Polysaccharides of Baker's Yeast. Part I. Glycogen.

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The method of "linkage analysis" of polysaccharides which involves the partial acid hydrolysis of a polysaccharide and the isolation (and characterisation) of the oligosaccharide fragments by charcoal chromatography has been applied to yeast glycogen and has resulted in the detection of only α-1 : 4- and α-1 : 6-types of glucosidic linkage. An essential part of the proof has consisted in the separation and isolation of maltose and *iso*maltose from the acid hydrolysate, readily accomplished on a charcoal column. The conclusion is reached that most, if not all, of the branch linkages are 1 : 6-links in the α-configuration.

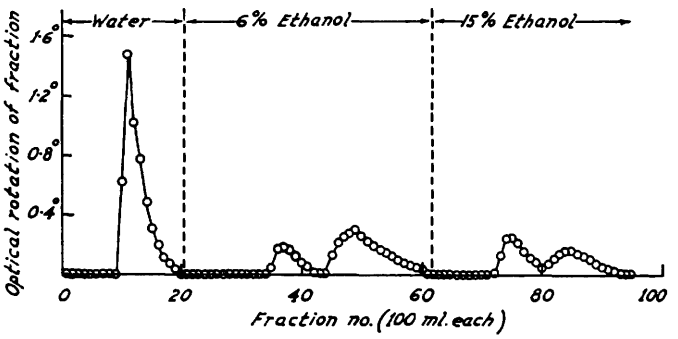
OUR researches over a number of years have been concerned with the metabolism of starch and related polysaccharides in the higher plants. Cognate studies of the maltase and invertase of yeast have renewed our interest in the polysaccharides of yeast and in particular in the problem of the inter-relation which might exist between the cell-wall polysaccharides, mannan and glucan, and the glycogen. This first communication reports the results of a structural analysis of the glycogen of baker's yeast. The earlier literature contains suggestions of the existence of two glycogens in yeast but Northcote (*Biochem. J.*, 1953, **53**, 348) has presented evidence which disposes of this misconception.

One of the most satisfactory methods of what might be called "linkage-analysis" of

branched polysaccharides consists in the partial hydrolysis of the polysaccharide by acid and the isolation and characterisation of the oligosaccharide fragments so produced. As has been pointed out by Whistler and Smart (" Polysaccharide Chemistry," Academic Press, New York, 1953) the method is free from the dubiety which attaches to assays based upon methylation or upon periodic acid oxidation. The classical method of hydrolysis of the methylated polysaccharide and characterisation of the resulting partly methylated monosaccharides has always been open to the very serious objection that it is rarely possible to achieve complete methylation of all the free hydroxyl groups in a large polysaccharide molecule. The objection to the less laborious periodate method lies in the difficulty experienced in controlling the reaction and estimating the extent of over- or under-oxidation in a given set of circumstances.

In the method of partial acid hydrolysis, the possibility of reversion synthesis of di- and tri-saccharides exists but does not detract from the value of the method since conditions of hydrolysis can usually be found under which reversion synthesis is insignificant in extent. The method obviously depends on quantitative separation of the oligosaccharides produced by fragmentation of the polysaccharides. The technique used for this purpose is

FIG. 1. *Fractionation of glycogen hydrolysate on charcoal–Celite.*



chromatographic separation on charcoal, originated by Whistler and Durso (*J. Amer. Chem. Soc.*, 1950, **72**, 677).

So far as we are aware, the only structural analysis hitherto made of yeast glycogen is that reported by Northcote (*loc. cit.*). The procedure adopted by this author included (i) end-group assay by the methylation (Haworth) and the periodate method, (ii) examination of the acid hydrolysate of the methylated glycogen, and (iii) study of the β-amylolysis of the polysaccharide. In none of these particulars was the yeast glycogen distinguishable from animal glycogens. It possesses a highly branched structure; the chain-forming linkages are 1 : 4-glucosidic and the average basal chain length * is 12 glucose units. Branching was indicated by the existence of a β-amylolysis limit at 50% conversion and by the isolation of a di-*O*-methylglucose fraction from the hydrolysate of the methylated glycogen. Paper chromatograms suggested the presence of two di-*O*-methylglucoses but neither was identified. The type or types to which the branch linkages belong remained therefore undetermined.

In our experiments the glycogen was extracted by acetic acid from fresh baker's yeast, by Northcote's procedure (*loc. cit.*), and was submitted to partial hydrolysis (with sulphuric acid) equivalent to 75% conversion into glucose. The neutralised hydrolysate was then separated on a charcoal column (cf. Whelan, Bailey, and Roberts, *J.*, 1953, 1293). Examination of the fractions (see Fig. 1) demonstrated the occurrence of one monosaccharide, two disaccharides, and at least three trisaccharides. The sugars were isolated and converted into the corresponding acetates, the properties of which are recorded in the

* The use of the term " chain length " in connection with a randomly branched polymer is misleading. The so-called chain length of a branched polysaccharide is measured by the percentage of non-reducing end groups and actually represents the number of monosaccharide units per end group. We describe this quantity as " basal chain length."

Table. The monosaccharide fraction, obtained by irrigation of the column with water, consisted of D-glucose only. The disaccharide fraction, eluted by 6% aqueous ethanol, contained two members which were identified by comparison with authentic specimens (see Table) as maltose and *iso*maltose (Fig. 2*b* and *c*). It should be observed (Fig. 1) that a sharp separation of these two sugars, not otherwise easily separable, is achieved on

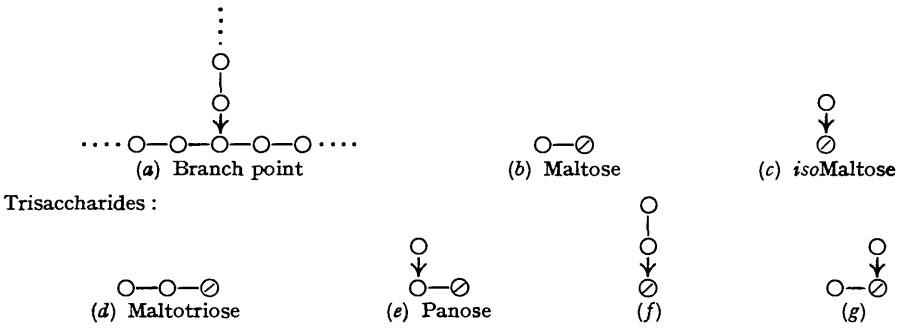
Properties of the products of hydrolysis of baker's yeast glycogen.

Sugar	Yield * (g.)	Characterising derivative	Properties of derivatives † $[\alpha]_D$	m. p.
[Glycogen]	[4·49]			
Glucose	2·45	Sugar	+52·7° (52·6°)	144° (146°)
*iso*Maltose	0·165	Octa-acetate	+96·8 (97)	143—144 (143—144)
Maltose	0·980	Octa-acetate	+62·7 (+63)	158—159 (159—160)
Panose	—	Panitol dodeca-acetate	+119 (+120)	146—147 (148—150)

* Calc. by summation of optical rotations of fractions obtained from the charcoal column (see Experimental section).

† Values found for authentic specimens are given in parentheses.

FIG. 2. *Oligosaccharides from Yeast Glycogen.*



○ = glucopyranose unit; ⊘ = reducing glucose unit; — = α-1 : 4-link; ↓ = α-1 : 6-link.

charcoal. These being the only disaccharide products it may be concluded that only two types of glycosidic linkage exist in yeast glycogen, namely, α-1 : 4- and α-1 : 6-linkages. The importance has already been stressed of providing proof that the oligosaccharides obtained in this type of experiment do not arise from the acid-catalysed polymerisation of the monosaccharide and we have satisfied ourselves by control experiments, which will be described in a later paper, that neither the maltose nor the *iso*maltose obtained from yeast glycogen is an artifact of this kind.

The yields (see Table) of maltose and *iso*maltose were in the approximate ratio of 6 : 1. The ratio of α-1 : 4- to α-1 : 6-linkages in yeast glycogen can be estimated from Northcote's end-group assay (*loc. cit.*) as approximately 11 : 1. The fact that *iso*maltose thus accumulates preferentially with respect to maltose is due to the greater stability of the α-1 : 6-linkage. Wolfrom, Lassettre, and O'Neill (*J. Amer. Chem. Soc.*, 1951, **73**, 595) have shown that at 100° the rates of hydrolysis of maltose and *iso*maltose by acid are in the ratio 4 : 1. These authors have also made calculations of the amounts of *iso*maltose to be expected at various stages in the hydrolysis of rabbit-liver glycogen which has the same proportion of branch linkages as the yeast glycogen. Their calculations show that at 75% hydrolysis (the limit to which our glycogen was taken) the yield of *iso*maltose should be 259 mg. from 4·49 g. We obtained only 165 mg. and this lower yield is known not to be caused by losses during fractionation. This suggests that the relative strength of the linkages in *iso*maltose and maltose is not the same as that of the α-1 : 6- and α-1 : 4-links in the polysaccharide.

The identification of maltose as one of the major products of partial hydrolysis agrees with Northcote's conclusions (*loc. cit.*) and proves that the 1 : 4-glucosidic links have the α-configuration. The isolation of *iso*maltose is consistent with the idea of a branched molecule in which the branch links are of the α-1 : 6-type. It should be noted, however,

that the isolation of maltose and *iso*maltose does not prove that the glycogen of yeast possesses a branched structure.

Conclusive evidence that α-1 : 4- and α-1 : 6-linkages actually occur together in the same molecule was afforded by the isolation and identification of panose (Fig. 2*e*) as a constituent of the trisaccharide fraction eluted from the charcoal column by 15% ethanol. Although panose could be derived from an unbranched polyglucose in which the glucosidic linkages were of two types (α-1 : 4 and α-1 : 6), nevertheless the clear demonstration by Northcote (*loc. cit.*) of the branched character of the yeast glycogen molecule makes it more reasonable to regard the panose which we have isolated as arising from the branched structure represented in Fig. 2*a*.

The number of trisaccharides theoretically derivable by random hydrolysis of a polysaccharide constituted as in Fig. 2*a* is four. One of these, maltotriose (Fig. 2*d*) contains α-1 : 4-links only. The remaining three trisaccharides contain both the α-1 : 4- and the α-1 : 6-link (Fig. 2*e*, *f*, and *g*). Indications were given in our experiments of the presence in the trisaccharide fraction of three of the four possible components and one of these was proved to be panose (Fig. 2*e*). Complete separation of the trisaccharides was not attempted on the charcoal column, and the panose component was ultimately separated from a second trisaccharide by taking advantage of the high electrophoretic mobility (on paper) of the latter sugar in borate buffer. The panose thus separated was identified by conversion into panitol dodeca-acetate (see Table).

While not essential to the present argument, some observations on the constitutions of the other components of the trisaccharide fraction are of interest. One fraction was shown, with some certainty, to be maltotriose (Fig. 2*d*) inasmuch as β-amylase exerted a limited action on some of the fractions in the trisaccharide band. This enzyme preparation is known to hydrolyse maltotriose slowly (Whelan *et al.*, *locc. cit.*) although it has no action on panose (unpublished observation) and it is therefore improbable that it will attack the compounds 2*f* or 2*g*. The trisaccharide which accompanies panose and which is separated from it electrophoretically probably has the structure 2*f*, since this would be expected to show the high electrophoretic mobility (in the presence of borate) of *iso*maltose rather than the low mobility of maltose (cf. Foster, *J.*, 1953, 982).

It is concluded from these experiments taken in conjunction with those of Northcote (*loc. cit.*) that the glycogen from baker's yeast possesses the branched structure which is now commonly accepted for glycogen whatever its source. A rigid experimental proof that the branch linkage is of the α-1 : 6-type has however been afforded by this method in only one other case, namely, for rabbit-liver glycogen. From the latter, by partial acid hydrolysis, maltose and *iso*maltose were obtained (Wolfrom *et al.*, *loc. cit.*). Panose was not isolated in this experiment; indeed, our preparation of panose is the first recorded isolation of this trisaccharide from a glycogen.

By an alternative procedure, namely, application of the method of Hirst, Jones, and Roudier (*J.*, 1948, 1779) to glycogen from a number of other biological sources, Bell and Manners (*J.*, 1954, 1891) have shown that more than 99% of the branch linkages in these glycogens are 1 : 6-links.

Experimental

Preparation of Glycogen.—The glycogen was prepared from fresh baker's yeast (Distillers Co. Ltd.). The alkali-insoluble residue was prepared from the yeast as by Bell and Northcote (*J.*, 1950, 1944) and glycogen was extracted from this with hot dilute acetic acid as follows. The alkali-insoluble residue (112 g.; from 6 kg. of pressed yeast) was treated with 0·5N-acetic acid (2 l.) at 75° for 1 hr., cooled, and centrifuged, and the gelatinous solid stirred with water (2 × 1·5 l.) at 75—80° for 1 hr. The combined extracts were treated with ethanol (2 vol.) at 4° and the precipitated glycogen was removed, washed with ethanol, and dried *in vacuo* over phosphoric oxide at room temperature (yield, 3·44 g.). The insoluble residue (67 g.), which was being used as a source of yeast glucan, was extracted a further four times with hot water as above but still appeared to contain some glycogen. It was therefore re-extracted with acetic acid as above and there was obtained a further 3·64 g. of glycogen which was combined with the first batch. Acidic hydrolysis of the polysaccharide after drying at 60° *in vacuo* over phosphoric oxide yielded 99·0% of glucose (Pirt and Whelan's method,

J. Sci. Food Agric., 1951, **2**, 224). The $[\alpha]_D$ in water was +188·7° (*c*, 0·1086, based on glucose content); ash content, nil (Found: N, nil).

Hydrolysis of the Glycogen.—Glycogen (approx. 5 g.; undried) was weighed into a 250-ml. flask, wetted with ethanol, and dissolved by gentle warming in 0·1N-sodium hydroxide (100 ml.). The solution was neutralised with sulphuric acid, 5N-acid (16·7 ml.) added, and the solution diluted to 250 ml., the final concentration of acid being 0·33N. A portion (1 ml.) was removed for determination of the glycogen content (Pirt and Whelan, *loc. cit.*) which was found to be 4·58 g. The remainder of the solution was heated in a boiling-water bath, portions (1 ml. each) were removed at intervals, neutralised, and diluted to 25 ml., and the reducing powers (as glucose) of 3 ml. portions were determined with the Somogyi copper reagent (*J. Biol. Chem.*, 1945, **160**, 61). After being heated for 170 min. the solution was cooled and at this stage the reducing power corresponded to an apparent conversion into glucose of 75%.

Fractionation of Products of Hydrolysis.—The acid hydrolysate (245 ml., equiv. to 4·49 g. of glycogen) was neutralised (6N-sodium hydroxide) and added to a charcoal–Celite column (adsorbent, 3·5 × 80 cm.) which was prepared and eluted as described by Whelan *et al.* (*loc. cit.*). The optical rotation of each fraction (100 ml.) was measured in a 4-dm. tube and the results are shown in Fig. 1. Fractions nos. 10—20, 34—41, 44—60, 71—75, 76—80, 81—82, 83—85, and 86—92 were combined and evaporated to dryness under reduced pressure.

Examination of Hydrolysis Products.—(a) *Monosaccharide.* Paper chromatography of combined fractions 10—20 revealed the presence of a single reducing sugar having the R_F value of glucose. The product was refluxed with methanol, filtered, and crystallised. The crystals showed $[\alpha]_D$ +87·1° (35 min.) ⟶ 52·7° (4·75 hr.) in water (see Table).

(b) *Disaccharides.* Paper chromatography of fractions 34—41 and 44—60 revealed the presence in each of one reducing sugar having the R_F values of *iso*maltose and maltose respectively. The β-octa-acetates were prepared by using sodium acetate–acetic anhydride, and the properties are reported in the Table (Found: *iso*maltose acetate, C, 49·4; H, 5·6; maltose acetate, C, 49·6; H, 5·7. Calc. for $C_{28}H_{38}O_{19}$: C, 49·5; H, 5·6%).

(c) *Trisaccharides.* Fractions 71—75 and 76—80 were examined by paper chromatography and their contents migrated as single spots having the same R_F value as panose. Fractionation by electrophoresis on paper in borate buffer (pH 8·7; 9·9 g. of H_3BO_3 + 22·88 g. of $Na_2B_4O_7,10H_2O$ per l.) at 400 v (current, 7·5 mA; temp. 4°) revealed the presence of two reducing sugars, the slower of which behaved as panose. The fractions were combined and the two sugars separated by electrophoresis on Whatman no. 3 filter paper, the sugar mixture (approx. 200 mg.) being applied as a narrow streak along a 40-cm. line at right angles to the electric field on each of two papers (46 × 57 cm.). The apparatus used in electrophoresis was constructed from "Perspex" according to Latner (*Biochem. J.*, 1952, **51**, xii). After electrophoresis for 48 hr. at 4° (400 v; 6·5—9 mA) the positions of the sugars were located by spraying guide strips with benzidine–trichloroacetic acid (Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114). The sugars were isolated by extracting the appropriate sections of the papers with water (3 × 200 ml.) and evaporating the extracts to dryness under reduced pressure. The faster-moving component was not further examined. The slower-moving component was dissolved in water (2 ml.), mixed with potassium borohydride solution (1 ml.; 30 mg.), and left for 2 hr. at room temperature. The solution was acidified with acetic acid to destroy excess of borohydride, neutralised, evaporated to dryness, and acetylated as above, yielding an acetate (94 mg.) having properties identical with those of panitol doceca-acetate (see Table) (Found: C, 50·2; H, 6·1. Calc. for $C_{42}H_{58}O_{28}$: C, 49·9; H, 5·8%).

Fractions 81—82, 83—85, and 86—92 were examined by paper chromatography; 81—82 contained two zones migrating with panose and maltotriose, fractions 83—85 and 86—92 each contained two zones migrating with maltotriose and maltotetraose. Each group of fractions was treated with a concentrated solution of crystalline sweet potato β-amylase (kindly provided by Dr. A. K. Balls) and left for 7 days at 35° under toluene. Paper-chromatographic examination of the digests revealed that substances having the R_F values of glucose and maltose had been produced from fractions 81—82 and 83—85; none of the original zones had completely disappeared.

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