2. The two methods of sedimentation gave identical results and 80–95 % of the enzyme activity was found in the precipitate.

3. As the medium was made increasingly hypotonic, a greater proportion of the enzyme activity was released from the particles. Particles of different sizes, separated by fractional sedimentation, behaved comparably in this respect.

4. The distribution of enzyme activity over particles of different sizes was the same in infant liver and in liver regenerating after partial hepatectomy as in the normal adult tissue, but prepara-

tions of the growing or regenerating tissue showed greater release of enzyme activity to the solution on dilution with water.

5. The homogenates in isotonic media did not display their full glucuronidase activity on direct assay, but partial activation occurred under assay conditions. Full activity was displayed when about half the enzyme present was brought into solution.

6. The possible mechanism of activation and its physiological significance are discussed.

The author is in receipt of a grant from the Agricultural Research Council.

REFERENCES

- Fishman, W. H. & Talalay, P. (1947). *Science*, **105**, 131.
 Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.
 Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. (1949). *Biochem. J.* **44**, 487.
 Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. (1950). *Biochem. J.* **46**, 278.
 Kerr, L. M. H. & Levvy, G. A. (1951). *Biochem. J.* **48**, 209.
 Levvy, G. A., Kerr, L. M. H. & Campbell, J. G. (1948). *Biochem. J.* **42**, 462.
 Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
 Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
 Schneider, W. C. & Hogeboom, G. H. (1951). *Cancer Res.* **11**, 1.
 Talalay, P., Fishman, W. H. & Huggins, C. (1946). *J. biol. Chem.* **166**, 757.
 Walker, P. G. & Levvy, G. A. (1951). *Biochem. J.* **49**, 620.

The Chemical Composition and Structure of the Yeast Cell Wall

By D. H. NORTHCOTE

Biochemical Laboratory, University of Cambridge

AND R. W. HORNE

Cavendish Laboratory, University of Cambridge

(Received 18 June 1951)

The chemical composition of the yeast cell wall was first studied by Salkowski (1894), who investigated the polysaccharide material which remained after intact cells had been digested with dilute sodium hydroxide solution. Zechmeister & Tóth (1934) continued the study, but again disrupted the cells by fairly vigorous chemical action. They suggested, however, that an enzymic method might do less damage to the cell wall, and indeed later they isolated the glucan component of the cell wall by the action of pepsin and amylase on an autolysed yeast suspension (Zechmeister & Tóth, 1936). These chemical and enzymic investigations have indicated that several polysaccharides may be present in the cell wall, and it has been suggested that, besides the glucan, a mannan (Haworth, Hirst & Isherwood, 1937) and possibly a 'glycogen' (Ling, Nanji & Panton, 1925) may form part of the structure; no direct evidence for this has been given. The structure of these polysaccharides has been investigated by

various workers. The general method of isolation has been extraction of whole yeast with 3 % sodium hydroxide solution at 100°, whereby the mannan and some glycogen go into solution, whereas the glucan and most of the glycogen remain as the insoluble material, which is seen under the microscope to retain the shape of the cell, and is therefore assumed to be part of the cell wall. The glucan was shown to contain only glucose with β -1:3 linkages between the radicals, by Zechmeister & Tóth (1934) and Hassid, Joslyn & McCready (1941); more recently Bell & Northcote (1950) found that this polysaccharide was highly branched and determined its average chain length. The structure of the mannan has been studied by Haworth *et al.* (1937), Haworth, Heath & Peat (1941) and Lindstedt (1945), but the constitution of the glycogen, and especially its possible existence in two forms (Ling *et al.* 1925; Daoud & Ling, 1931) has received little attention.

In the present work the yeast cell wall has been isolated in two ways:

A. By physical methods entailing mechanical breakage of the cell and isolation of the washed cell wall by differential centrifugation.

B. By chemical methods, entailing breakage and isolation, similar to those used by the previous workers, i.e. digestion of the whole cell with 3% sodium hydroxide.

In this way a more complete and quantitative survey of the substances making up the structural elements of the outer cell wall has been obtained than was hitherto possible. Both a chemical and a microscopical examination of the preparation have been made and in this latter connexion the material has been found to be very suitable for investigation by the electron microscope.

EXPERIMENTAL

Material used and general analytical methods

The yeast used was a commercial pressed baker's yeast (Ark Yeast, Distillers' Co. Ltd.). Its dry weight, determined under the same conditions as those used with the cell wall preparations, was 27.3% of the moist weight.

All the analyses were carried out on material dried at 0.01 mm. Hg over P_2O_5 at room temperature.

Total N (Kjeldahl) was determined using Nessler's reagent (Umbreit, Burris & Stauffer, 1949). Total P was determined according to Fiske & Subbarow (1925, cf. Umbreit *et al.* 1949).

Chromatography of sugars. Descending chromatograms were run on Whatman no. 1 papers with *n*-butanol/water at 37° for 60 hr. (Hough, Jones & Wadman, 1950). Glucose, galactose, glucosamine, mannose and arabinose were used as markers. The spots were coloured with aniline hydrogen phthalate (Partridge, 1949) and ammoniacal $AgNO_3$ on duplicate papers.

Chromatography of amino-acids. Descending chromatograms were run with phenol/0.3% (w/v) aqueous NH_3 at 20° in an atmosphere of coal gas (Consden, Gordon & Martin, 1944). The spots were coloured with 0.3% (w/v) triketohydrindene hydrate in *n*-butanol.

The solutions applied to the chromatograms were adjusted so that the approximate concentration of the sugars and the amino-acids was never less than 1%.

The work below is described in two parts, dealing with cell wall material obtained by mechanical breakage of the cell, preparation A, and that obtained by chemical cytolysis of the cell, preparation B.

PREPARATION A

Isolation of the cell wall material by mechanical breakage of the cell

Yeast (700 mg.) was suspended in 10 ml. of water with 4 g. of fine glass beads (Ballotini no. 12, Chance Bros Ltd., Birmingham) and the mixture placed in a vertical cup (internal measurements 5 × 2.2 cm.) of a Mickle cell disintegrator (Mickle, 1948). Vibration was continued for 30 min., after which the resultant suspension was decanted away

from the glass beads. The supernatant was centrifuged at 1500 g for 10 min. (height of tube 10 cm.), and the residue washed repeatedly with water until the final supernatant became clear; the residue comprised the required cell wall fraction. The initial supernatant and the washings were combined and centrifuged at 14 000 g for 20 min. (height of tube 10 cm.), when a residue of fine particles was obtained. The final supernatant and the two residues were freeze-dried. The yields of material from three representative fractionations are shown in Table 1. The beads cannot have contributed much to these fractions since their ash content never exceeded 3%.

Table 1. *Yield of fractions obtained by differential centrifugation of mechanically disintegrated yeast cells*

Exp. no.	mg./100 mg. dry wt. taken			
	Residue deposited at 1500 g (cell wall fraction)	Residue deposited at 14 000 g (small particle fraction)	Supernatant ('soluble' material)	Recovery (%)
1	15.3	30.1	44.4	89.8
2	14.1	28.4	41.8	84.3
3	14.5	30.3	42.0	86.8

Microscopical examination of cell wall

The cell walls isolated in the above manner are Gram-negative, whereas the whole yeast cells are Gram-positive. It is thus an easy matter to distinguish any unbroken cells in the preparations by a microscopical examination of stained films. Many fields of numerous preparations were examined and in no case was a whole cell detected. The cell walls were also examined by the phase-contrast microscope and here again no whole cells and very little debris could be detected. It is concluded that the material isolated does represent solely the outer membranes of the cell, and that chemical investigations carried out on it will in fact characterize the cell wall which is thought to be made up of these membranes.

Electron microscope. A comprehensive examination of the cell wall was made under the electron microscope using both Siemens and Radio Corporation of America (R.C.A.) instruments. The high tension voltages used ranged from 50 to 90 kV. The material was suspended in water and allowed to dry at room temperature in air on the filmed specimen grids. Films of two types were used as supporting membranes, these being mounted on standard Kodak copper grids. Early preparations were mounted on nitro-cellulose supporting films, but owing to the relatively large size of yeast cells these films frequently ruptured during investigation in the microscope. 'Formvar' (polyvinyl formal) films prepared from dioxan solution were found to be much stronger, particularly when large numbers of cells were present in the field under examination. Preparations were shadowed with an alloy of Au and Pd employing the usual shadow-casting techniques. The shadowing angle was 45°.

The most characteristic and obvious detail in the structure of the cell walls was the occurrence of scars on the surface; these appeared on the majority of the larger cells but not on the smaller cells. Some cells carried as many as sixteen such markings (Fig. 4a). By comparisons with those cells to which were attached parts of the cell wall of the bud, and

with that of an intact cell in the process of budding (Fig. 1), these markings were identified as budding scars and appeared as circular thickenings of the cell surface (Figs. 2 and 4a-d). The figures are metal-shadowed electron micrographs. The bud scars could also be seen on the cell walls under the phase-contrast microscope. It is of interest to note that Dorsten, Oosterkamp & Le Poole (1947) used whole yeast cells for testing the 400 kV. experimental electron microscope and the pictures taken by their instrument show indications of these budding scars in the whole cell. When the preparations were treated with 2N-HCl the scars appeared to be more resistant to chemical attack than the general cell surface. By observations of the edge of the cells and of the apparent peeling of the membranes which often occurred at the bud scar (Fig. 2) it could be seen that the cell envelope consisted of at least two membranes. When the preparations were extracted successively with methanol and ether (see below) to remove lipids, and re-examined, the division into two membranes became obvious (Fig. 5). However, after treatment with 3% (w/v) aqueous NaOH solution to remove mannan, protein, and lipid (see below) only one membrane is apparent at the bud scars (cf. Figs. 2 and 4).

The type of breakage resulting from disruption by glass beads, as applied to yeast cells, can be seen from the electron micrographs (Figs. 3 and 4d).

Chemical investigation of the cell wall

Elementary analysis. Total N, 2.1%, indicating a protein content of approx. 13%; total P, 0.31%.

Mineral content. When maintained at red heat in a platinum boat in a stream of clean air for 1 hr. 17.21 mg. yielded a white ash (0.55 mg., 3.21%).

Lipid content. The lipid was isolated by boiling the cell walls (17.39 mg.) in 95% (v/v) aqueous methanol (1 ml.) for 30 min. and subsequently extracting continuously with ether in a modified Soxhlet apparatus (Mitchell, 1951) at room temperature for 6 hr., the whole process being repeated three times. The ether was evaporated and the fat weighed directly. The yield was 1.48 mg. (8.5%). (Found: N, 0.1; P, 0.5%). The fat thus appears to contain little phospholipid.

Acid hydrolysis. The preparation (8.40 mg.) was hydrolysed for 6 hr. with 1 ml. of 2N-HCl in a sealed tube at 98°; it dissolved completely to give a very light brown solution. This was evaporated to dryness at 20° under reduced pressure and dissolved in 0.1 ml. of water. The resultant solution was investigated on paper chromatograms. Only two sugars were apparent in the hydrolysate, namely mannose and glucose; no glucosamine could be detected. Although hydrolysis of protein was probably incomplete, the hydrolysate also showed the presence of glutamic acid, aspartic acid, serine, glycine, threonine and alanine with only very faint indications of histidine, leucine and the other basic amino-acids.

Water content. The cell wall material (10.8 mg.) was dried over P_2O_5 at 100° and 0.01 mm. Hg for 8 hr.; there was no loss in weight. Dried material (100 mg.) exposed to air at room temperature for 48 hr. increased in weight by 11.2%.

Polysaccharides of the cell wall

Isolation and investigation of the glucan component. The cell wall material (97.21 mg.) was digested with 3% (w/v) aqueous NaOH (2.0 ml.) for 6 hr. at 100°. The supernatant obtained after centrifuging was retained for examination of the dissolved mannan (see below). The insoluble residue was extracted consecutively with 3% NaOH at 100° for 6 hr.,

0.5N-acetic acid (2 ml.) at 75° for 6 hr. and finally washed with ethanol (2 ml.) and ether (2 ml.). The white solid was dried over P_2O_5 at 0.1 mm. Hg. Yield 27.9 mg. (28.8% of dry matter taken). (Found: N, 0.3; P, 0.0%).

Hydrolysis of 10 mg. of this substance with 2N-HCl (1 ml.) in a sealed tube at 100° for 6 hr. gave a light-brown solution. This was freeze-dried and the resultant solid dissolved in 0.1 ml. of water. The chromatograms of this solution showed the presence of glucose only.

The isolated material contained 98% glucose when this was estimated by anthrone (Seifter, Dayston, Novic & Muntwyler, 1950). An equivalent amount of glucan isolated from whole yeast by the method of Bell & Northcote (1950) gave 97.4% glucose.

The substance isolated from the cell wall contained only glucose and was very insoluble in water, thus resembling the glucan isolated from whole yeast. It still retained in most cases the shape of the cell wall when examined under the microscope, and thus it constitutes at least part of a continuous membrane within the cell wall.

Isolation and investigation of the mannan component. The solution obtained in the above experiment was made just acid to methyl red with acetic acid, and the mannan was precipitated by 4 vol. of ethanol. The precipitate was centrifuged and separated from the supernatant, redissolved in water and reprecipitated. The white solid thus obtained was washed with ethanol and ether and dried in the normal manner. Yield, 30.1 mg. (31.0% of original dry matter). $[\alpha]_D^{20} + 87^\circ$ (l, 2; c, 1 in water). (Found: N, 1.3; P, 0.26%).

This material (20 mg.) was hydrolysed as above for the glucan and gave a colourless solution. The hydrolysate was freeze-dried and dissolved in 1 ml. of water. Mannose phenylhydrazine was prepared from this solution according to the method of Nowatnowna (1936). Yield 20.2 mg. (i.e. 60% mannose); m.p. 196° (uncorr.) not depressed on admixture of an authentic sample of mannose phenylhydrazine (m.p. 196°). The cell wall mannan (30.0 mg.) was further purified by precipitation and subsequent decomposition of the copper compound (Haworth *et al.* 1937). Yield 25.0 mg. (N, 1.0; P, 0.2%). $[\alpha]_D^{20} + 88^\circ$ (l, 2; c, 1.0 in water). The hydrolysed mannan showed only mannose on a paper chromatogram. The mannan could also be extracted from the cell wall material by water at 100°, although the rate of extraction was considerably lower; 25 mg. gave 2.5 mg. of mannan after 6 hr. and a further 2.4 mg. after 12 hr. The mannan isolated in this manner had a relatively high nitrogen content (N, 2.5; P, 0.23%) and gave a positive biuret reaction.

Glycogen. No glycogen could be isolated from the cell wall preparation by means of 0.5N-acetic acid (75° for 12 hr.) nor could any glycogen be detected in the cell wall by staining with iodine and subsequent microscopical examination. The small particle fraction isolated by differential centrifugation of the broken yeast cell did, however, stain a dark brown with iodine.

PREPARATION B

Isolation of the cell wall material by digestion of the cells with sodium hydroxide solution

The whole yeast (12.62 g.) was dispersed in 15 ml. of 3% (w/v) aqueous NaOH and heated on a boiling-water bath for 6 hr. The brown solution was centrifuged and the residue redigested with a further 15 ml. of 3% NaOH for 3 hr.; it was then washed with water, ethanol and ether and dried in the normal manner. Yield, 0.13 g.

Microscopical examination of cell wall

This cell wall material showed an electron-dense substance scattered in granular and particulate masses throughout the walls (Fig. 6). Part of this material seemed to be glycogen as it could be seen under the optical microscope to stain brown with iodine. It could be dissolved away with 0.5*N*-acetic acid at 75° (see below). Apart from this the electron microscope examination of this preparation showed little difference from that of preparation A.

Chemical investigation of the cell wall

Elementary analysis. Total N, 0.7%; total P, 0.07%.

Acid hydrolysis. The hydrolysis was carried out in the same manner as for preparation A. Only glucose was apparent in the hydrolysate when this was examined on paper chromatograms.

Polysaccharides of the cell wall

Isolation and investigation of the glycogen component. The cell wall material (130 mg.) was digested with 0.5*N*-acetic acid (5 ml.) for 6 hr. at 75°. The residue was centrifuged and re-extracted with acetic acid for a further 6 hr. The acetic acid solutions were combined and evaporated to small bulk under reduced pressure when the glycogen was precipitated from solution by the addition of 6 vol. of ethanol. Yield of glycogen 25.1 mg. (19.3% of the cell wall).

The glycogen could not be extracted with water from the cell wall, but after removal by dissolving in the dilute acetic acid and precipitation by ethanol the resultant material was very soluble in water to give a clear solution. This solution gave a deep red-brown colour with iodine.

Isolation and investigation of the glucan component. The residue obtained in the above experiment during the isolation of the glycogen corresponded to the glucan. It was washed with water, ethanol and ether and dried in the usual way. Yield, 93.3 mg. (71% of the cell wall). This glucan corresponded exactly in appearance, glucose content and solubility, with that isolated from whole yeast and from the cell wall preparation A.

DISCUSSION

The literature contains very little information concerning the chemical and enzymic nature of the outer cell walls of plant or animal cells, and it was with this in mind that we attempted in the present research to isolate a part at least of the cell wall of yeast which might lend itself to further studies. A preparation has been obtained by mechanical breakage and differential centrifugation which is not contaminated by whole cells or cell debris. This preparation can be made with a fairly constant composition, free from substances present in other fractions of the cell and thus a clear-cut separation of a definite morphological structure has probably been achieved. The wall as isolated is made up of two or more membranes; two in fact can be clearly

seen by means of the electron microscope. It has been possible by relating the chemical investigation to the microscopical studies to show that one of these two apparent membranes is, in part, composed of the glucan polysaccharide since this polysaccharide can be isolated free from other material, still retaining the general shape of the whole cell and obviously constituting a complete membrane. The other membrane remains intact after removal of lipid; but when the protein and mannan are removed from the cell wall preparation by means of dilute sodium hydroxide solution it is no longer visible and thus this second membrane is made up in part of protein or mannan or both of these substances. The general analysis of the cell wall shows an approximate chemical composition of glucan 29%, mannan 31%, protein 13%, lipid (mainly neutral fat) 8.5% and ash 3% which accounts for over 84% of the material. No other major constituent has been detected during the present investigation. Glycogen could only be obtained associated with the cell wall by using sodium hydroxide as a cytolysing agent. It is not present in the mechanically broken cell wall material, but appears among the fine particulate matter released by the breakage of the cell and the subsequent emptying of its contents. The glycogen present in the chemically prepared cell walls was seen to be present as scattered granules and it is thought that although it is probably closely associated with the cell wall in the living cell it does not form a structural part of the wall in the same way as the glucan and mannan.

The mannan can be extracted from the cell wall preparation A by means of hot water, and prepared in this manner it is found to be closely associated with protein which is extracted with it. This association in the cell wall had already been postulated by Garzuly-Janke (1940) from studies on the whole cell.

The observations carried out on the cell wall with the electron microscope and the phase-contrast microscope have shown clearly the existence on the cell surface of bud scars. These were first reported by Barton (1950) by microscopical examination of living and stained whole cells, although the observations were difficult and details of structure could not be obtained. The present work has shown that these scars are characterized by a circular thick edge slightly raised above the cell surface; the larger and presumably older cells carry a larger number of these scars. The edges of the scars are more resistant to chemical attack by dilute acid than is the general cell wall and this may indicate a difference in chemical composition. The existence of these scars must be important in relation to the process of budding and the behaviour of the cell wall as an osmotic boundary.

SUMMARY

1. A cell-wall fraction of yeast has been isolated after disintegrating the whole cells in a Mickle cell disintegrator and subsequently centrifuging.

2. The material isolated has been shown to be free of whole cells and cell debris.

3. A quantitative chemical analysis of the cell wall of yeast has shown it to be composed of protein, lipid and at least two polysaccharides, a mannan and a glucan. The glycogen associated with the cell wall preparation obtained by treatment of the whole cells with sodium hydroxide solution does not

function as a structural element and is not present in the fraction obtained by mechanical breakage.

4. The mannan is associated with a protein present in the cell wall.

5. The cell wall has been examined by the optical and electron microscopes and has been shown to consist of at least two membranes, one of which is made up in part of the glucan component.

6. The existence of bud scars on the cell wall has been confirmed and some details of their structure observed.

We wish to thank Dr V. E. Cosslett and Dr D. J. Bell for their advice and encouragement during this investigation.

REFERENCES

- Barton, A. A. (1950). *J. gen. Microbiol.* **4**, 84.
 Bell, D. J. & Northcote, D. H. (1950). *J. chem. Soc.* p. 1944.
 Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
 Daoud, K. & Ling, A. R. (1931). *J. Soc. chem. Ind., Lond.*, **50**, 365 T.
 Dorsten, A. C. van, Oosterkamp, W. J. & Le Poole, J. B. (1947). *Philips tech. Rev.* **9**, 193.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Garzuly-Janke, R. (1940). *J. prakt. Chem.* **156**, 45.
 Hassid, W. Z., Joslyn, M. A. & McCready, R. M. (1941). *J. Amer. chem. Soc.* **63**, 295.
 Haworth, W. N., Heath, R. L. & Peat, S. (1941). *J. chem. Soc.* p. 833.
 Haworth, W. N., Hirst, E. L. & Isherwood, F. A. (1937). *J. chem. Soc.* p. 784.
 Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
 Lindstedt, G. (1945). *Ark Kemi Min. Geol. A* **20**, No. 13.
 Ling, A. R., Nanji, R. D. & Pantou, F. J. (1925). *J. Inst. Brew.* **31**, 316.
 Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
 Mitchell, P. D. (1951). Personal communication.
 Nowatnowna, A. (1936). *Biochem. J.* **30**, 2177.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Salkowski, E. (1894). *Ber. dtsch. chem. Ges.* **27**, 3325.
 Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950). *Arch. Biochem.* **25**, 191.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd. ed. p. 190. Minneapolis: Burgess Publishing Co.
 Zechmeister, L. & Tóth, G. (1934). *Biochem. Z.* **270**, 309.
 Zechmeister, L. & Tóth, G. (1936). *Biochem. Z.* **284**, 133.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Intact yeast cell showing budding.

Fig. 2. Group of cell walls (preparation A) showing bud scars and peeling of two apparent membranes at the scars.

Fig. 3. Cell wall (preparation A) showing junction between two budding cells together with other bud scars.

Fig. 5. Fragment of cell wall (preparation A) after extraction of lipid showing two distinct membranes.

Fig. 6. Cell wall prepared by cytolysing whole cells with 3% NaOH (preparation B) showing the granular appearance of the glycogen.

PLATE 2

Fig. 4. Cell wall (preparation A) treated with 3% NaOH. (a) Cell membrane showing numerous bud scars and disappearance of double membrane apparent in Fig. 2. (b), (c) and (d) Showing, in particular, details of structure of cell membrane at the budding point; note thickening and raised appearance of the membrane.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 5.

Fig. 6.

D. H. NORTHCOTE AND R. W. HORNE—THE CHEMICAL COMPOSITION AND STRUCTURE
OF THE YEAST CELL WALL

Fig. 4 *a*.

Fig. 4 *b*.

Fig. 4 *c*.

Fig. 4 *d*.

D. H. NORTHCOTE AND R. W. HORNE—THE CHEMICAL COMPOSITION AND STRUCTURE
OF THE YEAST CELL WALL