Studies of Aspergillus niger. *Part IV.* The Synthesis of β-Linked Glucosaccharides.*

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A study has been made of the oligosaccharides formed from cellobiose by (*a*) growing cultures of *Aspergillus niger* (strain 152), (*b*) resting cells, and (*c*) cell-free extracts. It is shown that the enzyme system of the mould is able to transfer a glucose unit from cellobiose to another cellobiose molecule, to other β-linked disaccharides, or to glucose. The principal linkage synthesized is β-1 : 6, but β-1 : 2-, β-1 : 3-, and β-1 : 4-linkages are also formed. Indications have been obtained that D- and L-xylose, L-sorbose, and *N*-acetyl-D-glucosamine can also function as receptors in the transfer reaction.

As part of an investigation of the transglycosylation processes occurring in *Aspergillus niger* (strain 152, *i.e.*, the strain which synthesizes nigeran) (Barker, Bourne, and Stacey, *J.*, 1953, 3084), we have studied the metabolism of cellobiose by the mould. Just before publication of our preliminary results (Barker, Bourne, and Stacey, *Chem. and Ind.*, 1953, 1287), other workers reported that they had observed syntheses of oligosaccharides, of unknown constitution, during the action on cellobiose of enzymes obtained from *Aspergillus oryzae* (Jermyn and Thomas, *Austral. J. Biol. Sci.*, 1953, **6**, 70), *Aspergillus niger*, *Aspergillus aureus*, and *Myrothecium verrucaria*, and from the gut of the snail, *Helix pomatia* (Crook and Stone, *Biochem. J.*, 1953, **55**, xxv). More recently, Buston and Jabbar (*Chem. and Ind.*, 1954, 48) and Giri, Nigam, and Srinivasan (*Nature*, 1954, **173**, 953) have made similar observations with cell-free extracts of *Chaetomium globosum* and *Aspergillus flavus*, respectively. We now describe the isolation and proof of structure of some of the complex mixture of oligosaccharides synthesized by *Aspergillus niger* (152), during growth on cellobiose, and also by the action on cellobiose of resting cells of the same organism.

The oligosaccharides produced in the culture filtrate of *Aspergillus niger*, which had been grown for 7 days at 30° on cellobiose (12·5% w/v) and the mineral constituents of Currie's medium, were fractionated on charcoal (Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677). Part of the small amount of glucose (1·3% of the initial weight of cellobiose) eluted from the column with water was isolated as crystalline α-D-glucose, the remainder being characterized as its β-penta-acetate. The 10%-ethanol eluate contained unchanged cellobiose, isolated as the crystalline sugar and as its β-octa-acetate, and traces of gentiobiose.

A complex series of six components having the mobilities of trisaccharides was detected in fractions obtained by elution with gradually increasing concentrations of ethanol (10—20%). Four of the trisaccharides I, III, V, and VI (numbered in their order of elution) were isolated almost chromatographically pure (>95%) but the others (II and IV) were only obtained as impurities in other fractions. Electrophoresis in the presence of borate buffer (pH 10) (Foster, *J.*, 1953, 982) enabled the saccharides to be classified into two distinct groups; one group (I, II) had M_G values similar to that of laminaritriose, while the others (III, IV, V, VI) had mobilities corresponding more closely to that of cellotriose. Since, under these conditions, a glucosaccharide in which the reducing unit is linked through $C_{(3)}$ or $C_{(6)}$ migrates much faster than an isomeric glucosaccharide with the reducing unit linked through $C_{(2)}$ or $C_{(4)}$ (cf. Foster, *loc. cit.*), and since the only disaccharide receptors detectable in the culture filtrate were gentiobiose and cellobiose, it seems likely that trisaccharides I and II had their reducing unit linked through $C_{(6)}$ and trisaccharides III, IV, V, and VI through $C_{(4)}$.

Complete hydrolysis of trisaccharides I, III, V, and VI gave only glucose. Partial hydrolysis of the same trisaccharides, and chromatographic and ionophoretic identification of the disaccharides produced, provided evidence sufficient to allow the tentative assignments of structure shown in Table 1.

Such assignments are in agreement also with the known order of elution from charcoal

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TABLE 1.

Trisaccharide	Partial hydrolysis products	Probable point of linkage * A	Probable point of linkage * B
I	Gentiobiose, glucose	β-1 : 6	β-1 : 6
II †	—	?	β-1 : 6
III	Gentiobiose, cellobiose, glucose	β-1 : 6	β-1 : 4
IV †	—	?	β-1 : 4
V	Cellobiose, glucose	β-1 : 4	β-1 : 4
VI	Cellobiose, laminaribiose, glucose	β-1 : 3	β-1 : 4

* A = non-reducing end; B = reducing end. † Disaccharide?

(gentiobiose, sophorose, cellobiose, and laminaribiose) of the β-linked glucose disaccharides. The infrared spectra of the trisaccharide mixture, and of the individual trisaccharides III, V, and VI, indicated that the linkages were predominantly of the β-type (cf. Barker, Bourne, Stacey, and Whiffen, *J.*, 1954, 171), although this did not, of course, exclude the possibility that traces of α-linked saccharides were also present. The low specific rotations of trisaccharides I and III (approximately +4·1° and +10·2°, at equilibrium, respectively) were consistent with this conclusion; Lindberg and MacPherson (*Acta Chem. Scand.*, 1954, **8**, 985) reported $[\alpha]_D$ −6·5° for gentiotriose (I).

Trisaccharide III was produced in by far the largest amount (*ca.* 75% of the total trisaccharides) and so it was possible to obtain further confirmation of the structure already assigned to it. Partial hydrolysis of the aldonic acid produced by oxidation with hypoiodite gave a product with the same R_F value as gentiobiose, but no cellobiose. Trisaccharide III was reduced with sodium borohydride and the product was partially hydrolysed; specific spraying reagents showed that the products were gentiobiose, cellobiitol, glucose, and sorbitol. These observations indicated that III was indeed *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose. It follows that the corresponding glycitol should consume 7·0 mols. of periodate and give 3·0 mols. of formic acid and 2·0 mols. of formaldehyde and the observed figures (6·9, 2·9, and 1·8 mols., respectively) corresponded closely to these values. Of all the possible β-linked glucopyranose trisaccharides only one other [*O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→3)-D-glucopyranose] would have shown this behaviour during periodate oxidation of its alcohol.

Resting cells of *A. niger* incubated with cellobiose (10 g.; 20% w/v) for 7 days gave large amounts of glucose, and a series of disaccharides, whose R_F values were identical with those of gentiobiose, cellobiose, sophorose, and laminaribiose, as well as a complex mixture of trisaccharides. Fractionation on charcoal and elution with water and 5% ethanol led to the recovery of the glucose fraction (4·7 g.), most of which was obtained as the crystalline α-anomer.

Elution with 12·5% ethanol gave two fractions, one (0·6 g.) containing gentiobiose and a small amount of sophorose, and the other (0·7 g.) containing gentiobiose, cellobiose, and laminaribiose. Refractionation of the former on charcoal gave a fraction containing chromatographically-pure gentiobiose, which was characterized by conversion into its crystalline β-octa-acetate. The sophorose could be obtained in this way only in admixture with gentiobiose and so a portion of the mixture was separated on several ionophoretograms run in borate buffer (pH 10). Areas of paper containing sophorose were eluted under slightly acid conditions and the ions present were removed by using an ion-exchange column, electrodialysis, and co-distillation with methanol; the sophorose was isolated as a freeze-dried powder. When treated with phenylhydrazine it gave glucosazone; this reaction is shown only by disaccharides in which the sugar units are linked 1 : 2 and is typical in general of 2-substituted aldoses (*e.g.*, 2-*O*-methyl-D-glucose).

In order to facilitate the identification of laminaribiose, advantage was taken of the observation that the action of resting cells on cellobiose produced mainly laminaribiose during the initial stages and gentiobiose in the later stages. Accordingly, a cell-free extract of *A. niger* was incubated with cellobiose for three days and a crude cellobiose–laminaribiose fraction was isolated by using a charcoal column. The basis of the subsequent separation was that laminaribiose forms a furanoside mixture when treated with methanolic hydrogen chloride at room temperature (which cellobiose cannot do) and the glucosidic substituent exerts a retarding influence when the substance is passing through charcoal (cf. Barker, Bourne, and O'Mant, *Chem. and Ind.*, 1955, 425). In this way, methyl

αβ-laminaribiofuranoside was obtained free from cellobiose and freeze-dried. Oxidation with periodate confirmed the structure. All the disaccharides were hydrolysed readily by an almond emulsin preparation, which had negligible action on the α-linked disaccharides maltose and nigerose.

It is interesting that disaccharide formation by the resting cells shows such a marked contrast with the results obtained with growing cultures, which gave a trace of gentiobiose only. The reason seems to be that the cultures metabolize much of the glucose formed in the transfer reaction, so that it is not available as a receptor in disaccharide synthesis.

The complex mixture of at least nine trisaccharides synthesized by the resting cells from cellobiose was examined only by paper chromatography and paper ionophoresis. Most of the trisaccharides (I—VI) synthesized by the growing cells could be detected. The remainder presumably arose through the ability of laminaribiose, sophorose, and the increased quantity of gentiobiose to function as receptors of transferred glucose units. Confirmatory evidence that this was so was obtained when trisaccharides were detected following the incubation of enzyme extracts with gentiobiose and with laminaribiose.

Thus it is clear that the enzyme system of the 152 strain of *Aspergillus niger* catalyses the transfer of glucose from cellobiose to glucose, to cellobiose itself, and to other β-linked glucose disaccharides, with the formation of β-1 : 2-, β-1 : 3-, β-1 : 4-, and β-1 : 6-linkages, of which the last predominates; if α-linkages are produced then they constitute a very small proportion of the total. It is not essential for the receptor to be glucose or its higher homologues, because evidence has been obtained that D- and L-xylose, L-sorbose, and *N*-acetyl-D-glucosamine can function in the same way; structural examinations of the products are now in progress. The fact that D-xylose can serve as a receptor is interesting in view of its common natural occurrence in association with D-glucose, and the close stereochemical relationship between the two sugars. It is significant also that its optical enantiomorph, and also L-sorbose which differs only at the reducing position from L-xylose, should be receptors. These observations raise interesting problems regarding enzyme specificity, but it would be unwise to speculate further at this stage, since it remains to be proved whether all the reactions observed are due to the same enzyme or to several.

During the preparation of this manuscript, Peat, Whelan, and Hinson (*Chem. and Ind.*, 1955, 385) published a preliminary account of studies with an enzyme extract of *Aspergillus niger* NRRL 330 in which they demonstrated the formation from glucose of ten disaccharides, which belonged to both the α- and β-series. In addition, Pfanmüller and Noë (*Science*, 1952, **115**, 240) and Aso and Shibasaki (*Tohoku J. Agric. Res.*, 1953, **3**, 337) have found that *A. oryzae* and *A. niger* concentrates give *iso*maltose from glucose. These reports prompt the question whether the syntheses which we have observed from cellobiose were direct transfer reactions, or whether they entailed hydrolysis to glucose and subsequent resynthesis. We believe that the former alternative is the correct one in this case because, in contrast to the latter, it explains the formation of disaccharides by the resting cells, but not by the growing cultures, and also the fact that the main trisaccharide is *O*-β-D-glucopyranosyl-(1→6)-*O*-β-D-glucopyranosyl-(1→4)-D-glucopyranose. Moreover, cell-free extracts of *A. niger* (strain 152) synthesize principally *iso*maltose and panose from maltose (Barker and Carrington, *J.*, 1953, 3588), so that, with at least one of the two disaccharide substrates, hydrolysis is not an essential step in the synthesis of glucosaccharides. It will be recalled that Peat, Whelan, and Hinson (*Nature*, 1952, **170**, 1056) demonstrated that almond emulsin produces β-linked oligosaccharides from high concentrations of glucose; we have now shown that cellobiose is an alternative substrate, but it remains to be proved whether a direct transfer is involved. An interesting problem which is beginning to emerge is whether the synthesis of α- and β-oligosaccharides by moulds necessarily results from two different enzymes, or whether the anomeric character of the products is determined solely by that of the substrate (maltose, α-glucose; cellobiose, β-glucose).

Experimental

Production of Glucosaccharides by Growing Cells.—A sterile solution (160 c.c.), containing cellobiose (20 g.) and the mineral components of Currie's medium (*J. Biol. Chem.*, 1917, **31**, 15), was inoculated with *Aspergillus niger* " 152 " and incubated at 30°. Spots of the solution were

removed at intervals and analysed chromatographically as the free sugars and as their benzylamine derivatives (Bayly and Bourne, *Nature*, 1953, **171**, 385) with *n*-butanol (40%)–ethanol (10%)–water (49%)–ammonia (1%) as solvent. The disappearance of cellobiose and the gradual production of components having the mobilities of trisaccharides, together with traces of glucose and gentiobiose, were detectable after spraying with aniline hydrogen phthalate (Partridge, *Nature*, 1949, **164**, 443). After 7 days, the culture filtrate and mycelial washings were adjusted to pH 7 with sodium hydroxide and boiled for 20 min. to arrest enzyme action. The cooled solution was passed through charcoal (30 × 3·4 cm. diam.) and the glucosaccharides fractionated by elution with increasing concentrations of aqueous ethanol (Whistler and Durso, *loc. cit.*). After analysis by paper chromatography and by paper ionophoresis in borate buffer, pH 10 (Foster, *loc. cit.*), similar fractions were combined, concentrated, purified by methanol extraction, and obtained as syrups or freeze-dried powders.

Characterization of the Oligosaccharide Fractions.—Elution with water (1050 c.c.) gave a fraction (0·25 g.) which contained a single sugar component with an R_F value equal to that of glucose. Crystallization from methanol gave α-D-glucose (0·15 g.), m. p. and mixed m. p. 146°, $[\alpha]_D^{18}$ +52·0° (equil.; *c*, 0·56 in H_2O) (Found: C, 40·4; H, 6·9. Calc. for $C_6H_{12}O_6$: C, 40·0; H, 6·7%). The residual glucose was isolated as its β-penta-acetate (0·14 g.), m. p. and mixed m. p. 131°, $[\alpha]_D^{18}$ +3·7° (*c*, 0·43 in $CHCl_3$) (Found: C, 49·1; H, 5·6. Calc. for $C_{16}H_{22}O_{11}$: C, 49·2; H, 5·7%).

Elution with 10% ethanol (2 l.) gave fractions containing cellobiose and traces of gentiobiose. Crystallization from methanol afforded β-cellobiose (1·68 g.), m. p. and mixed m. p. 224—225°, $[\alpha]_D^{19}$ +35·1° (equil.; *c*, 0·84 in H_2O) (Found: C, 42·6; H, 6·9. Calc. for $C_{12}H_{22}O_{11}$: C, 42·1; H, 6·5%). Cellobiose (0·050 g.) was further characterized as its β-octa-acetate (0·072 g.), m. p. and mixed m. p. 200°, $[\alpha]_D^{18}$ −15·7° (*c*, 0·26 in $CHCl_3$) (Found: C, 49·35; H, 5·55. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6%).

Gradient elution with 10—20% ethanol (3100 c.c.) gave a series of fractions (total wt., 1·432 g.) which contained trisaccharides only. By paper ionophoresis and paper chromatography jointly, six different trisaccharides were distinguished; they were numbered I—VI in order of elution from the column. Their behaviour on chromatograms (as the free sugars) and on ionophoretograms (methods as above) is shown in the following Table.

Trisaccharide	$R_{cellotriose}$	$R_{laminaritriose}$	M_G *value approx. same as that of:*
I	0·8	0·4	Laminaritriose
II	2·3	1·1	Laminaritriose
III	1·3	0·6	Cellotriose
IV	3·0	1·4	Cellotriose
V	1·0	0·45	Cellotriose
VI	1·8	0·8	Cellotriose

Traces (5—15 mg.) only of II and IV were obtained as impurities in other fractions and these were not investigated further. Trisaccharide III was present in largest amount (*ca.* 1·1 g.), followed by trisaccharide I (190 mg.) and small quantities of trisaccharides V and VI (30—50 mg.).

Samples of trisaccharides I, III, V, and VI (5 mg. of each) were hydrolysed with N-sulphuric acid (1 c.c.) at 100° for 5 hr. Each hydrolysate was almost neutralized with barium carbonate and filtered, and the residual ions removed by electrodialysis (Consden, Gordon, and Martin, *Biochem. J.*, 1947, **41**, 590). Analysis by paper ionophoresis and paper chromatography, as previously described, revealed only one component, having R_F and M_G values identical with those of glucose, in each hydrolysate.

Partial hydrolysis of samples (5 mg.) of trisaccharides I, III, V, and VI was effected by heating them in N-sulphuric acid (0·5 c.c.) at 100° for 20 min. Removal of ions and analysis by the above methods indicated that trisaccharide I gave gentiobiose and glucose, trisaccharide III gave gentiobiose, cellobiose, and glucose, trisaccharide V gave cellobiose and glucose, and trisaccharide VI gave laminaribiose, cellobiose, and glucose.

Infrared analysis of trisaccharides III, V, and VI (as mulls in liquid paraffin) showed marked absorption at 896 $cm.^{-1}$, but little at 840 $cm.^{-1}$, indicating the presence of β-links in the three trisaccharides (Barker, Bourne, Stacey, and Whiffen, *loc. cit.*).

Further Examination of Trisaccharide III.—(a) *Partial hydrolysis of the aldonic acid of trisaccharide III.* The trisaccharide (10 mg.) was oxidized with a solution (8 c.c.) containing 0·1N-iodine (1 c.c.) and 0·2M-bicarbonate buffer (2 c.c.; pH 10·6). After 3 hr. at room temperature, the excess of iodine was destroyed with sodium thiosulphate, and the aldonic acid was partially hydrolysed by addition of N-sulphuric acid (1 c.c.) and heating the solution at 100°

for 25 min. Paper-chromatographic analysis revealed a component having the same mobility as gentiobiose; no cellobiose was detected.

(b) *Partial hydrolysis of the alcohol from trisaccharide III.* An aqueous solution (12 c.c.) of the trisaccharide (100 mg.) and sodium borohydride (50 mg.) was kept at room temperature until the optical rotation was constant (*ca.* 2 hr.). After destruction of the excess of borohydride with acetic acid, N-sulphuric acid (5 c.c.) was added. The solution was heated at 100° for 20 min. and then neutralized, filtered, and de-ionized in the usual manner.

Portions of the hydrolysate were separated on duplicate chromatograms run in the organic phase of the butanol solvent mixture previously described. One of these, having glucose, cellobiose, and gentiobiose as references, was sprayed with aniline hydrogen phthalate and showed the presence of components with the mobilities of gentiobiose and glucose. The other chromatogram, having cellobiitol and sorbitol as references, was sprayed with a solution of bromocresol purple (40 mg.) in 95% methyl alcohol (100 c.c.) containing boric acid (100 mg.) to which 1% borax solution (7·5 c.c.) had been added (Bradfield and Flood, *Nature*, 1950, **166**, 264). This showed the presence of components having the mobilities of cellobiitol and sorbitol.

(c) *Periodate oxidation of the alcohol from trisaccharide III.* A sample of trisaccharide III (50 mg.) was reduced with sodium borohydride then treated with acetic acid and electrodialysed, as described previously. The boric acid was removed by three distillations with methanol, and the residual sugar alcohol was dissolved in water and freeze-dried. Chromatographic analysis, and use of the bromocresol-purple spray (see above), showed one spot due to the trisaccharide alcohol, but no cellobiitol or sorbitol.

An aqueous solution (10 c.c.) of the alcohol of trisaccharide III (42 mg.) was added to 0·25M-sodium periodate (15 c.c.), diluted to 50 c.c., and kept at 17°. Aliquot portions (5 c.c.) of the solution were withdrawn at intervals and neutralized with solid sodium hydrogen carbonate, and the periodate determined by addition of excess of standard sodium arsenite solution and back-titration of the excess of arsenite with standard iodine solution (Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107, 196). The periodate content at zero time was determined in a suitable blank from which the carbohydrate had been omitted. The periodate consumption, expressed in moles per mole of alcohol was : 6·8 (2 hr.), 6·9 (20 hr.).

After 20 hr., a portion (5 c.c.) was treated with ethylene glycol and titrated against 0·02N-sodium hydroxide, methyl red being used as indicator; the result indicated that the formic acid production was 2·9 moles per mole of alcohol.

After 20 hr., the formaldehyde present in 5 c.c. of the solution was determined by addition of an excess of dimedone (10%) in alcohol. After filtration and drying at 110° to constant weight, the amount of dimedone derivative corresponded to 1·8 moles of formaldehyde per mole of alcohol taken.

Production of Glucosaccharides by Resting Cells.—An aqueous solution (50 c.c.) of cellobiose (10 g.) was incubated for 7 days with freeze-dried *Aspergillus niger* cells (1 g.) at 30°. Paper-chromatographic analysis showed the disappearance of cellobiose and the gradual production of glucose and trisaccharides, together with three disaccharides having the mobilities of gentiobiose, sophorose, and laminaribiose. In the initial stages, laminaribiose was the main disaccharide produced, but in the later stages of incubation by far the greatest proportion of the disaccharide fraction was gentiobiose. After 7 days the digest filtrate was adjusted to pH 7 with sodium hydroxide and enzyme action arrested by heating the solution at 100° for 20 min. The saccharide mixture was fractionated on charcoal (30 × 3·4 cm. diam.) by Whistler and Durso's method (*loc. cit.*) and fractions (200 c.c.) analysed and obtained as syrups or freeze-dried powders in the manner previously described.

Further Purification and Characterization of Oligosaccharide Fractions.—Elution with water (1600 c.c.) and 5% ethanol (600 c.c.) gave a fraction (4·691 g.) which contained glucose and a trace of gentiobiose. When crystallized from methanol, α-D-glucose (3·537 g.) was obtained, m. p. and mixed m. p. 140—142°.

Elution with 10% ethanol (800 c.c.) and 12·5% ethanol (800 c.c.) gave fraction *A* (0·630 g.) which contained gentiobiose, together with a small amount of sophorose. Further elution with 12·5% ethanol (2000 c.c.) gave a mixture (0·692 g.) of gentiobiose, cellobiose, and laminaribiose. Refractionation of *A* on charcoal gave one fraction containing pure gentiobiose and another containing a mixture of gentiobiose and sophorose. A portion (0·065 g.) of the chromatographically pure gentiobiose was acetylated at room temperature with acetic anhydride (1·5 c.c.) and pyridine (1·5 c.c.). Isolated in the usual way and crystallized from methanol, the β-gentiobiose octa-acetate (0·032 g.) had m. p. and mixed m. p. 193°, $[\alpha]_D^{17}$ −5·0° (*c*, 0·14 in $CHCl_3$) (Found : C, 50·0; H, 5·7. Calc. for $C_{28}H_{38}O_{19}$: C, 49·6; H, 5·6%).

A portion (20 mg.) of the gentiobiose–sophorose mixture, in water, was streaked across a Whatman No. 3 filter paper (57 × 13 cm.) and separated by ionophoresis in borate buffer (pH 10) at 500 volts for 6 hr. and the ionophoretogram left to dry in air. Marker strips (1 cm.) were cut off six such papers and sprayed with aniline hydrogen phthalate to indicate the positions of the two components. The total area (472·5 cm.²) of paper containing sophorose was eluted with 0·01N-hydrochloric acid (232 c.c.). The filtrate and washings of the paper pulp were passed down a column of Amberlite 120H directly on to silver carbonate. This procedure removed sodium, chloride, and most of the borate ions. The filtrate was concentrated to 5 c.c. and the residual silver and chloride ions removed by electrodialysis. The solution was then concentrated to a syrup and co-distilled three times with methanol *in vacuo* to remove all borate. After the residue had been dissolved in water the sophorose was freeze-dried (13 mg.); it was ionophoretically pure.

Part (2 mg.) of the sophorose was hydrolysed with N-sulphuric acid (1 c.c.) at 100° for 1 hr.; only one component, having an R_F value equal to that of glucose, could be detected in the hydrolysate. An aqueous solution (1 c.c.) of the remaining sophorose (10 mg.) was heated with a solution of freshly distilled phenylhydrazine (0·2 c.c.) in acetic acid (0·4 c.c. of acid in 1 c.c. of water) for 1·5 hr. at 100°. The recrystallized product (12 mg.) had m. p. 207° alone and in admixture with glucosazone (Found: C, 59·9; H, 6·25; N, 15·2. Calc. for $C_{18}H_{22}O_4N_4$: C, 60·3; H, 6·2; N, 15·6%).

Elution with 12·5—27·5% alcohol (5200 c.c.) gave a series of fractions (total yield, 0·967 g.) in which at least nine suspected trisaccharides could be detected by combining the evidence supplied by paper ionophoresis in borate buffer (pH 10) and paper chromatography in the butanol solvent mixture previously described.

Isolation of Laminaribiose.—Cellobiose (2 g.) and a cell-free extract of *Aspergillus niger* " 152 " were incubated at 30° for three days; the main disaccharide produced was then laminaribiose. Fractionation on charcoal (17 × 3·4 cm. diam.) gave a fraction (1·01 g.) containing only cellobiose and laminaribiose. This was treated with 4% methanolic hydrogen chloride (35 c.c.) at room temperature for 2 hr. After addition of excess of silver carbonate the pH rose to *ca.* 5. The suspension was filtered and the methanol removed *in vacuo* in the presence of a little freshly-washed barium carbonate. The syrupy residue was suspended in water, filtered, and passed through acid-free charcoal. Washing with increasing concentrations of aqueous ethanol removed the cellobiose first and later gave fractions which were non-reducing but contained a component which had an M_G value slightly greater than that of cellobiose when separated as its borate complex on a ionophoretogram. This component, presumably the mixed methyl furanoside of laminaribiose, could be detected with an alkaline silver nitrate spray (Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444).

A portion (79 mg.) of the freeze-dried glycoside was oxidized with periodate by the method previously described. After 20 hr., the periodate consumption was 3·3 moles per mole of furanoside; and the amounts of formic acid and formaldehyde produced were 0·8 and 1·3 moles per mole, respectively. The theoretical figures for a methyl furanoside of laminaribiose are 3·0, 1·0, and 1·0, respectively.

The Action of Almond Emulsin on Cellobiose.—Emulsin (10 mg.), prepared from bitter almonds by a method similar to that of Bourquelot and Bridel (*Ann. Chim. Phys.*, 1913, **29**, 145), was added to an aqueous solution (1 c.c.) of cellobiose (20% w/v) and aliquot portions submitted to paper-chromatographic analysis at intervals. Components were produced during the incubation which had R_F values identical with those of glucose, gentiobiose, laminaribiose, and higher saccharides.

Investigation of Possible Receptors in β-Transglucosylation.—A suspension of freeze-dried cells of *A. niger* (1·5 g.) in water (8 c.c.) was shaken with glass beads for several hours in a Mickle Tissue Disintegrator and the cell debris removed by centrifugation. Equal weights (150 mg.) of cellobiose and the possible receptor (D- and L-xylose, D-ribose, L-sorbose, L-arabinose, and *N*-acetyl-D-glucosamine) were dissolved severally in portions (1 c.c.) of the cell-free enzyme extract and chromatographic analyses carried out on each solution at frequent intervals during the first day and thereafter at daily intervals. All chromatograms were sprayed with aniline hydrogen phthalate and in the case of L-sorbose a duplicate was sprayed with naphtharesorcinol. In the cases of the cellobiose–D-xylose and cellobiose–L-xylose mixtures components, presumably two glucose-xylose disaccharides, were detected additional to those produced on incubation of the cell-free extracts and cellobiose alone. These additional components had R_F values intermediate between those of cellobiose and glucose when separated in the butanol solvent mixture and gave a pink colour with aniline hydrogen phthalate. There was evidence

also that L-sorbose and *N*-acetyl-D-glucosamine functioned as receptors in the formation of oligosaccharides.

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