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ISOLATION AND IDENTIFICATION OF YEAST PLASMA MEMBRANE

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SUMMARY

The heterogeneous nature of the membrane preparation, derived from lysis of yeast protoplasts, has been investigated by ultracentrifugation on a discontinuous sucrose gradient. Specific location of the plasma membrane fraction was made from experiments in which the outer membrane of intact protoplasts was tagged with isotopically labelled reagents. The results of studies on the incorporation of *N*-ethylmaleimide, and fluorodinitrobenzene, dansylation, and iodination revealed that the plasma membrane fraction possessed a buoyant density of 1.18–1.29 g/cm^3. No evidence for plasma membrane redistribution into other gradient fractions was obtained but entrapment of intracellular materials occurred, as determined from the stability of the membrane-labelled fraction and redistribution of contaminating ^{32}P-labelled intracellular components on washing and further ultracentrifugation. The yeast plasma membrane fraction, which accounted for approximately 25% of the total protein content of the membrane preparation, was found to consist of two major fractions with respective buoyant densities of 1.18–1.21 and 1.21–1.23 g/cm^3. Although Mg^{2+}-stimulated ATPase activity was associated with each of these plasma membrane fractions, the ease of release into the soluble fraction precluded its use as a satisfactory marker enzyme.

INTRODUCTION

Several important physiological functions have been assigned to the plasma membrane of yeast cells, but little information is available on the basis for its biological activity in terms of chemical composition and structure[1,2]. Generally, the preparation of the plasma membrane has involved enzymic removal of the cell wall carbohydrate[3], a procedure carried out either before[4] or after[2,5] rupture of the cell. Both of these techniques have been criticized for reasons of either possible membrane fragmentation and aggregation[4,6] or vesiculation (membrane entrapment)[7]. Furthermore the plasma membrane fraction subsequently isolated has been of ill-defined purity, and the significance of characterization from chemical composition analysis, enzyme marker assay and electron microscopy is uncertain. The advantages of chemically tagging the outer membrane of a cell as an aid to following its subsequent isolation have been

Abbreviation: FDNB, 1-fluoro-2,4-dinitrobenzene.

discussed[8]. Application of this general procedure with a variety of tagging compounds has now permitted a study on the purification and isolation of the plasma membrane fraction from a total yeast protoplast lysate.

METHODS

Protoplast membrane preparation

Saccharomyces cerevisiae, strain Y 95 (Department of Microbiology designation), was grown statically in a defined culture medium[9] containing 5% glucose at 30 °C under anaerobic conditions, and harvested in the log phase. Washed cells (200–300 mg/ml) were treated with 0.2 M β-mercaptoethanol in 50 mM McIlvaine's buffer, pH 8.0, for 30 min at 30 °C, and then collected and washed with water. The cells were further incubated in the presence of 0.5% pronase in buffer, pH 8.0, for 30 min at 30 °C, collected and washed once with water and once with 15% mannitol[10] in 50 mM McIlvaine's buffer, pH 5.2. A final incubation of the cells was made with 1.5% (w/v) helicase (L'industrie Biologique Française, Gennevilliers, France) in 15% mannitol at pH 5.2 at 30 °C. Conversion of yeast cells to protoplasts was determined to be essentially complete within 2.0–2.5 h. Protoplast formation was followed both by phase-contrast microscopy and by the decrease in absorbance at 600 nm following lysis of the suspension on dilution. The protoplasts were recovered on centrifugation at $600 \times g$ for 10 min at 4 °C and washed twice with 15% mannitol in 50 mM McIlvaine's buffer, pH 5.2. Lysis of the protoplasts was made in 0.1 M phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$[4,10] and the membrane preparation isolated by centrifugation at $29000 \times g$ (av.) for 20 min at 4 °C.

Fractionation of membrane preparation

The recovered membrane preparation was layered on a discontinuous density gradient with steps of 60, 40, 36, 32, 30 and 25% (w/v) sucrose and then centrifuged in a Spinco SW 40 rotor at $189000 \times g$ (av.) for 2.5 h at 4 °C. Fractions (5 drops/fraction) were collected and detected by upward flow displacement of the gradient using an ISCO upward flow-cell equipped with a model UA-2 ultraviolet analyzer (Instrumentation Specialities Co., Lincoln, Nebr.).

Tagging of membranes

Yeast cells were grown in the presence of ^{32}P-labelled orthophosphate (Amersham/Searle Corp., Don Mills, Ontario). The membrane preparation was obtained in the described manner and the material subjected to sucrose density gradient centrifugation, followed by separation of fractions and re-centrifugation.

Studies on the labelling of the outer membrane of intact protoplasts were conducted with reagents selective for various amino acid residues in the protein component. $Na^{131}I$ was supplied by New England Nuclear, Dorval, Quebec, while the other isotopically labelled reagents were obtained from Amersham/Searle Corp. The various reactions were carried out with a protoplast suspension of 10^7–10^8 cells/ml at 30 °C in a buffered medium containing 15% mannitol. The tagged cells were recovered on centrifugation at $600 \times g$ and washed three times in buffered mannitol solution.

(*i*) *N-[2,3-$^{14}C_2$]Ethylmaleimide incorporation*[11]. The reaction was carried out

for 20 min in 0.1 M phosphate buffer, pH 7.0, using 1 mM *N*-ethylmaleimide supplemented with 8 μCi of *N*-[2,3-$^{14}C_2$]ethylmaleimide.

(*ii*) *Dansylation*[12,13]. Protoplasts suspended in 50 mM McIlvaine's buffer, pH 8.0, were dialyzed against 15% mannitol in 10 mM $NaHCO_3$, pH 9.0, containing 50 μM 1-dimethylamino-naphthalene-5-sulphonyl chloride (dansyl chloride) and 8 μCi/ml of [G-^{3}H]dansyl chloride. The reaction was permitted to continue with constant shaking for 30–45 min and then terminated by removing the dialysis bag which was washed with water.

(*iii*) *1-Fluoro-2,4-dinitrobenzene (FDNB) incorporation*[14,15]. Freshly prepared 15 μM FDNB in 120 mM $NaHCO_3$ solution containing 10 μCi/ml of [3,5-$^3H_2$] FDNB was incubated with a protoplast suspension in 120 mM $NaHCO_3$ for 10–15 min.

(*iv*) *Iodination*[16,17]. The reaction mixture consisted of protoplast suspension, $1.0 \cdot 10^{-7}$ M lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) and 0.1 μCi/ml $Na^{131}I$ in 50 mM McIlvaine's buffer, pH 7.4. Iodination was initiated by the addition of 8 μM H_2O_2. Sequential addition of 8 μM H_2O_2 was made at 1-min intervals over an incubation period of 5–10 min.

Membrane preparations were obtained from the washed, tagged cells on osmotic lysis and were then subjected to centrifugation on the sucrose density gradient.

Scintillation measurements of the incorporated radioisotope were made with a Packard liquid scintillation spectrometer model 3003 following incubation of the collected fractions with 0.5 ml NCS (Amersham/Searle Corp.) at 50 °C for 20 min and dilution with toluene-based scintillation fluid. The counting of ^{131}I was made using a Nuclear Chicago γ-spectrometer (model 4233).

Electron microscopy

Washed membrane samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.6, containing 10 mM $CaCl_2$ at 4 °C for 2 h and then subjected to four successive rinses of 30 min. The samples were post-fixed in 1% OsO_4 in 56 mM barbital–acetate buffer, pH 6.1, containing 10 mM $CaCl_2$ and bactotryptone (1 mg/ml) for 4 h at 4 °C, followed by four successive washings of 20 min. Concentration of the samples was carried out by sucking a suspension of the sample in 2% agar in buffer, pH 6.1, at 45–50 °C into a 1-mm capillary tube, cooling and storing overnight at 4 °C. The resulting agar rods were extruded and stained in the dark by shaking with 0.5% uranyl acetate in 56 mM barbital–acetate buffer, pH 6.1, at 20 °C for 2 h. Excess stain was removed by washing with the buffer solution.

Dehydration was performed in a graded series of acetone (25, 50, 75, 90 and 100%, v/v). The samples were dried twice in 100% acetone (stored over $CaSO_4$ to maintain anhydrous) and then infiltrated with embedding medium–acetone mixture (1:1, v/v) at room temperature for 30 min followed by 1 h at 37 °C. The embedding medium consisted of equal weight quantities of resin A (Epon 812, 80 g; dodecenyl succinic anhydride, 91 g) and resin B (Epon 812, 100 g; methyl nadic anhydride*, 76 g).

The agar rods were cut into small blocks and placed in BEEM capsules (Electron Microscopy Sciences, Fort Washington, Pa.) to which was added, with removal of all air bubbles, embedding medium containing 1.4% (v/v) DMP-30 (dimethyl-

* Methylbicyclo[2.2.1]heptene-2,3-dicarboxylic anhydride.

aminomethyl phenol) as catalyst. The material was polymerized at 37 °C for 12 h followed by 12 h at 45 °C and finally for 16 h at 60 °C.

The blocks were removed and silver sections cut with a diamond knife on a Sorvall Porter Blum MT-2 ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.). Following staining with lead citrate[18], the sections were viewed and electron micrographs taken using a Philips EM 300 electron microscope. Further examination of the electron micrographs was made with a Beckman Analytrol Model RB densitometer.

Analytical procedures

Colorimetric methods were employed for the quantitative determination of protein[19], carbohydrate[20], fast-acting sterol[21] and lipid phosphorus[22]. Assay of RNA was made spectrophotometrically[23].

Mg^{2+}-stimulated ATPase activity was measured under described conditions[24], employing the modified Berenblum and Chain method[25] for the measurement of the released phosphate.

RESULTS AND DISCUSSION

The formation of protoplasts from the strain of *S. cerevisiae* investigated could not be achieved by direct incubation with helicase, but required pretreatment of the cells with β-mercaptoethanol and pronase. Considerable variation in the susceptibility of different species and strains of *Saccharomyces* to the action of helicase has been reported[26,27]. The use of pronase has not been found to influence either the appearance[28] or physiological behaviour[29] of protoplast membrane isolated following helicase action. The protoplasts obtained in the present work were determined to be osmotically stable when suspended in 15% mannitol solution over a period of several days.

Electron microscopic examination (Fig. 1) of the membrane preparation confirmed its membranous nature and gave no apparent evidence for the presence of cell wall. The low carbohydrate content of the preparation was also suggestive of its origin from true protoplasts. The total membrane preparation was found to have the same general chemical composition as previously reported[4,30] for comparable preparations obtained from *S. cerevisiae* (Table I). Yeast grown anaerobically has been noted[31] to have a lower cell content of sterol than occurs under aerobic conditions.

The fractionation of the membrane preparation into 7 centrifugally different fractions (Figs 2 and 3) was reproducible and demonstrated the heterogeneous nature of the material. A similar fractionation was obtained on examination of a membrane preparation derived from helicase treatment of washed yeast cell envelopes. A variety of fractions has been isolated previously from extracts of whole yeast cells[6,32] and yeast envelopes[7], and confirms the general complexity of yeast membrane preparations irrespective of the source of the material.

Examination of the membrane preparation derived from protoplasts tagged with radioactive-labelled *N*-ethylmaleimide, FDNB, dansyl chloride and iodine revealed, except for the large quantity of presumably hydrolyzed dansyl chloride in Fraction 1, the predominant occurrence of the label in Fraction 7 (Fig. 4). The

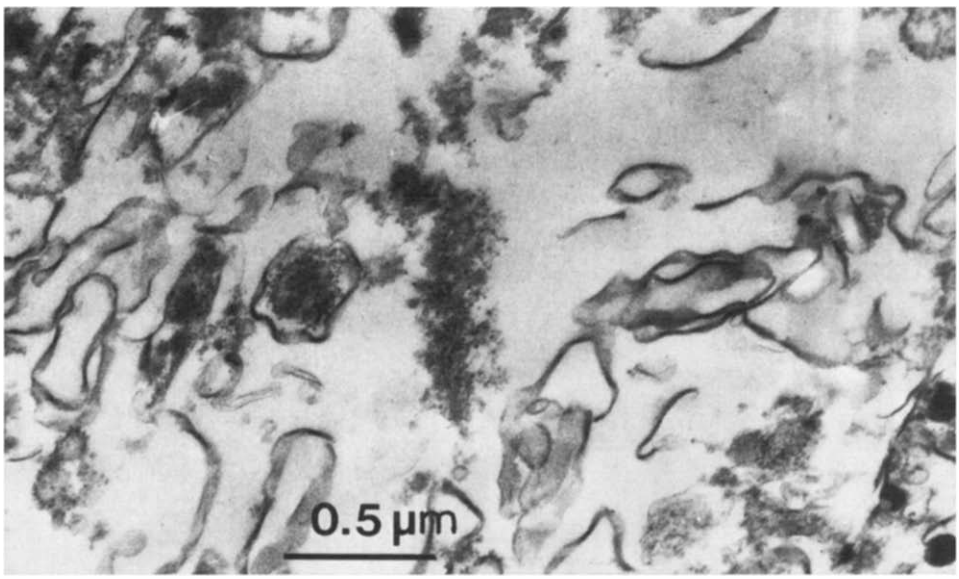

Fig. 1. Lysed plasma membrane prior to washing and fractionation. The essentially symmetrical appearance of the membranes indicates that little or no cell wall remains.

TABLE I

CHEMICAL COMPOSITION OF PLASMA MEMBRANE PREPARATIONS DERIVED FROM YEAST PROTOPLASTS

Values are expressed as percentage of dry wt of membrane preparation. The total membrane and Fraction 7 were obtained from a protoplast lysate, and its subsequent fractionation by ultracentrifugation on a discontinuous sucrose density gradient as detailed in the text. Protoplast membranes and 1.5 p 5 and 20 p 30 fractions were reported to be derived from a lysate[30] and centrifugation of a lysate[4], respectively.

Component	*Total membrane*	*Fraction 7*	*Protoplast membranes*	*1.5 p 5 fraction*	*20 p 30 fraction*
Protein	34.3	39.7	49.3	36.2–40.0	46.0–47.5
Carbohydrate	7.0	7.1	4.0–6.0	4.4	3.2
RNA	5.2	4.6	7.0	4.3	6.7
Sterol	2.4	1.8	6.0	5.7–6.4	5.1–6.2
Lipid phosphorus	0.40	0.57	0.25	0.52–0.58	0.36–0.43

reaction of these various tagging agents would be expected to involve specific, but different, groupings on the protein (Table II). In addition, FDNB has been found[15] to tag erythrocyte membrane amino phospholipids, and although a similar reaction has not been observed[12] on dansylation of membranes, dansyl phospholipid derivatives can be prepared[36]. The variable quantity of isotope associated with Fraction 1 may be assumed to represent unreacted tagging agent. The permeability of the protoplast membrane to the various tagging compounds has not been established, but,

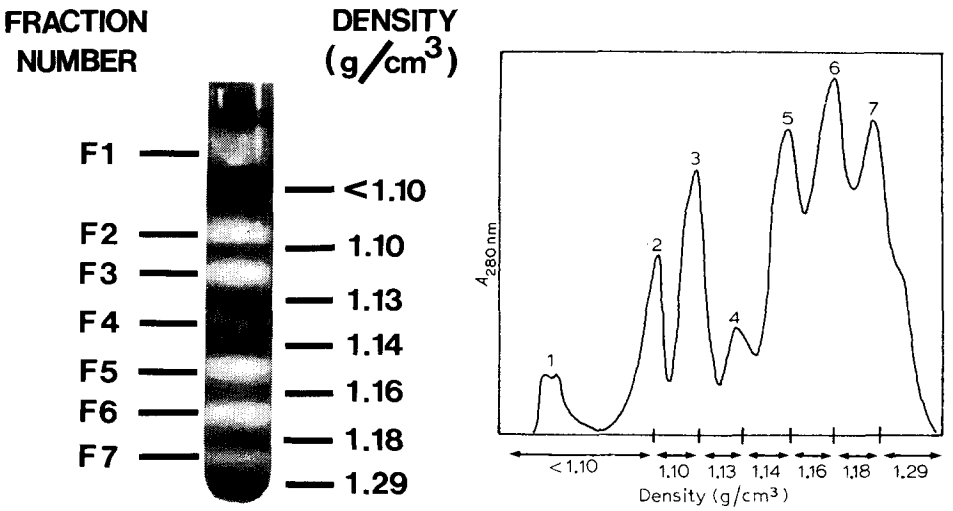

Fig. 2. Fractionation of a yeast protoplast membrane preparation by ultracentrifugation on a discontinuous sucrose density gradient. The designated fractions banded at the interfaces of the various gradient steps and had buoyant densities corresponding to the ranges indicated.

Fig. 3. Light scattering profile of sucrose density gradient obtained on ultracentrifugation of the yeast protoplast membrane preparation shown in Fig. 2. The peak numbers correspond to the fractions with buoyant densities as designated previously.

TABLE II

THE GENERAL SPECIFICITY OF REAGENTS EMPLOYED IN THE LABELLING OF THE SURFACE MEMBRANE OF CELLS

Appropriate references to studies on membranous systems have been selected from the literature.

Reagent	*Reactive site*	*Refs*
Iodide:		
lactoperoxidase	tyrosinyl; imidazolyl	16, 33
N-Ethylmaleimide	sulphydryl	11, 34
Dansyl chloride	free amino groups*; phenolic and serinyl hydroxyl; imidazolyl; sulphydryl	12, 35, 36
FDNB	free amino groups; phenolic hydroxyl; imidazolyl; sulphydryl	14, 15, 37

* Dansyl chloride does not label phospholipids in membranes[12], but dansylated phospholipid derivatives have been used[36] in model membrane studies.

in general, *N*-ethylmaleimide appeared to be excluded. The lack of significant iodination of the intracellular fractions in the presence of soluble iodide is presumably due to the impermeability of lactoperoxidase[16,17]. Application of FDNB resulted in the apparent, partial labelling of all fractions. The lytic action of this reagent has been noted[14] in erythrocytes and may reflect the interaction[15] between FDNB and amino phospholipid, which may in turn influence permeability factors. It was noted in the present study that FDNB-tagged protoplasts were less osmotically

stable. Based on the results of the several labelling studies it may be therefore concluded that the plasma membrane is associated with Fraction 7. The buoyant density of 1.18–1.29 g/cm^3 would encompass the value reported for the plasma membrane of amoeba[38], but is greater than the values reported[39,40] in other tissues.

Re-centrifugation of the isolated, variously labelled preparations of Fraction 7 showed no major re-distribution of the label except in the case of [^{3}H]FDNB and ^{32}P-labelled orthophosphate (Fig. 5). Both of these labels were recognized as being associated with all the fractions obtained across the sucrose density gradient. Thus it can be concluded, based on these general findings and the relatively high initial concentration of label in Fraction 7, that the plasma membrane entity was stable to the centrifugation procedure but tended to be contaminated with other materials which could be removed by careful washing. This general problem of vesiculation, involving entrapment of sub-cellular elements and fragments by the plasma membrane, has been convincingly demonstrated from investigations by electron microscopy[7].

Fraction 7, which constituted approximately 23% of the centrifugally fractionated preparation (Fig. 3), was observed by electron microscopy to possess a membraneous nature (Fig. 6). The material accounted for 28% of the protein and 30%

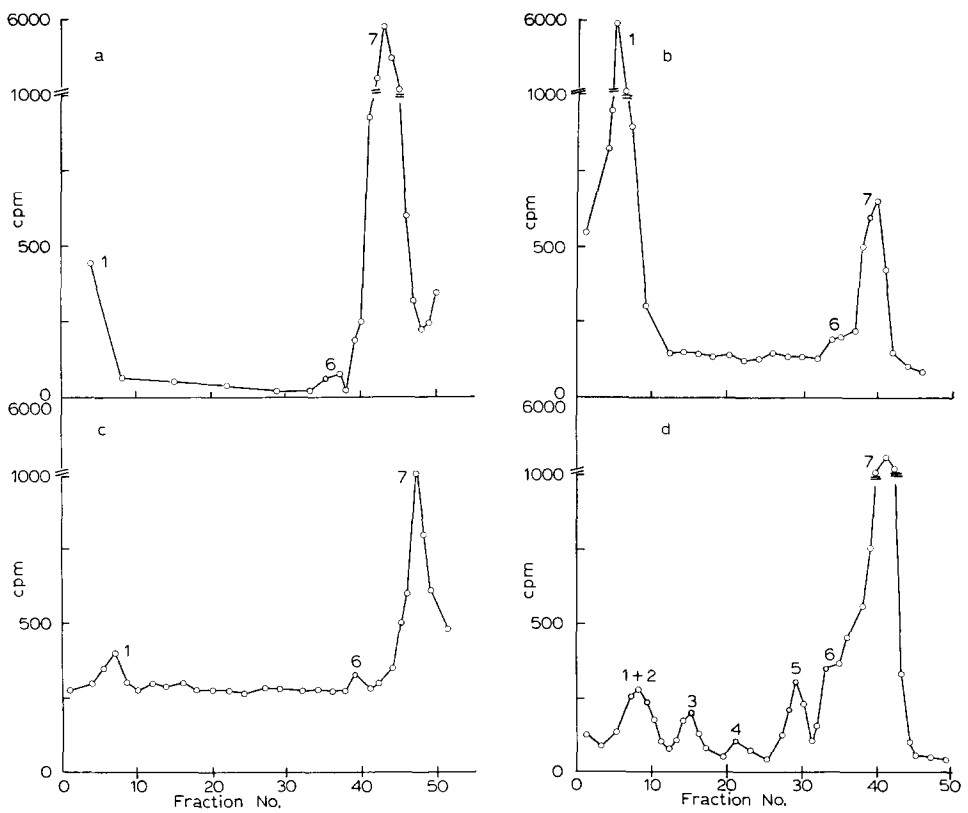

Fig. 4. Distribution of label obtained on the ultracentrifugation of membrane preparations derived from variously tagged yeast protoplasts (Peak numbers correspond to the various bands designated in Fig. 3). (a) *N*-[^{14}C]Ethylmaleimide, (b) ^{3}H-labelled dansyl, (c) ^{131}I, (d) [^{3}H]FDNB.

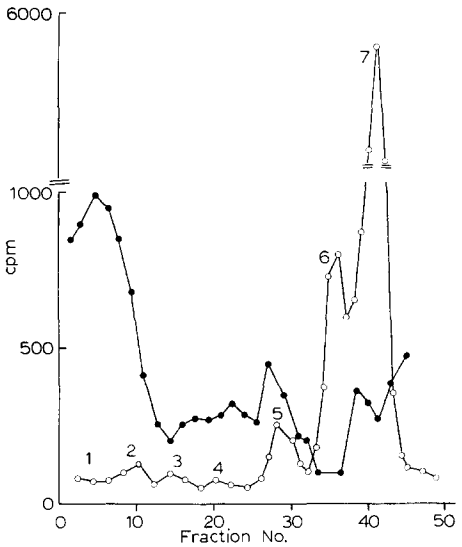

Fig. 5. Distribution of label on re-centrifugation of isolated Fraction 7 obtained from membrane preparations of tagged yeast protoplasts (●—●, ^{32}P; ○—○, [^{3}H]FDNB. Peak numbers correspond to the various bands designated in Fig. 2).

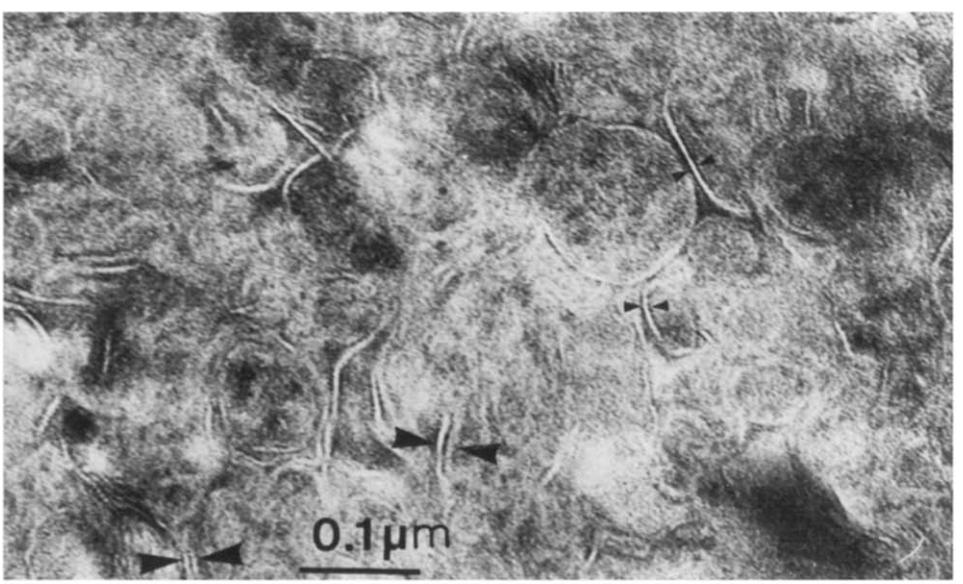

Fig. 6. Purified plasma membrane. The triple-layered structures (large arrows) occur where membranes overlap and may originate from the overlap of broken membranes. The non-overlapping membrane exhibits the usual double-layered appearance (small arrows).

of the lipid phosphorus content of the total membrane preparation. The calculated value of 0.36 mg phospholipid/mg protein agrees with the values available[38] for the plasma membrane of animal cells, but a markedly lower ratio of sterol: phospho-

lipid was obtained. The general composition of Fraction 7 is summarized in Table I. A quantitatively varying but primary occurrence of Mg^{2+}-ATPase was found in Fraction 7; washing of the preparation resulted in the apparent solubilization of the enzyme activity and its re-distribution into Fraction 1. Considerable leaching of membrane-bound Mg^{2+}-ATPase from yeast cell envelopes has been previously noted[41]. A further resolution of *N*-ethylmaleimide-labelled Fraction 7 was achieved on a modified sucrose density gradient and yielded two major fractions and a minor component with respective buoyant densities of 1.18–1.21, 1.21–1.23 and 1.23–1.29 g/cm^3 (Fig. 7). The Mg^{2+}-ATPase activity was found to be distributed between all three fractions, but to be especially concentrated in the most dense component (Fig. 7). An ATPase-rich double membrane with a buoyant density of 1.23 g/cm^3 has been isolated from yeast[42]. The components of Fraction 7 do not appear to correspond to fractions 1.5 p 5 and 20 p 30 described[4] previously (see Table I), one of which showed considerable vesiculation and varied with the conditions of lysis of the protoplasts. On the other hand, studies[38] on the plasma membrane of amoeba have shown that it can be resolved into two fractions with buoyant densities of 1.18–1.195 and 1.195–1.21 g/cm^3, and even distribution of Mg^{2+}-ATPase activity.

The use of outer membrane tags, specific for differing amino acid residues in the protein component, has permitted easy location of the plasma membrane and an unequivocal method for its identification. Each tagging agent employed in the present study provided an independent means of detection. *N*-Ethylmaleimide appears to be the reagent of choice even though the cysteine content of yeast protoplast membrane is low[30]. The technique of tagging has provided self-sustaining evidence for the occurrence of the plasma membrane without having to resort to reliance on suspected characteristic chemical composition and enzyme marker systems[39,43]. The ^{32}P-labelled orthophosphate and [^{3}H]FDNB labelling of sub-cellular fragments provides a sensitive method of detecting cross-contamination which can be deduced from the re-distribution of the label on re-centrifugation of the isolated fractions. Previous

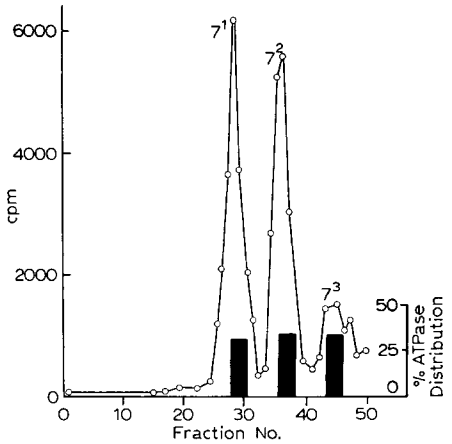

Fig. 7. Distribution of label and ATPase activity obtained on the ultracentrifugation of isolated Fraction 7 derived from *N*-[^{14}C]ethylmaleimide tagged yeast protoplasts. (The discontinuous gradient was modified to steps of 60, 50, 44, 40, 36 and 25% sucrose and ultracentrifugation was conducted at 189000 × *g* (av.) for 25 h at 4 °C.).

attempts to study the contamination of rat liver plasma membrane with other subcellular components have involved detailed analyses for lipid composition and enzyme activities[43]. It would appear from the present results that the tagging technique provides the most sensitive procedure for following the isolation and purification of the yeast plasma membrane.

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