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The Structure of a β-(1→3)-D-Glucan from Yeast Cell Walls

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Yeast glucan as normally prepared by various treatments of yeast (*Saccharomyces cerevisiae*) cell walls to remove mannan and glycogen is still heterogeneous. The major component (about 85%) is a branched β-(1→3)-glucan of high molecular weight (about 240000) containing 3% of β-(1→6)-glucosidic interchain linkages. The minor component is a branched β-(1→6)-glucan. A comparison of our results with those of other workers suggests that different glucan preparations may differ in the degree of heterogeneity and that the major β-(1→3)-glucan component may vary considerably in degree of branching.

The structure of the polysaccharide components of yeast cell walls has been the subject of numerous studies (for reviews, see Phaff, 1963, 1971; MacWilliam, 1970). In this earlier work, the cell wall of baker's yeast (*Saccharomyces cerevisiae*) has been regarded as a complex containing glucan, mannan, small amounts of chitin, phosphate, lipid and protein, part of the latter being enzymic. On this basis, some schematic structures for the yeast cell wall and associated enzymes have been proposed (Lampen, 1968; Kidby & Davies, 1970). However, Bacon and his co-workers have shown that yeast glucan as normally prepared is heterogeneous and contains a minor β-(1→6)-D-glucan component, in addition to a major β-(1→3)-D-glucan (Bacon & Farmer, 1968; Bacon *et al.*, 1969), so that any discussion of yeast cell-wall structure must take account of this fact.

Before this discovery, which we have confirmed (Manners & Masson, 1969), chemical analysis of yeast glucan had given conflicting results. A methylation analysis carried out by Bell & Northcote (1950) indicated a highly branched structure in which chains of (1→3)-linked D-glucose residues were interlinked by about 10% of (1→2)-D-glucosidic linkages. By contrast, a partial acid-hydrolysis study by Peat *et al.* (1958*a*) led to the conclusion that the glucan had a linear structure with both β-(1→3)- and β-(1→6)-D-glucosidic linkages. The presence of about 10–20% of (1→6)-linkages in yeast glucan was also shown chemically by tosylation followed by iodination of the primary hydroxyl groups (Peat *et al.*, 1958*b*). In an attempt to resolve this discrepancy, Manners & Patterson (1966) carried out methylation, periodate-oxidation and enzymic-degradation experiments, and concluded that yeast glucan contained main chains of (1→6)-linked β-D-glucose residues to which were attached linear side chains of (1→3)-linked β-D-glucose residues. In view of the heterogeneity of yeast glucan, which was later observed, this structure is also incorrect. We now describe a reassessment of our previous experimental results (Manners & Patterson, 1966), together with additional information, which leads to the conclusion that the major glucan component of yeast cell walls has a branched structure, containing about 3% of β-(1→6)-D-glucosidic interchain linkages. The results of an examination of the minor glucan is described in the following paper (Manners *et al.*, 1973). In these two papers, the term 'yeast glucan' will denote the mixture of the two polysaccharides, in accord with the previous literature, and the individual components will be referred to as β-(1→3)-glucan and β-(1→6)-glucan respectively. In addition, the D-configuration of the glucose will be assumed.

Methods and Materials

Analytical methods

Total acid hydrolysis of oligosaccharides was effected by heating with 1 M-H_2SO_4 at 100°C for 2h. For the total acid hydrolysis of glucan samples at 100°C, 90% (v/v) formic acid for 2h followed by 1.5 M-H_2SO_4 for 3h was used, as described by Peat *et al.* (1958*a*). For the partial acid hydrolysis of glucans, 90% formic acid for 30–60min, followed by 0.17 or 0.22 M-H_2SO_4 for 1h was used. In the hydrolyses with formic acid, this was effectively removed by evaporating the solution to dryness several times with water before the treatment with H_2SO_4. Hydrolysates were neutralized with $BaCO_3$, and if necessary deionized with mixed Amberlite IR-120 and IR-45 resins, before concentration and paper-chromatographic analysis. All solutions were concentrated at reduced pressure by using a rotary film evaporator with water-bath temperatures not exceeding 40°C.

Reducing sugars were measured as glucose by using a colorimetric alkaline copper reagent (Nelson,

1944; Somogyi, 1945). Glucose, either free or glycosidically bound, was also determined by a phenol–H_2SO_4 method (Dubois *et al.*, 1956). The nitrogen content of glucan preparations was determined by the micro-Kjeldahl method.

Qualitative paper chromatography was carried out with the following solvents: *A*, ethyl acetate–pyridine–water (10:4:3, by vol.); *B*, methyl ethyl ketone–water–NH_3 (sp.gr. 0.88) (200:17:1, by vol.); *C*, butan-1-ol–ethanol–water (4:1:5, by vol.); *D*, ethyl acetate–acetic acid–90% formic acid–water (18:3:1:4, by vol.); *E*, propan-1-ol–ethanol–water (14:2:7, by vol.). Solvents *A*, *D* and *E* were mainly used for unmodified sugars, which were detected with an alkaline $AgNO_3$ reagent (Trevelyan *et al.*, 1950). Methylated sugars were normally separated by using solvents *B* and *C* and detected with aniline oxalate (Partridge, 1949). Paper-chromatographic mobilities (R_{Glc}) are expressed relative to D-glucose (R_{Glc} = 1.00).

The degree of polymerization of glucans was determined from the sorbitol content of an acid hydrolysate of the borohydride-reduced glucan by using sorbitol dehydrogenase (Manners *et al.*, 1971).

Methylation analysis

The various conditions used for the methylation of yeast glucan are given in the Results section. The methoxyl contents were determined by a semi-micro Zeisel method (Belcher & Godbert, 1954). Oligosaccharides (2mg) were methylated on the micro-scale with methyl iodide and Ag_2O in dimethylformamide as described by Kuhn *et al.* (1955).

Total acid hydrolysis of methylated glucan was effected at 100°C by using 90% formic acid for 1h, followed by 1M-H_2SO_4 for 2h, the hydrolysate then being neutralized with $BaCO_3$. Methylated oligosaccharides and glucan were also methanolysed by heating at 100°C in a sealed tube for 4–6h and 18–24h respectively with 3% methanolic HCl. After cooling, the solution was evaporated to dryness five times with dry methanol to remove HCl.

The mixtures of methyl glycosides were then analysed by g.l.c. as described by Aspinall (1963) on a Pye–Argon chromatograph by using either butane-1,4-diol succinate at 175°C or 10% neopentyl glycol adipate at 195°C as liquid phases. Retention times were measured relative to methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside. A comprehensive range of authentic methylated sugar glycosides was available from previous work on methylated soluble laminarin (Fleming *et al.*, 1966), which also contain β-(1→3)- and β-(1→6)-glucosidic linkages.

The total acid hydrolysate of methylated glucan A1 was fractionated on a column (45cm×3cm) of Whatman cellulose powder, which had been washed successively with water and various mixtures of butan-1-ol (half-saturated with water) and light petroleum (b.p. 100–120°C) to remove soluble impurities. The column was finally washed with the initial eluting solvent, a mixture of butan-1-ol (half-saturated with water) and light petroleum (b.p. 100–120°C; 1:4, v/v) before application of the neutralized hydrolysate. The column was eluted at 14ml/h, 9ml fractions being collected. After fractions 308 and 501, the composition of the eluting solvent was changed to 3:7 and 2:3, v/v, respectively.

The proportion of the various methylated sugars in hydrolysates was also determined by quantitative paper chromatography by using an alkaline hypoiodite reagent (Hirst *et al.*, 1949).

Periodate oxidation analysis

Suspensions of glucan, whose concentrations were determined by the phenol–H_2SO_4 method, were oxidized with shaking at room temperature (20±2°C) with unbuffered sodium metaperiodate in the absence of light. The reduction of periodate, measured spectrophotometrically (Aspinall & Ferrier, 1957), and the production of formic acid, titrated with standard solutions of $Ba(OH)_2$ or NaOH to an end-point of pH5.8, were plotted against time; after about 25h all the graphs were linear. This portion was extrapolated to zero time, to correct for overoxidation (Manners & Wright, 1961); all results reported have been corrected. In control experiments under these conditions, methyl α-D-glucoside reduced the theoretical amount of periodate (2.0mol.prop.) and a sample of amylopectin and various samples of glycogen had average chain lengths of 24 and 13–15 respectively, in good agreement with previous determinations that used potassium metaperiodate oxidation.

For the Smith-degradation procedure, glucan A2 (3.85g) was oxidized at 20°C with 0.3M-sodium metaperiodate (200ml) in the dark. The initial reduction of periodate was 0.26mol.prop. The oxidation was continued for 5 days to ensure that all the α-glycol groups in the glucan had been cleaved, and the reaction was then terminated by the addition of ethylene glycol (6ml) followed by dialysis against running tap water for 48h. The dialysed suspension (300ml) was made 2% (w/v) with respect to KBH_4, and after being stirred at 20°C for 24h the mixture was neutralized to pH6.8 with acetic acid, and dialysed for 3 days against tap water. The suspension was then made 0.06M with respect to H_2SO_4 and stirred at 20°C for 24h. The residual glucan was isolated by centrifugation, washed with water, ethanol and ether and then air-dried at 37°C to give 3.0g of degraded glucan A2. The supernatant solution and water washings were combined, neutralized (with $BaCO_3$), and concentrated to a syrup after removal of the $BaSO_4$.

In a repetition of the degradation, 3.6g of glucan A2 yielded 3.1g of degraded polysaccharide.

Enzymic-degradation analysis

Glucan A1 or A2 (20mg) was incubated at 40°C under toluene with 10mg of a bacterial laminarinase preparation (supplied by Glaxo Research Ltd., Greenford, Middx., U.K.) dissolved in 1ml of 0.05M-sodium citrate buffer, pH5.0. Samples were removed at intervals for paper-chromatographic analysis (solvent *A*) or measurement of reducing power. Glucan A1 (15mg) was also incubated at 30°C with 1ml of culture fluid of *Cytophaga johnsonii* (see Bacon *et al.*, 1970) and 0.8ml of Tris–HCl buffer, pH7.5, which was prepared containing either 0 or 20mM-2-mercaptoethanol. Paper-chromatographic analysis was carried out at intervals up to 10 days. The results were the same in both digests.

Glucan A2 (2mg) was also incubated with 0.5mg of β-(1→6)-glucanase from *Penicillium brefeldianum* dissolved in 0.5ml of 0.05M-sodium citrate buffer, pH4.8. The enzyme digest was examined by paper chromatography after incubation at 37°C under toluene for 1, 7 and 14 days.

Materials

Yeast glucan samples. Two samples (A1 and A2) were prepared from pressed baker's yeast (*S. cerevisiae*) essentially by the method of Peat *et al.* (1958*a*). This differs from the procedure of Bell & Northcote (1950) in that glycogen was removed by autoclaving rather than by extraction with acetic acid. Sample A1, prepared by our former colleague Dr. W. D. Annan, was obtained from 1830g of yeast; yield 39g; ash, 0.3%; N, 0.24%, equivalent to about 1.5% of denatured protein, if chitin is neglected; apparent glucose content 80% (this low value was later attributed to some lipid impurity). Sample A2 isolated from 1800g of yeast; yield, 37g; ash, 1.2%; N, 0.30%, equivalent to about 1.9% of apparent protein; apparent glucose content, 96%. The two samples of glucan were very similar, if not identical, as shown by subsequent partial acid and enzymic hydrolysis, and by periodate-oxidation analysis.

Samples B and P were those used by Bell & Northcote (1950) and Peat *et al.* (1958*a*) respectively. The apparent glucose content of the latter was 90%; that of the former is not known.

β-(1→6)-D-*Glucans.* Pustulan was kindly provided by Professor B. Lindberg (Lindberg & McPherson, 1954). Luteose of high molecular weight was prepared by the method of Nakamura & Tanake (1963) from a culture of *Penicillium aculeatum* var. *apiculatum* grown on a liquid medium containing glucose as carbon source. The polysaccharide was freed from malonic acid by treatment with NaOH.

β-(1→3)-D-*Glucans.* Various samples of laminarin (see Fleming *et al.*, 1966) and pachyman (see Warsi & Whelan, 1957) were available from previous work in this laboratory.

Enzyme preparations. A sample of bacterial endo-β-(1→3)-glucanase (laminarinase) was kindly provided by Glaxo Research Ltd., Greenford, Middx., U.K. Specificity studies, described in detail in the preceding paper (Manners & Wilson, 1973), showed that the enzyme had strong endo-β-(1→3)-glucanase activity, and had no action on gentiobiose, pustulan or luteose.

An endo-β-(1→6)-glucanase was isolated from the culture filtrate of *P. brefeldianum* grown on a medium containing both glucose and luteose as carbon sources (Reese *et al.*, 1962). The enzyme preparation was freed from weak laminarinase and β-glucosidase activity by passage down a column of pachyman (Noble & Sturgeon, 1968). The appropriate column fractions were combined, dialysed to remove reducing sugars, and freeze-dried in 0.1M-sodium citrate buffer, pH4.5. The enzyme preparation caused random hydrolysis of luteose, giving the gentiobiose series of oligosaccharides, but had no action on laminarin or *p*-nitrophenyl β-D-glucoside.

Human saliva was used as a source of α-amylase. It was diluted with an equal volume of water and centrifuged. The supernatant solution, to which a trace of NaCl had been added, was then used without further treatment.

Extraction and purification of the glucans from baker's yeast. Compressed yeast (2kg) was dispersed in 1.6 litres of 6% (w/v) NaOH and stirred at room temperature for 24h. Distilled water (12 litres) was then added and the insoluble material collected by centrifugation at 1200*g* for 15min. This material was suspended in 3 litres of 3% (w/v) NaOH and heated at 75°C for 3h, then allowed to cool, and the suspension was stirred for 16h at room temperature. After dilution with 3 litres of distilled water, the insoluble material was again collected by centrifugation at 1200*g* for 15min. The residue was extracted again with hot 3% NaOH, adjusted to pH4.5 (with HCl) and collected by centrifugation. The insoluble cell-wall material was washed thrice with water, once with ethanol and twice with ether. On drying in air at 37°C, the yield was 47.9g, or 2.4% of the weight of the compressed yeast.

The whole of this material was extracted with 2 litres of 0.5M-acetic acid at 90°C for 3h. After cooling, the suspension was centrifuged and the supernatant solution neutralized (with NaOH), dialysed, concentrated to a small volume, and polysaccharide was precipitated by the addition of 2vol. of ethanol. The precipitate was washed with ethanol and ether and air-dried at 37°C (yield 2.00g).

The insoluble residue was extracted a further 15 times in the same manner. The yield of acetic acid-soluble polysaccharide decreased rapidly with the number of extractions, being 0.36, 0.25 and 0.15g after 6, 12 and 15 extractions. Twelve further extractions with 5 litres of 0.5M-acetic acid were carried out, the residue being finally recovered by centrifugation, dialysed against running water for 3 days, and freeze-dried to give 6.9g of an off-white powder, glucan M1.

To examine the possible degradation effect of hot 0.5M-acetic acid on β-(1→3)-glucan, laminarin was heated under similar conditions. Samples were withdrawn at intervals, neutralized (with NaOH), and the reducing power was measured. The E_{600} was 0.450, 0.465 and 0.470 after 0, 1 and 2h, showing that a barely significant increase in reducing power had occurred. Wolfrom *et al.* (1963) have shown that with disaccharides, the β-(1→2)- and β-(1→3)-linkages were hydrolysed at very similar rates, and that β-(1→6)-linkages were more stable to acid, so that the acetic acid treatment was unlikely to cause selective degradation of only one of the constituent types of β-glucosidic linkage.

Subsequent analysis showed that glucan M1 was still contaminated with traces of the β-(1→6)-glucan. This was removed by incubation with the β-(1→6)-glucanase from *P. brefeldianum*, under conditions which had been devised in preliminary experiments. Glucan M1 (0.47g) was suspended in 50ml of 0.01M-sodium citrate buffer, pH5.0, and incubated, with stirring, for 24h at 37°C with 5mg of enzyme. A further 5mg of enzyme in 1ml of buffer was then added, and the incubation continued for a further 48h. The glucan was collected by centrifugation, washed thrice with water, dialysed against running water for 24h and isolated by freeze-drying. The yield of glucan M2 was 0.43g, and represents the purified β-(1→3)-glucan (see Scheme 2 for summary). It should be noted that there were unavoidable experimental losses during the various purification steps, and the final yields of glucans M1 and M2 are not necessarily representative of their content in the initial pressed yeast.

Results

Studies on yeast glucan

Nature of the minor glucosidic linkages. Since the methylation analysis of Bell & Northcote (1950) indicated the presence of (1→2)-glucosidic linkages, whereas the later studies of Peat *et al.* (1958*a*) showed (1→6)-glucosidic linkages to be present, various samples of yeast glucan were subjected to partial acid hydrolysis, and the resultant mixture of disaccharides separated and identified by micro-scale methylation. Dr. D. J. Bell and Professor S. Peat kindly provided samples of their yeast glucan preparations (samples B and P, respectively) for comparative purposes.

Yeast glucan (samples A1, A2, B and P; 90–100mg) was partially hydrolysed at 100°C essentially as described by Peat *et al.* (1958*a*), by using 90% formic acid for 32min followed by 0.22M-H_2SO_4 for 1h. Paper-chromatographic analysis (solvent *A*) gave identical results with all the glucan samples, and showed the presence of sugars with the R_{Glc} values of D-glucose, laminaribiose, laminaritriose and gentiobiose, and an unidentified sugar with an R_{Glc} value (0.57) similar to, but not identical with, that of the β-(1→2)-linked disaccharide sophorose (0.59). The hydrolysates were fractionated by preparative paper chromatography (solvent *A*).

Authentic samples of laminaribiose, sophorose and gentiobiose were methylated on the micro-scale, methanolysed, and the resulting mixture of methyl glycosides was examined by g.l.c. Each disaccharide gave peaks corresponding to 2,3,4,6-tetra-*O*-methylglucose and a characteristic tri-*O*-methylglucose. The hydrolysate fractions from glucans A1 and B were treated similarly and identified as glucose, laminaribiose, laminaritriose and gentiobiose. None of the disaccharides gave rise to 3,4,6-tri-*O*-methylglucose. The unknown sugar was identified as maltose, and presumably arose from a small amount of glycogen, which was shown to contaminate the glucan samples. Glucans A1 and B were incubated with salivary α-amylase for 10h; paper-chromatographic analysis showed the production of some glucose and maltose, indicating that even with exhaustive extraction it was difficult to remove all of the glycogen from a yeast glucan preparation.

The possibility that the gentiobiose had arisen by acid reversion from glucose or by acid-catalysed transglucosylation from laminaribiose was ruled out, since a partial acid hydrolysate of pachyman, prepared and analysed under similar conditions, did not contain gentiobiose, although the expected mixture of glucose and laminarisaccharides was present.

We conclude that none of the four glucan samples yield sophorose on partial acid hydrolysis, and therefore do not contain (1→2)-glucosidic linkages.

Methylation analysis of yeast glucan. Yeast glucan A1 (2.0g) was successively methylated five times by the method of Haworth (1915), once by that of Kuhn & Trischmann (1963) and ten times by the procedure of Purdie & Irvine (1903). After 13 methylations, the yield of methylated glucan, which had methoxyl content of 40.0%, was 0.69g; after 16 methylations, the yield was 0.33g and the product had methoxyl content 41.5%. Since further treatment with the methylating reagents had little effect on the methoxyl content, and experimental losses had become unacceptable, the methylated glucan was examined at this stage, on the assumption that it was representative of the whole glucan, and that no change in com-

position had occurred during the treatment by the method of Purdie & Irvine (1903). Previous methylation studies on the related β-(1→3)-glucan, soluble laminarin, had shown that increasing the methoxyl content from 37.0 to 43.1% did not alter the number of methylated sugars produced on subsequent hydrolysis (Fleming *et al.*, 1966).

Paper-chromatographic analysis (solvents *B* and *C*) of an acid hydrolysate indicated the presence of tetra-, tri- and di-*O*-methylglucose and small amounts of mono-*O*-methylglucoses and glucose. Multiple development in solvent *B* separated the tri-*O*-methylglucose into two compounds, the slower of which chromatographed at the same rate as 2,4,6-tri-*O*-methylglucose. By quantitative paper chromatography (solvent *B*) of a hydrolysate of 50mg of methylated glucan, hypoiodite oxidation being used to determine the sugars, the corresponding yields were 4.1, 64.4, 28.0 and 3.5%. It was not possible to separate the tri-*O*-methyl sugars in this experiment.

Methylated yeast glucan (266mg) was hydrolysed as described above. A small sample of the hydrolysate was treated with methanolic HCl and examined by g.l.c. on a column containing butane-1,4-diol succinate polyester. Peaks were obtained which corresponded to the methyl glucosides of 2,3,4,6-tetra-, 2,3,4-tri- and 2,4,6-tri-*O*-methylglucose. The major part of the hydrolysate (equivalent to 230mg of methylated glucan) was chromatographed on a column (45cm × 3cm) of Whatman cellulose powder, eluted with butan-1-ol saturated with water–light petroleum (b.p. 100–120°C) mixtures. Appropriate fractions were combined for analysis by hypoiodite oxidation, by paper chromatography in solvents *B* and *C*, and by g.l.c. A total of 890 fractions were collected, from which two major and one minor isomer of tri-*O*-methylglucose and three isomeric di-*O*-methylglucoses were separated and identified. The latter were also characterized by paper-electrophoretic mobility in 0.1M-sodium borate buffer, pH 10.0, with 2,4- and 4,6-di-*O*-methylglucose as reference compounds. The amounts of the various methylated sugars identified was as follows: 2,3,4,6-tetra-, 6.4%; 2,4,6-tri-, 63.4%; 2,3,4-tri-, 7.5%; 3,4,6-tri-, 0.8%; 2,4-di- 8.3%; 4.6-di-*O*-methylglucose, 5.8%. The third di-*O*-methyl sugar (5.7%) was probably the 2,6-isomer. In addition 2.1% of a mixture of glucose and three mono-*O*-methylglucoses was present. The overall recovery of methylated sugars from the cellulose column was 92%. These results clearly established the presence in the yeast glucan of (a) about 6% of non-reducing terminal glucopyranose residues, (b) about 63% of glucose residues linked solely through C-1 and C-3, and (c) about 8% of glucose residues linked solely through C-1 and C-6. The structural implications of the other methylated sugars will be considered below, in relation to the extent of methylation of the original glucan.

In a previous report (Manners & Patterson, 1966), the above methylated sugars were considered to arise from only one glucan. It is now evident that part of the tetra-*O*-methylglucose and all the 2,3,4-tri-*O*-methylglucose are derived from the minor β-(1→6)-glucan (cf. Manners *et al.*, 1973), so that our views on the structure of yeast glucan have had to be modified accordingly.

Periodate-oxidation analysis of yeast glucan. Samples of yeast glucan A1 (122mg) and A2 (64mg) were oxidized with sodium metaperiodate (75ml of 0.1M and 50ml of 0.05M solution respectively). The initial production of formic acid indicated the presence of one triol group per 6.8 glucose residues in glucan A1, and one per 7.0 residues in glucan A2.

Yeast glucan A2 (140–150mg) was also oxidized with 20ml of 0.03M-sodium metaperiodate solution. In duplicate oxidations, 0.26 and 0.27 mol of periodate per anhydroglucose residue were reduced.

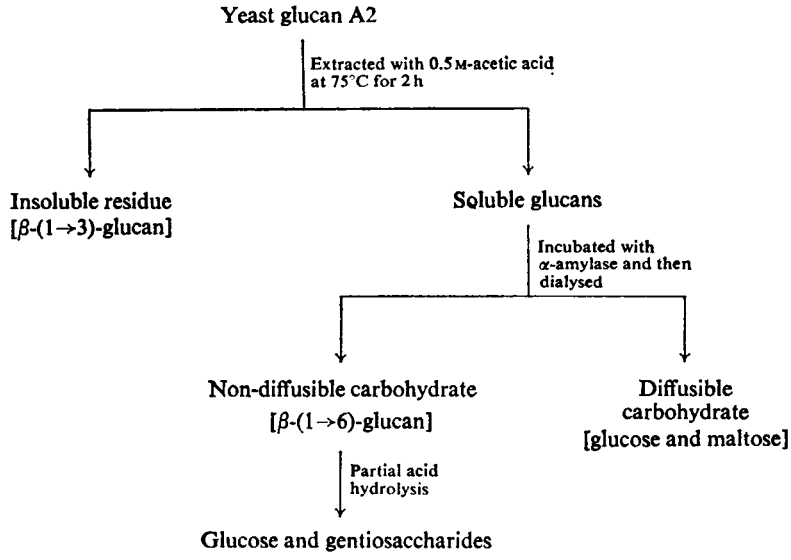
Enzymic degradation of yeast glucan. When samples of glucan A1 or A2 were incubated with the bacterial laminarinase preparation, paper-chromatographic analysis showed that glucose and laminarisaccharides were detectable within 12h and that the final products, after incubation for 4 days, were glucose, laminaribiose and laminaritriose. Gentiobiose was not detected.

The action of a specific β-(1→6)-glucanase from *P. brefeldianum* on glucan A2 was also examined. Within 24h glucose and a mixture of gentiobiose, gentiotriose and gentiotetraose were detected. After 7 days, a further addition of enzyme was made. After 14 days, the glucan was still insoluble and its appearance was unchanged, indicating that extensive degradation such as debranching or random hydrolysis had not taken place.

Yeast glucan A1 was also incubated with a culture fluid of the myxobacterium *C. johnsonii*, kindly provided by Dr. D. M. Webley (see Bacon *et al.*, 1970). Glucose, the (1→3)-β-linked and the (1→6)-β-linked series of oligosaccharides were produced, showing that the mixture of β-glucanases present in the culture fluid could hydrolyse both glucan components.

Demonstration of the heterogeneity of yeast glucan. The presence of a predominantly β-(1→6)-glucan component in yeast glucan was first observed by Bacon & Farmer (1968), who used hot dilute acetic acid to extract this polysaccharide selectively from a normal preparation of yeast glucan. A similar procedure was applied to glucan A2 (2g) as shown in Scheme 1, by using 50ml of acetic acid. After extraction, the residual material was isolated by centrifugation and washed twice with distilled water. These washings and the acetic acid extract were combined, neutralized to pH 7.0 (with NaOH), deionized (mixed IR-120 and IR-45 resins) and evaporated to dryness.

The acetic acid extract was dissolved in 5ml of water. With iodine, a portion (1ml) gave a light



Scheme 1. *Fractionation of yeast glucan A2 by extraction with acetic acid*

red–brown solution with $\lambda_{max.}$ 430nm. Under similar conditions, a 1% solution of glycogen gave a deep red–brown colour and had $\lambda_{max.}$ 440nm. The remainder (4ml) was incubated with an equal volume of diluted saliva at 37°C under toluene for 24h and then dialysed against water. The diffusible carbohydrate was collected, concentrated, deionized and finally evaporated to a syrup, which contained glucose and maltose.

The non-diffusible carbohydrate was concentrated to a small volume, made 0.5M with respect to H_2SO_4 and heated to 100°C for 1h. Paper-chromatographic analysis (solvent *A*) of the neutralized partial acid hydrolysate showed the presence of glucose, gentiosaccharides of degree of polymerization 2–5, with traces of mannose and galactose and unknown sugars of R_{Glc} 1.37 and 1.74.

This experiment showed clearly that glucan A2 was contaminated with a trace of glycogen, and with a significant amount of polysaccharide that on partial acid hydrolysis gave gentiosaccharides, i.e. a β-(1→6)-glucan. The traces of mannose and galactose presumably arose from other contaminating polysaccharide material.

Smith degradation of yeast glucan. The Smith-degradation procedure, i.e. the sequence of reactions involving periodate oxidation and borohydride reduction followed by mild acid hydrolysis causing the hydrolysis of acetal, but not glycosidic, linkages, has been widely used for the analysis of glucans containing (1→3)- and either (1→4)- or (1→6)-glucosidic linkages (Goldstein *et al.*, 1965). Residues that are linked through C-1 and C-3, or through C-1, C-3 and C-6 as at branch points, are resistant to periodate oxidation, and are isolated as a derivative of glucose. By contrast, residues which are linked through C-1 and C-4 or C-1 and C-6 are oxidized, and the remaining fragments are isolated as derivatives of erythritol or glycerol, and glycollic aldehyde. The application of the procedure to a mixture of a β-(1→3)- and a β-(1→6)-glucan should therefore result in the selective oxidation and fragmentation of the latter glucan. The residual polysaccharide should be the (1→3)-glucan from which the original non-reducing terminal residues have been removed.

Yeast glucan A2 (3.85g) was subjected to the Smith-degradation procedure (see the Methods and Materials section), giving a residual polysaccharide (3.0g) and a mixture of low-molecular-weight carbohydrates. These were separated by preparative paper chromatography (solvent *D*) and characterized as far as possible (Table 1). Fraction 1 had the same R_{Glc} value as authentic glycerol, and the derived *p*-nitrobenzoate had m.p. 193°C, which was not depressed on admixture with glycerol tri-*p*-nitrobenzoate. Fractions 2 and 3 could not be clearly separated in solvents *A*, *D* or *E*, but contained about equal amounts of carbohydrates with the R_{Glc} values of glycollic aldehyde and erythritol. Fractions 4 and 5 could not be identified; they both gave rise to glycerol and erythritol on borohydride reduction followed by acid hydrolysis. Fractions 6 and 7 were not entirely pure, but on acid hydrolysis gave glucose and glycerol as the major products. Fraction

Table 1. *Composition of soluble carbohydrates from Smith degradation of yeast glucan A2*

The identity of fractions 1, 2 and 3 is established, but that of fractions 6–9 is only tentative, since they were mixtures containing carbohydrates of similar R_{Glc} mobilities. The probable identity of the major component of these fractions is given, but the structural implications of the tri- and tetra-saccharide are not known.

Fraction no.	R_{Glc} in solvent *D*	Yield (mg)	Probable identity
1	2.6	67.7	Glycerol
2	2.3	16.0	Glycollic aldehyde +
3	2.2		erythritol
4	1.6	10.0	Unknown
5	1.35	5.1	Unknown
6	1.0	10.7	Glucosylglycerol etc.
7	0.85–1.00	14.3	Glucosylglycerol etc.
8	0.50	6.5	Laminaribiosylglycerol etc.
9	0.34	3.1	Laminaritriosylglycerol etc.

8 was only weakly detected with aniline oxalate and had an R_{Glc} value similar to that of laminaribiose; however, it was not homogeneous, and on acid hydrolysis glucose was the major product, with smaller amounts of glycerol and some erythritol. Fraction 9 was also a mixture; it resembled fraction 8 except that the R_{Glc} value was similar to that of laminaritriose. The presence of erythritol and derivatives in some of the above fractions presumably arose from the Smith degradation of the trace of contaminating glycogen in the original glucan A2 preparation. This suggestion was later verified experimentally (Masson, 1969) by carrying out a Smith degradation on a glucan sample that had been incubated with salivary α-amylase. In this case, 2.70g of glucan gave 2.26g of polysaccharide, and erythritol was absent from the various carbohydrate fractions. The presence of glycerol, glycollic aldehyde, glucosylglycerol and glycerol-containing oligosaccharides was confirmed.

The above results show that extensive fragmentation of part of the glucan preparation had occurred, but the major portion was resistant to periodate oxidation and was therefore a β-(1→3)-glucan. This polysaccharide was characterized as follows.

(*a*) A comparison (see Table 2) of partial acid hydrolysates of this polysaccharide and the original yeast glucan, by quantitative paper chromatography, showed that the gentiobiose content had been greatly decreased by the Smith degradation, although the presence of this disaccharide indicated that some (1→6)-linked glucose residues were not oxidized by periodate, and must therefore have been triply linked at C-1, C-3 and C-6. These residues therefore serve as branch points. The gentiotriose content of the glucan had been eliminated by the degradation procedure.

(*b*) The Smith-degraded glucan was extensively hydrolysed by the bacterial laminarinase preparation. In one experiment, the increase in reducing power corresponded to 38% apparent conversion into glucose under conditions in which the original glucan gave 32%, i.e. the extent of hydrolysis, which must be related to the relative proportion of β-(1→3)-glucosidic linkages, was significantly greater than in the parent polysaccharide.

(*c*) On periodate oxidation, the Smith-degraded glucan reduced 0.13mol of periodate per anhydroglucose residue, whereas under the same conditions the original glucan reduced 0.27mol.prop. The initial production of formic acid indicated the presence of one triol group per 30.6 glucose residues. In a second experiment, one triol group per 33.3 glucose residues was present. Since any glucose residues linked solely through C-1 and C-6 would have been eliminated during the Smith degradation, these results represent the production of formic acid arising only from non-reducing terminal glucose residues, and indicate an average chain length of about 32 glucose residues in the degraded glucan, and hence a chain length of 33 in the original polysaccharide, equivalent to about 3% of β-(1→6)-interchain linkages.

Isolation of β-(1→3)-glucan from baker's yeast. In view of the results obtained with yeast glucan, and its subsequent extraction with hot dilute acetic acid, a combined procedure was devised for the isolation of the β-(1→3)-glucan. This is summarized in Scheme 2. From 47.9g of alkali-insoluble carbohydrates, 6.9g of glucan M1 was finally obtained. The overall yield was low, owing to inadvertent experimental losses during the fractionation.

The i.r. spectrum of glucan M1 was typical of a β-(1→3)-glucan, although there was a slight shoulder at 920cm^{-1}, which is characteristic of β-(1→6)-glucans. A partial acid hydrolysate contained glucose and substantial amounts of laminarisaccharides of degree of polymerization 2–5 with smaller amounts of gentiosaccharides of degree of polymerization 2–5. It was evident that even the prolonged acetic acid extraction had not removed all the β-(1→6)-glucan.

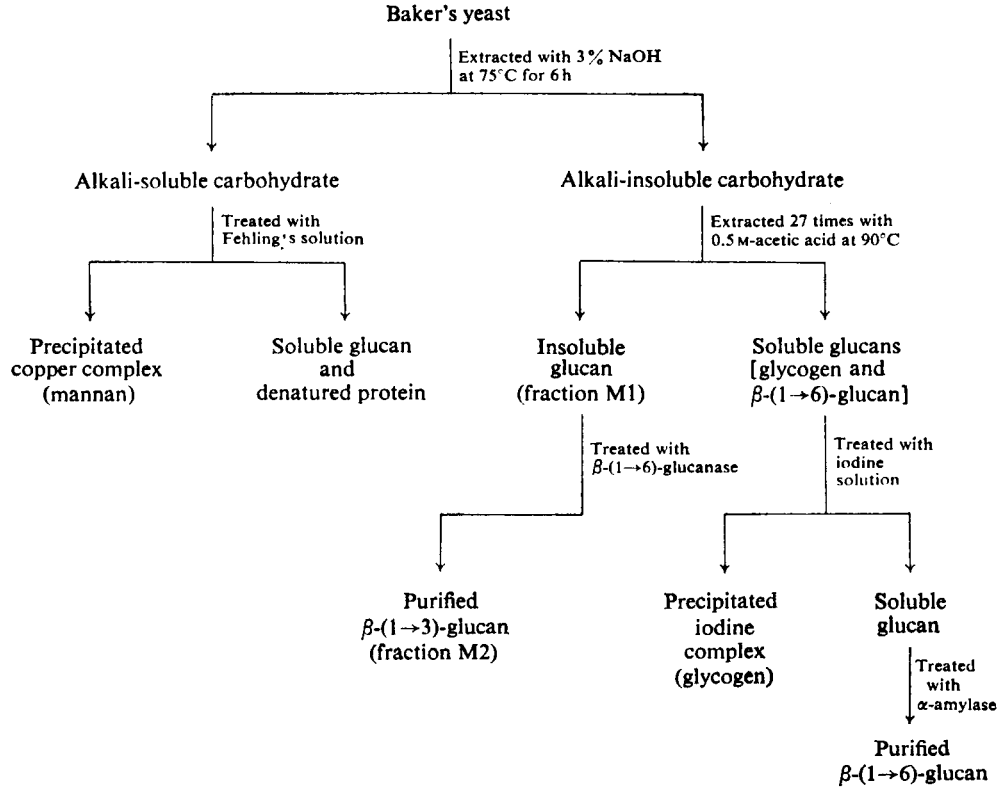
This view was confirmed by periodate oxidation

Table 2. *Products of partial acid hydrolysis of yeast glucan A2 before and after the Smith degradation*

For details see the text.

	Original glucan		Smith-degraded glucan	
Fraction no.	Identity	Relative amount (%)	Identity	Relative amount (%)
1	Laminaribiose*	23	Laminaribiose	26
2	Gentiobiose+laminaritriose (trace)	5	Gentiobiose+laminaritriose†	2
3	Laminaritriose	18	Laminaritriose	22
4	Gentiotriose (major)+ laminaritetraose (minor)	16	Laminaritetraose‡	19
5	Higher oligosaccharides	39	Higher oligosaccharides	30

* This fraction also contained some maltose.
† Relative amounts about 3:1 gentiobiose/laminaritriose, from visual estimation of paper chromatograms.
‡ This fraction also contained some unidentified oligosaccharides.



Scheme 2. *Fractionation of baker's yeast to give purified β-(1→3)- and β-(1→6)-glucans*

The alkali-soluble carbohydrate is included for completeness, but is not considered further in this discussion

analysis of glucan M1. The initial reduction of periodate amounted to 0.17 mol.prop. and the initial production of formic acid indicated the presence of one triol group per 7.8 glucose residues.

The contaminating β-(1→6)-glucan was finally removed by selective enzymolysis, by using the purified β-(1→6)-glucanase preparation from *P. brefeldianum*. During this digestion, no physical changes were observed in the glucan, which remained as an insoluble suspension. Solubilization, which would be expected if debranching had occurred, did not therefore take place. Purified glucan M2 was finally obtained in 92% yield from glucan M1.

Properties of the purified β-(1→3)-glucan. Paper-chromatographic analysis (solvent *A*) of a partial acid hydrolysate showed the presence of glucose and laminarisaccharides of degree of polymerization 2–5, together with a trace of gentiobiose. Gentiotriose and higher gentiosaccharides could not be detected, under conditions in which their presence in a partial acid hydrolysate of glucan M1 was observed. The glucan became solubilized during incubation with the bacterial laminarinase preparation. After 7 days, glucose, gentiobiose and laminarisaccharides of degree of polymerization 2–4 were produced. Other oligosaccharides of lower R_{Glc} value were also present, but these were not gentiotriose, gentiotetraose or gentiopentaose.

On periodate oxidation, the initial reduction was 0.12 mol.prop. and the initial production of formic acid indicated the presence of one triol group per 30.5 glucose residues. The degree of polymerization of the glucan, determined by the sorbitol dehydrogenase method, was 1450±150. In view of the insolubility of the glucan, and consequent difficulty with the reactions, this result is regarded as indicating the order of magnitude of the glucan molecules. Nevertheless, since the degree of polymerization is very much greater than the average chain length, the glucan has a branched structure. The overall results therefore show the presence of about 3% of β-(1→6)-glucosidic interchain linkages.

Discussion

The partial acid-hydrolysis studies on four different samples of yeast glucan show that they contain a major proportion of β-(1→3)-glucosidic linkages and a minor proportion of β-(1→6)-glucosidic linkages, in agreement with the previous results of Peat *et al.* (1958*a*). The absence of sophorose from the hydrolysate, including the sample originally analysed by Bell & Northcote (1950), indicates that β-(1→2)-linkages are absent. The apparent discrepancy between the methylation analysis of these authors, and the results obtained by Peat *et al.* (1958*a*) and those recorded here, is not readily explained, but we are indebted to the late Dr. D. J. Bell for helpful discussions of this problem. Bell & Northcote (1950) emphasize that 'methylation of yeast glucan is difficult', a view with which we entirely agree. After 31 methylations, their product had a methoxyl content of 41.7%; the theoretical value for a tri-*O*-methyl glucan is 45.6%. By chromatography on silica gel, these authors obtained 2,3,4,6-tetra-, 2,4,6-tri- and 4,6-di-*O*-methylglucose in the molar proportions of 1:7:1, in an aggregate yield of 90%. However, with the observed extent of methylation, the amount of di-*O*-methylglucose should have been considerably greater than that of tetra-*O*-methylglucose, as we have found, and it seems likely that not all of the di-*O*-methylglucose was accounted for, or characterized. The 4,6-isomer is the least soluble of the di-*O*-methylglucoses and may crystallize from a mixture with other isomers, so that the latter would be undetected. Moreover, at the time the original work was carried out (1949–1950), the simple solvent used for paper-chromatographic analysis (butan-1-ol–water) would not have separated the isomeric di-*O*-methylglucoses from each other. In addition, the key reference compound 2,4-di-*O*-methylglucose was not known then, and only became available some years later when its preparation was first described by Bell & Manners (1954).

It should also be emphasized that sample B used by Bell & Northcote (1950) had a significantly higher degree of branching than sample A1. The proportion of tetra-*O*-methylglucose corresponded to one terminal non-reducing residue per 9 and 16 glucose residues respectively. Since periodate-oxidation analysis of sample B indicated the presence of one triol group per 10 glucose residues, it follows that the degree of contamination of sample B with a β-(1→6)-glucan must have been minimal. Similar analysis of sample A1 showed the presence of one triol group per 6.8 glucose residues. This represents the total production of formic acid from (*a*) non-reducing terminal residues and (*b*) glucose residues linked solely through C-1 and C-6. By methylation analysis, these amounted to 6.4 and 7.5% respectively, i.e. a total of 13.9%, which compares favourably with the value of 14.7% by periodate oxidation. In sample P examined by Peat *et al.* (1958*a*), the proportion of triol groups was one per 10.3 glucose residues. This emphasizes that different samples of yeast glucan show significant differences in this property, which reflects (*a*) the relative proportion of the β-(1→3)- and β-(1→6)-D-glucan and (*b*) the relative degree of branching in both components.

The heterogeneous nature of yeast glucan, as normally prepared, is shown clearly by the results obtained by enzymic degradation, and by the fractionation Schemes 1 and 2. The major component (about 85%) is a β-(1→3)-glucan with a low degree of branching, which may be selectively hydrolysed by a

bacterial endo-β-(1→3)-glucanase, leaving unattached the minor component (see also Manners & Masson, 1969). Alternatively, the minor component may be preferentially degraded either enzymically by a fungal β-(1→6)-glucanase, or chemically by the Smith-degradation method. This latter procedure yields a product which is virtually identical with that obtained by the fractionation Scheme 2. Thus, the initial reduction of periodate is 0.13 and 0.12 mol.prop. respectively and the initial production of formic acid indicates the presence of one triol group per 33 and 31 glucose residues respectively. In the absence of glucose residues linked solely through C-1 and C-6, both of these formic acid results indicate a degree of branching of 3%. The interchain linkages are identified as β-(1→6)-glucosidic, on the basis of partial acid hydrolysis studies on (*a*) the original yeast glucan samples, (*b*) the glucan remaining after the Smith degradation (Table 2) and (*c*) the purified glucan M2. The methylation analysis of glucan A1, which shows that 2,4-di-*O*-methylglucose is the major di-*O*-methyl sugar, is in accord with this view, although owing to the extent of undermethylation of the glucan, it does not provide definitive proof. It should also be noted that the insoluble nature of the Smith-degraded glucan eliminates any possibility of a linear structure containing certain sequences of β-(1→3)- and β-(1→6)- glucosidic linkages (Peat *et al.*, 1958*a*), since this would have been converted into a mixture of soluble mono- and oligo-saccharide derivatives.

Although the observed degree of branching in the β-(1→3)-glucan is much lower than that reported by other workers for yeast glucan (e.g. Bell & Northcote, 1950; Misaki *et al.*, 1968), it is in agreement with the results of Barry & Dillon (1943), who obtained an average chain length of 28 by a periodate-oxidation method. On the assumption that there was no inherent error in either the preparation of the polysaccharide, or the method of analysis, these latter results provide further evidence for the view that different samples of yeast glucan may differ significantly in degree of branching.

The molecular weight of glucan M2 (about 240000) represents the highest value yet recorded for any yeast glucan preparation, and the order of magnitude has been confirmed by using a sample of glucan soluble in dimethyl sulphoxide (kindly provided by Dr. J. S. D. Bacon; see Bacon *et al.*, 1969), which had a degree of polymerization of 1580±150 (equivalent to a mol.wt. of 256000). This result also implies that little, if any, degradation of glucan M1 had occurred during the extraction with hot acetic acid, since the latter reagent was not used by Bacon *et al.* (1969). Misaki *et al.* (1968) obtained a degree of polymerization of 410 for glucan prepared from alkali-extracted yeast by extraction with acetic acid followed by solution in dimethyl sulphoxide. However, this result was based on the yield of formaldehyde released by periodate oxidation of the borohydride-reduced glucan; this procedure does not give quantitative results (Annan *et al.*, 1965; Manners *et al.*, 1971).

The structure of yeast glucan has been independently examined by F. Smith and his co-workers (Misaki & Smith, 1963; Misaki *et al.*, 1968), who postulated the structure shown in Fig. 1.

Methylation analysis of glucan (degree of polymerization 410) gave 2,3,4,6-tetra-, 2,4,6-tri- and 2,4-di-*O*-methylglucose in the molar proportions of 1:7:1, together with a trace of the 2,3,4-tri-*O*-methyl derivative. After Smith degradation, the glucan had a degree of polymerization of 150, and after methylation analysis, the molar proportions of the methylated sugars were 1:6:1. 2,3,4-Tri-*O*-methylglucose was not detected. The proposed structure is not consistent with our results, but their experimental results could be explained by assuming that the glucan preparation was contaminated with a small amount of β-(1→6)-glucan. This would account for the second tri-*O*-methyl sugar produced on methylation of the original, but not the degraded, glucan. It would also account for the small amounts of glycerol and β-glucosylglycerol isolated during the Smith-degradation procedure. The degree of polymerization of the degraded glucan was measured by a different method from that used with the original glucan. This depended on the yield of glycerol, which was attached to the reducing

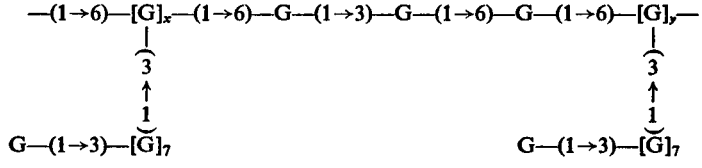


Fig. 1. *Partial structure of yeast glucan proposed by Misaki et al.* (1968)

G = β-D-glucopyranose residue, and $x+y = 40$–50.

end of a polysaccharide chain, and would arise from any residues linked through C-1 and C-6. However, it should be noted that the terminal reducing residue of a branched β-(1→3)-glucan will also yield 1 mol.prop. of glycerol under these experimental conditions. In general, periodate-oxidation methods for the determination of the degree of polymerization of glucans are not always satisfactory, and their accuracy decreases rapidly with increase in molecular weight (cf. Manners *et al.*, 1971). Our interpretation of the results of Misaki *et al.* (1968) is that their particular strain of *S. cerevisiae* had produced a β-(1→3)-glucan, which had a much higher degree of branching than our strain, but comparable with that found by Bell & Northcote (1950), and that their glucan preparation contained a small amount of a β-(1→6)-glucan.

The special properties of yeast glucan in providing an insoluble 'envelope' for the cells have been discussed in detail (Bacon *et al.*, 1969; MacWilliam, 1970; Phaff, 1971) and need not be repeated here. Our present view is that a β-(1→3)-glucan of high molecular weight and relatively low degree of branching could form an essentially two-dimensional randomly branched tree-type structure of the kind originally postulated for amylopectin. Some restriction from a wholly three-dimensional arrangement is necessary to account for the probable layer of glucan in the cell walls (cf. MacWilliam, 1970). Certain portions of adjacent chains, from different molecules, could be closely associated to form a continuous structure. An essential feature of a tree-type structure is multiple branching, and it is recognized that our present results do not distinguish between structures having single or multiple branching. Alternatively, the glucan could have a comb-type structure with a long chain to which were attached, at intervals of unknown distance, linear side chains containing, on average, about 30 glucose residues. Since the degree of polymerization of the β-(1→3)-glucan is about 1500, each molecule would have to contain about 50 side chains. These two structural types resemble those postulated for amylopectin- and glycogen-type polysaccharides (for a review, see Manners, 1957).

It has been shown that an algal β-(1→3)-xylan may assume a helical conformation with three chains together forming a triple helix (Atkins & Parker, 1969). Rees (1973) has suggested that certain β-(1→3)-glucans could assume similar conformations, since the replacement of the equatorial H at C-5 of D-xylose by $-CH_2OH$ should not affect the tendency to form helical structures. We have no direct evidence that yeast β-(1→3)-glucan exists even partly in the triple-helix state, but this conformation could provide a means of linking together, partly by hydrogen bonding and partly by other forces of attraction, adjacent molecules to give a macromolecular structure with a substantial degree of rigidity and insolubility in water. The presence of a low degree of branching in the glucan would be expected to affect the packing of helices and their alignment, but need not necessarily prevent the formation of a helix. At the same time, the structure could be 'loosened' by ultrasonic treatment or suspension in certain solvents such as dimethyl sulphoxide (cf. Misaki *et al.*, 1968; Bacon *et al.*, 1969). It should be noted that in β-(1→3)-glucans the length of unbranched chains of glucose residues has a marked effect on solubility properties. Soluble laminarin has a low degree of branching and an average chain length ranging from 7 to 10 glucose residues (Fleming *et al.*, 1966). By contrast, the molecules of insoluble laminarin are essentially linear, and contain about 15–20 residues. By analogy, the degree of branching in the relatively large yeast β-(1→3)-glucan molecules is so low that substantial linear segments of β-(1→3)-linked glucose

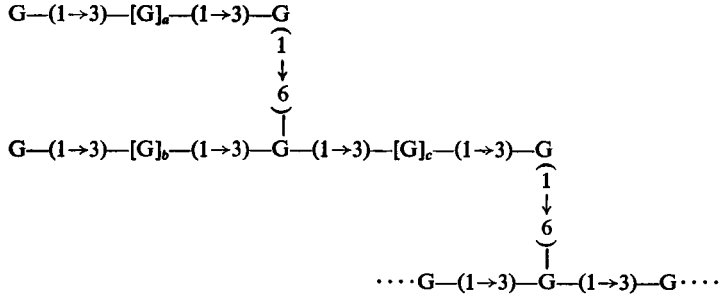


Fig. 2. *Partial structure of a segment of yeast* β-(1→3)-*glucan*

a+*b*+*c* comprise about 60 glucose residues, although the extract lengths of *a*, *b* and *c* are unknown.

residues must be present, and at least some of these could be aligned together, both inter- and intramolecularly, to form an insoluble aggregate or gel, depending upon the potential solvent and temperature (see Fig. 2).

Our overall conclusion is that for the successful structural analysis of even the apparently simplest homopolysaccharide, a combination of experimental methods must be employed.

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