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Distribution of Membranes, especially of Plasma-Membrane Fragments, during Zonal Centrifugations of Homogenates from Glucose-Repressed *Saccharomyces cerevisiae*

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1. The distributions of several enzymes and other marker components were examined after zonal centrifugations of whole homogenates from glucose-repressed *Saccharomyces cerevisiae* on sucrose and iso-osmotic Ficoll, and the composition and morphology of the fractions were investigated. 2. After high-speed zonal centrifugation most of the protein, acid and alkaline phosphatases, alkaline pyrophosphatase, adenosine monophosphatase, β-fructofuranosidase, α-mannosidase, NADPH–cytochrome *c* oxidoreductase and an appreciable amount of phospholipid and sterol were non-sedimentable, i.e. were at densities below 1.09 (g/cm^3). Most of the RNA was at $\rho = 1.06$–1.08 in Ficoll and at $\rho = 1.09$–1.11 in sucrose. 3. The bulk of the Mg^{2+}-dependent adenosine triphosphatase (Mg-ATPase) was coincident with the main peak of phospholipid and sterol, at median density 1.10, which was also rich in smooth-membrane vesicles. In Ficoll, a minor peak of phospholipid and sterol at $\rho = 1.12$–1.15 contained a smaller part of the oligomycin-insensitive Mg-ATPase and heavy membrane fragments. In sucrose, several minor peaks of Mg-ATPase were in the mitochondrial density range, and a peak of oligomycin-insensitive Mg-ATPase coincident with a minor peak of phospholipid and sterol at around $\rho = 1.25$ contained heavy membrane fragments of high carbohydrate content, especially mannose. 4. Further purification of the oligomycin-insensitive Mg-ATPase containing membrane preparations was performed on Urografin gradients. 5. It is argued that the oligomycin-insensitive Mg-ATPase containing membranes are fragments of the plasma membrane, but have different densities because they contain different amounts of glycoprotein particles.

The great separation capacity of zonal centrifugation developed by Anderson and co-workers (e.g. Anderson *et al.*, 1966, 1967) has often been used to obtain more detailed information about the localization of enzymes or to isolate subcellular fractions on a larger scale than is possible by other methods. Only Cartledge & Lloyd (1972*a*,*b*,*c*, 1973) have investigated fractions obtained by the zonal centrifugation of yeast homogenates over a wide density range. They traced the distribution of various enzymes and, in particular, isolated mitochondria at different development stages from the spheroplasts of a *Saccharomyces carlsbergensis* strain.

The isolation of larger amounts of purer membrane than has previously been obtained would allow a closer biochemical study of the yeast plasma membrane. The principal aim of the present work was to study the behaviour of plasma-membrane fragments during zonal centrifugations of homogenates from *Saccharomyces cerevisiae*. The Mg-ATPase (Mg^{2+}-dependent, oligomycin-insensitive adenosine triphosphatase) is the only marker enzyme whose presence in yeast plasma membrane has been confirmed by all reported isolation methods, i.e. from spheroplast lysates (Boulton, 1965; Altieri, 1971; Schibeci *et al.*, 1973), or by using mechanically disrupted whole cells, from microsomal fractions (Matile *et al.*, 1967; Matile, 1970), cell-envelope fractions (Nurminen *et al.*, 1968, 1970), and fractions sedimenting between 2000 and 5000*g* (Fuhrmann *et al.*, 1974). Because Mg-ATPases do not occur only in the plasma membrane, and because *S. cerevisiae* has not previously been systematically fractionated by zonal centrifugation, we examined by many different methods fractions obtained over a wide density range. This was also necessary because of the considerable differences in the composition, especially in the content of carbohydrate and nucleic acids, and density between the plasma-membrane preparations isolated by previous workers.

Whole homogenates of glucose-repressed *S. cerevisiae* were fractionated by high-speed or low-speed zonal centrifugation through continuous gradients of sucrose or Ficoll. The membrane

fractions obtained by high-speed zonal centrifugation through sucrose gradients were further studied by swing-out centrifugation on a Urografin gradient. The results are discussed with special reference to the behaviour of the fragments of plasma membrane. Preliminary reports of some of this work have been published (Suomalainen & Nurminen, 1972, 1973; Nurminen & Suomalainen, 1973).

Experimental

Growth of yeast

An inoculum of a pure culture of *S. cerevisiae* maintained on wort/agar slant was transferred to 300ml of sterilized medium containing Difco Bacto Yeast Nitrogen Base (6.7g/l), glucose (50g/l) and potassium lactate buffer, pH5.5 (10ml/l), and shaken overnight at 30°C. The shaking culture from the late-exponential growth phase was cultured further in 6-litre batches of the fresh sterilized medium in an autoclaved 12-litre Biotec FL-110 fermenter (Biotec AB, Stockholm, Sweden). N_2+O_2 (95:5) was bubbled through the culture maintained at 28°C. The stirring speed was 650rev./min and the gas flow rate 5litres/h per litre of medium. After 8h the culture reached the mid-exponential growth phase under catabolite repression, and was harvested and washed free of medium by centrifugation.

Disruption of cells

Cells (10g fresh wt.) were suspended in 25ml of chilled 0.25M-sucrose/25mM-Tris/HCl, pH7.2 (referred to below as ST medium). Glass beads (40ml) (diam. 0.2mm) were added and the cells were ruptured at 3300rev./min in a Mini-mill disintegrator (Gifford-Wood Co., Hudson, NY, U.S.A.). Mill gap position 45 was used and the cup was placed in an ice bath (see Guarnieri *et al.*, 1970). The suspension of disrupted cells was separated from the glass beads by filtering through a glass sinter type G1, the pH was adjusted to 7.2 by adding 0.25M-Tris base and the volume of total homogenate was adjusted to 100ml with ST medium.

Zonal centrifugations

All the subsequent steps were performed immediately at 0–4°C. The continuous gradients of buffered sucrose or Ficoll were formed by mixing 20% (w/w) and 60% (w/w) sucrose, made up in the Tris buffer described above, or 20% (w/w) and 50% (w/w) solutions of Ficoll, made up in ST medium. The heavier solution was pumped from a container to a mixing vessel containing about 700ml of the lighter solution by using an adjustable series II Hughes micropump (F. A. Hughes Ltd., Epsom, Surrey, U.K.) equipped with a size-4 long-stroke pumping-head (see Hinton & Dobrota, 1969). The gradient solutions were magnetically stirred. The density gradient formed was pumped by a controlled peristaltic Hiloflow pump (F. A. Hughes Ltd.) from the mixing vessel through a pressure regulator into a B XV aluminium rotor (Beckman Instruments, Munich, West Germany) in a Spinco L-50 centrifuge (cf. Anderson *et al.*, 1967). The rotor was loaded at 3000–4000rev./min. The volume of whole homogenate was 100ml, the overlay was 100ml of 25mM-Tris/HCl, pH7.2, the density gradient amounted to 1400ml, and cushion [60% (w/w) sucrose buffered with Tris] filled up the rest of the rotor volume. The high-speed zonal centrifugation was at 20000rev./min for 6h, corresponding to a minimum of 5×10^6g-min at the sample zone, and the low-speed zonal centrifugation was at 5000rev./min for 1h, corresponding to a minimum of 5×10^4g-min at the sample zone. The contents of the rotor were displaced at 3000–4000rev./min by pumping 60% (w/w) sucrose into the outer edge of the rotor. Fractions (normally 40ml) were collected at 0–4°C and their sucrose concentrations were measured at 20°C by using a refractometer. The corresponding densities (20°C/4°C) were calculated from tables. The densities of the Ficoll solutions were measured areometrically at 20°C. In cases when fractions larger or smaller than 40ml were required, the gradient was introduced into a glass column equipped with a stopcock at the lower end. The separated bands could be seen and suitably sized volumes were collected.

Swing-out centrifugations

Some selected zonal fractions from the high-speed zonal centrifugations in the sucrose density gradient were further studied by swing-out centrifugation on a stepwise Urografin gradient. The samples were diluted with double the volume of ST medium and spun down at 30000rev./min for 5h (Spinco 30 rotor, $r_{av.}$ 7.8cm) twice. The sediments were suspended in 4ml of ST medium and applied on to a discontinuous Urografin density gradient consisting of 5ml portions of density steps 1.07, 1.11, 1.15, 1.19 and 1.24, or 4ml portions of Urografin solutions having the densities 1.09, 1.13, 1.17, 1.21, 1.25 and 1.29. After centrifugation at 25000rev./min for 5h in a Spinco SW 25.1 rotor, selected bands were carefully removed, diluted with ST medium and their materials were sedimented and Urografin was removed by centrifugations in a Spinco 30 rotor at 30000rev./min for 5h.

Enzyme assays

Incubations for enzyme assays were made at 30°C unless otherwise stated. Controls without sample or without substrate were carried out, and corrections made for any changes observed in the incomplete test system. Acid phosphatase (EC 3.1.3.2) and alkaline phosphatase (EC 3.1.3.1) were determined with

2mM-*p*-nitrophenyl phosphate as substrate. The reaction mixture contained 2mM-$MgCl_2$, 10mM-KCl and 25mM-sodium succinate (pH3.5) or Tris/HCl (pH9.0) buffer. The determinations were made by using essentially the modifications of Nurminen *et al.* (1970) of the method described by Torriani (1960). Other phosphohydrolases were assayed as the rate of P_i release from substrate, determined essentially as described by Nurminen *et al.* (1970). Alkaline pyrophosphatase (EC 3.6.1.1) was assayed by the method of Weimberg & Orton (1964), and Co^{2+}-activated 5'-AMPase (adenosine monophosphatase; EC 3.1.3.5) by using 100mM-sodium acetate buffer (pH6.0) as described by Neu (1967). The activities of the Mg^{2+}-dependent ATPase (adenosine triphosphatase; EC 3.6.1.4) were measured with and without oligomycin (10μg/ml) in the reaction mixture, which contained 2mM-Tris/ATP, 2mM-$MgCl_2$, 10mM-KCl and 25mM-Tris/maleate buffer (pH6.8), by using essentially the method described by Nurminen *et al.* (1970). Glucose 6-phosphatase (EC 3.1.3.9) was determined as described by Imai *et al.* (1966). β-Fructofuranosidase (EC 3.2.1.26) was determined polarimetrically by the method of Weidenhagen (1941) as modified by Nurminen *et al.* (1970), by using the samples from which sucrose had been removed by dialysis. α-Mannosidase (EC 3.2.1.24) was assayed as described by van der Wilden *et al.* (1973), except that the pH of the reaction mixture was 7.2. NADPH–cytochrome *c* oxidoreductase (EC 1.6.2.4), succinate–ferricyanide oxidoreductase (EC 1.3.99.1) and cytochrome *c* oxidase (EC 1.9.3.1) were determined spectrophotometrically at 22–24°C and at pH6.8, the first two by the methods of Green & Ziegler (1963) and Rabinowitz & de Bernard (1957) respectively. Cytochrome *c* oxidase was measured by observing the rate of enzymic oxidation of cytochrome *c* (Cooperstein & Lazarow, 1951; Yonetani & Ray, 1965) reduced by the method of Chantrenne (1955).

Analytical methods

The samples were freed from gradient material by precipitation with 10% (w/v) trichloroacetic acid and washed twice by centrifugation. The lipid components were extracted from the sedimented samples by shaking overnight under N_2 at 22–24°C in 20vol. of chloroform/methanol (2:1, v/v) and washed as described by Folch *et al.* (1957) by adding 0.2vol. of 0.2M-NaCl. The lipid-free residue was homogenized in 1M-NaOH and divided into portions for protein and nucleic acid determinations. Protein was determined by the method of Lowry *et al.* (1951) after solubilization with 1M-KOH for 10min at 100°C. DNA was separated from RNA as described by Schmidt & Thannhäuser (1945) by adding 6M-HCl/5% (w/v) trichloroacetic acid (1:5, v/v). The precipitated DNA was determined by the diphenylamine method (Burton, 1956) and RNA in the supernatants by the orcinol method (Mejbaum, 1939). The total lipid in the lipid extracts was determined gravimetrically, and the lipid-soluble P was determined after digestion of the organic material with H_2SO_4 at 180°C for 2h as described by Kolb *et al.* (1963). The phospholipid content was obtained by multiplying the lipid-P content by 25. The total sterol was determined by a modified Lieberman–Burchard reaction (Sackett, 1925), with ergosterol as standard. The total content of carbohydrates was measured by the anthrone method (Pfäffli & Suomalainen, 1960). The yeast dry matter was determined gravimetrically after drying the washed cells overnight at 105°C. During the cultivations the glucose content in the medium was followed by using the glucose oxidase reaction with Boehringer kit no. 15755, and the growth by measurements of the turbidity at 600nm in suitably diluted suspensions.

For g.l.c. analysis of the carbohydrates, the samples were hydrolysed in 2M-HCl for 3h at 100°C. After hydrolysis, the lipophilic material was removed, the aqueous phase was neutralized and, after addition of sodium metaborate, the remaining lipophilic material was extracted essentially as described by Työrinoja *et al.* (1974). Sugars in the aqueous phase were reduced with $NaBH_4$, borate was removed and the reduced sugars were acetylated essentially as described by Työrinoja *et al.* (1974). Standard sugars were reduced and derivatives prepared in the same way. Sugars were analysed as their alditol acetates by g.l.c. by using 3% ECNSS-M on Gas-Chrom Q, 100–120 mesh. The column temperature was programmed from 150°C to 170°C at 0.5°C/min and from 170°C to 195°C at 1°C/min, and finally held isothermally at 195°C for 10min. Some samples were also examined using a column packed with 1.5% OV-225 and 1.5% butanediol succinate on Chromosorb (W AW, dimethyldichlorosilane-treated, 80–100 mesh). The temperature of this column was held isothermally for 8min at 185°C, then programmed from 185°C to 220°C at 4°C/min and finally held at 220°C for 30min. The instrument used was a Hewlett–Packard 7620 A Research Chromatograph (Hewlett–Packard, Avondale, PA, U.S.A.) equipped with flame-ionization detectors. Helium was used as carrier gas for all analyses. The percentage composition of the neutral sugars was calculated from the peak areas by using the same detector response for all the analysed sugars.

Recovery

Validity of the assay methods was indicated by the satisfactory recoveries after the fractionations. These were: protein, 105%; acid phosphatase, 82%; alkaline phosphatase, 105%; Mg-ATPase without oligomycin, 127%; Mg-ATPase with oligomycin, 101%; β-fructofuranosidase, 99%; α-mannosidase, 109%; NADPH–cytochrome *c* oxidoreductase, 83%;

succinate–ferricyanide oxidoreductase, 78%; RNA, 103%; phospholipid, 117%; and sterol, 105%. These values are averages of two to five fractionations, except for alkaline phosphatase and succinate–ferricyanide oxidoreductase, for which only one test was made.

Electron microscopy

The sedimented samples were prefixed with 2.5% (v/v) glutaraldehyde in 0.2M-sucrose/0.2M-sodium phosphate buffer (pH7.2) for 90min at 4°C, post-fixed with 1% osmium tetroxide in 0.2M-sucrose/0.2M-sodium phosphate buffer (pH7.2) for 90min at 4°C and then dehydrated with successively increasing concentrations of ethanol and propylene oxide before being embedded in epoxy resin. After polymerization of the resin, the thin sections prepared with a Porter–Bloom microtome were post-stained with 1% lead citrate or with 1% lead citrate and 3% uranyl acetate. Electron micrographs were taken with a Philips EM 300, Philips EM 200 or JEM-100 B (JEOL Ltd., Tokyo, Japan) electron microscope in the electron-microscopy laboratory of the University of Helsinki.

Chemicals

The gradient materials used were sucrose from BDH Ltd., Poole, Dorset, U.K., Ficoll from Pharmacia Fine Chemicals AB, Uppsala, Sweden, and Urografin (methylglucamine salt of *NN'*-diacetyl-3,5-diamino-2,4,6-tri-iodobenzoic acid) from Schering A.G., Berlin, Germany. Difco Bacto Yeast Nitrogen Base was from Difco Laboratories, Detroit, MI, U.S.A. Enzyme substrates, *p*-nitrophenyl phosphate, *p*-nitrophenyl α-D-mannoside and cytochrome *c* (from horse heart), and oligomycin (15% oligomycin A and 85% oligomycin B), bovine serum albumin (fraction V), Tris base, yeast RNA, DNA (from calf thymus) and ergosterol were from Sigma Chemical Co., St. Louis, MO, U.S.A. 5'-ATP, 5'-AMP, NADH and NADPH were from Boehringer und Soehne G.m.b.H. (Mannheim, West Germany) and propylene oxide and Dowex 50W (×8) from Fluka A.G., Buchs, Switzerland. The column packing materials used were ECNSS-M, OV-225, butanediol succinate and Gas-Chrom Q from Applied Science Laboratories, State College, PA, U.S.A., Chromosorb W AW dimethyldichlorosilane-treated from Johns–Manville Products Corp., Manville, NJ, U.S.A., silicic acid from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A., Duolite S-10 from Diamond Alkali Co., Redwood City, CA, U.S.A., and ion-exchange resin, Amberlite IR-45 (OH^-) from BDH Ltd. Reference sugars were obtained from Fluka A. G., and E. Merck A. G., Darmstadt, West Germany, or from Supelco, Bellefonte, PA, U.S.A., and Epon 812 DDSA–Epon 812 MNA was from Ladd, Burlington, VT, U.S.A. Other reagents were

Table 1. *Contents of some marker components in the whole homogenates from glucose-repressed and aerobic S. cerevisiae*

The glucose-repressed yeast was grown to the mid-exponential phase [from 5% to 2% (w/v) glucose concentration in the growth medium] and the aerobic yeast at a glucose concentration kept at less than 0.1% under strong aeration which was continued after the exhaustion of glucose. Unless stated otherwise specific activities are expressed as μmol/min per mg of homogenate protein and chemical compositions as mg/100mg of homogenate protein. Results are expressed as mean±S.D. Numbers of individual yeast samples analysed are given in parentheses. Experimental details are given in the text.

Component	Content		Ratio aerobic/repressed
	Repressed yeast	Aerobic yeast	
Acid phosphatase	0.044±0.022 (6)	0.100±0.028 (4)	2.3
Alkaline phosphatase	0.069±0.006 (4)	0.072±0.012 (4)	1.0
Alkaline pyrophosphatase	0.355±0.030 (2)	0.464±0.150 (4)	1.3
AMPase*	1.80 ±0.64 (4)	3.19 ±0.47 (4)	1.8
Mg-ATPase without oligomycin	0.051±0.019 (12)	0.155±0.063 (10)	3.0
Mg-ATPase with oligomycin	0.026±0.011 (12)	0.040±0.016 (10)	1.5
β-Fructofuranosidase	0.467±0.216 (4)	2.66 ±0.65 (4)	5.7
α-Mannosidase*	0.132±0.030 (4)	1.40 ±0.14 (4)	10.6
NADPH–cytochrome *c* oxidoreductase	0.017±0.007 (10)	0.036±0.007 (4)	2.1
Succinate–ferricyanide oxidoreductase	0.008±0.004 (2)	0.083±0.014 (5)	10.4
Cytochrome *c* oxidase	0.007±0.002 (3)	0.227±0.094 (10)	32.4
RNA	18.4 ±2.6 (7)	15.6 ±2.9 (6)	0.8
DNA	0.34 ±0.08 (7)	0.40 ±0.08 (7)	1.2
Phospholipid	4.6 ±0.7 (7)	7.7 ±1.1 (7)	1.7
Sterol	1.5 ±0.4 (6)	2.8 ±0.5 (7)	1.9
Total lipid	18.9 ±1.6 (4)	24.4 ±2.5 (5)	1.3

* nmol/min per mg of homogenate protein.

from E. Merck A.G. The reagents were of analytical grade or of the highest purity available.

Results and Discussion

Content of marker components in whole homogenates of glucose-repressed yeast

To characterize the repressed yeast being studied, the contents of some marker components in glucose-repressed yeast are compared (Table 1) with those in aerobic baker's yeast. The mitochondrial cytochrome *c* oxidase was very sensitive to catabolite repression, and only traces were detected in repressed yeast. Succinate dehydrogenase was also highly repressed but still present in the primitive mitochondrial structures of repressed yeast. Glucose strongly inhibited also the synthesis of both β-fructofuranosidase (cf. Lampen, 1968) and α-mannosidase. The total Mg-ATPase activity was at least three times as high in aerobic as in repressed yeast, evidently because of mitochondria, and was probably even higher, because the assay conditions were optimal for the oligomycin-insensitive ATPase of plasma membrane but not for the oligomycin-sensitive mitochondrial enzyme. The oligomycin-insensitive Mg-ATPase activity was only 50% higher in aerobic yeast. Acid phosphatase and NADPH–cytochrome *c* oxidoreductase activities in aerobic yeast were rather more than double those in repressed yeast, and all the other enzymes listed were slightly more active in aerobic yeast. The activity of glucose 6-phosphatase, which is often used as a marker for the endoplasmic reticulum, was very low in our yeast, and further, other phosphatases had a perturbing effect on its determination; consequently it could not be used as a marker for the yeast endoplasmic reticulum.

The higher contents of phospholipid and sterol in aerobic yeast reflect the increased amount of membranes, particularly mitochondrial, in aerobic yeast (cf. Jollow *et al.*, 1968).

Fractionation by low-speed zonal centrifugation in sucrose gradients

During the low-speed centrifugations most components do not reach their equilibrium densities, and the fractionation is therefore by rate-zonal centrifugation. The distribution of important components through the sucrose gradients is shown in Table 2 (lines RZ). More of the oligomycin-insensitive Mg-ATPase remained in the gradient

Table 2. *Percentage distribution by density range of some marker components from homogenates of glucose-repressed S. cerevisiae after zonal centrifugation on a buffered sucrose gradient*

When more than one experiment was performed, the values presented are means and the number of experiments is given in parentheses. Abbreviations: RZ, rate-zonal centrifugation (approx. $5\times10^4 g$-min at the sample zone); IB, isopycnic banding (approx. $5\times10^6 g$-min at the sample zone).

Component	Method	Density range ...	Distribution (%) 1.00–1.09	1.09–1.13	1.13–1.20	over 1.20
Protein	RZ (2)		94.3	1.5	1.3	2.9
	IB (7)		67.9	21.5	7.0	3.6
Acid phosphatase	RZ		90.5	6.5	0.8	9.9
	IB (3)		56.6	17.2	16.3	2.2
Alkaline phosphatase	RZ		98.6	1.3	0.1	0.0
	IB		72.6	10.6	11.8	5.0
Alkaline pyrophosphatase	IB		95.0	4.0	0.8	0.2
AMPase	IB		62.4	16.8	18.3	2.5
Mg-ATPase (without oligomycin)	RZ (2)		64.2	14.8	10.9	10.1
	IB (7)		13.1	28.7	33.0	25.2
Mg-ATPase (with oligomycin)	RZ (2)		71.6	9.3	10.6	8.5
	IB (7)		9.8	31.8	39.6	18.8
β-Fructofuranosidase	RZ		87.5	0.0	0.0	12.5
	IB (3)		92.1	7.9	0.0	0.0
α-Mannosidase	RZ (2)		53.0	14.5	19.3	13.2
	IB		55.4	17.2	5.6	21.8
NADPH–cytochrome *c* oxidoreductase	RZ (2)		93.5	3.8	1.5	1.2
	IB (3)		55.7	19.6	16.7	7.9
Succinate–ferricyanide oxidoreductase	IB		15.4	26.9	53.9	3.8
RNA	RZ (2)		93.1	1.7	2.1	3.1
	IB (6)		26.2	63.4	7.2	3.2
Phospholipid	RZ (2)		81.4	6.6	6.2	5.8
	IB (6)		20.7	40.7	28.8	9.8
Sterol	RZ (2)		80.3	4.8	6.6	8.3
	IB (6)		29.4	40.1	14.0	16.5

at low speeds than in the 'high-speed' experiments. If the amount found in the gradient is taken as 100%, the amount which sedimented on the rotor walls was 35% in low-speed centrifugation and 80% in high-speed centrifugation.

Over 80% of phospholipid and sterol, and 65–70% of the Mg-ATPase (without or with oligomycin) were at $\rho < 1.09$ after low-speed centrifugation. However, although not evident in Table 2, most of the membranes, including oligomycin-insensitive Mg-ATPase-rich membranes, had moved to slightly higher densities than the soluble cytosol and the rough endoplasmic reticulum. Thus, the main protein maximum at $\rho = 1.03$–1.04 also contained most of the acid and alkaline phosphatase, β-fructofuranosidase, α-mannosidase, NADPH–cytochrome *c* oxidoreductase and RNA, whereas most of the sterol, phospholipid and Mg-ATPase occurred at higher densities, with maxima at $\rho = 1.07$–1.08, and a smaller peak of phospholipid at $\rho = 1.04$–1.05. However, there was definite overlapping of these zones. An electron micrograph of material from the main phospholipid peak is shown in Plate 1*a*. At the heavy end of the gradient, there was a minor peak of protein, phospholipid, sterol, Mg-ATPases and β-fructofuranosidase at $\rho = 1.26$–1.28.

β-Fructofuranosidase (Sutton & Lampen, 1962; Islam & Lampen, 1962; Millbank, 1963; Burger *et al.*, 1965; Suomalainen *et al.*, 1967) and acid phosphatase (McLellan & Lampen, 1963; Tonino & Steyn-Parvé, 1963; Suomalainen *et al.*, 1967) are mainly located outside the plasma membrane, for they are released into the surrounding medium during the preparation of yeast spheroplasts. These exoenzymes are synthesized inside the yeast cell (e.g. Lampen, 1968) and small amounts of them have been found in plasma-membrane preparations (Matile, 1970; Nurminen *et al.*, 1970). The results of Bauer & Sigarlakie (1973) suggest that the main part of acid phosphatase is located throughout the cell wall itself, whereas Arnold (1972) has suggested that it is mainly situated in the periplasmic space, between the cell wall and plasma membrane. Our results show that this enzyme is anyway not strongly bound to the cell wall, and are consistent with a periplasmic site. Thus, although electron microscopy showed that some pieces of cell walls were still present at high densities (over 1.20) after low-speed centrifugation (but not after high-speed zonal centrifugation, when all of the cell walls sedimented on to the walls of the rotor) only 2.2% of the enzyme was found in this density range, and 90% was at densities below 1.09. By contrast, 12.5% of the β-fructofuranosidase activity was at $\rho > 1.20$ after low-speed centrifugation. This part of the activity is evidently retained in the pieces of cell walls (or cell envelopes, i.e. cell walls containing fragments of the plasma membrane) present at these high densities. The finding that most of the β-fructofuranosidase is non-sedimentable, however, supports the suggestion of Arnold (1972) that this enzyme is located in the periplasmic space, and suggests that most of it is not covalently bound to the cell wall, as was proposed by Lampen (1968).

Increasing the centrifugation conditions to $5 \times 10^5 g$-min at the sample zone improved the resolution at the lighter end of the gradient. The peak of soluble proteins was at $\rho = 1.02$–1.04, but another high peak of protein at $\rho = 1.06$ with a shoulder extending to $\rho = 1.10$ had begun to separate from it. Phospholipid and sterol had their maxima at $\rho = 1.09$–1.10, with a high shoulder at $\rho = 1.06$ and a lower one at $\rho = 1.11$. At densities over $\rho = 1.11$ they were uniformly distributed along the gradient, with a small peak at $\rho = 1.13$–1.14. The density distribution of Mg-ATPase was similar to that of phospholipid and sterol. However, the separation of different subcellular fractions at $5 \times 10^5 g$-min was still incomplete. High-speed zonal centrifugation (about $5 \times 10^6 g$-min at the sample zone) was therefore investigated. This corresponds to isopycnic banding of the particles of mitochondrial size.

Fractionation by high-speed zonal centrifugation in Ficoll gradient

An advantage of Ficoll as a gradient material is that it has a relatively small effect on the density of subcellular particles because their membranes are impermeable to it. Only densities up to $\rho = 1.20$ in iso-osmotic Ficoll gradient were accurately studied, because with Ficoll alone the higher densities cannot be obtained. However, observations showed that the bulk of the subcellular material was distributed below this density.

Typical density distributions of some marker components in Ficoll are shown in Table 3 and Fig. 1. The components of the soluble cytosol and the main part of the protein were at $\rho = 1.03$–1.05, and most of the RNA, and thus most of the ribosomal components at $\rho = 1.06$–1.08. Sterol and phospholipid showed two main peaks at densities of about 1.06–1.08 and 1.09–1.10. The first of these was coincident with the maxima of RNA and NADPH–cytochrome *c* oxidoreductase. Electron microscopy revealed that this fraction contained free and membrane-bound ribosomes, and small plates or vesicles probably originating from the rough endoplasmic reticulum. Several kinds of small smooth-surfaced membranes, plates and particles, including some electron-dense particles were also present.

The second, and larger, phospholipid peak at $\rho = 1.09$–1.10 was coincident with the major peaks of Mg-ATPase measured with or without added

Table 3. *Percentage distribution by density range (from 1.00 to 1.20) of some marker components from homogenates of glucose-repressed S. cerevisiae after zonal centrifugation on a buffered iso-osmotic Ficoll gradient*

Centrifugation was about $5 \times 10^6 g$-min at the sample zone.

Component	Density range ...	Distribution (%) 1.00–1.09	1.09–1.13	1.13–1.20
Protein*		77.6	20.7	1.7
Acid phosphatase*		50.4	38.2	11.4
Alkaline phosphatase*		77.8	20.0	2.2
AMPase*		64.9	31.2	3.9
Mg-ATPase (without oligomycin)*		27.8	61.2	11.0
Mg-ATPase (with oligomycin)*		20.9	67.6	11.5
β-Fructofuranosidase		93.6	6.4	0.0
NADPH–cytochrome *c* oxidoreductase		67.3	32.7	0.0
RNA*		73.6	25.8	0.6
Phospholipid*		36.7	49.0	14.3
Sterol*		48.9	45.9	5.2

* Mean of two fractionations.

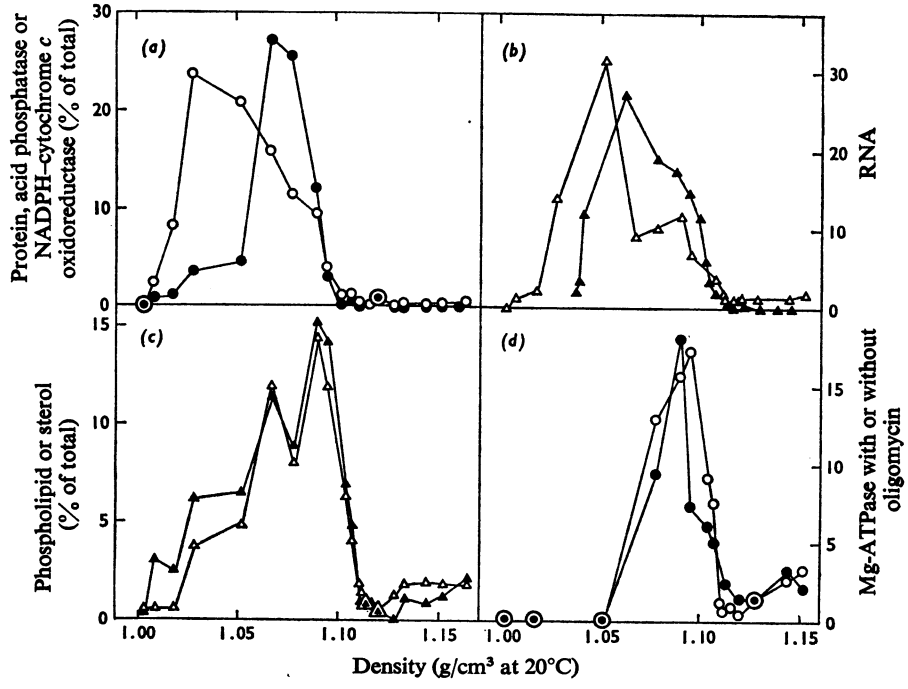


Fig. 1. *Fractionation of a whole homogenate of glucose-repressed S. cerevisiae by high-speed zonal centrifugation in gradient of buffered iso-osmotic Ficoll*

Centrifugation was at 20000rev./min for 360min in a B XV rotor (approx. $5 \times 10^6 g$-min at the sample zone). The density distributions of the following components are shown: (*a*) protein (○) and RNA (●); (*b*) acid phosphatase (△) and NADPH–cytochrome *c* oxidoreductase (▲); (*c*) phospholipid (△) and sterol (▲); (*d*) Mg-ATPase without (○) and with (●) oligomycin. Results are expressed as percentages of the total amounts recovered in the density range 1.00–1.20.

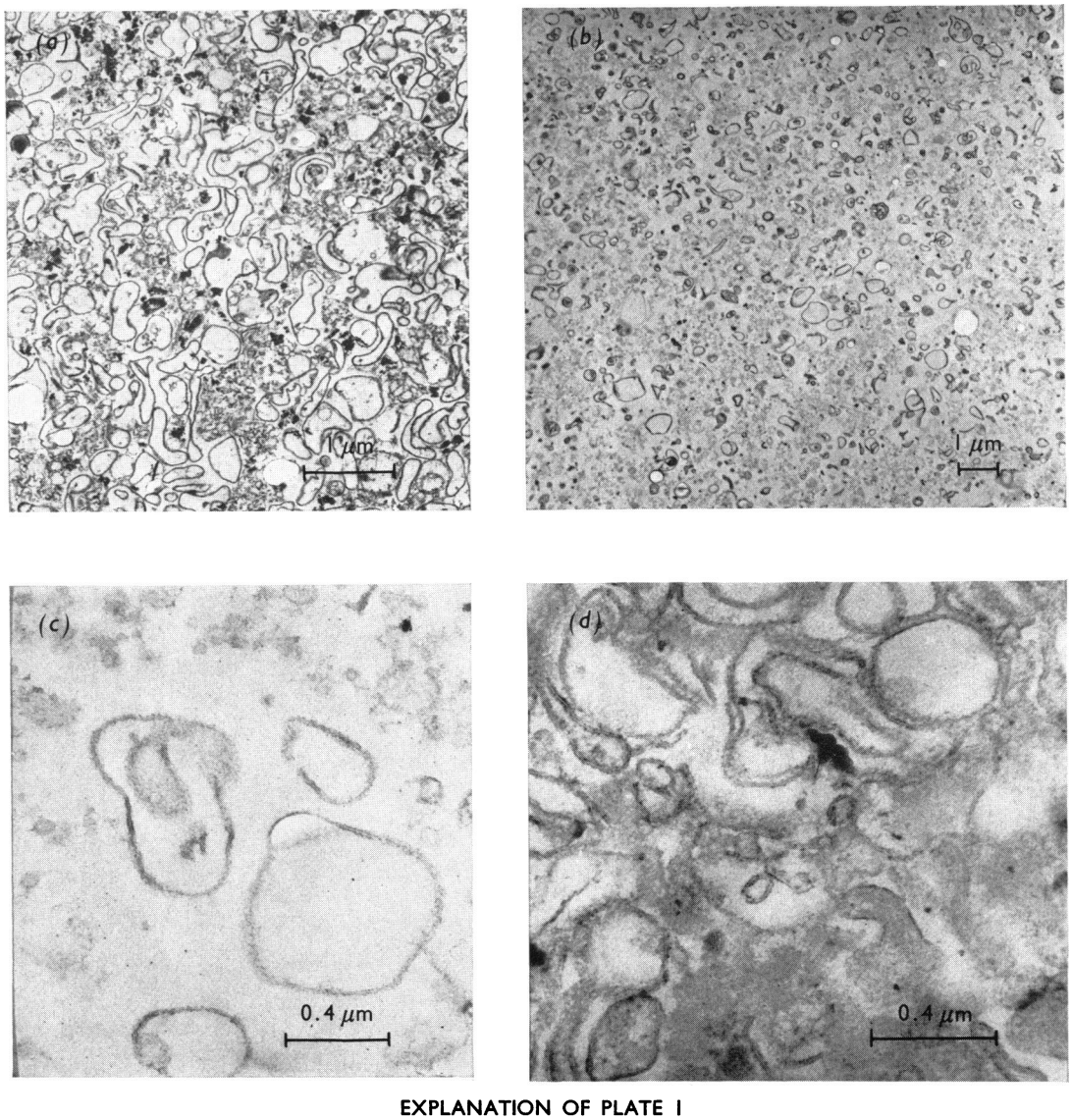
oligomycin. Indeed, very little Mg-ATPase was found anywhere else, except for a small peak at $\rho = 1.12$–1.15, accompanied by a minor peak of phospholipid and sterol. The oligomycin-insensitivity of Mg-ATPase increased towards the higher densities, being about 40% at $\rho = 1.08$, about 60% around $\rho = 1.10$ and at $\rho = 1.12$ and above, generally almost 100%. Electron microscopy showed vesicles, some with visible inclusions, but most apparently empty and with a smooth-surfaced, triple-layered single-unit membrane structure, as the dominant objects around a density of 1.10 (Plate 1*b* and 1*c*). Larger membrane sheets, coiled membrane fragments, single-unit membrane vesicles and heavy double membranes, the last-mentioned similar to those found by Schatz *et al.* (1963) in sucrose at a density of 1.23, became the dominant materials at a density of 1.12 (Plate 1*d*). These Mg-ATPase-containing density ranges must contain fragments of the plasma membrane, since this contains oligomycin-insensitive Mg-ATPase. Additional evidence is provided by the distribution of phospholipid and sterol, since the plasma membrane in glucose-repressed yeast is a very large proportion of all membrane material. But it is clear from the electron-microscopic and enzymic analyses that other membranes are also present, and probably different kinds of fragments of the plasma membrane, too. The densest material, at $\rho = 1.12$–1.15, was evidently free from mitochondrial membranes, because its Mg-ATPase was insensitive to oligomycin. Plasma-membrane fragments retaining their original carbohydrate content, and therefore heavy, are probably present here, together with other, unidentified, structures such as the heavy double membranes noted above.

The Mg-ATPase in the major phospholipid peak at $\rho = 1.09$–1.10 was partially inhibited by oligomycin, so membranes containing oligomycin-sensitive Mg-ATPase (possibly mitochondrial) are present, together with membranes containing oligomycin-insensitive Mg-ATPase. The latter are very probably fragments of the plasma membrane which have lost part of their carbohydrate content, and consequently become less dense. They may be tentatively identified with the larger vesicles visible in Plate 1(*c*). The smaller vesicles in Plate 1(*b*) may be the secretory vesicles containing β-glucanase and presumably derived from endoplasmic reticulum (Cortat *et al.*, 1972). β-Glucanase is present both in these secretory vesicles and in plasma membrane, and possibly also in the fragments of endoplasmic reticulum. Moreover, the fusion of the secretory vesicles with plasma membrane may occur. However, according to Cortat *et al.* (1972), they are smaller than vesicles of plasma membrane (Plate 1*c*), having a diameter of approx. 0.1 μm, compared with 0.2–0.4 μm. Of these three populations of particles containing β-glucanase, Mg-ATPase has been detected only in the plasma membrane.

This region also contained 20–40% of the alkaline and acid phosphatase, Co^{2+}-dependent AMPase, and NADPH–cytochrome *c* oxidoreductase activities (Table 3). A part of these may have been trapped in artificial vesicles formed during disruption of the yeast cells, but some may be naturally associated with real intracellular particles. However, the solubility of about 65% of the Co^{2+}-dependent AMPase shows that this enzyme cannot be used as a marker for the yeast plasma membrane. This contrasts with the situation in animal cells, where AMPase (5′-nucleotidase) is commonly used as a plasma-membrane marker. Some evidence exists for the presence of several kinds of sedimentable particles containing acid and alkaline phosphatases which are distinct from low-density vacuoles (cf. Cartledge & Lloyd, 1972*b*). The results of Bauer & Sigarlakie (1973) suggest, interestingly, that a small part of the acid phosphatase is located in the cytoplasmic vesicles near the plasma membrane, and a part of the alkaline phosphatase in budding cells within the plasma-membrane invaginations. Further, Cartledge & Lloyd (1972*a*,*c*) have demonstrated, with homogenates of *S. carlsbergensis*, the presence of small sedimentable particles containing NADPH–cytochrome *c* oxidoreductase that are distinct from the microsomal fraction. Our results obtained by fractionation on Ficoll suggest that about two-thirds of the NADPH–cytochrome *c* oxidoreductase in our strain of *S. cerevisiae* is located in microsomal material, in accordance with the report of Schatz & Klima (1964), and that the roughly one-third found among the membranes at $\rho = 1.09$–1.10 is particulate.

Fractionation by high-speed zonal centrifugation in sucrose gradients

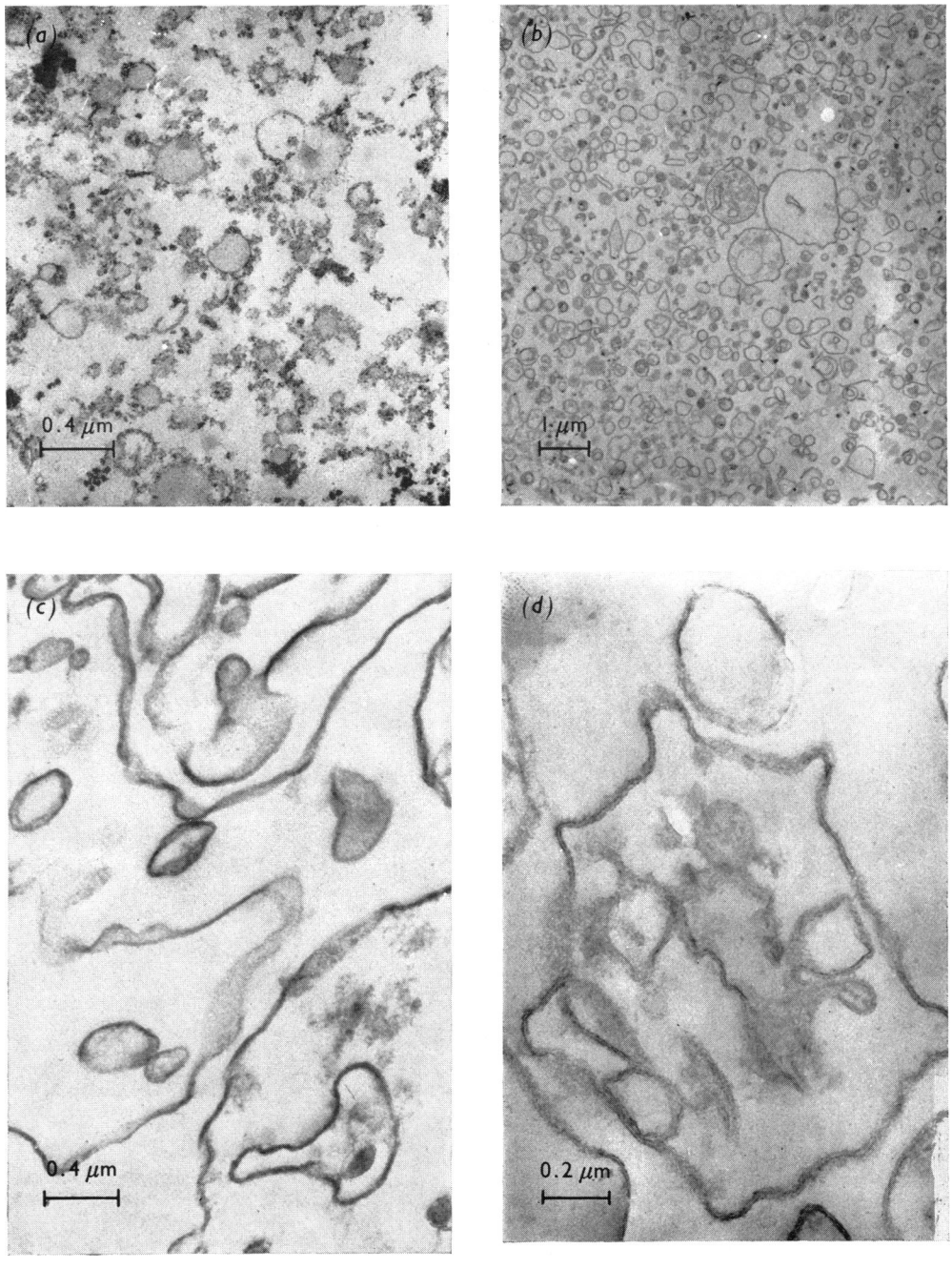
Typical density distributions of some marker components after high-speed zonal fractionation in buffered sucrose gradients are shown in Fig. 2 and in Table 2, lines IB. The bulk of the soluble proteins and soluble or solubilized enzymes, including, for example, acid and alkaline phosphatase, alkaline pyrophosphatase, AMPase and β-fructofuranosidase, was found within the lowest range of density, at $\rho = 1.03$–1.06. The second protein peak at a median density of 1.10 was coincident with the bulk of the RNA. In sucrose, compared with the corresponding density distribution in Ficoll, clearly more RNA was at $\rho = 1.09$–1.13 (63% in sucrose and 26% in Ficoll). Some 55% of NADPH–cytochrome *c* oxidoreductase was among microsomal material in sucrose, mainly at $\rho = 1.05$–1.07, and a smaller part, about 20%, formed a shoulder of the main peak extending to $\rho = 1.11$ and was evidently associated with the rough endoplasmic reticulum. The remainder of NADPH–cytochrome *c* oxidoreductase was rather evenly



EXPLANATION OF PLATE 1

Electron micrographs of sections of material from density 1.09 *after low-speed zonal centrifugation in sucrose* (*a*), *from density* 1.10 *after high-speed zonal centrifugation in Ficoll* (*b*, *c*) *and from density* 1.12 *after high-speed zonal centrifugation in Ficoll* (*d*)

Various forms of membrane vesicles are present in (*a*)–(*d*); (*c*) shows triple-layered single-unit-membrane structures and (*d*) larger membrane sheets and double membranes in addition. All material was fixed with glutaraldehyde and OsO_4 and stained with lead citrate in (*b*)–(*d*) and with lead citrate and uranyl acetate in (*a*). The bars in (*a*) and (*b*) represent 1 μm and in (*c*) and (*d*) 0.4 μm.



EXPLANATION OF PLATE 2

Electron micrographs of sections of material from density 1.10 (*a*), *from density* 1.20 (*b*) *and from density* 1.25 (*c*, *d*) *after high-speed zonal centrifugation in sucrose*

Various forms of membrane vesicles are present in (*a*)–(*d*); (*a*) shows pieces of endoplasmic reticulum, (*d*) shows the triple-layered single-unit structure of membranes present in (*c*). All material was fixed with glutaraldehyde and OsO_4 and stained with lead citrate in (*a*) and (*b*) and with lead citrate and uranyl acetate in (*c*) and (*d*). The bars in (*a*) and (*c*) represent 0.4 μm, in (*b*) 1 μm and in (*d*) 0.2 μm.

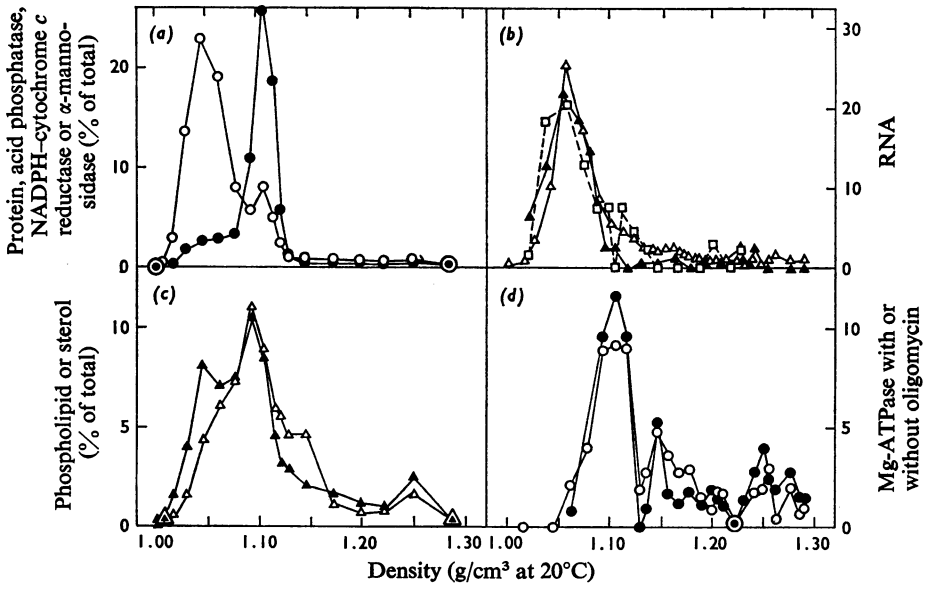


Fig. 2. *Fractionation of a whole homogenate of glucose-repressed S. cerevisiae by high-speed zonal centrifugation in gradient of buffered sucrose*

Centrifugation was at 20000rev./min for 360min in a B XV rotor (approx. $5\times10^6 g$-min at the sample zone). The density distributions of the following components are shown: (*a*) protein (○) and RNA (●); (*b*) acid phosphatase (△), NADPH–cytochrome *c* oxidoreductase (▲) and α-mannosidase (□); (*c*) phospholipid (△) and sterol (▲); (*d*) Mg-ATPase without (○) and with (●) oligomycin. Results are expressed as percentages of the total amounts recovered in the density range 1.00–1.29.

distributed at the higher densities, showing a flat peak in the mitochondrial range. It is noticeable that no activity of this enzyme was present at $\rho = 1.25$ or at higher densities.

According to Schatz (1965) and Criddle & Schatz (1969), succinate dehydrogenase is present even in the pro-mitochondria of anaerobic yeast. Consequently its density distribution also reveals the position of the primitive mitochondrial structures of the glucose-repressed yeast studied. Most of this enzyme was found at $\rho = 1.13$–1.20.

α-Mannosidase, a suitable marker for vacuolar membranes (van der Wilden *et al.*, 1973), had its main peak (55%) at $\rho = 1.04$–1.08, with a lower shoulder at $\rho = 1.09$–1.10 and a smaller peak at $\rho = 1.11$–1.13. The first major peak of this enzyme and the first peak of sterol were coincident, and these fractions contained low-density vesicles similar to those described by Hossack *et al.* (1973) and Cartledge & Rose (1973), and which probably originate from the primary vacuole. According to Hossack *et al.* (1973) and Cartledge & Rose (1973), a high content of sterol esters is a characteristic of these low-density vesicles. The remaining α-mannosidase activity at $\rho = 1.09$–1.13 was probably associated exclusively with the membranes of the main vacuole.

The main peaks of phospholipid and sterol at $\rho = 1.09$–1.11 again show the position of the major part of the membranes. A shoulder of the main phospholipid peak covers the mitochondrial range, $\rho = 1.13$–1.16. Both sterol and phospholipid have a smaller peak at $\rho = 1.25$.

Mg-ATPase had a complex density distribution in sucrose. Only about 10% was non-sedimentable (at $\rho < 1.09$). The major peak was at $\rho = 1.09$–1.11, 50% of this activity being oligomycin-insensitive. The second peak of Mg-ATPase, at $\rho = 1.15$, was oligomycin-insensitive to an extent of 60%, as was the Mg-ATPase activity covering the wide density range $\rho = 1.16$–1.21. The oligomycin-insensitivity of Mg-ATPase increased towards the higher densities in sucrose: its activities in the peaks at $\rho = 1.25$ and 1.28 were completely oligomycin-insensitive at the high concentrations of oligomycin relative to protein.

As could be inferred from the density distributions of phospholipid, sterol and Mg-ATPase, electron microscopy revealed that the second protein peak at a median density of 1.10 was the richest density region

for membranes. In accordance with the presence of a high amount of RNA, numerous rough-surfaced membranes of endoplasmic reticulum were seen (Plate 2*a*). Also present were larger smooth-surfaced single-unit membranes similar to those visible after low-speed zonal centrifugation in sucrose at $\rho = 1.09$ (Plate 1*a*). On the basis of their high Mg-ATPase content, most of these membranes are evidently light vesicles formed during disruption of the plasma membrane.

The second Mg-ATPase peak at $\rho = 1.15$ is at least partly associated with mitochondrial structures. According to Schatz (1965) and Criddle & Schatz (1969) the pro-mitochondria of anaerobic *S. cerevisiae* reach their isopycnic equilibrium density in sucrose at $\rho = 1.15$. Much of the Mg-ATPase activity existing at $\rho = 1.16$–1.21 must also be attributed to mitochondria, for the bulk of the succinate dehydrogenase was in the density range 1.13–1.20. According to Perlman & Mahler (1974), the relatively few, often dilated and fragile, primitive mitochondrial structures of glucose-repressed yeast equilibrate in sucrose at about the same density as the fully developed mitochondria of aerobic yeast, i.e. at $\rho = 1.16$–1.18. However, it is very likely that some intermediate forms of plasma membrane, carrying variable amounts of their original carbohydrate content, were also present, and both kinds of these structures are seen in Plate 2(*b*).

At the heavy end of the sucrose gradient, at $\rho = 1.25$, the last peaks of phospholipid and sterol were coincident with a peak of oligomycin-insensitive Mg-ATPase (Fig. 2). Electron microscopy (Plates 2*c* and 2*d*) revealed the presence of smooth-surfaced single-unit membranes as the dominating objects. Because Mg-ATPase was fully oligomycin-insensitive and practically no NADPH–cytochrome *c* oxidoreductase or succinate dehydrogenase was present, it is reasonable to conclude that these membranes are heavy fragments of plasma membrane. Schatz *et al.* (1963) detected heavy double membranes showing Mg-ATPase activity in sucrose at $\rho = 1.23$, and proposed that they were membranes of endoplasmic reticulum, whereas Matile *et al.* (1967) suggested that they are probably fragments of plasma membrane. Their exact origin, however, has never been clarified. In sucrose we found double membranes similar to those visible in Plate 1(*d*), mainly among the membranes at $\rho = 1.21$.

The total carbohydrate was distributed in the sucrose density gradient in three main peaks, the first two being coincident with the first and second protein peaks, and the third at the high density of 1.25. The amount of carbohydrate sedimented on the walls of the rotor was about ten times higher than the total carbohydrate found in the gradient, indicating that during high-speed zonal centrifugation the polysaccharide-rich cell walls, and most of the glycogen, were sedimented through the gradient.

Subfractionation of zonal fractions on Urografin gradient

Because both biochemical and morphological investigations showed that after zonal centrifugation several subcellular organelles may still come into the same fractions, further attempts were made to purify the zonal fractions. Several membraneous fractions obtained by high-speed zonal centrifugation in density gradients of buffered sucrose were applied on to discontinuous gradients of iso-osmotic Urografin, and the main bands formed during isopycnic banding were collected and analysed (Table 4).

Sedimentation through Urografin to $\rho = 1.11$–1.15 increased the total lipid, phospholipid and sterol contents and decreased the RNA, DNA and protein contents of membranes obtained first in sucrose at $\rho = 1.09$–1.11 or 1.11–1.14. As regards yield, the density range $\rho = 1.09$–1.11 in sucrose is important, because it was the richest in membranes (the maxima of phospholipid and sterol in Fig. 2). In addition to the light vesicles of plasma membrane (the maximum peaks of Mg-ATPase in Fig. 2), membranes of rough endoplasmic reticulum (the maximum of RNA in Fig. 2) and vacuolar membranes (a minor peak of α-mannosidase in Fig. 2) were present in sucrose. The removal of rough endoplasmic reticulum from the preparations is indicated by the marked decrease in both nucleic acids and NADPH–cytochrome *c* oxidoreductase. The specific activity of this enzyme in sucrose at $\rho = 1.09$–1.11 and 1.11–1.14 was 0.014 and 0.011 μmol/min per mg of protein respectively, and in the corresponding membrane preparations recovered at $\rho = 1.11$–1.15 after banding in Urografin only 0.004 and 0.002 μmol/min per mg of protein. The presence of vacuolar membranes in the preparations after banding in Urografin is unlikely, for these membranes have been shown to move slowly even through a flat Urografin gradient (cf. van der Wilden *et al.*, 1973). The conclusion that the purified preparations of light vesicles of plasma membranes are in fact obtained is further supported by the increased oligomycin-insensitivity of Mg-ATPase. When the membrane preparations were obtained by high-speed zonal centrifugation in sucrose at $\rho = 1.09$–1.11 or 1.11–1.14, only 50% of their Mg-ATPase was insensitive to oligomycin; after a further purification on Urografin gradient, the Mg-ATPase of membranes recovered at $\rho = 1.11$–1.15 was completely oligomycin-insensitive.

In sucrose (lines S1–S8 in Table 4), the proportion of carbohydrate in the preparations isolated generally increases towards the higher densities having a maximum value at $\rho = 1.24$–1.26 coincident with the heavy peaks of phospholipid, sterol and Mg-

Table 4. *Chemical compositions and oligomycin-insensitivity of Mg-ATPase of some membrane fractions isolated from whole homogenates of glucose-repressed S. cerevisiae by high-speed zonal centrifugation in buffered sucrose before and after subfractionation by swing-out centrifugation on Urografin gradient*

Fractions S1–S8, isolated at the densities shown by high-speed zonal centrifugation in sucrose gradients, were further purified by swing-out centrifugation on Urografin gradients. Details are given in the Experimental section. The chemical compositions (expressed as mg/mg of protein) and the oligomycin-insensitivity of Mg-ATPase (expressed as units with oligomycin/units without oligomycin × 100) of each starting fraction (S1 etc.) and the main visible bands obtained from it on Urografin (U) are shown. Total carbohydrate contents are expressed as mg of glucose equivalent/mg of protein. Mean values are given, when two to four samples of similar preparations have been analysed. N.D., not determined.

Gradient material	Density range	RNA	DNA	Phospho-lipid	Sterol	Total lipid	Carbohydrate*	Oligomycin-insensitivity of Mg-ATPase
S1	1.09–1.11	0.37	0.001	0.05	0.01	0.31	0.053 (0.063)	51
U	1.11–1.15	0.12	0.001	0.26	0.11	1.34	N.D.	106
S2	1.11–1.14	0.17	0.004	0.20	0.04	0.45	0.062	50
U	1.11–1.15	0.05	0.001	0.25	0.10	1.01	N.D.	128
S3	1.14–1.16	0.08	0.004	0.70	0.05	2.04	0.079	59
U	1.11–1.15	0.01	0.002	0.28	0.11	1.41	N.D.	63
U	1.13–1.17	0.10	0.001	0.28	0.10	N.D.	0.173	N.D.
S4	1.16–1.18	0.12	0.004	0.25	0.06	N.D.	0.173	56
U	1.14–1.18	0.07	0.004	0.15	0.06	N.D.	0.141	N.D.
S5	1.18–1.19	0.03	0.002	0.04	0.02	N.D.	N.D.	46
U	1.19–1.24	0.03	0.002	0.08	0.05	N.D.	N.D.	69
S6	1.19–1.21	0.11	0.006	0.13	0.02	N.D.	0.289 (0.356)	58
U	1.17–1.21	0.06	0.006	0.12	0.08	N.D.	0.359	N.D.
S7	1.21–1.24	0.08	0.004	0.13	0.03	N.D.	0.109 (0.134)	82
U	1.21–1.25	0.16	0.007	0.18	0.14	1.43	1.009	N.D.
S8	1.24–1.26	0.14	0.005	0.24	0.06	N.D.	0.501 (0.702)	117
U	1.21–1.25	0.16	0.007	0.15	0.11	1.61	0.902	N.D.

* The values obtained by the anthrone method used are low by a factor of 1/1.55 for mannose (Pfäffli & Suomalainen, 1960). Results corrected on the basis of Table 5 are given in parentheses.

ATPase (Fig. 2). According to the cytochemical investigation of Voříšek & Pokorný (1975), in *S. cerevisiae* the polysaccharide lamellae were visible on the surface of the endoplasmic-reticulum membranes and the plasma membrane; their yeast strain also exhibited polysaccharide deposits on the membranes of small vacuoles and in glucanase vesicles. It is noteworthy that, in contrast, no polysaccharides were detected on the surface of nuclear and mitochondrial membranes. Consequently, it is evident that some fragments of plasma membrane carrying variable amounts of their original carbohydrate content are also present in the mitochondrial range, at about $\rho = 1.14$–1.21 in sucrose, accounting for a part of its carbohydrate and Mg-ATPase contents.

At a high density of 1.24–1.26 in sucrose, the last peaks of phospholipid and sterol were coincident with a peak of Mg-ATPase (Fig. 2). Because no microsomal or mitochondrial oxidoreductases were present, electron microscopy revealed the presence of smooth-surfaced single-unit membranes, Mg-ATPase was completely oligomycin-insensitive, the nucleic acid content was low and the carbohydrate content high (Table 4), it can be concluded that this density in sucrose contains heavy fragments of plasma membrane that have retained their original carbohydrate content. After a further centrifugation in Urografin, a band recovered at $\rho = 1.21$–1.25 contained roughly equal amounts of carbohydrate and protein (Table 4).

The finding that the fractions at $\rho = 1.24$–1.26 in sucrose contained the heavy fragments of plasma membrane is in agreement with the report of Fuhrmann *et al.* (1974), who concluded that plasma-membrane vesicles with mitochondrial membranes were between $\rho = 1.12$ and 1.18 and purer plasma-membrane fragments were concentrated at the interface of the sucrose solutions of $\rho = 1.23$ and 1.29. By contrast, Hossack *et al.* (1973) considered the material derived from spheroplast lysates with densities in the range 1.13–1.16 in sucrose to be mainly plasma membrane. However, Schibeci *et al.* (1973) found that a plasma-membrane preparation prepared essentially as described by Boulton (1965), but from lysates of spheroplasts tagged chemically with isotopically labelled reagents, became distributed from below $\rho = 1.10$ to 1.29 on a discon-

tinuous sucrose gradient. On the basis of the chemical tagging, they concluded that plasma membranes consisted of two major fractions with densities of 1.18–1.21 and 1.21–1.23, and a minor fraction with density in the range 1.23–1.29, and accounted for about 25% of the total protein of the membrane preparation.

The percentage contents of hexoses and inositol found by g.l.c. after acid hydrolysis of carbohydrates present in various membrane preparations are given in Table 5. As regards plasma membrane, the most important preparations are those isolated by high-speed zonal centrifugation in sucrose at densities of 1.09–1.11 and 1.24–1.26. The proportion of mannose is much higher in the latter preparation than in the former, namely 72% of the total of hexoses and inositol, compared with 30%. When the latter preparation was further centrifuged on Urografin, mannose was again the dominant sugar present in a band recovered at $\rho = 1.21$–1.25. Electron microscopy of freeze-etched specimens of intact yeast cells has revealed that the outer surface of the yeast plasma membrane is densely covered with globular particles embedded in the membrane at a variable depth (Moor & Mühlethaler, 1963). Evidence has been presented that the plasma-membrane particles are mannan–protein in nature (Matile *et al.*, 1967). Matile *et al.* (1967) found mannose as the only hexose after acid hydrolysis of their plasma-membrane preparation, whereas the plasma-membrane preparation of Fuhrmann *et al.* (1974) contained, in addition to mannan, a considerably smaller amount of glycogen. The presence of a certain amount of mannose, glucose, galactose and inositol in our preparations is explained by the presence in the plasma membrane of glycolipids, in which they are the main carbohydrate components (Nurminen & Suomalainen, 1971; Työrinoja *et al.*, 1974). Most of the inositol in the plasma membrane is present as phosphatidylinositol (Työrinoja *et al.*, 1974), which is the dominant phospholipid of the plasma membrane (Longley *et al.*, 1968; Suomalainen & Nurminen, 1970).

The obviously easy detachment of glycoprotein particles during fractionation is unexpected, for it has been possible to isolate reasonably well preserved preparations of mitochondria by the same homogenization method (cf. Guarnieri *et al.*, 1970; T. Nurminen, L. Taskinen & H. Suomalainen, unpublished work). The distribution of glycoprotein particles in the intact plasma membrane may not be uniform, and the ease with which these particles are removed may also vary, since they are embedded at different depths in the membrane. Thus the plasma-membrane fragments found in different density ranges may possibly originate from functionally different areas of the membrane. It is also very likely that during the preparation of yeast spheroplasts a certain amount of the glycoprotein particles on the outer surface of the plasma membrane is enzymically digested, because the plasma-membrane preparations obtained from spheroplast lysates have shown a considerably lower carbohydrate content than the preparations obtained from mechanical homogenates. Thus the following carbohydrate contents (as mg per mg of protein) have been reported for preparations from spheroplast lysates: 0.06–0.07, Boulton (1965); 0.13, Garcia Mendoza & Villanueva (1967); 0.08–0.12, Longley *et al.* (1968); 0.18–0.20, Schibeci *et al.* (1973); and the following for preparations from mechanical homogenates: 0.75, Matile *et al.* (1967); 1.16, Matile (1970); 0.75, Nurminen *et al.* (1970); 1.02, Cortat *et al.* (1972); 0.29, Fuhrmann *et al.* (1974). In this respect the membrane preparations obtained in sucrose at $\rho = 1.10$ resemble the former preparations, whereas the heavy-membrane fragments found at $\rho = 1.25$ in sucrose and purified on Urografin had a carbohydrate content of 0.70–0.90mg per mg of protein, similar to most of the latter preparations. It is therefore reasonable to conclude that the plasma-membrane preparations obtained in sucrose at density 1.24–1.26 have retained the heavy glycoprotein particles, whereas in the plasma-membrane preparations found in sucrose at $\rho = 1.09$–1.11 their amount is greatly diminished. The very different amounts of total carbohydrate (Table 4), especially that of mannose (Table 5), in these preparations support this conclusion. Consequently, it is evident that the buoyant density and thus the behaviour of plasma-membrane fragments during density-gradient centrifugations depends greatly on the amount of these heavy glycoprotein particles present.

Table 5. *Content of mannose, galactose, glucose and inositol in some membrane fractions isolated from whole homogenates of glucose-repressed S. cerevisiae by high-speed zonal centrifugation in buffered sucrose*

Centrifugation was at 20000rev./min for 360min in a B XV rotor (about $5 \times 10^6 g$-min at the sample zone). Other experimental details are given in the text. The results are expressed as percentages of the sum of mannose, galactose, glucose and inositol.

Density range	Mannose	Galactose	Glucose	Inositol
1.09–1.11	30.0	25.0	35.0	10.0
1.19–1.21	39.9	8.3	47.2	4.6
1.21–1.24	36.0	12.0	40.0	12.0
1.24–1.26	72.0	5.4	18.3	4.3
1.27–1.29	66.7	5.1	24.2	4.0

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