids were separated as described by Broekhuyse [14]. Lipids were detected by charring with sulfuric acid (5% in ethanol solution). PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.

absent in plasmalemma. A more detailed analysis of the major classes of phospholipids is given in Table II. Besides the above mentioned differences, which have been quantified in the table, there exists also a quantitative difference in phosphatidylserine and phosphatidylinositol. The percentage of these two phospholipids was 28% of total lipid phosphorus in plasmalemma against 43% in tonoplasts. All other phospholipids were similar in magnitude.

In Fig. 4 a thin-layer chromatogram of the neutral lipids of plasmalemma and tonoplasts from *S. cerevisiae* H 1022 is given; the individual lipid spots being only tentatively identified by comparison with known lipid standards. In both types of membranes a prominent spot numbered "2" is seen which dem-

TABLE II

DISTRIBUTION OF THE MAJOR CLASSES OF PHOSPHOLIPIDS IN PLASMALEMMA AND TONOPLAST

	Lipid phosphorus (%)	
	Plasmalemma	Tonoplast
Phosphatidylcholine	34	33
Lysophosphatidylcholine	0	5
Phosphatidylethanolamine	20	15
Phosphatidylserine + Phosphatidylinositol	28	43
Phosphatidic acid	15	0
Other phospholipids	3	4

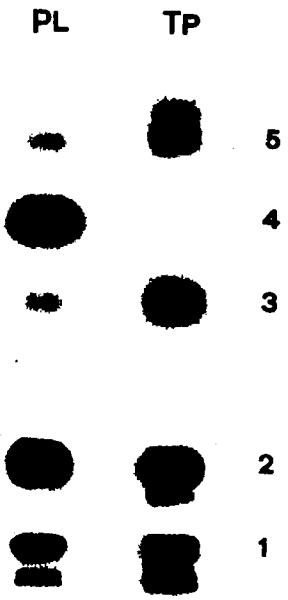

Fig. 4. Neutral lipids of yeast plasmalemma (PL) and tonoplasts (TP). The solvent system for thin-layer chromatography was petroleum ether/diethyl ether/acetic acid (75 : 25 : 1, v/v). Lipids were detected by charring with sulfuric acid (5% in ethanol solution). 1, sterols, diglycerides; 2, free fatty acids; 3, triglycerides; 4, sterol esters; 5, hydrocarbons.

onstrates a high content of free fatty acids. Differences in the two membranes are the higher content of sterol ester, "4" in plasmalemma and a higher content of triglycerides "3", in the tonoplasts.

The last figure (Fig. 5) shows the pattern of plasmalemma proteins and of tonoplast proteins from *S. cerevisiae* H 1022 which have been obtained by sodium dodecyl sulphate polyacrylamide gel electrophoresis. In order to show the similarity in membrane protein profile the scans and protein patterns of the two membranes have been placed in normal (plasmalemma) and reversed (tonoplast) position. The molecular weight of the plasmalemma proteins in both kinds of membranes ranges from 20 000 to 200 000. In plasmalemma there are 20 protein bands clearly distinguishable, whereas in tonoplasts 17 protein bands are seen. 15 protein bands in plasmalemma and tonoplasts overlap and they are not to be distinguished by means of statistics in their R_F values. However, 3 protein bands are significantly different in the two membrane preparations. The dotted lines in Fig. 5 represent the periodic acid-Schiff positive carbohydrate staining area of the two gels. Two peaks of glycoproteins are seen around 160 000 and 240 000 daltons in both gels. At the front of the gels glycolipid peaks are visible which match the lipid peaks from the protein scan.

The effect of 50 μg/ml concanavalin A on agglutination of tonoplasts was followed by observation of the vacuoles with a phase contrast microscope. Whereas tonoplasts prepared as above, after deep freezing, showed agglutination by concanavalin A, this phenomenon was absent in tonoplasts which were freshly prepared by an isotonic method [27].

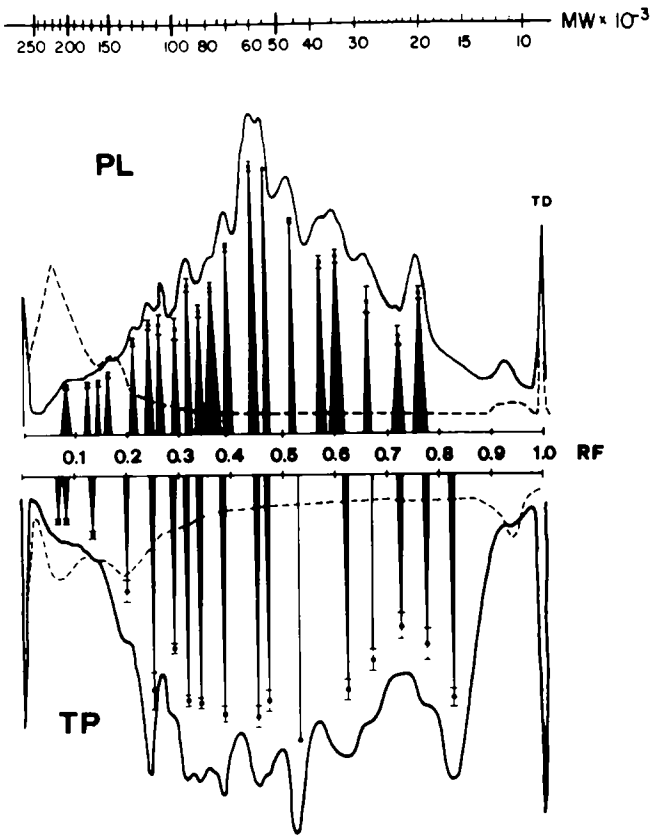

Fig. 5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of plasmalemma (PL) and tonoplasts (TP). The solid lines represent the protein scans in the two membranes, whereas the broken lines show the periodic acid-Schiff positive area. The triangles depict the single protein bands. The base of the triangles represents the standard deviation of the mean R_F values of 10 experiments. The tips of the triangles symbolize the relative protein concentration including the standard deviations of the means. TD, tracking dye.

Discussion

The plasmalemma is the limiting membrane of the yeast cell, which is surrounded by an exoskeleton cell wall. The cell wall has mainly supporting functions, whereas the plasmalemma is highly involved in transport functions. Such functions were also found in the tonoplast [23,24], which is the limiting membrane of the yeast vacuole. The vacuole is known to accumulate certain metabolites in high concentrations [5,8,25,26]. The tonoplast has the ability to shrink and swell considerably [8,27]. In this respect it is of physiological interest that small lipid granules are in close connection with the tonoplast and that these granules can fuse with this membrane [8,28,29]. In addition to the fusing mechanism the tonoplasts show division into smaller vesicles and also the opposite reaction has been described [8,28].

A comparative study of the two types of membranes, the plasmalemma and the tonoplast, is of interest in several respects. First, the exact analysis of the chemical composition of proteins and lipids in the membranes might contribute to the understanding of their above cited physiological functions and secondly,

this analysis might provide suggestions for the biosynthesis of membrane components.

The existence of differences between the two membranes is already obvious from their electron micrographs. The arrangement of particles in Fig. 1, a, à, b and b̀ is typical for both kinds of membranes [30]. The internal particles of the membrane revealed after freeze fracturing represent protein molecules in a lipid matrix [31]. Both membranes show an asymmetric distribution of their particle populations, there are more particles on the plasmatic face (PF) than on the complementary extraplasmatic face (EF). These particles are normally arranged at random. The plasmalemma particles, however, are mostly found in paracrystalline patches [30]. Other conspicuous structures are the invaginations [30] demonstrated in Fig. 1, a and à. In tonoplasts no such structures are found. In our view the obvious difference in particle density between plasmalemma and tonoplast coincides well with the difference found in biochemical analysis in which a three times higher value for the protein/total lipid ratio was found in plasmalemma. The smooth layers in both membranes are expected to consist mainly of lipids. For the plasmalemma the smooth outer layer has been shown by deep etching to be extractable by detergents or organic solvents [32].

A difference between the two membranes was also found after silver staining of protoplasts (Fig. 2). The dense silver granules in the plasmalemma area are due to the presence of carbohydrates. In tonoplasts the silver staining of carbohydrates is not significant. However, from periodic acid-Schiff staining of the two types of membranes the presence of carbohydrates can be assumed (Fig. 5). The difference in silver staining in the two membranes could be due to qualitative differences. Boller et al. [33] described an asymmetric distribution of concanavalin A binding sites on yeast plasmalemma and tonoplasts. It is concluded that both membranes carry concanavalin A binding sites exclusively on the surface opposite to the cytoplasma. The binding sites for concanavalin A are thought to be carbohydrates; binding to the tonoplast could only be shown after disruption of the vacuole. Our results on concanavalin A binding to the two kinds of membranes agree with the results of Boller et al. [33]. Cortat et al. [34] have described the presence of mannan synthetase in fragments of plasmalemma. Localization of mannan at the surface of yeast protoplasts by scanning electron microscopy has been shown by Horisberger et al. [35]. It also has been found chemically in plasmalemma, as demonstrated and reviewed by Fuhrmann et al. [1]. There are, however, no data about the chemical composition of carbohydrates in tonoplasts.

According to Hunter and Rose [36] the various membranes in yeast contain the bulk of the cellular lipids. The lipid composition of plasmalemma, especially the neutral lipids under aerobic and anaerobic conditions, were examined by Nurminen et al. [37]. Compared to the anaerobic plasmalemma, the aerobic plasmalemma contained more unsaturated fatty acids and more than ten times as much ergosterol. A condensing effect of ergosterol which might limit the mobility of fatty acid chains, especially in the case of unsaturated phosphoglycerides, with implications in permeation processes, has been discussed. Another interesting suggestion about biosynthesis of plasmalemma was made by Cartledge and Rose [38]. Since wall growth is intimately associated with plasmalemma growth, the possibility is evaluated of whether lipid granules may

transfer new material to the growing plasmalemma.

Our results on lipid composition in plasmalemma and tonoplasts show statistical differences which might be a reflection of their different biological functions. The significantly higher lipid and lower protein content in tonoplasts might be related to differences in permeation processes and enzymatic activities of the two membranes. The higher ergosterol concentration in plasmalemma, on the other hand, could be in addition an expression of the higher stiffness in that membrane as compared to the tonoplasts which can shrink and swell considerably [27]. However, we are aware of the fact that after the purification process our tonoplast membranes might still be contaminated by a small amount of lipid granules. Therefore, we would cautiously discuss only tendencies in chemical composition of the two membranes. This applies especially to comparing the differences in phospholipids which are six times higher in tonoplasts. As Cartledge and Rose [38] suggested, we like to stress the idea that the lipid granules are physiologically connected with other membranes, possibly transferring material for the biosynthesis of the membrane. The complete absence of phosphatidic acid in the tonoplasts and its presence in plasmalemma are conclusive evidence for the achievement of complete separation of the two membranes by our technique.

Difficulties exist in explaining the high concentration of free fatty acids in our membrane preparations. One explanation could be that this might be due to lipolytic activities of phospholipases and unspecific lipases, which have been demonstrated in plasmalemma preparations of yeast [39]. In this respect it is important to note that there was only minor evidence for lyso-compounds in the thin-layer chromatograms of the phospholipids. Moreover, when isolation and extraction performed in the presence of EDTA, which inhibits the membrane-bound phospholipases of the A_2-type, we did not find any difference in the phospholipid content of the membranes and still observed about the same amount of free fatty acids. A short-time lipid extraction, according to Folch [11], for 15 min did not influence the phospholipids extractable from the membranes, but yielded a lower amount of neutral lipids. The composition of the neutral lipids, however, remained unchanged, as judged by qualitative thin-layer chromatography. Therefore, the question remains open whether the high amount of free fatty acids present in both the plasmalemma and the tonoplast is an artefact of preparation or if it is of any biological significance.

The similarity in the protein profiles as shown by sodium dodecyl sulphate acrylamide gel electrophoresis suggests at least a partly common origin of membrane material for the two types of membranes investigated.

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