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The Enzymic Composition of the Isolated Cell Wall and Plasma Membrane of Baker's Yeast

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A study was made of the enzyme content of the isolated cell walls and of a plasma-membrane preparation obtained by centrifugation after enzymic digestion of the cell walls of baker's yeast. The isolated cell walls showed no hexokinase, alkaline phosphatase, esterase or NADH oxidase activity. It was concluded that these enzymes exist only in the interior of the cell. Further, only a negligible activity of deamidase was detectable in the cell walls. Noticeable amounts of saccharase, phosphatases hydrolysing *p*-nitrophenyl phosphate, ATP, ADP, thiamin pyrophosphate and PP_i, with optimum activity at pH 3–4, and an activity of Mg^{2+}-dependent adenosine triphosphatase at neutral pH, were found in the isolated cell walls. During enzymic digestion, the other activities appearing in the cell walls were mostly released into the medium, but the bulk of the Mg^{2+}-dependent adenosine triphosphatase remained in the plasma-membrane preparation. Accordingly, it may be assumed that the enzymes released into the medium during digestion are located in the cell wall outside the plasma membrane, whereas the Mg^{2+}-dependent adenosine triphosphatase is an enzyme of the plasma membrane. This enzyme differs from the phosphatases with pH optima in the range pH 3–4 with regard to location, pH optimum, substrate specificity and different requirement of activators.

Little is known of the chemical and enzymic composition of the cell surface of the yeast cell, particularly of the chemical structure of the plasma membrane and of the mechanisms by which it functions. It is known that the cell walls of baker's yeast (*Saccharomyces cerevisiae*) are composed of large amounts of polysaccharides, glucan and mannan, but contain little protein and lipids (cf. Northcote & Horne, 1952; Falcone & Nickerson, 1956; Eddy, 1958; Suomalainen, Nurminen & Oura, 1967*c*). On the other hand, the osmotic barrier, the plasma membrane, in yeast cells is usually assumed to be lipoprotein in nature, as it is in bacteria, but no direct evidence for this has been presented. Preparations of yeast membranes have been obtained by lysis of yeast protoplasts (Boulton, 1965; Garcia Mendoza & Villanueva, 1967). The major components of these preparations were protein and lipids. However, the usefulness of such preparations, for instance in analytical work, may be somewhat questionable, as they appear to be heterogeneous. For example, Boulton (1965) has demonstrated that washed preparations of protoplast 'ghosts' contain noticeable amounts of RNA and DNA, in addition to lipids and proteins. Further, electron micrographs of this type of preparation have attested to the heterogeneity of this material. To avoid contamination by various internal-membrane materials, we have examined the possibility of isolating a fraction, rich in plasma-membrane fragments, by starting from the carefully isolated cell walls of baker's yeast (Suomalainen, Nurminen & Oura, 1967*b*,*c*; Nurminen, Oura & Suomalainen, 1968). The bulk of the carbohydrates of the cell wall can be removed by enzymic digestion and then the fraction in which the fragments of plasma membrane are enriched can be isolated by centrifugation.

This paper presents results on the enzymic composition of the cell envelope, and some observations on its chemical composition. A preliminary account of a part of this work has been presented (Nurminen *et al.* 1968; Nurminen & Suomalainen, 1969) and certain aspects have been treated in detail elsewhere (Suomalainen *et al.* 1967*b*,*c*; Suomalainen & Nurminen, 1969*a*,*b*).

EXPERIMENTAL

Preparation of subcellular fractions. The yeast used was commercial baker's yeast, produced by the Rajamäki Factories of the Finnish State Alcohol Monopoly. For some experiments, the yeast was cultured further on a laboratory scale, under aerobic conditions in a medium containing Difco Bacto Yeast Nitrogen Base (6.7g/l), sucrose (50g/l), D-biotin (8μg/l) and potassium lactate

buffer, pH 5.5. The amount of inoculum was 0.6 g/l. After 15 h the yeast reached the end of the exponential growth phase, was harvested by centrifugation and washed twice with chilled water.

All the subsequent steps were done at 0–4°C. Portions (6 ml) of a suspension of yeast containing 1 g fresh wt. of cells, usually in ice-cold 25 mM-tris–HCl buffer, pH 7.2 (other solutions used are mentioned in the text), were added to cooled Mickle vessels containing 6 ml of Ballotini beads (grade 12). The vessels were then placed in the Mickle (1948) disintegrator and shaken at maximum amplitude for 5 min. Longer disruption times were avoided in order to get unsplintered cell walls. The suspension of disrupted cells was separated from the glass beads by filtering through a glass sinter type G1 and washing the beads with 5 ml of medium. The sample taken from this step is referred to below as 'disintegrated preparation'. The combined material from several disintegrations was centrifuged at 1000*g* for 10 min and the supernatant, later supplemented by the washings of sediments, was retained for analysis of material representing the 'cell interior'. The mixture of unbroken cells and cell walls was washed twice with the same medium, by resuspension and centrifugation at 3000*g* for 10 min. The pellet was made up of two distinct layers; the upper white layer contained the cell walls. This material was separated from the cream-coloured bottom layer (unemptied cells) by the gradual addition of medium to the pellet and gentle shaking until the material in the upper layer was suspended. The supernatant was poured off and the cell walls were spun down at 3000*g* for 10 min. The supernatants from the first two washings were perceptibly turbid but subsequently relatively clear, and the dry matter released into the medium in the last washing was negligible (less than 0.1% of the starting material). A routine check of the purity of the isolated cell walls was made by phase-contrast microscopy; the washings were repeated if necessary.

The rupture of large quantities of yeast was effected by grinding with Ballotini beads in a peristaltic pump (Phillips, Lamanna & Mallette, 1965). About 250 g fresh wt. of cells was suspended in 500 ml of 0.1 M-potassium phosphate buffer, pH 7, containing 10 mM-$MgSO_4$ and mixed under continuous agitation with 1250 ml of Ballotini beads (grade 12) in a glass container immersed in an ice bath. The slurry suspension was pumped continuously from the bottom of the container through a silicone tube and back into the reservoir vessel. The disintegrated material was washed into a measuring cylinder with the medium, the glass beads were allowed to settle, the supernatant was decanted, and the beads were washed with the medium. The combined cell material was then centrifuged as above.

In some cases fractionation in a dextran gradient (Lieblová, Beran & Streiblová, 1964) was employed for the separation of cell walls and unbroken cells. Yeast suspension (10 ml, containing about 1 g fresh wt. of cells) was applied to the surface of a continuous linear 9–21% (w/v) dextran gradient (200 ml) and centrifuged at 200*g* for 10 min. The upper layers were removed and used for isolation of the cell walls.

Enzymic digestion of the isolated cell walls was effected with snail gut enzymes. 'Suc digestif d'*Helix pomatia* stabilisé' or 'Helicase' (L'Industrie Biologique Française, Gennevilliers, Seine, France) was clarified by centrifugation at 15000*g* for 15 min. The lipase and phosphatases present in the snail gut enzyme preparations were removed by gel filtration on a Sephadex G-100 column in 0.85% NaCl, by the method of Anderson & Millbank (1966). The collected fractions were desalted on a Sephadex G-25 column in deionized water and freeze-dried. The cell walls (10 mg dry wt./ml), together with enzyme in 5 mM-sodium citrate buffer, pH 5.8, usually containing 1–10 mM-Mg^{2+} (as sulphate or chloride), were incubated with gentle shaking at 30°C. For the cell walls isolated from resting cells, the digestion time was 4 h and the amount of enzyme, 10–50 mg dry wt./ml. The digestion could be promoted by the addition of 10 mM-2-mercaptoethanol. When the cell walls were isolated from growing cells, the digestion time was 1 h and the concentration of enzyme 10 mg dry wt./ml. The progress of digestion was followed by measurement of the turbidity of the test samples at 600 nm. The digested preparation was collected by centrifugation at 15000*g* for 15 min and washed by resuspension and centrifugation in chilled citrate–Mg^{2+} buffer. The supernatant solution, supplemented by washings, was retained for analysis of the compounds released into the medium during digestion. The pellet was gently resuspended in the same buffer solution and centrifuged at 1000*g* for 5 min. The supernatant was separated from the pellet (heavy sediment) and centrifuged at 10000*g* for 15 min; by this means a further pellet (light sediment) and the last supernatant were obtained. Sometimes the centrifugations were repeated with a view to ensuring a better separation of sediments. The sediments were washed by suspension in the cold buffer solution and by centrifugation at 15000*g* for 15 min.

The subcellular preparations were either analysed or further fractionated immediately, or, when necessary, suspended in a small volume of water or appropriate buffer in stoppered tubes, and stored at −20°C or, when possible, freeze-dried.

Analytical methods. The total content of carbohydrates was determined by the anthrone method (Pfäffli & Suomalainen, 1960). The total nitrogen content was determined by the micro-Kjeldahl method. The protein content was calculated by multiplying the value for the nitrogen content by 6.25. The protein was also determined by the biuret method (Racusen & Johnstone, 1961) or by the Folin phenol method (Lowry, Rosebrough, Farr & Randall, 1951). The total phosphorus and the lipid-soluble phosphorus were determined by the methods of Allen (1940) and Kolb, Weidner & Toennies (1963). RNA and DNA were determined in the $HClO_4$ extract by the orcinol and diphenylamine methods (Mejbaum, 1939; Burton, 1956). The sterols were determined by a modified Lieberman–Burchard reaction (Sackett, 1925), with ergosterol as standard. The total lipid was determined gravimetrically, after extraction as proposed by Folch, Lees & Sloane-Stanley (1957). The dry matter was determined gravimetrically.

Enzyme assays. The enzyme activities were measured either immediately after fractionation, or the fractions were kept overnight at −20°C and thawed only once. Enzyme assays were done at 30°C unless otherwise stated. Controls without sample or without substrate were carried out, correction being made for any changes observed in the incomplete test system. Hexokinase was determined

spectrophotometrically at 25°C by studying the reduction of $NADP^+$ (0.2mM final concn.) in the presence of excess of glucose 6-phosphate dehydrogenase, 3.3mM-glucose, 6.6mM-$MgCl_2$, 0.33mM-ATP and 33mM-tris–HCl buffer, pH7.4 (cf. Hommes, 1966). The NADH oxidase system was determined spectrophotometrically at 25°C, by the method of Green & Ziegler (1963), with or without added cytochrome *c* (oxidized form, 100μg/ml). Saccharase was determined polarimetrically (Weidenhagen, 1941). Convenient dilutions of the sample to be tested were added to a solution at 30°C containing 125mM-sucrose, 20mM-sodium acetate buffer, pH 4.6, and water to a final volume of 30ml. A sample (7ml) was at once pipetted into 7ml of 5% (w/v) Na_2CO_3 and filtered with a little Hyflo Supercel (Johns–Manville Co., New York, N.Y., U.S.A.); its optical rotation was read in a 20cm tube in a polarimeter within 2h. Further samples (7ml) were taken and similarly tested at intervals (5–30min), depending on the sample being tested. The extent of hydrolysis of the sucrose was calculated from the decrease in optical rotation. The esterase activity was determined colorimetrically at pH8, by studying the formation of β-naphthol with 0.25mM-β-naphthyl acetate as substrate (Nachlas & Seligman, 1949), or the diappearance of ester with 1mM-triacetin or 5mM-phenyl propionate as substrate (Hestrin, 1949). The deamidase activity was determined by incubating the sample at pH8 and 30°C, with 10mM-L-asparagine or 10mM-nicotinamide, in the presence of toluene (2%, v/v). The samples taken from the reaction mixture at appropriate times were centrifuged and the ammonium nitrogen produced in the supernatant was removed by vacuum distillation at pH10 and 40°C, and, after the addition of concentrated alkali, the remaining amide nitrogen was removed by steam-distillation at 100°C and titrated with 5mM-H_2SO_4 (Varner, Bulen, Vanecko & Burrell, 1953). Acid phosphatase and alkaline phosphatase were assayed with *p*-nitrophenyl phosphate as substrate (Torriani, 1960) at pH3.5 and at pH9.0. A 1ml portion of sample suspension was added to 9ml of pre-warmed medium containing 2mM-*p*-nitrophenyl phosphate and 25mM-sodium succinate or tris–HCl buffer, with additions of $MgCl_2$ and KCl as indicated for each experiment. Each tube was incubated separately at 30°C with continuous shaking and the reaction was followed by taking samples (1ml) at appropriate time-intervals (15s–15min) into 10ml of 20mM-NaOH. After centrifugation, the amount of *p*-nitrophenol formed in the supernatant was determined from the extinction at 400nm. The ATPase* activities were measured as the rate of P_i release from ATP within the zero-order portion of the reaction. The samples (1ml, 0.9–1.1mg of dry matter/ml of reaction mixture) were incubated at 30°C in a reaction mixture containing 1–2mM-ATP, 25mM-sodium succinate, tris–maleate or tris–HCl buffer (at the pH values shown in the legends, along with the other additions) in a total volume of 10ml. The samples were withdrawn at intervals (15s–10min), the reaction was stopped by the addition of $HClO_4$ (5%, w/v), and the P_i liberated was assayed by measurement of the extinction at 310nm of the phosphomolybdic acid complex after extraction with butyl acetate (Wadelin & Mellon, 1953). The values were corrected for non-enzymic degradation of the ATP and for the liberation of P_i in the absence of ATP. The breakdown of ADP, TPP and PP_i ($Na_4P_2O_7$) induced by the samples was determined in a similar way.

Electron microscopy. After fixation with osmium tetroxide, the samples were dehydrated with successive concentrations of ethanol and propylene oxide before being embedded in epoxy resin. Electron micrographs of thin sections were taken with a Philips EM200 electron microscope. Some preparations were also shadowed with an alloy of platinum and palladium by application of the normal shadow-casting technique.

* Abbreviations: ATPase, adenosine triphosphatase; TPP, thiamin pyrophosphate.

RESULTS AND DISCUSSION

Overall composition. The chemical composition of the isolated cell walls and the changes that occur during enzymic digestion have been presented in detail (Suomalainen *et al.* 1967*b*,*c*). The isolated cell walls were found to contain about 83% carbohydrate, 10% protein, 3% lipid, including 0.7% phospholipid, 0.45% sterol, 0.2% RNA and 0.04% DNA (percentages of dry weight of cell walls). The cell walls accounted for about 20% of the dry weight of the cells. The content of phospholipids and sterols supports the view that membrane material is present. The amount of nucleic acids is low. This fact was also established by comparing the effect of ribonuclease on the isolated cell walls with its effect on intact cells and partly emptied cells under the same conditions (Fig. 1). The amount of u.v.-absorbing compounds

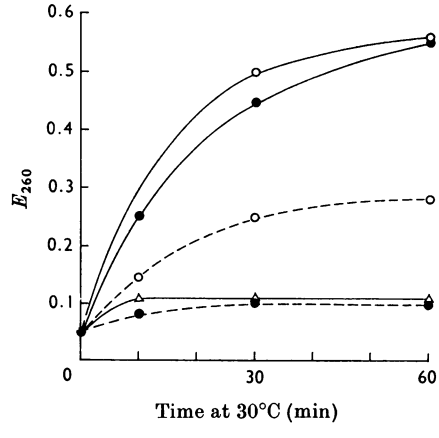

Fig. 1. Release of u.v.-absorbing compounds by ribonuclease from intact yeast cells (●), partly emptied cells (○) and cell wall fraction (△) with (----) or without (——) 10mM-Mg^{2+}. The incubation mixture contained 0.5mg dry wt. of sample and 0.2mg of ribonuclease/ml of water with or without 10mM-Mg^{2+}. After incubation at 30°C, the test samples were clarified by centrifugation, and the extinction at 260nm was measured (cf. Alper, Dainko & Schlenk, 1967). The same curve (△) was obtained for cell walls with and without added Mg^{2+}.

released from the cell walls was very low, the results with and without added Mg^{2+} being almost identical. The effect of added Mg^{2+} is clearly observable with intact and partly emptied cells.

When the cell walls were digested with snail gut enzymes, until the preparation contained about 15% of the carbohydrates originally present in the cell walls, it was found that the protein and lipid content as a percentage of dry weight increased (Table 1). The light sediment, obtained by centrifugation, amounted to about 5% of the dry weight of the original cell walls and consisted largely of protein and lipids. Further, it contained an appreciable amount of carbohydrates.

Hexokinase, alkaline phosphatase, esterase, NADH oxidase and deamidase. An extensive study of the distribution of various enzymes among the subcellular fractions obtained by isolation of the cell walls revealed the absence, or the presence of no more than a negligible activity, of hexokinase, alkaline phosphatase, esterase and the NADH oxidase system in the cell walls (Table 2). Moreover, only a negligible activity of deamidase was detectable in the cell walls. The bulk of activities was found in the cell interior fraction separated during the first centrifugation after the disruption of cells. The amounts found in both the washings (added to the cell interior) and the unemptied cells were low. The activities of esterase and deamidase were also tested in fractions prepared in tris buffer without additions, with 10mM-Mg^{2+} or with 1mM-EDTA. The distribution was similar in all cases. These enzymes have been found inside the yeast cell. However, special attention has been paid to the distribution of hexokinase, in view of the claim that it is superficially located in yeast (Rothstein, Jennings, Demis & Bruce, 1959). The present results are in agreement with those obtained by Boulton (1965) who found that no more than a small proportion of the total hexokinase activity was associated with the membranes proper, e.g. with the plasma membrane. Nevertheless, the relatively high activity of NADH oxidase found by Boulton (1965) in membranous fractions isolated from protoplast lysate may be derived from mitochondrial membranes. The isolated cell walls represented about 20% of the total dry matter of the cells, and thus it can be considered that their isolation was almost quantitative. It may consequently be assumed, on the basis of the results presented in Table 2, that these enzyme activities exist only in the interior of the cell and are not present in the cell wall or plasma membrane of yeast. The results can also be used as a criterion for the purity of the isolated cell walls, which were thus practically free from contamination originating from the cell interior.

Saccharase. The saccharase content of the isolated cell walls is particularly noteworthy in relation to the

Table 1. *Overall chemical composition of isolated cell walls and fractions obtained after partial digestion*

The results are expressed as mg/100mg dry wt. of original cell walls.

Preparation	Carbohydrates	Protein	Lipids
Cell walls	83	10	3.1
Digested preparation before fractionation	14.3	5.5	3.8
Heavy sediment	12.6	4.2	2.4
Light sediment	1.2	1.6	1.4
Supernatant	<0.5	<0.1	<0.5

Table 2. *Distribution of the total activities of hexokinase, alkaline phosphatase, esterase, the NADH oxidase system and deamidase between cell walls and cell interior*

Total activities are expressed as μmol/min per fraction obtained from 1g fresh wt. of cells (values in parentheses refer to percentage of total activity found in the fraction). The 'cell interior' is the 10min 1000*g* supernatant separated by the first centrifugation after the disruption of cells including washings. Recovery was calculated from the amount found in the disintegrated preparation after removing the unemptied cells. N.D., not determined.

Enzyme	Substrate	Total activity: Cell walls	Total activity: Cell interior	Recovery after fractionation (%)
Hexokinase	D-Glucose	0 (0)	34.9 (100)	97
Alkaline phosphatase	*p*-Nitrophenyl phosphate	0.03 (1)	3.32 (99)	116
Esterase	β-Naphthyl acetate	0 (0)	1.11 (100)	71
	Phenyl propionate	0.05 (2)	2.6 (98)	81
	Triacetin	0 (0)	0.032 (100)	81
NADH oxidase system	NADH			
without added cytochrome *c*		0.14 (2)	6.34 (98)	N.D.
with added cytochrome *c*		0.18 (2)	9.36 (98)	N.D.
Deamidase	L-Asparagine	0.008 (2)	0.385 (98)	112
	Nicotinamide	0.004 (3)	0.145 (97)	98

Table 3. *Effect of the medium on the distribution of saccharase during disintegration of cells*

Total activities are expressed as μmol/min per fraction obtained from 1g fresh wt. of cells (values in parentheses refer to percentages of total activity found in the fraction). Saccharase activity of intact cells was in Expt. I 4640 and in Expt. II 4430. The duration of disruption of cells was 5min by our standard procedure; when longer disruption times (10 or 15min) were used the amount of saccharase found in the 10min 1000*g* supernatant increased by 2 and 3% respectively. BPA, bovine plasma albumin.

Medium	Additions to medium	Expt. no.	Disintegrated preparation	Total activity: 10min 1000*g* supernatant	Total activity: 10min 1000*g* sediment
Water	None	I	4720	3060 (65)	1640 (35)
	0.2% BPA	I	4800	2880 (60)	1870 (39)
	1mM-EDTA	I	4640	2780 (60)	1890 (41)
	10mM-Mg^{2+}	I	4760	2780 (58)	1930 (41)
0.1M-Sodium acetate buffer, pH4.6	None	I	4520	2640 (58)	1860 (41)
0.1M-Potassium phosphate buffer, pH7.0	None	I	4480	3280 (73)	1160 (26)
	10mM-Mg^{2+}	I	4560	3100 (68)	1380 (30)
25mM-Tris–HCl buffer, pH7.2	None	I	4640	2860 (62)	1890 (41)*
50mM-Tris–HCl buffer, pH7.2, containing 0.2% BPA	None	II	4440	2780 (63)	1690 (38)
	1mM-EDTA	II	4040	2620 (65)	1620 (40)
	10mM-Mg^{2+}	II	4240	2460 (58)	1820 (43)

* When the sediment was fractionated further and the unemptied cells were removed by using (*a*) 25mM-tris–HCl buffer, pH7.2, (*b*) 25mM-tris–HCl buffer, pH7.2, containing 1mM-EDTA, or (*c*)25 mM-tris–HCl buffer, pH7.2, containing 10mM-Mg^{2+}, the total activities found in the pure cell-wall fraction were (*a*) 9%, (*b*) 10% and (*c*) 12% of the original activities.

preservation of the plasma membrane in isolated cell walls. If it is correct that saccharase is located mostly in the space between the yeast cell wall and the plasma membrane (cf. Burger, Bacon & Bacon, 1961; Gascón & Ottolenghi, 1967), one logical consequence would be that the saccharase content of the isolated cell walls would indicate that parts of the plasma membrane are still attached to the isolated cell walls. Accordingly, an investigation was made of the saccharase content of cell walls, by using different media to isolate the cell walls. As Table 3 shows, in our experiments about 60–70% of the total activity of saccharase was found in the medium after disruption of the cells. The rest, 30–40%, was sedimentable on centrifugation for 10min at 1000*g*. The saccharase content remaining in the isolated cell walls represented about 10% of the total activity. The distribution of saccharase during the mechanical disruption of the cells and the isolation of the cell walls, was hardly affected by the saccharase content of the yeast used, the pH of the medium within the range used, the presence of EDTA or the bivalent cation Mg^{2+}, which is known to stabilize membrane structures. The largest amounts that remained in the 10min 1000*g* sediment and further in the isolated cell walls were attained in the presence of Mg^{2+}.

On preparation of yeast protoplasts, the bulk of the saccharase is released into the surrounding medium (Sutton & Lampen, 1962; Islam & Lampen, 1962; Millbank, 1963; Burger, Bacon, Bacon & Millbank, 1965; Suomalainen, Nurminen & Oura, 1967*a*), although there is some disagreement on the extent to which protoplasts retain saccharase activity. Nevertheless, it has been found that the saccharase activity of the isolated cell walls of yeast represents no more than a small percentage of the total activity of the whole cell (Eddy & Williamson, 1959; McMurrough & Rose, 1967). Saccharase is not eluted from intact cells of *S. cerevisiae* by potassium chloride or 2-mercaptoethanol (Weimberg & Orton, 1966), and is accordingly most probably not attached to the cell wall by a combination of electrostatic forces and disulphide bonds. Our experience has indicated that carefully prepared cell walls contain a considerable proportion of the total activity of saccharase, but, the more the cell walls are disintegrated and washed, the lower is the amount of saccharase remaining in the cell walls (cf. Suomalainen & Arkima, 1961). This observation supports the view that damage, or even the complete removal, of the plasma membrane is at least an important factor that exercises an effect on the leakage of saccharase during the preparation of cell walls.

During enzymic digestion and the following fractionation, the bulk of the saccharase of the isolated cell-wall preparation was released into the surrounding medium (Table 4). This observation corresponds with that noted in the preparation of

Table 4. *Behaviour of the saccharase activity of the isolated cell walls during enzymic digestion and fractionation*

Total activities are expressed as μmoles/min per fraction obtained from 1 g fresh wt. of cells (values in parentheses refer to percentage of total activity found in the preparation).

Preparation	Total activity	
Cell walls	532	(100)
Released into medium	450	(85)
Heavy sediment	73	(14)
Light sediment	23	(4)
Supernatant	25	(5)
Recovery (%)		108

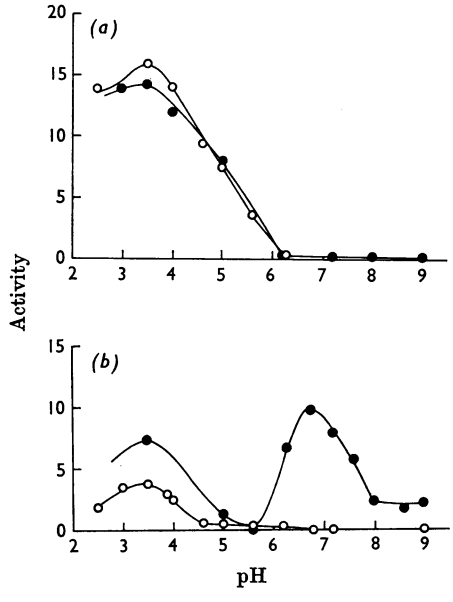

Fig. 2. pH–activity curves of the hydrolysis of externally added *p*-nitrophenyl phosphate (*a*) and of ATP (*b*) by intact cells (○) and by isolated cell walls (●). The reaction mixture contained 2 mM-*p*-nitrophenyl phosphate (*a*) or 1 mM-ATP (*b*) buffered with 25 mM-sodium succinate (pH 2.5–5.6) or 25 mM-tris (pH 6.2–9.0) and 0.9–1.1 mg dry wt. of yeast preparation/ml; 3 mM-Mg^{2+} and 10 mM-K^+ were added within the pH range 6.2–9.0. At pH values below 6.2 these additions did not increase the activities. Activity is expressed as nmol/min per mg dry wt.

yeast protoplasts, suggesting that saccharase may be for the most part located in the cell wall outside the plasma membrane.

Acid phosphatase, ATPases and related activities. Experiments with intact and disintegrated cells and

Table 5. *Hydrolysis of ATP, ADP, TPP and PP_i at pH 3–4 by intact yeast cells and by isolated cell walls*

The reaction mixture contained 1 mM-substrate, 25 mM-sodium succinate buffer with or without 3 mM-$MgCl_2$ and 10 mM-KCl, and 0.9–1.1 mg dry wt. of yeast preparation/ml. The activities are expressed as nmol of P_i/min per mg dry wt.

		Activity			
		Intact cells			Cell walls
Substrate	Additions (Mg^{2+}+K^+) pH ...	3.0	3.5	4.0	3.5
ATP	–	3.5	3.8	2.5	7.4
	+	4.0	4.0	2.5	5.5
ADP	–	0.7	2.7	2.2	3.2
	+	3.0	2.7	1.5	2.8
TPP	–	4.2	4.4	2.9	4.0
	+	4.0	5.2	2.7	5.3
$Na_4P_2O_7$	–	3.9	3.5	2.6	7.1
	+	3.7	3.2	1.9	6.4

application of the protoplast technique have demonstrated that the acid phosphatase of baker's yeast is mainly located on the surface of the cell, whereas the alkaline phosphatase is found inside the cell (Suomalainen, Linko & Oura, 1960; McLellan & Lampen, 1963; Tonino & Steyn-Parvé, 1963; Suomalainen *et al.* 1967*a*). It has been reported that the acid phosphatase is of low substrate specificity (Tonino & Steyn-Parvé, 1963). However, the opinion has been expressed that a number of different acid phosphatases are located on the cell surface of yeast (Rothstein & Meier, 1948).

The pH–activity curves of phosphatase and ATPase activities obtained in these experiments with intact cells and isolated cell walls are illustrated in Fig. 2. In both cases the acid phosphatase activity, with a pH optimum of 3.5 and almost zero activity at pH 6 and above, is observed with *p*-nitrophenyl phosphate as substrate. Alkaline phosphatase does not occur in the preparations, as neither intact cells nor isolated cell walls exhibit any activity towards *p*-nitrophenyl phosphate at alkaline pH values. Both preparations show ATPase activity at pH 3.5, but the isolated cell walls additionally contain ATPase activity at neutral pH values, where the intact cells have almost zero activity. At pH 3–4, also ADP, TPP and PP_i are hydrolysed at about the same rate as ATP (Table 5). The pH–activity curves for ATP, ADP and TPP were similar, with an optimum at about 3.5 in each case. However, the PP_i had a lower pH optimum, at about 3.0. In each experiment, the rate of hydrolysis of ADP was also lower than that of ATP. Additions of Mg^{2+} and K^+ had no or only little effect on the rate of hydrolysis of externally

Table 6. *Behaviour of phosphomonoesterase, pyrophosphatase and ATPase activities appearing at* pH 3.5 *in the isolated cell walls during enzymic digestion and fractionation*

Expt. I used baker's yeast from the end of the exponential growth phase, and Expts. II and III used commercial baker's yeast. The activities were determined as described in Table 5 with 2mM-*p*-nitrophenyl phosphate, 1mM-ATP, 1mM-TPP or 1mM-$Na_4P_2O_7$ as substrate at pH 3.5 without added Mg^{2+} and K^+. Total activities are expressed as nmol/min per fraction obtained from 1g fresh wt. of cells (values in parentheses refer to percentage of total activity recovered found in the preparation).

	Activity				
Substrate ...	*p*-Nitrophenyl phosphate		$Na_4P_2O_7$	ATP	
Preparation	Expt. I	Expt. II	Expt. II	Expt. II	Expt. III
Cell walls	805 (100)	795 (100)	560 (100)	360 (100)	638 (100)
Released into medium	625 (78)	400 (50)	460 (82)	380 (105)	688 (108)
Heavy sediment	133 (16)	196 (25)	270 (48)	60 (17)	87 (14)
Light sediment	53 (7)	99 (12)	20 (4)	43 (12)	85 (13)
Supernatant	13 (2)	10 (1)	12 (2)	0 (0)	0 (0)
Recovery (%)	103	89	135	134	135

Table 7. *Behaviour of* Mg^{2+}*-dependent ATPase of the isolated cell walls during enzymic digestion and the following fractionation*

The reaction mixture contained 1mM-ATP, 3mM-Mg^{2+}, 10mM-K^+ and 25mM-tris–maleate buffer, pH 6.8. In Expt. II 10μg of oligomycin/ml was added to the reaction mixture. Total activities are expressed as nmol/min per fraction obtained from 1g fresh wt. of cells (values in parentheses refer to percentage of total activity found in the preparation).

	Activity			
Preparation	Expt. I		Expt. II	
Cell walls	525	(100)	170	(100)
Released into medium	0	(0)	0	(0)
Heavy sediment	88	(17)	55	(32)
Light sediment	262	(50)	173	(102)
Supernatant	0	(0)	0	(0)
Recovery (%)		67		134

added substrates studied at pH 3–4; the hydrolysis of ADP at pH 3.0 was an exception. About 20–25% of the total enzyme activities appearing at pH 3.5 remained bound in the cell wall on disintegration of the cells and isolation of the cell walls. As with saccharase, the composition of the medium did not exert any major effect on the distribution.

During enzymic digestion and the following fractionation the activities appearing in the cell walls at pH 3.5 behaved in the way indicated in Table 6. As with saccharase, the bulk of these enzyme activities was released into the medium. The residual activity found in the sediments might be attributable to the remnants of the cell walls rather than being a characteristic of the plasma membrane.

In addition to the activities that appeared at pH 3–4, the isolated cell walls contained ATPase activity at neutral pH that was not detected with intact cells. About 15% of the total activity of ATPase at pH 6.8 in disintegrated preparation was found in the isolated cell walls. The distribution of Mg^{2+}-dependent ATPase at pH 6.8, after the enzymic digestion of the isolated cell walls, is presented in Table 7. The bulk of the total activity was found in the isolated light sediment, where also the specific activity was highest. By the use in the reaction mixture of a high concentration of oligomycin, which inhibits a mitochondrial ATPase of aerobic yeast (Schatz, 1965), it was found that in particular the oligomycin-insensitive part of the ATPase activity was a characteristic of the isolated light sediment. In contrast with the ATPase active at pH 3–4, which was mostly solubilized during digestion, the ATPase active at neutral pH must be located in the plasma membrane itself, rather than in the cell wall or in the space between the cell wall and plasma membrane (cf. Boulton, 1965). This ATPase has, also in contrast with the enzyme active at pH 3–4, an absolute Mg^{2+} requirement, as is shown in Table 8. The optimum concentration of Mg^{2+} was about equivalent to that of tris–ATP. At higher Mg^{2+} concentrations the activity diminished. When added alone K^+ had a stimulatory effect, although this was less marked when K^+ was added together with Mg^{2+}. When the substrate was the disodium salt of ATP Na^+ had no effect, or even an inhibitory one, but when ATP was used as its tris salt Na^+ also slightly increased the activity. However, Na^+ could not increase the activity when Na^+ was added together with Mg^{2+} and K^+. In this respect the ATPase of the plasma membrane in yeast obviously differs from the ATPase activity that requires Na^+, K^+ and Mg^{2+}, and is a part of the enzyme system for the transport of Na^+

Table 8. *Properties of the plasma-membrane-bound ATPase*

The reaction mixture contained 2mM-tris–ATP, 25mM-tris–maleate or tris–HCl buffer, with the additions indicated and the cell-wall preparation (0.9–1.1mg dry wt./ml). The activities are expressed as nmol of of P_i/mg dry wt. Results are given as individual values, for two experiments, or as a range when more than two experiments were carried out (mean values are given in parentheses).

pH	Additions to medium	Activity
6.2	None	0–0.3 (0.1)
	2mM-Mg^{2+}, 10mM-K^+	1.6–2.5 (2.1)
6.8	None	0–0.3 (0.1)
	1mM-Mg^{2+}	5.4
	2mM-Mg^{2+}	7.5, 7.6 (7.6)
	6mM-Mg^{2+}	4.8
	10mM-K^+	2.0
	10mM-Na^+	0.9–1.3 (1.1)
	10mM-K^+, 10mM-Na^+	1.1
	2mM-Mg^{2+}, 10mM-K^+	7.8, 8.0 (7.9)
	2mM-Mg^{2+}, 10mM-Na^+	7.9, 8.3 (8.1)
	2mM-Mg^{2+}, 10mM-K^+, 10mM-Na^+	4.9, 5.4 (5.2)
	2mM-Mg^{2+}, 10mM-K^+, oligomycin (10μg/ml)	1.3, 1.5 (1.4)
7.2	None	0
	2mM-Mg^{2+}, 10mM-K^+	6.4
7.6	None	0.9
	2mM-Mg^{2+}, 10mM-K^+	2.4

Table 9. *Effect of the composition of medium used during isolation of cell walls on the activity of* Mg^{2+}*-dependent ATPase*

The reaction mixture contained 2mM-tris–ATP, 25mM-tris–maleate buffer, pH6.8, with additions indicated, and the cell wall preparation (0.9–1.1mg dry wt./ml). The cell walls were isolated at the same time in 25mM-tris–HCl buffer, pH7.2, without additions (Expt. I), with 1mM-EDTA (Expt. II) and with 10mM-$MgCl_2$ (Expt. III). Activities are expressed as nmol of P_i/min per mg of dry wt.

	Activity		
Additions to medium	Expt. I	Expt. II	Expt. III
None	0	0	14.2
1mM-Mg^{2+}, 10mM-K^+			15.6
2mM-Mg^{2+}, 10mM-K^+	5.0	4.9	15.2
3mM-Mg^{2+}, 10mM-K^+	6.0	4.5	11.7
4mM-Mg^{2+}, 10mM-K^+	5.9		

outward and K^+ inward across plasma membranes in many animal tissues.

Slight activity with TPP or ADP as substrate was also noted at neutral pH values, although this activity was lower than that appearing at pH3–4. It was much lower than the activity observed under the same conditions with ATP as substrate, and Mg^{2+} did not exert any noteworthy effect on it.

By isolation of the cell walls in the presence of Mg^{2+} it was possible to increase the amount of Mg^{2+}-dependent ATPase remaining in the cell walls (Table 9). When the cell walls were isolated in the presence of Mg^{2+} a high activity was detectable without the addition of Mg^{2+} to the reaction mixture. It was impossible, by the addition of Mg^{2+}, to raise the activity appearing in the cell walls isolated in the absence of Mg^{2+} to the same value. This suggests that Mg^{2+} promotes the preservation of the plasma membrane in the cell walls during the course of isolation.

The fate of the plasma membrane of yeast when mechanically disintegrated cells are used has been problematical. The extent to which various preparations, such as mitochondrial fractions, made by the fractionation of mechanically disrupted cells, includes fragments of the plasma membrane, has never been clarified. A fraction has been isolated from the microsomal fraction of a mechanically prepared cell-free extract of anaerobic yeast (Matile, Moor & Mühlethaler, 1967), a fraction that should, in accordance with the surface structure, contain fragments of plasma membrane. This does not involve any real discrepancy. Our working hypothesis is based on the well-established assumption that saccharase and acid phosphatase are located on the cell surface region, and the results obtained with regard to the saccharase content of isolated cell walls, for instance, agree with the view that under favourable conditions the plasma membrane remains only partially in the isolated cell wall. Consequently, various possibilities must exist for the acquisition of

further information on the plasma membrane and the cell surface region of yeast.

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