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The Glucan Components of the Cell Wall of Baker's Yeast (*Saccharomyces cerevisiae*) Considered in Relation to its Ultrastructure

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1. Commercial pressed baker's yeast, and cell walls prepared from it, were extracted in various ways and the products examined by a number of techniques, including infrared spectroscopy and electron microscopy. 2. The glucan components of the walls cannot be extracted from intact yeast cells by 3% (w/v) sodium hydroxide at 75°, but at least one-third of the glucan of cell wall preparations is dissolved under these conditions, and more will dissolve after ultrasonic treatment. 3. If intact cells are given a preliminary treatment with acid the wall glucans dissolve in dilute aqueous alkali. 4. Acid conditions as mild as sodium acetate buffer, pH 5·0, for 3 hr. at 75° are sufficient for this preliminary treatment; the glucan then dissolves in 3% sodium hydroxide at 75° leaving a very small residue, which contains chitin and about 1% of the initial glucan of the wall. Dissolution is hindered by exclusion of air, or by a preliminary reduction with sodium borohydride, suggesting that some degradation of the glucan by alkali is taking place. 5. After treatment with 0·5 M-acetic acid for 24 hr. at 90° the glucan dissolves slowly at room temperature in 3% sodium hydroxide, or in dimethyl sulphoxide. The extraction with acetic acid removes glycogen and a predominantly β-(1→6)-linked glucan (not hitherto recognized as a component of baker's yeast), but none of the β-(1→3)-glucan, which remains water-insoluble. 6. Without treatment with acid, the glucan is not significantly soluble in dimethyl sulphoxide, but can be induced to dissolve by ultrasonic treatment. 7. These results are interpreted by postulating the presence of an enclosing membrane, composed of chitin and glucan, that when intact acts as a semi-permeable membrane preventing the escape of the alkali- and dimethyl sulphoxide-soluble fraction of the glucan. Mild acid treatments damage this membrane, and ultrasonic and ballistic disintegration disrupt it. 8. Some support for this hypothesis is given by the effects of certain enzyme preparations, which have been found to render a substantial part of the glucan extractable by dimethyl sulphoxide.

Two developments that have increased our understanding of the structure of the yeast cell wall in recent years are the use of wall fractions prepared by mechanical disruption of the cells and the application of various techniques of electron microscopy. The results have been reviewed by Northcote (1963*a*,*b*), Nickerson (1963), Phaff (1963) and Rogers & Perkins (1968).

During work on the degradation of microbial cell walls by microbial enzymes, some accounts of which have appeared (Jones & Webley, 1967, 1968; Webley, Follett & Taylor, 1967; Jones, Bacon, Farmer & Webley, 1968; Bacon, Jones, Farmer & Webley, 1968; and references below), we have made observations on the yeast cell wall that cannot be explained adequately by existing ideas of its structure. We now present these observations and give some reasons, mainly chemical, for postulating the existence of an alkali-stable layer composed of chitin and glucan. We have also (J. S. D. Bacon, D. Jones, I. F. Taylor & D. M. Webley, unpublished work) studied the action of hydrolytic enzymes on the cell wall, particularly as exemplified by the lytic action of culture filtrates of *Cytophaga johnsonii*; these studies give additional support to the tentative conclusions expressed here.

MATERIALS AND METHODS

Yeast. A commercial pressed baker's yeast (Distillers Co. Ltd., Glenochil, Clackmannan) was used throughout these experiments.

Yeast cell walls. Pressed yeast (50 g.) was suspended in water and centrifuged at 16000*g* at 1°. A thin dark layer was scraped off the top of the pellet and the remainder was

mixed with 65ml. of glass beads (Ballotini no. 9; Jencons Ltd., Hemel Hempstead, Herts.) in the 100ml. container of a Vibrogen Cell-mill (E. Bühler, Tübingen, W. Germany). Cooling fluid at 2° was circulated round the container for 10min., and vibration was applied for two 5min. periods, with a 5min. pause between to control the temperature. The mixture of beads and broken cells was filtered through Miracloth (Calbiochem Ltd., London W.1,) which retained the beads, with a total of 400ml. of ice-cold water. The suspension was centrifuged lightly (maximum of 2500*g* during a total of 15min.) and the supernatant discarded. The walls were then washed four times with water by centrifugation at 16000*g*. These treatments broke a large proportion of the cells and washed away much of the cell contents, reducing the bulk to about half. This was now mixed with 35ml. of glass beads in the 50ml. container and disintegrated for the minimum period (usually 2–3min.) needed to achieve complete breakage.

The walls were then separated and centrifuged as before, except that after each centrifugation at 16000*g* the pellet was cautiously resuspended in a mechanical shaker (Turbula Mixing Pulsator; Glen Creston Ltd., Stanmore, Middlesex) so that the heaviest part (which included dark particles of dirt) could be discarded. The removal of contents from the walls was checked microscopically, and when a satisfactory state was reached the walls were finally suspended in 50–70ml. of water. This was heated to 75° (in 5min.) and held there for 30min. to inactivate endogenous glucanases, then stored at 4° in the presence of toluene. A typical yield was 2g., i.e. 16% of the initial dry matter.

Alkali-extracted yeast. The procedure described by Bacon, Davidson, Jones & Taylor (1966), based on that of Bell & Northcote (1950), was used throughout, except that a fourth extraction was sometimes omitted because the third alkali extract already contained as little as 50mg. of carbohydrate/100g. of pressed yeast.

Enzyme preparations. Chitinase preparations were purchased from Koch–Light Laboratories Ltd., Colnbrook, Bucks., who kindly informed us that they were prepared from the culture filtrate of an unidentified actinomycete. They were slightly active against laminarin, but completely without action on lutean.

Other preparations are described in the text.

Polysaccharides. Insoluble laminarin was a gift from the Seaweed Research Institute, Musselburgh, Midlothian. Chitin was a commercial crustacean preparation from BDH (Chemicals) Ltd., Poole, Dorset. Lichenin was provided by Professor D. J. Manners, Heriot–Watt University, Edinburgh; a sample of the fungus *Poria cocos*, a source of pachyman (Warsi & Whelan, 1957), by Professor W. J. Whelan, University of Miami, Miami, Fla., U.S.A.; a glucan from *Candida parapsilosis* by Dr C. T. Bishop, National Research Council, Ottawa, Ont., Canada; and curdlan (Harada, Masada, Fujimori & Maeda, 1966; Saito, Misaki & Harada, 1968) by Professor T. Harada, Osaka University, Japan.

Lutean was prepared from the culture fluid of *Penicillium luteum* Zukal I.M.I. 44338 (kindly provided by the Commonwealth Mycological Insitute, Kew), which had been grown on Czapek–Dox medium containing 5% glucose for 4 weeks at 25°. The viscous fluid was separated from the mycelium by filtration through Miracloth, adjusted to pH10 with NaOH and warmed to 80°. Any drift of pH to acid values, due to hydrolysis of the malonyl-ester linkages in luteic acid, was corrected by further additions of alkali and heating was continued for 30min. The pH was then brought to 6 with acetic acid and 0·5vol. of ethanol was added. A fibrous precipitate formed, which was purified by suspending it in hot water, undissolved material being rejected, and reprecipitation was hastened by the addition of 0·5vol. of ethanol. The process was repeated and the product finally dissolved in hot water and freeze-dried. A typical yield was 1·7g. from 3l. of culture fluid; a further 0·5g. was obtained by washing the gelatinous mycelium at 20° with water.

Detection and analysis of carbohydrates. For comparisons of the quantities of polysaccharides extracted by various procedures the anthrone method of Fairbairn (1953) was used, with glucose standards; chitin gives practically no colour with this reagent. Reducing sugars were measured in a Technicon AutoAnalyzer with alkaline ferricyanide (Hoffman, 1937).

Chromatographic methods have been described previously (see Bacon, 1959). We use *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) as a general spraying reagent now that a carcinogenic hazard has been shown to be associated with the use of benzidine.

For qualitative examination of sugar composition, samples were hydrolysed at 100° for 5hr. in sealed tubes with 0·5M-H_2SO_4 (for neutral sugars) or 6M-HCl (for amino sugars). For quantitative determinations, they were hydrolysed by the procedure of Crook & Johnston (1962) or Johnston (1965) and the final hydrolysates applied without deionization to a column of ion-exchange resin for separation of the sugars as described by Mundie, Cheshire & Bacon (1968).

Nitrogen was determined by the Kjeldahl method or in a C,H,N analyser (Hewlett–Packard no. 185).

Ultrasonic treatment. A Vibrason ultrasonic generator and transducer with a 0·5in.-diam. titanium probe (Kerry's Ultrasonics Ltd., Hitchin, Herts.) was used at its highest setting (100w) for 5min. periods. The suspension was held in a 100ml. beaker, and cooled by ice as necessary.

Infrared spectra. These were measured, preferably on freeze-dried samples (1mg.), in a 12mm. KBr disc.

Microscopy. Shadow casts and ultra-thin sections were prepared as described by Jones & Webley (1967) and Jones & McHardy (1967), and examined in a model EM6 electron microscope (A.E.I. Ltd., Manchester).

RESULTS

The following account deals particularly with the proportion of glucan solubilized by various treatments of whole yeast cells or of cell-wall preparations, and so it would clearly be of advantage to begin by stating how much wall glucan is present in an intact cell. This information could be calculated from a knowledge of (*a*) the glucan content of a representative sample of cell wall, and (*b*) the proportion of the whole cell constituted by the wall. Some difficulties in this approach can be seen at once if one considers the case of yeast mannan, which is considered to be exclusively a wall component. The mannan contents of various samples of whole yeast cells have been given as 13–16% by Trevelyan & Harrison (1952, 1956) and 12–15% by

Suomalainen, Christiansen & Oura (1967) (cf. 13% for a commercial yeast of Pfäffli & Suomalainen, 1960). Taken in conjunction with a mannan content of 31% for yeast cell walls (Northcote & Horne, 1952), or the rather higher value of 43% found in the present work (and also by Mill, 1966), these values seem to indicate that the wall makes up 35–50% of the yeast cell, but Northcote & Horne (1952) by direct measurement of the yields of walls suggested a value of 15%, which has not so far been questioned (see Rogers & Perkins, 1968). In the present work yields of up to 16% of well-washed walls have been obtained. Mill (1966) obtained by the method of Trevelyan & Harrison (1956) somewhat lower values for the mannan content of his yeast strain, 9·6, 9·9 and 8·5%, from which one may calculate the wall to be about 22% of the cell. From this result and his value for wall glucan content (47%), the glucan content of the whole cell should be about 10%, but his direct determinations showed 5·3, 6·0 and 5·3%. Calculations from the latter values thus make the wall only about 12% of the cell.

Although some of the discrepancies referred to might possibly be attributed to differences in strains and in conditions of cultivation, the results of Mill (1966) make it more likely that serious errors are being introduced somewhere in the analytical procedures: either cell walls have lost a considerable part of their mannan during preparation, or the estimates of whole-cell glucan content are too low. Both might be true. McMurrough & Rose (1967) noticed that appreciable amounts of mannan (up to 2·5% of the whole-cell dry weight) were lost during ballistic disintegration, and, as the present paper shows, one cannot be certain that all the wall glucan remains insoluble in dilute aqueous alkali at 100° if the cells have previously been subjected to a treatment with acid, however mild.

For these reasons it is not possible to obtain from previous work any firm indication of the glucan content to be expected in the yeast we have used. However, our own experience confirms the general impression that yeast that has been extracted only with hot dilute aqueous alkali still contains the greater part of the wall glucan. We therefore propose to use the glucan content of this material as a point of reference.

Extraction with aqueous alkali. When Distillers Co. Ltd. pressed yeast is extracted repeatedly with hot dilute aqueous alkali (e.g. 3% sodium hydroxide at 75°; see Bell & Northcote, 1950) 83% of the dry matter is dissolved. Thus 100g. of yeast, which has a dry weight of 27g., yields 4·5g. of residue, which contains 3·5g. of carbohydrate (as glucose equivalents, determined by anthrone) and about 1g. of lipid. The nitrogen content is 0·5%. The polysaccharides present include glycogen, derived from the cytoplasmic contents, the glucans of the cell wall, and a little chitin (35mg.; see below); none of these is present in the alkaline extract (cf. Northcote, 1953*b*). From the amount isolated by procedures described below it is unlikely that the glycogen amounts to more than 0·5g. The wall glucan would thus be 3·0g., representing 11% of the whole cell; the glucan content of wall preparations is 44%, which would indicate that the cell wall constitutes 25% of the cell.

Some confirmation of these values was obtained by determinations of mannose and glucose in hydrolysates of freeze-dried pressed yeast. Cells containing 25% of wall material with a mannan content of 43% should have 12% of mannose; we found 11–12% of mannose.

The 3·5g. of carbohydrate left in 100g. of pressed yeast after extraction with alkali corresponds to 13% of glucose in the original cells. We found 14–15%, which implies that some glucose is lost during extraction with alkali. This would be expected if trehalose were present, and the glucose could well have been underestimated if the trehalose contents quoted by Trevelyan & Harrison (1952) are typical.

Bearing in mind batch-to-batch variations, we propose, as a rough guide to yields of glucan fractions, to take the glucan content of pressed yeast as 3·0g./100g., and of cell walls as 44mg./100mg., thus implying that the wall is 25% of the cell.

When yeast cell-wall preparations are extracted with alkali under the same conditions a considerable part of the glucan passes into solution. The following experiment is typical: an unheated suspension of walls (90ml.; containing 850mg. dry wt.) that had been prepared the previous day and stored overnight at 4° was mixed with 50ml. of 3% sodium hydroxide at 75° and the temperature quickly raised again. Extraction with stirring was continued for 1hr., the suspension was centrifuged and the residue again extracted. Three such extractions removed 378, 125 and 18mg. respectively of anthrone-determined carbohydrate (from an original total of 670mg.), leaving 123mg. in the residue (illustrated in Plate 2*c*). The alkali extracts were combined and neutralized, and 5vol. of ethanol was added. The precipitate weighed 484mg., and on hydrolysis yielded 45% of glucose and 41% of mannose.

Extraction of cell-wall preparations was carried out with stirring in open beakers, or in closed vessels in a stream of nitrogen. In all cases there was a slow but appreciable dissolution of glucan and the final residue represented only a small fraction of the original dry matter. Infrared spectra showed the residues to consist mainly of a β-glucan of the laminarin type, but it was sometimes possible to see the absorption bands characteristic of chitin,

which would be expected to remain insoluble under these conditions.

Treatment with sodium borohydride before extraction with alkali, and subsequent incorporation of sodium borohydride into the extracting medium, delayed the extraction of glucan from cell walls. Thus 300mg. dry wt. of walls treated in this way lost 126mg. of anthrone-determined carbohydrate in four extractions, leaving 97mg. undissolved (32% of the original wall); a control sample without borohydride gave 63mg. of carbohydrate in the residue (21% of initial dry weight; cf. 14% in the extraction above).

Another sample (300mg. dry wt.) was treated with ultrasonics for 10min. in aqueous suspension (15ml.) and extracted three times with alkali (127, 57 and 4mg. removed), leaving a residue of 37mg. of carbohydrate (12%), suggesting that, although the degree of physical damage may influence glucan extraction, part of the glucan resists hot 3% sodium hydroxide indefinitely.

Extraction under neutral and acid conditions. It is known that when whole yeast is autoclaved (e.g. in 19mM-citrate buffer, pH7·0, for 2hr. at 140°; Peat, Whelan & Edwards, 1961) most of the cell-wall mannan passes into solution. We found the same with cell walls, but even after repeated treatments some of the mannan remained in the residue. Incubation in buffer solutions for several days at 30° with or without β-mercaptoethanol does not extract much mannan.

Mundkur (1960) found that boiling whole cells with hydrochloric acid for 2hr. abolished the periodate–leucofuchsin staining properties, but that a thin double membrane still surrounded the cell when studied in ultra-thin section. In confirmation of this we found that the outline of the cell was still distinguishable under phase contrast and in shadow-cast specimens. If cell walls are boiled in 2% (w/v) hydrochloric acid for 3hr. (Houwink & Kreger, 1953) part of the glucan dissolves, and the remainder, representing about a quarter of the original dry matter, assumes a fibrillar structure [the 'hydroglucan' illustrated by Houwink & Kreger (1953) and Kreger (1967)]. Glucan preparations (described in detail below) obtained by extraction (*a*) with 3% sodium hydroxide at 75° under nitrogen (after heating at pH5) or (*b*) with dimethyl sulphoxide (with simultaneous ultrasonic treatment) also yielded these fibrils when boiled with acid (Plate 1*a*), although no fibrous structure at all could be discerned before the treatment; the yields of dry weights in the insoluble residue from 50mg. of glucan were (*a*) 17mg. and (*b*) 12mg. Hydroglucan, although clearly a β-(1→3)-glucan, shows minor but significant differences in its i.r. absorption spectrum in the 8–9μm. region (Fig. 1*e*) from that of laminarin (Fig. 1*d*), indicating a difference in chain structure

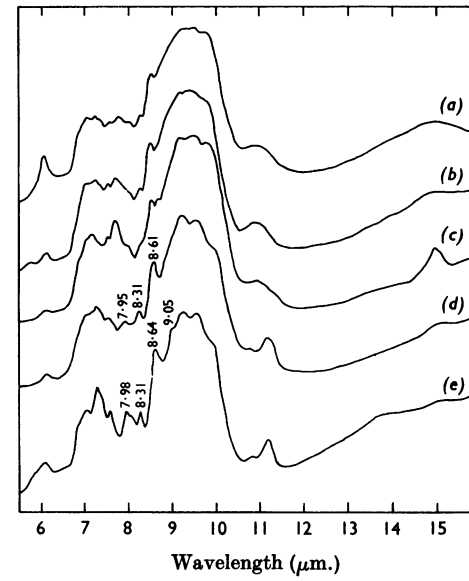


Fig. 1. Infrared spectra of glucans (1mg.) in KBr discs (1·2cm. diam.): (*a*) β-(1→6)-glucan liberated from alkali-extracted yeast by chitinase action; (*b*) β-(1→6)-glucan from *Candida parapsilosis*; (*c*) lutean; (*d*) laminarin; (*e*) 'hydroglucan' prepared from an alkali-soluble glucan, sample (*a*) in the text.

or packing. The i.r. spectrum of laminarin is not distinguishable from those of curdlan and pachyman.

Combinations of treatments with alkali and acid. When treatments with alkali and acid are combined, the results depend on the sequence and the severity of the treatments. The most drastic treatment that needs to be mentioned is that of Houwink & Kreger (1953), who boiled yeast with 2% hydrochloric acid for 1hr. and then briefly with 3% sodium hydroxide. The resulting residue is very small, e.g. from 100g. of pressed yeast we obtained 35mg. of dry material. Houwink & Kreger (1953) showed, and we confirmed, that it has a granular appearance under the electron microscope. They also showed that it was chitin by its X-ray-diffraction pattern, and we confirmed this by the i.r. spectrum. Treatment of cell walls by this procedure yields the same product, and the yield (3·7mg. from 506mg. of cell wall) is rather more than that which would be expected if the wall were 25% of the cell.

If whole cells previously extracted with dilute aqueous alkali are treated with 0·5M-acetic acid at 80° the glycogen is extracted (Bell & Northcote, 1950), but with some difficulty (Peat, Whelan & Edwards, 1958). Mineral acid is no longer used for this purpose, although frequently employed in the

past (cf. McAnally & Smedley-Maclean, 1937; Hassid, Joslyn & McCready, 1941). The glycogen extracted by treatment with acetic acid does not appear to have suffered much degradation (Northcote, 1953*b*).

However, if the insoluble residue from this treatment is subjected again to the action of 3% sodium hydroxide at 75° almost all of it dissolves, leaving a very small residue, half of which is chitin (Bacon *et al.* 1966). Electron microsopy shows that it consists mainly of crater-like structures, evidently derived from the bud-scars of the original wall. If the treatment with 0·5M-acetic acid is more intense, the alkali-solubility of the glucan is evident at room temperature. Thus when alkali-extracted yeast was treated for 24hr. at 90° (cf. Misaki, Johnson, Kirkwood, Scaletti & Smith, 1968) the greater part of the glucan then dissolved during 3hr. stirring with 3% sodium hydroxide at 20°. The almost colourless extract was neutralized, dialysed and freeze-dried, in a yield equivalent to 2·44g./100g. of pressed yeast. The alkali-insoluble residue consisted mainly of bud-scar residues and debris.

The difficulties met by Trevelyan & Harrison (1952, 1956) with their earlier method of determining yeast carbohydrates were probably due to some glucan dissolving in hot alkali as a result of previous treatment with acid. We found that, after being shaken for 3hr. with 10% (w/v) trichloroacetic acid at 22°, yeast cells dissolved almost completely when heated in 30% (w/v) potassium hydroxide at 100° for 30min.

Treatment with acid in organic solvents has a similar effect. Pressed yeast (50g.) suspended in 500ml. of ethanol–ether (1:1, v/v) containing 1% (w/v) of hydrochloric acid and heated at 50° for 5hr. (cf. Kessler & Nickerson, 1959) was solubilized when subsequently heated at 75° in 3% sodium hydroxide, leaving only some debris and bud-scars.

A milder treatment with acid is effective. Bacon *et al.* (1966) showed that treatment with sodium acetate buffer, pH 5·0, for 3hr. at 75° is sufficient to effect a change in alkali-solubility. Two periods of autoclaving for 30min. at 15lb./in.^{2} in phosphate buffer, pH 7·0 (falling to 6·5), did not do so.

Soluble products of treatment with acid of alkali-extracted whole cells. Glycogen is present in the acid extracts, even when the mildest treatment (acetate buffer, pH 5) is used. If the acetate-buffer extract is made alkaline a white flocculent precipitate containing magnesium hydroxide and glycogen settles out. Treatment with acetic acid (0·5M) (for 24hr. at 90°) extracts glycogen and also a β-(1→6)-glucan: the addition of 1vol. of ethanol precipitates the glycogen, and a further 1vol. of ethanol gives a polysaccharide with the same i.r. spectrum as that liberated by treatment with enzyme (see below). The yield of this material corresponds to 106mg./100g. of pressed yeast. When the mixed polysaccharides were precipitated with 3vol. of ethanol and the glycogen was degraded by incubation with salivary amylase the yield was 190mg. Absorption characteristic of β-(1→3)-glucan was not seen in the preparation.

Extraction of glucan with alkali after mild treatments with acid. Dissolution of the main part of the glucan in alkali after treatment at pH 5 is rather slow, requiring about 3hr. at 75° when the suspension is exposed to air, and longer if oxygen is excluded by passing nitrogen through the mixture.

Various glucan fractions dissolved by hot alkali after treatment at pH 5 were isolated by precipitation and washing at pH 8 followed by solution in alkali and dialysis for several days against distilled water. The resulting gels were freeze-dried, yielding spongy products, usually brown in colour, but almost colourless when prepared by extraction under nitrogen. The yield was very variable, the highest being 1·87g., the lowest 0·33g./100g. of pressed yeast. Careful exclusion of air while the alkaline solution was hot gave a yield of 1·04g./100g. If the treatment with hot buffer is followed by treatment with sodium borohydride the subsequent extraction with hot alkali seems to remove mainly the glycogen and leaves a large part of the cell-wall glucan; after neutralization the residues swell and once again assume the shape of the original cells. Pressed yeast (150g.) was extracted with 3% sodium hydroxide and treated with acetate buffer as described by Bacon *et al.* (1966). The residue was washed twice by centrifugation with 750ml. of water and then suspended in 250ml. of water, 1g. of sodium borohydride was added and the mixture was stirred for 16hr. at 22°. The wet residue (65g.) was extracted three times at 75° with 250ml. of 3% sodium hydroxide containing 0·2g. of sodium borohydride. Determinations by the anthrone method showed that a total of 1·43g. of carbohydrate had been removed, of which 0·21g. was precipitated on neutralization. The residue was suspended in 200ml. of water and neutralized with acetic acid. It swelled greatly so that even after prolonged centrifuging at 10000*g* the gelatinous precipitate still weighed 88g. It was washed four times with 800ml. of water and suspended in water to 200ml., and small portions were freeze-dried, indicating a total yield of dry matter of 2·8g. (1·9g./100g. of pressed yeast). It had the i.r. spectrum of a β-(1→3)-glucan, but glycogen was absent. Microscopic observation showed phase-dark cell shapes, some without contents.

The variable yield, the colour of the products, the effect of reduction with borohydride and the known susceptibility of β-(1→3)-glucans to degradation by alkali suggested that the extraction of the glucan, which requires heating, might be accompanied by

some degradation initiated by the exposure of free reducing groups during the treatment under mildly acid conditions. In confirmation of this, after a more drastic treatment (heating twice in acetic acid at 80° for 1hr.), the yield of glucan precipitable at pH8 was only 0·19g./100g. of pressed yeast. In the hope that this degradation was not an essential part of the process by which the solubility had been modified, other methods of bringing the glucan into solution were sought.

Extraction with dimethyl sulphoxide. Misaki *et al.* (1968) used a yeast glucan preparation soluble in dimethyl sulphoxide. We found that alkali-extracted whole yeast swells considerably, but does not dissolve to any significant extent in this solvent; this is true both of freeze-dried material and also of preparations that have remained in aqueous suspension throughout their preparation. However, if the suspension in dimethyl sulphoxide is given ultrasonic treatment most of it can be induced to dissolve. The mixed glycogen and glucan can then be precipitated directly with ethanol, washed with ethanol and dehydrated in the usual way. Alternatively water is added to the solution, the glucan forms a precipitate and glycogen can be recovered from the supernatant.

By the latter procedure, glycogen and water-insoluble β-(1→3)-glucan were obtained in yields corresponding to 0·6g. and 2·04g./100g. of pressed yeast respectively. The insoluble residue (0·2g./100g.), like others obtained in this way, consisted mainly of glucan, with chitin detectable in the i.r. spectrum, and had the appearance of the bud-scar residues already described (Bacon *et al.* 1966).

The solubility of the preparation of Misaki *et al.* (1968) is probably explained by our finding that after treatment with 0·5M-acetic acid at 90° for 24hr. the greater part of alkali-extracted yeast was dissolved by dimethyl sulphoxide at room temperature. This fraction, after dialysis and freeze-drying, corresponded to 2·6g./100g. of pressed yeast; only 2% of it was soluble in water. The material insoluble in dimethyl sulphoxide corresponded to 0·95g./100g.

Thus changes in the solubility of the major glucan component can be demonstrated under circumstances in which no degradation by alkali can occur.

Ultrasonic treatment in the presence of alkali. When alkali-extracted yeast was suspended in 3% sodium hydroxide, treated ultrasonically for 10min. and shaken for 1hr. at room temperature, an appreciable part of the glucan passed into solution, especially in the second of three such treatments. On neutralization of the extracts much glucan was precipitated, but polysaccharide equivalent to 230mg./100g. of pressed yeast was recovered from solution. This consisted of about equal parts of glycogen and β-(1→6)-glucan, with traces of β-(1→3)-glucan. Some glycogen (60mg.) could still be extracted with acetic acid from the alkali-insoluble residues.

Effect of enzymes on alkali-extracted whole cells. Alkali-extracted yeast was treated with a commercial chitinase preparation (Koch–Light Laboratories Ltd.) to observe the effects of removing chitin from the cell-wall residues. The visible effects were not consistent; in several incubation mixtures the dark rings of the bud-scars seen under phase contrast disappeared, but in others no marked change was seen. However, paper chromatography showed that *N*-acetylglucosamine and oligosaccharides containing amino sugars were formed, and the typical chitinous bud-scar residues could no longer be prepared from the treated residues.

Treatment with chitinase had a marked effect on the solubility of most of the glucan, which could then be extracted with dimethyl sulphoxide at room temperature, leaving a small residue with no recognizable structure; for the maximum effect, 1mg. of the chitinase preparation was used/g. of pressed yeast, and the incubation was for 3–6 days at 30° and pH6·0. It is significant that when the chitinase-treated material was first suspended in dimethyl sulphoxide the outlines of the cells could be seen under phase contrast and were no larger than in a control suspension.

In two experiments, material corresponding to 2·07g. and 2·13g./100g. of pressed yeast was recovered from the dimethyl sulphoxide extract by precipitation with ethanol.

The incubation medium was examined for the presence of glycogen: 5ml., corresponding to 5g. of original pressed yeast, was evaporated to dryness and dissolved in 0·5ml. of water, and 1·0ml. of ethanol was added. A precipitate that appeared (18·9mg.; 0·38g./100g. of pressed yeast) was mainly carbohydrate in nature, but did not contain glycogen. The i.r. spectrum (Fig. 1*a*) indicated a glucan with a preponderance of β-(1→6)-linkages, distinguishable from lutean (Fig. 1*c*), but not from a β-(1→6)-glucan from *Candida parapsilosis*, shown by Yu, Bishop, Cooper, Blank & Hasenclever (1967) to contain about 15% of β-(1→3)-linkages in linear regions of the molecule (Fig. 1*b*).

The carbohydrates soluble in aq. 65% (w/v) ethanol included some glucose and a mixture of oligosaccharides, among which the following were tentatively identified by paper chromatography: maltose, laminaribiose, gentiobiose and the corresponding trisaccharides. The β-(1→3)-linked glucose residues, which are clearly distinguishable by colour reactions from the β-(1→6)- and α-(1→4)-linked glucose residues, represented only a small proportion of the total. Nevertheless, these results raised the suspicion that the action of the enzyme preparation was due to the contaminating β-(1→3)-glucan-

Table 1. *Effects of treatment of alkali-extracted whole yeast with enzyme preparations*

In each incubation mixture 5ml. of a suspension of alkali-extracted yeast (see the text for details), equivalent to 5g. of pressed yeast, was incubated at pH 6·0 in citrate–phosphate buffer at 30°. The purified *Streptomyces* chitinase and the purified *Cytophaga* laminarinase were used in amounts calculated to give the activities of these two enzymes in the commercial chitinase preparation, of which 5mg. was used. Afterwards the incubation mixture was centrifuged at 16000*g* and the residue washed twice with water, before extraction with dimethyl sulphoxide at room temperature. The supernatant fluid and washings were concentrated by evaporation at reduced pressure and 2vol. of ethanol was added.

		Extraction of residue with dimethyl sulphoxide		Supernatant	
Nature and source of enzyme	Duration of incubation (days)	Insoluble (mg.)	Soluble* (mg.)	Total carbohydrate by anthrone (mg.)	Ethanol-insoluble material (mg.)
Chitinase (Koch–Light)	6	—	112	49·3	18·9†
Control: no enzyme	6	—	—	3·5	2·7‡
Chitinase (*Streptomyces albidoflavus*)	3	111	33	20·4	4·8‡
Control: no enzyme	3	154	0	4·3	3·2‡
Laminarinase (*Cytophaga johnsonii*)	3	39	89	—	19·4†
	6	32	34	—	14·6†
Control: no enzyme	6	177	0	—	3·1‡

* Recovered from extract by precipitation with 2vol. of ethanol.
† Infrared spectra showed principally β-(1→6)-glucan.
‡ Infrared spectra showed indeterminate mixtures, containing some carbohydrate.

ase, and not to the chitinase. Dr R. J. Sturgeon of Heriot–Watt University very kindly supplied us with a chitinase preparation completely free from β-(1→3)- and β-(1→6)-glucanase (Noble & Sturgeon, 1968) and Mr A. H. Gordon provided a β-(1→3)-glucanase free from β-(1→6)-glucanase, prepared from a culture filtrate of *Cytophaga johnsonii*. The results in Table 1 show that weak β-(1→3)-glucanase activity will release β-(1→6)-glucan and solubilize the β-(1→3)-glucan, but that the purified chitinase did neither. However, the treatment with chitinase removed only half of the chitin, as was shown by recovery of the residue after treatment with acid and alkali.

When alkali-extracted yeast was treated with a β-(1→6)-glucanase preparation free from β-(1→3)-glucanase (also prepared from a *Cytophaga johnsonii* culture filtrate by Mr A. H. Gordon) changes could not be seen under the microscope, but a series of β-(1→6)-linked oligosaccharides was produced. Of 45mg. taken, 31mg. remained insoluble, and anthrone determinations indicated that 8·3mg. of carbohydrate (0·83g./100g. of pressed yeast) had gone into solution. Part of this soluble carbohydrate was maltose and maltotriose, but laminaribiose was not seen, and β-(1→3)-glucan could not be recovered by ethanol precipitation. It appeared that only a β-(1→6)-glucan component and glycogen were degraded by the enzyme preparation; by difference, β-(1→3)-glucan amounting to 2·7g./100g. of pressed yeast must have remained insoluble in water. Most of this now dissolved in dimethyl sulphoxide, leaving 2·5mg. (0·25g./100g. of pressed yeast) of a residue, chiefly chitin, showing bud-scars and amorphous material.

β-(1→6)-Glucan component. This substance is a cell-wall component, as can be demonstrated by treating cell-wall preparations with β-(1→6)-glucanase, when the characteristic oligosaccharides are formed.

Extraction of cell walls with hot acetic acid removes considerable amounts of mannan together with the β-(1→6)-glucan; the latter may then be estimated in the mixture by degrading it with a β-(1→6)-glucanase, this treatment indicating about 100mg./100g. of pressed yeast. Separation of the polysaccharides, by the addition of the minimum volume of Fehling's solution needed to precipitate the mannan, yields rather less of the glucan (about 80mg.). Hot-acetic acid extracts of the residues left after treatment of walls with alkali give even smaller yields (about 70mg. from a residue prepared in the presence of sodium borohydride, and only 20mg. for one made without it).

The discrepancy between these yields and those from treatment of alkali-extracted yeast with chitinase is partly due to the presence of a water-insoluble form of the β-(1→6)-glucan, which is not readily solubilized by acetic acid. Water-insoluble glucan fractions, prepared by (*a*) extraction with alkali after treatment with 0·5M-acetic acid for 24hr. at 90° and (*b*) extraction with dimethyl sulphoxide and ultrasonic treatment, were treated with chitinase, and yielded (*a*) 94mg. and (*b*) 114mg.

of β-(1→6)-glucan/100g. of original yeast; no β-(1→3)-glucan was detected in the fractions solubilized.

Microscopy of alkali-extracted preparations. Northcote & Horne (1952) advanced the view that the yeast cell wall consists of two layers; such a structure seemed to be visible in some shadow-cast specimens, particularly of walls that had been extracted with methanol and ether. After extraction with 3% sodium hydroxide at 100°, only one layer could be seen and they therefore suggested that the other was made up 'in part of protein or mannan or both of these substances'. Ultra-thin sections of whole yeast (Agar & Douglas, 1955; Bartholomew & Levin, 1955; Mundkur, 1960; Vitols, North & Linnane, 1961; Hagedorn, 1964; and many others) do not wholly support this view (Northcote, 1963*b*), although the outer region of the wall is more readily stained than the inner. Sections through cell-wall preparations, on the other hand, rarely show any layering.

Under phase contrast, alkali-extracted whole cells have a well-defined outer boundary (see also shadow-cast specimens; Plate 1*b*), but the wall appears much thicker relative to the cell diameter than in living cells and its inner boundary is indistinct.

Ultra-thin sections confirm this, and suggest that the structure is denser at the outer boundary of the wall. The bud-scar regions show in section horn-like projections (Plate 1*c*), which presumably correspond to the crater rim of the scar. Within the crater there is a dense layer, thickest in the middle, and apparently not continuous with the horns; instead it passes beneath them in a more attenuated form.

After treatment with the purified chitinase from *Streptomyces albidoflavus* there is little change in appearance. The bud scars in shadow-cast specimens still have a sharp crater rim. Many cells now show in profile what may be a birth scar (arrowed in Plate 2*a*); this is not visible before treatment with the enzyme.

After treatment with β-(1→6)-glucanase, alkali-extracted yeast still has very prominent bud-scar structures (Plate 2*b*), and the wall boundary is still fairly sharp.

Bud-scar preparations (see Bacon *et al.* 1966) always have a small field surrounding them, and are sometimes linked together in groups by this membrane. In extreme cases almost the entire outline of a cell wall can be seen, with many bud-scar regions [see left-hand side of Plate 1(*a*) in Bacon *et al.* (1966)]. In ultra-thin sections all parts of the structure are seen as dense layers. The inner area [corresponding to the bud-scar 'plug' referred to by some authors, e.g. Mundkur (1960)] is rarely lost during chemical or enzymic digestion.

Various very small residues referred to above are indistinguishable from bud-scar preparations. In residues from extraction of cell walls with alkali, bud-scars are very prominent, but the outlines of many walls are distinguishable (Plate 2*c*). Where the extraction procedure is incomplete the residues of alkali-extracted whole cells still contain remains of the cell contents. No fibrils have ever been seen in the products of alkaline treatments.

DISCUSSION

Although the main constituents of the baker's yeast cell wall have been characterized in a general way as glucan, mannan, protein, lipid and chitin, the detailed structure of each and its place in the architecture of the wall have yet to be determined. The results reported here refer particularly to the glucan and chitin.

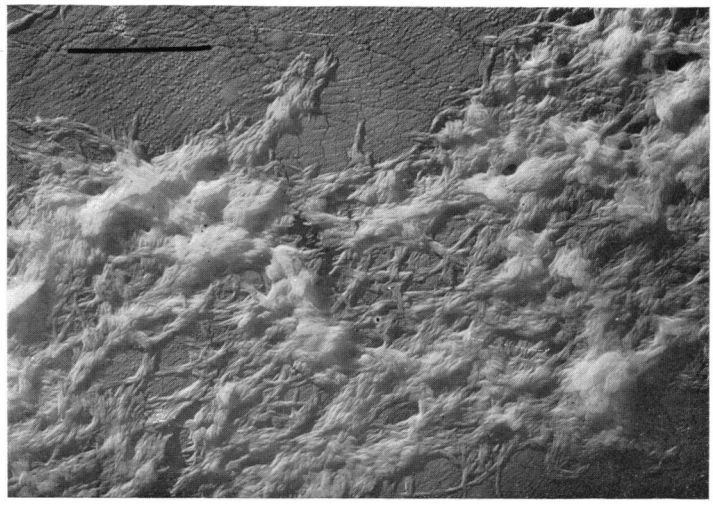
The outstanding problem with regard to the glucan is to find an explanation for its solubility properties; in the intact cell it appears to be quite insoluble in alkali, but in cell-wall preparations, as this and other studies show, a large part is alkali-soluble. Chemical studies seem to indicate a highly branched structure, which is difficult to reconcile with insolubility in alkali, and Northcote (1953*a*) failed to account for its 'very insoluble nature' by internal hydrogen-bonding. He therefore concluded that it was due to its very large molecular weight.

We consider that a more satisfactory explanation is one based on cell morphology, namely that during extraction of whole yeast with alkali the greater part of the glucan, like the glycogen (Northcote, 1953*b*), is retained by a semi-permeable membrane formed by some elements of the original wall. This membrane may be tentatively considered to contain chitin and one or more β-glucan components. Until now we have not been able to remove the chitin selectively without degrading the glucan, presumably because the two substances are very intimately associated; Domanski & Miller (1968) made a similar observation with *Candida albicans*.

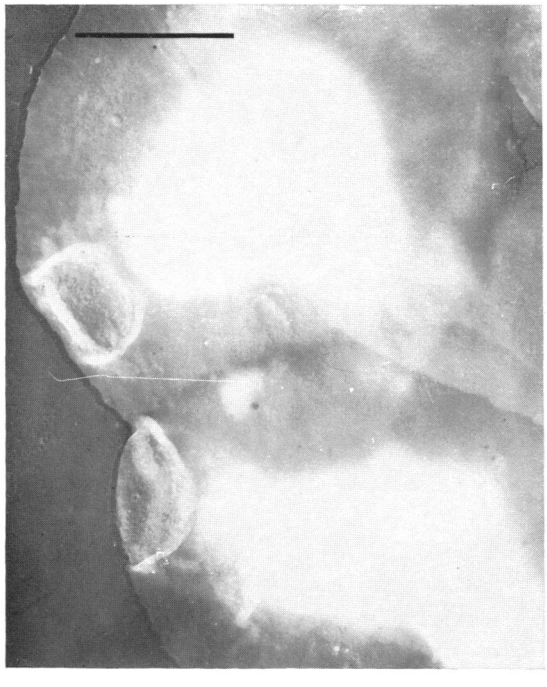
During preparation of cell walls the continuity of the membrane will be broken, allowing extraction of the glucan through holes. Treatment with ultrasonics or mildly acid conditions may be assumed to increase the permeability of the membrane.

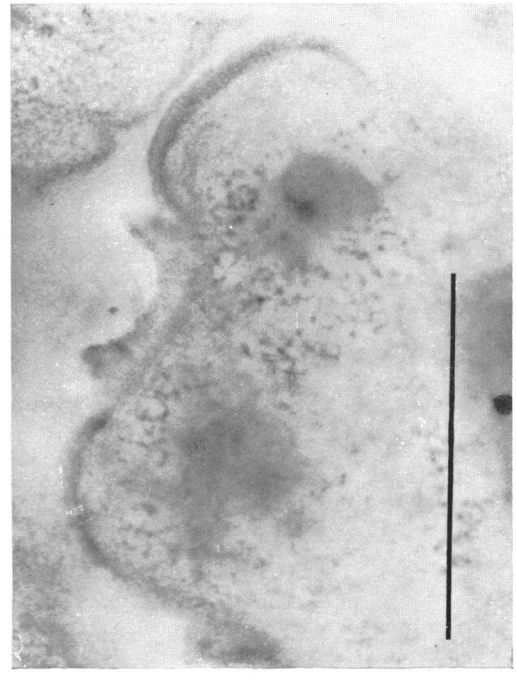
It is difficult to demonstrate the existence of this membrane directly because it collapses during the preparation of shadow-cast specimens for electron microscopy. Elements of the bud-scar structure seem always to be associated with the membrane and help to distinguish it, but the position is further complicated by the persistence inside the membrane of insoluble residues of the protoplast (partly lipid). Preliminary isolation of cell walls eliminates most of the cell contents, but undoubtedly damages the outer layers of the wall (see McMurrough & Rose,

(*a*)



(*b*)



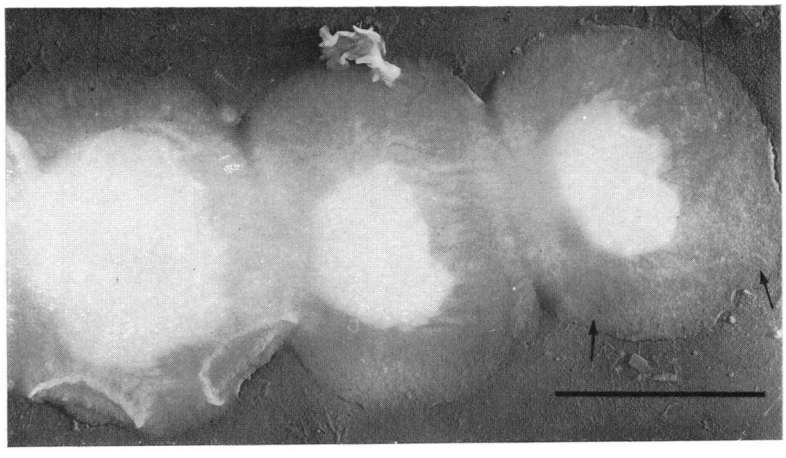


(*c*)

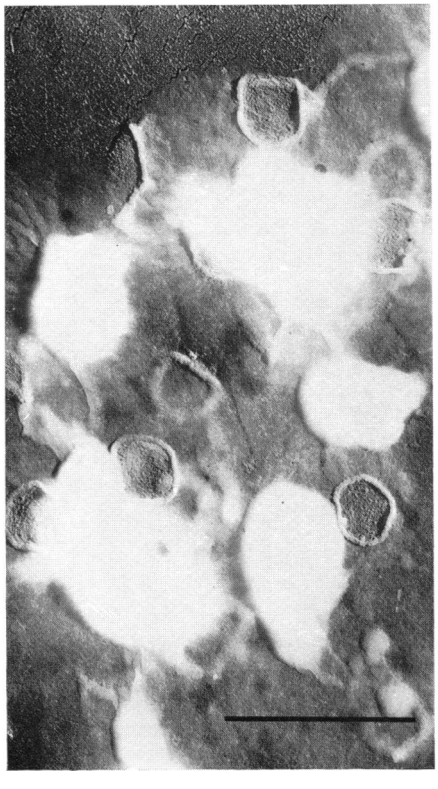
EXPLANATION OF PLATE I

(*a*) 'Hydroglucan' prepared by treatment with acid of a glucan fraction, sample (*b*) in the text; shadowed with nickel–palladium. (*b*) Alkali-extracted whole cells, shadowed with nickel–palladium. (*c*) An ultra-thin section of the same sample as (*b*), fixed with osmic acid and stained with uranyl acetate, showing two bud-scars. (Magnification is indicated by a line 1 μm. long.)

(*a*)



(*b*)



(*c*)

EXPLANATION OF PLATE 2

All preparations were shadowed with nickel–palladium. (*a*) Alkali-extracted yeast treated with purified chitinase from *Streptomyces albidoflavus* for 3 days (see Table 1). (*b*) Alkali-extracted yeast treated with purified β-(1→6)-glucanase from *Cytophaga johnsonii* for 12 days (see the text). (*c*) Residue of yeast cell wall after three extractions with 3% NaOH at 75° (See under 'Extraction with aqueous alkali' in the Results section). (Magnification is indicated by a line 2 μm. long.)

1967) and so may destroy the membrane to some extent.

Mundkur (1960) found that acid-treated whole cells showed a thin double membrane in ultra-thin section, and suggested that this was chitinous. We find that alkali-extracted cells are about the same size as living ones, but that the wall has swollen inwards: in ultra-thin section a denser outer layer can be seen. The residues of the original cell contents occupy a central position, but after treatment with dimethyl sulphoxide and subsequent washing with water they become displaced, suggesting that the glucan has dissolved and been reprecipitated.

The chemistry of the membrane is best studied in alkali-extracted yeast, where by definition it should form a continuous retaining envelope. Its stability to alkali is consistent with the presence of chitin and glucan, but the effects of mild treatment with acid are not easily explained. After heating with acetate buffer the glucan is not extractable by dimethyl sulphoxide, and its dissolution in hot aqueous alkali is largely prevented by treatment with sodium borohydride. This would imply that some reducing end groups, necessary for degradation of the β-(1$\rightarrow$3)-glucan chains with alkali (cf. Corbett & Kenner, 1955), have been exposed by the treatment with buffer, although it is not usually considered that β-(1$\rightarrow$3)-glucopyranosidic linkages would be hydrolysed at pH 5 and 75°. We were therefore led to consider the possibility that a phosphodiester linkage (like that in the phosphomannans) might be present in the wall; if so, this would involve only glucan components, because all the mannan is removed by extraction with alkali.

Dr Slodki kindly provided us with a sample of the phosphomannan from *Hansenula holstii* N.R.R.L. Y-2448, and also tested a phosphomannan from *Pichia pinus* N.R.R.L. Y-2579 (formerly *Saccharomyces pini*) that has glucosyl-1-phospho-6-mannan end groups, but neither substance was appreciably hydrolysed under the conditions of the treatment with acetate buffer. Yeast glucan preparations have very low phosphorus contents, most of the phosphate being associated with the mannan (Eddy, 1958; Mill, 1966), but it remains a slight possibility that there are enough phosphodiester linkages, rather more susceptible to acid conditions, to permit the dissociation of the structure in acetate buffer at 75°.

The structure proposed by Lampen (1968), in which 80% of the mannan, linked by phosphodiester bonds, forms a continuous outer layer of the wall, is difficult to reconcile with the ease with which mannan is removed by aqueous alkali; the phosphoryl linkages in the *Hansenula* phosphomannan are stable to 0·1 M-potassium hydroxide for 10 min. at 100° (Slodki, 1962).

The more drastic treatment with acetic acid (Misaki *et al.* 1968), which makes a major part of the glucan soluble in aqueous alkali and in dimethyl sulphoxide, degrades the *Hansenula* phosphomannan noticeably, as judged by both viscosity and i.r. spectrum, but it may equally be capable of breaking a significant number of sugar–sugar glycosidic linkages.

The discovery of a β-(1$\rightarrow$6)-glucan in solution in two situations in which the extractability of the glucan is increased (treatment with acetic acid or β-(1$\rightarrow$3)-glucanase) suggests either that it is trapped by the same structures that retain the major part of the glucan, or that it is part of these retaining structures. The effect of the purified β-(1$\rightarrow$6)-glucanase shows that β-(1$\rightarrow$6)-linkages are somehow involved in the insolubility of glucans, but they might be present as a minor structural component in the β-(1$\rightarrow$3)-glucan. At first sight it seems that we have not made much progress, because the clue to glucan solubility still lies somewhere in glucan structure.

However, if our hypothesis is accepted a new appreciation of the complexity of the glucan components of the wall is possible. It was early realized that some glucan could be extracted from cell-wall preparations by the use of hot dilute aqueous alkali (Roelofsen, 1953; Eddy, 1958). McMurrough & Rose (1967) have shown that the proportion so extracted varies with the conditions under which the cells are grown. This might be taken to show that this extractability is an intrinsic property of the glucan fraction, and not the consequence of mechanical damage during the preparation of the walls. Eddy & Woodhead (1968) have found that in *Saccharomyces carlsbergensis* a part of the glucan of wall preparations is dissolved by 3% sodium hydroxide at 4° under nitrogen, a limit corresponding to 20% of the wall dry weight being reached after 4 days. All this evidence points to considerable heterogeneity in the β-(1$\rightarrow$3)-glucan, and in this paper we demonstrate that a β-(1$\rightarrow$6)-component is also present.

How the materials previously studied chemically under the name 'yeast glucan' are to be related to this picture is an open question, but it must be noted that the final yields of glucan obtained from 100g. of pressed yeast (after treatment with acid to remove glycogen) were always much lower than the 3·0g. we have taken as a reference point, namely 0·55g. (Bell & Northcote, 1950), 0·65g. (Peat *et al.* 1958) and 0·84g. (Misaki *et al.* 1968), and also much lower than the yields of water-insoluble glucan we have obtained by extraction with dimethyl sulphoxide (2·04 and 2·60g.).

The relation of 'hydroglucan' (Houwink & Kreger, 1953) to these components is also not clear, but we have shown here that soluble glucan fractions, featureless under the electron microscope,

may be converted in 30% yield into hydroglucan fibrils. This and other observations (Kreger, 1967) suggest that hydroglucan is an artifact. By this we do not mean that linear β-(1→3)-glucan chains do not occur in the intact wall, but that the process of boiling in acid liberates them from a more complex structure and allows them to associate as microcrystals. This idea is expressed less directly by Kreger (1967) when he speaks of hydroglucan as 'an individual structural unit comparable to e.g. chitin and cellulose . . . . linked in some way to a fraction which is easily hydrolysed'. The presence of a predominantly β-(1→6)-glucan component in the wall was not known until recently to those studying glucan structure by chemical methods (methylation, partial acid hydrolysis), and so the structures put forward (Manners & Patterson, 1966; Misaki *et al.* 1968) cannot be used to interpret the effects of acid hydrolysis. We suggested (Bacon & Farmer, 1968) that many of the β-(1→3)-glucan chains might be much longer than the 8–15 units proposed, and by the elimination of branch points might be enabled to aggregate. Support for this view has now come from a study of the structure of the glucan fraction that remains insoluble after exhaustive removal of the β-(1→6)-component by treatment with acetic acid and glucanase (Manners & Masson, 1969).

The association of one or more glucan fractions with wall protein is not excluded by our results. However, although there is good evidence for mannan–protein complexes (Falcone & Nickerson, 1956; Eddy, 1958; Korn & Northcote, 1960; Sentandreu & Northcote, 1968), the presumptive glucomannan–protein complexes, fractions 'GMP-I' and 'GMP-II' of Kessler & Nickerson (1959) and fraction B of Korn & Northcote (1960), have not been sufficiently characterized. In particular it has not been shown that the glucan migrates with the protein during electrophoresis or ultracentrifugation. Fraction 'GMP-I' from baker's yeast (Kessler & Nickerson, 1959) is mainly (about 80%) protein, so that only 10% of contaminating glucan would be needed to account for its composition. Similarly the presence of 30% of glucan might explain the 'small amount of relatively uncharged material' in fraction 'GMP-II'; the remainder could be the mannan–protein complex, which for some reason is not mentioned as a product of this fractionation. Fraction B is that material precipitated from the ethylenediamine extract which does not redissolve in water. In our experience the readily extracted glucan is insoluble at neutral pH, and can be completely separated from the mannan by this property; it would therefore be expected to accumulate in fraction B.

Of the components so far identified in the wall only the β-(1→6)-glucan would be expected to react extensively with periodate; Mundkur (1960) thought that only the mannan would do so, but he misunderstood its structure. Trevelyan & Harrison (1952) found that the periodate–Schiff technique (Hotchkiss, 1948) stained the walls of intact yeast cells; extraction with cold 10% (w/v) trichloroacetic acid did not abolish the reaction, but 30% (w/v) potassium hydroxide at 100° for 15–180min. did. Mundkur (1960) examined ultra-thin sections cut from freeze-dried cells stained with periodate–Schiff reagent. He found that the outer layer of the wall was more dense, and ascribed this to the presence of mannan; it would seem equally possible that it was due to β-(1→6)-glucan.

The concept of a wall composed of two layers, one of mannan–protein, the other containing the glucan, is now seen to be too simple, and seems in any case to have been based partly on a misunderstanding of the bud-scar structure (Fig. 8 of Northcote, 1963*b*). Ultra-thin sections show that the floor of the crater is not an exposed part of an inner layer, but is a separate 'plug', developed to seal off the connexion between mother and daughter cells (Hagedorn, 1964; Beran, 1968; Marchant & Smith, 1968). Structures like that arrowed in Fig. 8 of Northcote (1963*b*) can be seen in alkali-extracted cell walls as well.

For reasons we have partly explained (Bacon, Milne, Taylor & Webley, 1965) we consider that some of the mannan must occur in close association with glucan, and it would follow that this fraction would lie inside the membrane we are now postulating. Under alkaline conditions the membrane must then be permeable to this mannan fraction, which might have a maximum molecular weight of 60000 (Korn & Northcote, 1960). Eddy & Woodhead (1968) estimate that the molecular weight of their alkali-soluble glucan from *Saccharomyces carlsbergensis* is about 500000, and brewer's yeast glycogen is even larger (mol.wt. about 2×10^6; Manners & Khin Maung, 1955); these figures promise reasonable scope for differential permeability in baker's yeast.

It seems possible that part of the mannan and protein lies outside the glucan, and this may explain the resistance of living yeast cells to attack by the enzyme mixtures elaborated by some microorganisms.

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