The Enzymic Hydrolysis of β-Glucosides

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The present work arose out of an attempt to understand the course of cellulose breakdown in preparations of cellulolytic enzymes which also contained cellobiase activity. Previous investigations of the β-glucosidases have been of three kinds—an examination of their specificity ranges (reviewed by Veibel, 1950), the demonstration of their reversibility and the study of glucosyl transfer. Croft-Hill (1898), in demonstrating the reversibility of enzyme reactions, showed the formation of disaccharides from glucose in the presence of yeast enzymes. Gentiobiose was later found to be the chief product of this type of reaction catalysed by β-glucosidases (Bourquelot & Bridel, 1919; Bourquelot, Hérissey & Coirre, 1913), and it is now a recognized method of preparing this sugar (Helferich & Leete, 1942). More recently, Peat, Whelan & Hinson (1952) have shown that five β-linked glucose disaccharides can be obtained by incubating 60% glucose with almond emulsin over a period of 5 weeks. Aliphatic and aromatic alcohols, too, can condense with glucose in the presence of β-glucosidases (Bourquelot & Bridel, 1913; Veibel, 1936; Vintilescu, Ionescu & Kizyk, 1934, 1935*a*, *b*; Vintilescu, Ionescu & Solomon, 1935).

The first to describe transglycosylation and to realize the complementary role of the reactants was Rabaté (1935), who showed that methyl β-glucoside was formed when piceoside (*p*-hydroxyacetophenone β-glucoside) was incubated with methanol and extracts of *Salix purpurea*, and that glucosidically linked glucose was necessary for the reaction. Miwa, Takano, Mafune & Furutani (1949), Miwa & Takano (1950), Takano & Miwa (1950) and Jermyn & Thomas (1953) confirmed these results and extended them, using a series of fungal and plant extracts acting upon several aromatic β-glucosides in the presence of methanol, ethanol or butanol. In 1953 Crook & Stone reported the formation of a series of oligosaccharides from cellobiose in the presence of various enzyme preparations. Since then Barker, Bourne & Stacey (1953), Barker, Bourne, Hewitt & Stacey (1955), Buston & Jabbar (1954), Giri, Nigam & Srinivasan (1954) and Kooiman, Roelofsen & Sweeris (1954) have described similar reactions with various mould enzyme preparations. Manners (1955) has observed the formation of higher saccharides during the action of a barley β-glucosidase preparation on cellobiose. The present paper is a fuller account of the work previously reported (Crook & Stone, 1953) and an attempt to elucidate some of the reactions occurring. Its substance has already formed part of a thesis (Stone, 1954).

EXPERIMENTAL

Materials

Substrates. Salicin and cellobiose were products of L. Light and Co., Colnbrook, Bucks. Methyl β-glucoside was prepared by the method of Koenigs & Knorr (1901). The acetobromoglucose required for this preparation was obtained according to Bárczai-Martos & Kőrösy (1950), and the product was recrystallized from ethyl acetate, m.p. 110·0–110·5°. Laminaribiose was a gift of Dr W. J. Whelan. Gentiobiose was extracted from gentian root (*Gentiana lutea* L.) according to the method of Haworth & Wylam (1923). From gentian root (400 g.) was obtained 20 g. of a brown gum which was dissolved in 75 ml. of water and fractionated on a charcoal column (3·2 cm. × 33·5 cm.; 77 g. of Speakman no. 130, sieved charcoal). Gentiobiose was obtained from the pooled 5% and 7·5% ethanol fractions by preparative paper electrophoresis.

Oligosaccharides from laminarin. Laminarin (2·5 g.) from *Laminaria cloustoni*, a gift of Professor T. Dillon, was heated with 240 ml. of N oxalic acid at 95° for 5 hr. This time was calculated from the data of Connell, Hirst & Percival (1950) to give the maximum yield of oligosaccharides of 3–5 glucose units. The acid was neutralized with $CaCO_3$ and the calcium oxalate filtered off. The filtrate was heated at 90° for 1 hr., refiltered and freeze-dried to a white powder. When chromatographed in *n*-butanol–pyridine–water (6:4:3, by vol.), this material showed a series of spots, the lowest of which coincided with glucose and laminaribiose markers, and which gave a straight line on plotting log R_G against the spot number up to seven as required for a homologous series according to French & Knapp (1950). It may thus be assumed that a negligible amount of transglycosylation occurred during hydrolysis and that the material consisted of a series of oligosaccharides ranging from glucose through laminaribiose and laminaritriose to laminariheptaose.

Lutean (luteose) and oligosaccharides from lutean. Luteic acid, the malonic acid ester of lutean, was prepared according to the method of Anderson & Raistrick (1936) from *Penicillium luteum* Zuckal (L.S.H.T.M. no. P42) obtained from Mr G. Smith of the London School of Hygiene and Tropical Medicine. The lutean was isolated from the luteic acid according to the method of Stacey (Anderson, Haworth, Raistrick & Stacey, 1939). A series of β-1:6-linked oligo-

glucosaccharides was prepared by hydrolysing 0·5 g. of lutean in 0·5 N-H_2SO_4 at 95° for 4·5 hr. Under these conditions a trial experiment showed that the maximum amount of trisaccharide would be formed in this time. Chromatography as for the laminarin hydrolysate gave a similar result except that, as these oligosaccharides move more slowly, the largest observed after 2 days' irrigation was luteotetraose (gentiotetraose).

Enzyme preparations. (*a*) *Myrothecium verrucaria* (Alb. & Schwz.) Ditm. ex Fr., I.M.I. 45541 = QM 460 = U.S.D.A. 1334.2, from the Commonwealth Mycological Institute, Kew, Surrey, was grown on slopes of potato–carrot agar plus filter-paper strips for 9 days at 30°. The spore suspension obtained from these slopes was used to inoculate 25 ml. of culture medium (Siu, 1951) dispensed in 125 ml. flasks with cotton linters (1%) as the only carbon source. The flasks were incubated on a reciprocating shaker (120 strokes/min.) for 5–6 days. At the end of this time the mycelial-cellulose mat was removed and the culture filtrate centrifuged and dialysed in nitrocellulose sacs. A white flocculent precipitate appeared which was spun off and the clear fluid was freeze-dried to a white powder.

(*b*) The crop juice of *Helix pomatia* was collected and freeze-dried.

(*c*) Emulsin was prepared from sweet almonds according to Edman & Jorpes (1941) to the stage of the first ammonium sulphate precipitate.

(*d*) Fresh gentian roots (*Gentiana lutea* L.) were obtained from France through the courtesy of Wheeler and Huisking Ltd.. London. Root sections (200 g.) were mixed in a Waring Blendor with 300 ml. of water, squeezed through cotton cloth and the insoluble material was removed by centrifuging. The supernatant (260 ml.) was dialysed for 48 hr. on a rocking dialyser against tap water and then 24 hr. against distilled water in the cold room. A slight precipitate was spun off and the supernatant freeze-dried and stored in a desiccator.

(*e*) A commercial preparation of glucose oxidase ('DeeO'; Takamine Laboratory Inc., Clifton, New Jersey, U.S.A.) (7·5 g.) was dissolved in 125 ml. of water, dialysed, centrifuged at 20000 *g* and freeze-dried.

(*f*) Commercial mould enzyme preparations from *Aspergillus niger* 'E19' (Rohm and Haas Co., Philadelphia 5, Penn., U.S.A.); Carterzyme (H. W. Carter and Co., Coleford, Glos.); Saccharozyme and Clarase (Takamine Laboratory Inc., Clifton, N.J., U.S.A.); *Aspergillus aureus*, *A. flavus* and *A. niger* (Norman Evans and Rais Ltd., Manchester); and Pectinol 100D (Rohm and Haas Co., Philadelphia 5, U.S.A.) were obtained as stable powders. Before use they were dissolved in water to give 10% (w/v) solutions and allowed to stand several hours in a refrigerator with occasional stirring. The insoluble material was spun off and the supernatant dialysed until free from reducing sugars. The solutions were then freeze-dried and the powders stored in a desiccator under vacuum. Many of these enzyme preparations also contain α-glucosidase activity.

Methods

Paper partition chromatography. Qualitative and quantitative chromatograms were run on Whatman no. 1 paper by downward development in tanks similar to those described by Dent (1948). Two solvent systems were used: *n*-butanol–pyridine–water (6:4:3, by vol.) (Chargaff, Levine & Green, 1948) and *n*-propanol–ethyl acetate–water (6:1:3, by vol.) (Albon & Gross, 1952). The latter solvent mixture had a high resolving power for the higher oligosaccharides. Sugars were detected on the chromatograms by the benzidine–trichloroacetic acid reagent of Bacon & Edelman (1951). Silver nitrate was used according to Trevelyan, Procter & Harrison (1950). The lead tetra-acetate method of Buchanan, Dekker & Long (1950) was used to detect non-reducing derivatives of sugars. These are also detected by the $AgNO_3$ spray.

Quantitative chromatography was carried out essentially according to Bacon & Edelman (1951). The eluted sugars were estimated as glucose by the anthrone method (Trevelyan & Harrison, 1952) in solutions diluted to contain not more than 100 μg. of glucose/ml.

Paper electrophoresis of oligosaccharides. The apparatus used was essentially that of Consden & Stanier (1952). The paper was immersed in chlorobenzene to prevent evaporation and improve cooling. Electrophoresis was carried out on Whatman no. 1 paper in pH 10 borate buffer (7·44 g. of boric acid in 1 l. of 0·1 N-NaOH). A current of 1·5 mA/cm. width of paper was applied during 2·5 hr. The findings of Foster (1953) and of Aso & Hamada (1955) were used as an aid in the identification of compounds found.

For preparative work Whatman no. 3 MM paper was used. After spraying a guide strip the paper containing the sugars was cut out and eluted with water. The extracts were passed through a short column of Zeo-Karb 215 (The Permutit Co. Ltd., London) to remove the cations, and the boric acid was then removed by evaporating to dryness several times with 200 ml. portions of absolute methanol (Zill, Khym & Cheniae, 1953). Finally, the dry residue was dissolved in water and freeze-dried.

Chromatography on charcoal or charcoal–Celite mixtures. Mixtures of oligosaccharides were separated on charcoal or charcoal–Celite (Whistler & Durso, 1950) columns. The sugars adsorbed on the columns were eluted by a continuous gradient of aqueous ethanol produced by the method of Alm, Williams & Tiselius (1952), which gives an exponential rate of increase of ethanol concentration, or by the method of Parr (1954), which gives a linear rate of increase. Activated charcoal (B.D.H. Ltd.) or Speakman no. 130 (Sutcliffe, Speakman and Co. Ltd., Leigh, Lancs.) and Celite no. 513 (Johns-Manville and Co. Ltd., London) were used in preparing the columns. The preparation of the columns was greatly simplified by grading the charcoal and omitting the Celite. Sieving to give a cut between 50 and 200 B.S. mesh yielded a column which had approximately twice the capacity of the charcoal–Celite mixture was simpler to prepare, and ran more rapidly. Fractions were collected automatically (Crook & Datta, 1951) and analysed for total carbohydrate by the anthrone method. Fractions were concentrated under reduced pressure and finally freeze-dried and the dry powders stored in a desiccator.

Incubation procedure. The enzyme–substrate–buffer solutions were incubated in stoppered tubes in a water bath at 40°. Toluene was added if the experiment was to continue for longer than 8 hr. Samples to be analysed were removed by micropipette and applied in 30–50 μl. amounts, 5 μl. at a time, to the starting line of a paper chromatogram. To inactivate the enzyme 5 μl. portions of 0·01 M-$HgCl_2$ were previously applied at the starting points (Bealing & Bacon, 1953).

When the products were to be analysed quantitatively, 0·5 ml. samples were removed at suitable intervals from the

reaction mixture, 0·05 ml. of 0·04 M-$HgCl_2$ was added and the solution frozen in acetone–solid CO_2. The frozen solutions were stored at −20° in stoppered tubes and at a convenient time were analysed by quantitative chromatography.

RESULTS

Except where indicated, the *Aspergillus niger* preparation, 'E 19', was used as the β-glucosidase source in the following experiments.

Action on cellobiose

To 0·8 ml. of 0·01 M acetate buffer, pH 5·0, containing 50 mg. of cellobiose was added 0·2 ml. of enzyme solution (10 mg. of freeze-dried preparation/ml.) and the mixture incubated at 40°. Samples (30 μl.) were removed at intervals and spotted on a chromatogram which was run as described, usually in butanol–pyridine–water. The chromatogram, when developed with benzidine–trichloroacetic acid reagent, showed at least six reducing compounds with R_G values greater or smaller than that of cellobiose (Fig. 1). The fastest-moving compound corresponded in position to the glucose marker.

Representative R_G values in butanol–pyridine–water of the most prominent spots, together with the designations adopted in this paper, are indicated in Fig. 2.

Chromatograms sprayed with the more sensitive $AgNO_3$ reagent (Fig. 5*a*) detected at least two other compounds with R_G values lower than the δ-compound, a third compound (β_1) with an R_G slightly greater than β and a fourth (ω_1) with R_G slightly greater than cellobiose. The β_1-compound was more evident when higher concentrations of cellobiose were used (see below).

Most of the enzyme solutions themselves showed only a faint reducing spot with benzidine–trichloroacetic acid reagent and this spot did not move from the starting line. A few, however, showed traces of a compound with the same mobility as glucose.

It can be seen from the chromatogram in Fig. 1 that the cellobiose gradually disappears, whereas the glucose spot increases in intensity. The concentrations of the low-R_G compounds and the ω-compound, on the other hand, reach a maximum and then decrease, so that after 3 hr. glucose and the α-compound were the only ones remaining in

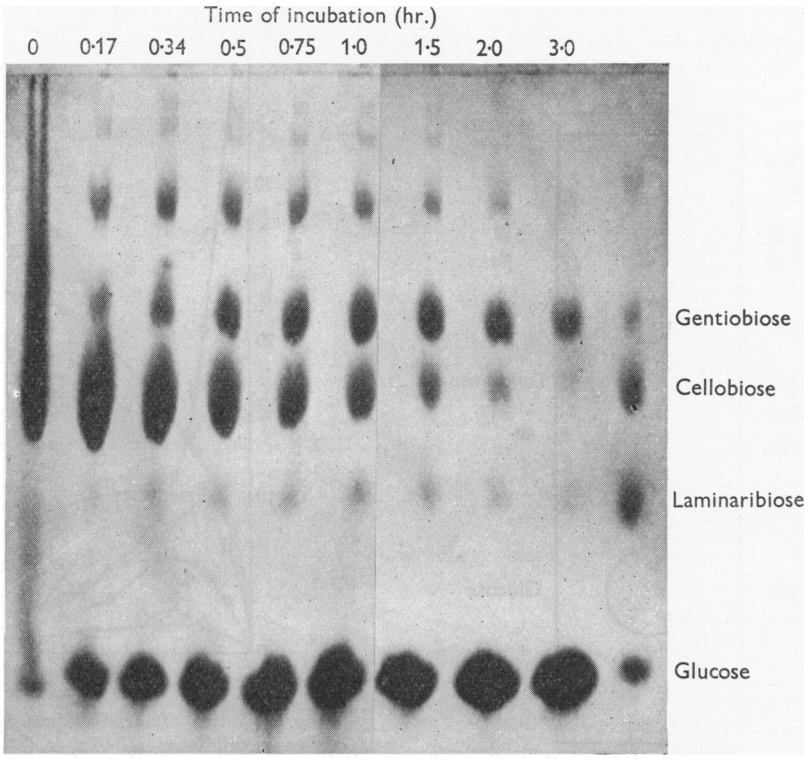

Fig. 1. Paper chromatogram of successive samples of the reaction mixture during the incubation of *A. niger* preparation ('E 19') with 5% (w/v) cellobiose in 0·01 M acetate buffer (pH 5·0) at 40°. Chromatogram developed with *n*-butanol–pyridine–water, sugars detected with benzidine–trichloroacetic acid reagent.

appreciable amounts. With 7·5 % (w/v) cellobiose as substrate and under the same conditions as before the reaction was followed quantitatively and the curves in Fig. 3 illustrate its course. The γ- and the δ-compounds were estimated together because of the small concentration of the latter. Glucose appeared rapidly, and almost simultaneously the β-compound, reaching a maximum early, to be followed by the $(\gamma+\delta)$- and later by the α- and ω-compounds. The β- and $(\gamma+\delta)$-compounds decreased most rapidly; the α- and ω-compounds were slower. Addition of 5 % glucose, as well as 7·5 % cellobiose, retarded the disappearance of the cellobiose and depressed the formation of the β-compound while increasing the α- and ω-compounds (Fig. 4). These observations suggest that α- and ω-compounds were formed by transfer to glucose, whereas the β-compound was formed by transfer to an oligosaccharide present in greatest concentration at the earliest stages of the reaction, namely cellobiose. The β-compound would then be a trisaccharide and the α- and ω-compounds disaccharides, a suggestion compatible with the mobilities of the α- and ω-compounds, which are identical with gentiobiose and laminaribiose markers.

Action on glucose

Oligosaccharides were also formed synthetically. Thus Fig. 5*c* shows the results of incubating 7·5 % (w/v) glucose under the same conditions as above with the enzyme for 28 hr. and spraying the chromatogram of the reaction mixture with $AgNO_3$. Cellobiose and the α- and ω-compounds (and ω_1-?) were present, but no component with an R_G lower than the α-compound; thus this synthesis does not account for all the oligosaccharides formed from cellobiose. A comparison of the time course of the formation of the α-compound from 7·5 % cellobiose, from 7·5 % cellobiose plus 5 % glucose and from 12·5 % glucose (Fig. 6) showed that the synthesis of the α-compound via glucose could not account for its rapid formation when cellobiose or a mixture of cellobiose and glucose was the substrate. Equilibrium was reached after 25–30 hr., and the final concentration of α-compound depended only on the total concentration of free and combined glucose.

Effect of glucose oxidase

Cellobiose (5 %, w/v) was treated under the conditions described above with the addition of 10 mg. of glucose oxidase and the reaction followed by paper chromatography. The glucose was converted almost entirely into gluconic acid, which was not detected by the benzidine–trichloroacetic acid reagent.

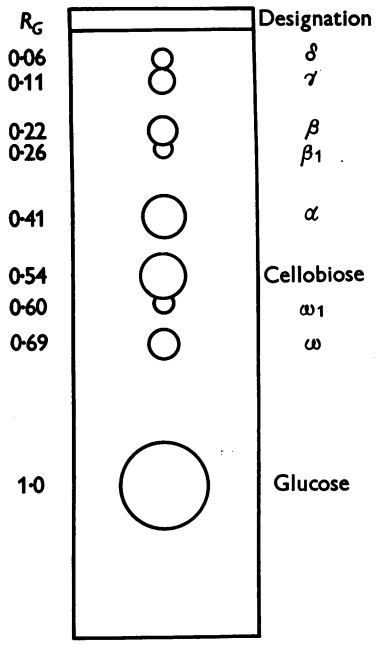

Fig. 2. Diagram of a chromatogram of the reaction products obtained by incubating cellobiose with *A. niger* preparation ('E19'), showing their R_G values in *n*-butanol–pyridine–water and the designations used in the text.

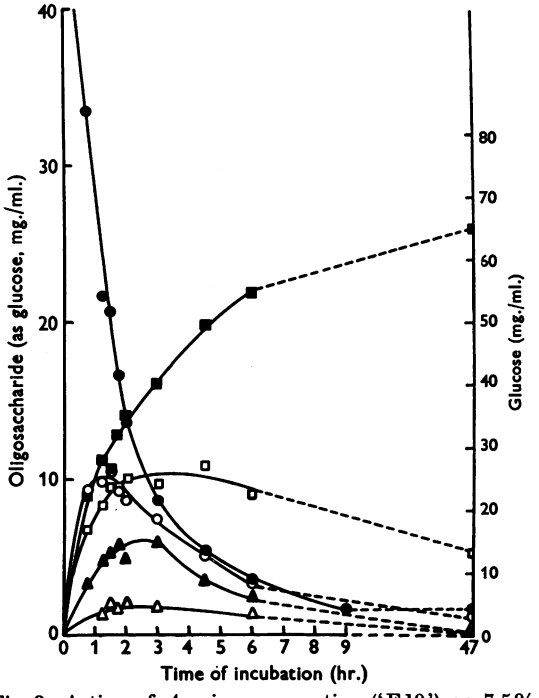

Fig. 3. Action of *A. niger* preparation ('E19') on 7·5 % (w/v) cellobiose in 0·01 M acetate buffer (pH 5·0) at 40°. Oligosaccharides estimated as glucose. Concentrations in mg./ml. of incubation mixture. ●, Cellobiose; ■, glucose; □, α-compound; ○, β-compound; ▲, $(\gamma+\delta)$-compounds; △, ω-compound.

Spraying with lead tetra-acetate showed that the gluconic acid has an R_G similar to that of the γ-compound. To prevent its interference with the upper spots, therefore, it was removed by shaking the solution with the ion-exchange resin Biodeminrolit (The Permutit Co. Ltd., London). It was difficult to prevent the accumulation of glucose during the first 2 hr. of reaction, but it was reduced to a small fraction of the usual amount. At the same time, the α- and ω-compounds were almost absent, and so also was the γ-compound. Cellobiose remained in high concentration and the β- and δ-compounds appeared early in the reaction as usual. These results confirm that the α- and ω-compounds are formed by transfer to glucose, and suggest that, as the γ-compound disappears when the α-compound does, it is normally formed by transfer to the α-compound, whereas the δ-compound may be formed by transfer to cellobiose or, more probably, to the β-compound.

Effect of cellobiose concentration

These conclusions were further strengthened by the results of an experiment in which 0·2 ml. of enzyme (10 mg./ml.) was incubated at 40° with

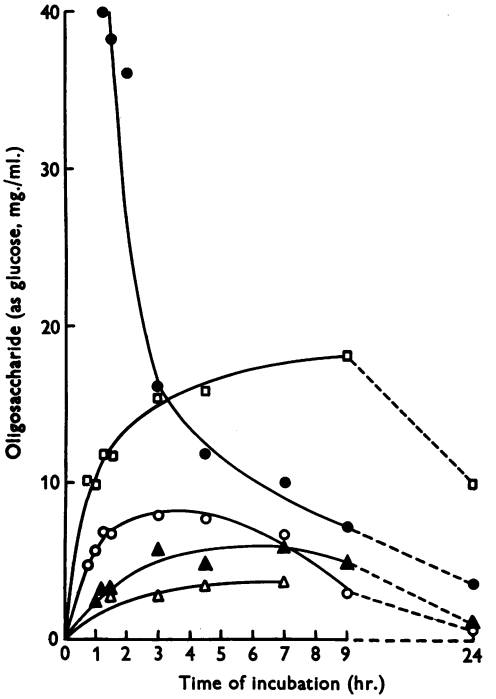

Fig. 4. Action of *A. niger* preparation ('E 19') on 7·5% (w/v) cellobiose plus 5·0% (w/v) glucose in 0·01 M acetate buffer (pH 5·0) at 40°. Oligosaccharides estimated as glucose. Concentrations in mg./ml. of the incubation mixture. Glucose not estimated. ●, Cellobiose; □, α-compound; ○, β-compound; ▲, (γ+δ)-compounds; △ ω-compound.

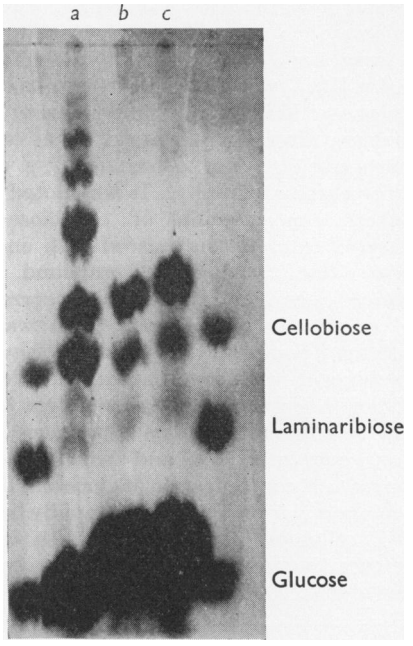

Fig. 5. Chromatogram comparing the composition of reaction mixtures obtained when *A. niger* preparation ('E 19') is incubated with (*a*) cellobiose, 5% (w/v), 1 hr.; (*b*) cellobiose, 5% (w/v), 24 hr.; (*c*) glucose 7·5% (w/v), 28 hr. in 0·01 M acetate buffer (pH 5·0) at 40°. Chromatograms developed in *n*-propanol–ethyl acetate–water for 4 days at 25°; sugars detected with $AgNO_3$ spray.

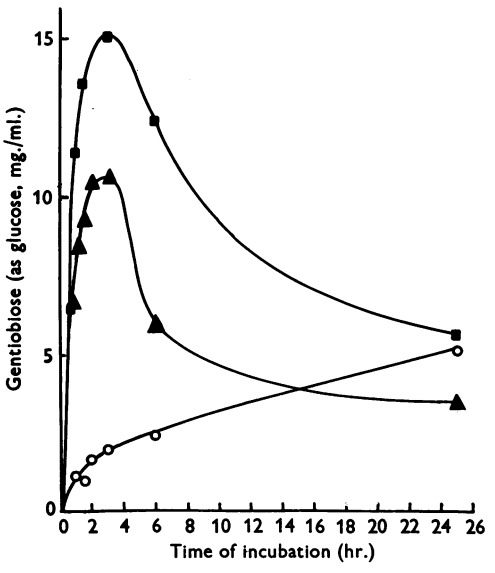

Fig. 6. Time course of gentiobiose formation during the incubation of *A. niger* enzyme ('E 19') with 12·5% (w/v) glucose (○), 7·5% (w/v) glucose plus 5% (w/v) cellobiose (■) and 5% cellobiose (▲) in 0·01 M acetate buffer (pH 5·0) at 40°. Concentrations in mg./ml. of incubation mixture.

0·8 ml. of 0·01 M acetate buffer (pH 5·0) containing cellobiose to give final concentrations ranging between 1 and 30 % (w/v). After 60 min. 0·05 ml. of 0·04 M-$HgCl_2$ was added and the mixture frozen in acetone–solid CO_2. The composition of the reaction mixture, after appropriate dilution of the higher concentrations, was determined by quantitative paper chromatography. It was noted that at the highest concentration of cellobiose an easily detected extra spot appeared with an R_G slightly greater than that of the β-compound. It has been referred to as β_1 and has a mobility corresponding to cellotriose. In this experiment it was estimated with the β-compound. Fig. 7 shows that glucose was liberated at very low concentrations of cellobiose whereas oligosaccharides were absent below 2·5 %. The α-compound made its appearance at low cellobiose concentrations and when there was an appreciable concentration of glucose. The amount of α-compound formed increased rapidly between 1 and 10 % cellobiose concentration with only a small increase thereafter; this course corresponded with that of glucose liberation, i.e. it behaved as though its concentration depended on the concentration of glucose rather than on that of cellobiose. On the other hand, the β-compound was not formed below 2·5 % cellobiose but continued to increase after the glucose and α-compound had ceased to accumulate rapidly. Again, these observations would be expected if the α-compound were formed by transfer to glucose and the β-compound by transfer to cellobiose.

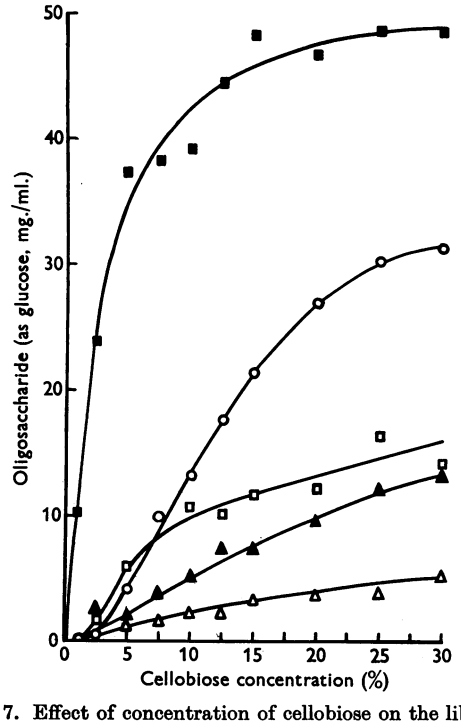

Fig. 7. Effect of concentration of cellobiose on the liberation of glucose and the formation of oligosaccharides. *A. niger* ('E19') preparation incubated for 1 hr. with cellobiose solutions in 0·01 M acetate buffer (pH 5·0) at 40°. Oligosaccharides estimated as glucose. Concentrations in mg./ml. of the incubation mixture. ■, Glucose; □, α-compound; ○, β-compound; ▲, $(\gamma+\delta)$-compounds; △, ω-compound.

Separation and isolation of oligosaccharides

To 15 g. of cellobiose in 80 ml. of 0·01 M acetate buffer (pH 5·0) 20 ml. of enzyme solution (20 mg./ml.) was added, and the mixture incubated at 40°. After 2 hr. the reaction was stopped by the addition of 1 ml. of 0·04 M-$HgCl_2$ and heating at 100° for 3 min. The inactivated mixture was applied to a column of charcoal–Celite (250 g.:250 g.; 54·5 cm. × 5 cm.). Water (5 l.) was run through to remove the glucose formed. A linear gradient of ethanol (0–20 % ethanol in 20 l.) was then applied to the column. Fractions (50 ml.) were collected and the course of the elution was followed by anthrone estimation of the sugars in the fractions. The fractions were pooled on the basis of the elution curve, evaporated to a small volume and freeze-dried. Three fractions were obtained in this way: fraction I (0–5 % ethanol approx.), fraction II (5–10 % ethanol approx.), fraction III (10–20 % ethanol approx.). The three fractions were analysed by paper chromatography and electrophoresis in borate buffer.

Fraction I. Paper chromatography showed two components: one with the R_G (0·69) of the ω-compound and laminaribiose, the other with an R_G (0·60) slightly higher than cellobiose (0·54) and identical with that of the ω_1-compound and with sophorose. Electrophoresis showed two components: one migrating to the same position as laminaribiose, the second moving more slowly and to a position intermediate between laminaribiose and cellobiose, as does sophorose (cf. Aso & Hamada, 1955). As the amount of this fraction was small, no attempt was made to separate the components.

Fraction II. Paper chromatography showed two components: one with the R_G of cellobiose and the other with the R_G of the α-compound and gentiobiose. Electrophoresis also showed two components with mobilities consistent with gentiobiose and cellobiose. The high mobility of the borate complex of gentiobiose enabled it to be separated from the cellobiose complex, which moves only slowly. Fraction II (500 mg.) was fractionated by preparative electrophoresis as described. The fast-moving component (II*a*) was isolated and corresponded to gentiobiose and the α-compound on electrophoresis and chromatography, and showed $[\alpha]_D^{20}+8°$ in water (*c*, 4) (gentiobiose has $[\alpha]_D^{20}+9·6°$).

The acetate (acetic anhydride–pyridine) melted at 195° (corr.) and had $[\alpha]_D^{20} - 5°$ in $CHCl_3$ (*c*, 3). Gentiobiose β-octa-acetate has m.p. 196° (corr.) and $[\alpha]_D^{20} = -5{\cdot}4°$ in $CHCl_3$. The second slow-moving component (II*b*) corresponded to cellobiose in chromatography and electrophoresis.

Fraction III. Paper chromatography showed two main components with the same R_G values as the β- and γ-compounds. Electrophoresis showed one immobile and one fast-moving component. Fraction III (500 mg.) was separated into two components on the basis of their electrophoretic mobilities. The immobile component III*a* had the same mobility as the β-compound on chromatography but did not correspond in R_G to any member of the laminarin or lutean series. On complete acid hydrolysis (1·5 N-H_2SO_4, 5 hr. at 100°) it yielded only glucose (chromatographically); partial acid hydrolysis (0·33 N-H_2SO_4, 1 hr. at 80°) at concentrations which preclude transfer showed components corresponding chromatographically and electrophoretically to cellobiose, gentiobiose and glucose. Component III*a* showed $[\alpha]_D^{20} + 9{\cdot}2°$ in water (*c*, 6·8). It formed an acetate with m.p. approx. 205° and $[\alpha]_D^{20}$ approx. −13° in $CHCl_3$. The fast-moving component, III*b*, had the same R_G as the γ-compound and corresponded electrophoretically and chromatographically to the second member of the series of oligosaccharides obtained by the acid hydrolysis of lutean, namely gentiotriose. It was completely hydrolysed by acid to glucose (1·5 N-H_2SO_4, 5 hr. at 100°) and on partial acid hydrolysis (0·33 N-H_2SO_4, 1 hr. at 80°) gentiobiose and glucose were identified as the only products by chromatography and electrophoresis.

Action on other β-glucosides

Laminaribiose. Laminaribiose (5 %, w/v) was incubated with the enzyme under the same conditions as for cellobiose and the reaction followed chromatographically. The laminaribiose disappeared with the concomitant formation of glucose. Oligosaccharides appeared and disappeared during the reaction, as with cellobiose. The most rapidly formed was a compound with an R_G somewhat greater than the β-compound and intermediate between laminaritriose and laminaritetraose. By analogy with the β-compound it would be a β-1:6–1:3 trisaccharide and its R_G was compatible with this. Its appearance was closely followed by a compound, with the R_G of gentiobiose, which was formed in relatively large amounts. Both of these compounds practically disappeared after 24 hr. Traces of a compound with the R_G of cellobiose could be detected. Also detectable were compounds with lower R_G values.

Gentiobiose. Gentiobiose (5 %, w/v) was incubated under the same conditions as for cellobiose and the reaction followed chromatographically. The disappearance of gentiobiose was accompanied by the appearance of glucose. A trisaccharide with an R_G corresponding to that of the γ-compound and of gentiotriose had a transient appearance.

Methyl β-glucoside and salicin (saligenin β-glucoside). Methyl β-glucoside (5 %, w/v) and salicin (5 %, w/v) were incubated with the enzyme under the same conditions as for cellobiose and the course of the reaction was followed chromatographically. The benzidine–trichloroacetic acid reagent detected spots corresponding in mobility to glucose and gentiobiose. The silver nitrate reagent, which also detects glycosides, showed additional spots above gentiobiose. These were not methyl β-glucoside or salicin, since these compounds have mobilities greater than glucose.

Effect of the addition of galactose and xylose

Cellobiose (7·5 %, w/v) was incubated separately with D-galactose (5 %, w/v) and D-xylose (5 %, w/v), under the same conditions as for cellobiose; the reactions were followed by paper chromatography. In the presence of galactose an extra compound running between gentiobiose and β-compound was detectable. This persisted together with the α-compound when no cellobiose could be detected. The R_G of this compound is compatible with the structure 6-glucosylgalactose, which would be formed in a manner analogous to the α-compound. The amount of β-compound was reduced, as compared with that formed when cellobiose was incubated alone with the enzyme. In the presence of xylose a small amount of a compound running between laminaribiose and cellobiose appeared, but there was no other alteration in the usual pattern of spots.

Action of other enzyme preparations on cellobiose

Preparations from various microfungi, from the snail *Helix pomatia*, from sweet almonds and from fresh gentian roots (see Materials) were incubated with 5 % (w/v) cellobiose in 0·01 M acetate buffer (pH 5·0) at 40°. Toluene was added as antiseptic. Samples were removed at intervals up to 7 days, and analysed by paper chromatography. All these preparations were able to hydrolyse cellobiose and produce a series of compounds identical, as far as can be judged by chromatographic mobilities, with those produced by the *A. niger* preparation when incubated with cellobiose. The *Myrothecium verrucaria* preparation was unique in giving rise to very small amounts of gentiobiose and γ-compound, thus adding to the evidence that these two are closely related. The *Helix pomatia* preparation had the same order of activity as the *A. niger* ('E 19'), whereas of the other mould preparations *A. aureus* gave the next highest activity. The β-glucosidase activities

of most of the mould preparations were low. Emulsin had a high activity and upper spots could be detected only when appropriately diluted enzyme was used.

DISCUSSION

Identity of oligosaccharides

The ω-compound may be tentatively identified from electrophoretic and chromatographic data as laminaribiose (3-*O*-β-D-glucopyranosyl-D-glucose). The other component (ω_1) of fraction I had an electrophoretic and chromatographic mobility compatible with sophorose (2-*O*-β-D-glucopyranosyl-D-glucose). The α-compound agrees in properties with gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose).

The β-compound behaves chromatographically as a trisaccharide and its kinetics of formation are compatible with this formulation. It has an electrophoretic mobility compatible only with the reducing glucose moiety being linked through the 4-position (cf. mobility of cellobiose). The products of partial hydrolysis indicate that the other glycosidic linkage must be 1:6. The low specific rotation suggests that the glycosidic linkages are of the β-configuration, as does the rotation of the acetate, which is also too negative for the known compound with β-1:4- and β-1:6-linkages, namely 4^2-β-glucosylgentiobiose* [*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-glucopyranosyl-(1 → 6)-D-glucose] whose $[\alpha]_D^{20}$ is $-10{\cdot}3°$ (Helferich & Schäfer, 1926). The β-compound would then have the structure 6^2-β-glucosylcellobiose (*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose). After the action of *A. niger* (strain 152) on cellobiose Barker *et al.* (1955) have isolated a trisaccharide with $[\alpha]_D^{20} + 10{\cdot}2°$ and with similar properties to the β-compound and have proposed the same structure. They also provide evidence that their compound must be 6^2- and not 6^1-β-glucosylcellobiose. The evidence cited here allows no such unambiguous formulation of the β-compound. However, on the basis of the general finding that most transfers are to non-reducing terminal residues and by analogy with the findings of Barker *et al.* the 6^2-β-glucosylcellobiose has been preferred.

The γ-compound is also a trisaccharide with properties compatible with the following structure: *O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-D-glucose, i.e. gentiotriose. This compound has previously been reported by Lindberg & Macpherson (1954). Helferich & Schäfer (1926) and Helferich & Gootz (1931) have synthesized the hendeca-acetate of gentiotriose. The δ-compound (see above) has not been isolated, but chromatographically it behaves as a tetrasaccharide (not gentiotetraose), and its electrophoretic behaviour suggests that the reducing glucose moiety is linked through the 4-position. The order of appearance of the lower R_g compounds under certain conditions, e.g. at pH 6·8 and 7·5, suggests that the β- and δ-compounds are related. The experiments with glucose oxidase also confirm that the δ-compound depends on the β- and not on the α- or γ-compounds, i.e. that it is probably formed by transfer from cellobiose to the β-compound. The most probable structure for the δ-compound on the basis of the mechanism put forward below is 6^2-β-gentiobiosylcellobiose (*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 6)-*O*-β-D-glucopyranosyl-(1 → 4)-D-glucopyranose).

* The shortened names are coined by assuming the sugar to be a derivative of the largest oligosaccharide within the molecule which contains the same type of linkage and the free reducing-end group. The superscript numeral refers to the glucose unit undergoing substitution, the reducing-end member being numbered 1, etc.

General

Enzymes from various sources catalyse the transient formation of glucose oligosaccharides during the hydrolysis of cellobiose and other β-glucosides. The oligosaccharides appear to be all β-linked, i.e. the steric configuration is retained. During the hydrolysis of methyl β-glucoside by emulsin (Bunton, Lewis, Llewellyn, Tristram & Vernon, 1954) and of salicin by an unspecified β-glucosidase (Springhorn & Koshland, 1955) cleavage of the glucosidic link occurs between the glucose carbon and the oxygen. Transfer from these compounds have been demonstrated with most of the enzymes used in the present work, and it is therefore likely that the same mechanism can be extended to cover them, i.e. that they are transglycosylations (cf. Hehre, 1951).

Transfer could proceed to any carbon atom on the acceptor with a free hydroxyl group, and if the acceptor happens to be free glucose any one of the six possible glucopyranose disaccharides with a β-linkage could be formed. Transfer to higher saccharides is of course restricted by the elimination of those hydroxyls already occupied by glucosidic links. It has also been generally observed that transfers are restricted to the non-reducing end of a chain, although the recent work of Bailey, Barker, Bourne & Stacey (1955) shows that other possibilities must be kept in mind.

In the present work direct evidence was obtained for transfer to all positions of glucose acceptor except C-1. The proportions of the various isomers formed at equilibrium (47 hr. values, Fig. 3) appear to fall in the same order as observed by Peat *et al.* (1952) for the five isomeric β-linked glucose disaccharides based on the yield in the condensation reaction (1:6 > 1:4 > 1:3 > 1:2 > 1:1). An identical

series is obtained on the basis of yields of acid-reversion products from glucose (Thompson, Anno, Wolfrom & Inatome, 1954; Wolfrom & Thompson, 1955). In the present work the position of sophorose in the series depends on the observation that sophorose is no longer detectable at equilibrium and hence must follow laminaribiose. The present data suggest that the relative rates of formation of the linkages is approximately in the same order, 1:6-linkages being formed faster than any others, whereas the rate of formation of 1:3-links is low. It is difficult to obtain good evidence for the relative speed of formation of 1:4-links. A tentative conclusion may be drawn from a comparison of Figs. 3 and 4. In the initial stages of the reaction, addition of glucose has retarded the disappearance of cellobiose without appreciably increasing the rate of appearance of laminaribiose. This suggests that the retardation is due to the transfer more rapidly to the 4-position of glucose than to the 3-position. The same predominance in transfer to the primary hydroxyl at C-6 on a pyranose residue occurs in other enzyme transfers, e.g. amylose → amylopectin, dextrin → dextran, sucrose → dextran, maltose → panose (6^2-α-glucosylmaltose), and lactose → 6^{Gal}-α-galactosyl-lactose, and also in the transfer of fructose residues by yeast invertase to the primary hydroxyl group of glucose or to one of the three primary hydroxyl groups of sucrose. However, in many instances it was not clear whether transfer was to primary hydroxyl groups exclusively or whether the analytical methods were not sensitive enough to detect other products. Thus recently Wilham, Alexander & Jeanes (1955) have shown, using periodate oxidation techniques, that enzymically synthesized dextrans also contain varying proportions of 1:4- and 1:3-like linkages, and Wolfrom & Thompson (1955) have detected α-1:3-links (nigerose) in hydrolysates of amylopectin. 1:3-Links have also been detected in the reaction products of enzymes on maltose (Barker & Carrington, 1953; Matsuda & Aso, 1953; see also the chromatograms of Pazur & French, 1952, which show a compound in the correct position for a 1:3-linked disaccharide although these authors do not comment on it).

On the basis of these conclusions, and taking into account the structures which have been deduced for the various compounds observed on the chromatograms, the following tentative scheme may be drawn up to account for the reactions occurring when cellobiose is broken down.

Initially cellobiose is the only glucose donor available, but both cellobiose and water are available acceptors:

$$G\beta\text{—}4G + H_2O \rightarrow 2G, \quad (1)$$

$$G\beta\text{—}4G + G\beta\text{—}4G \rightarrow G\beta\text{—}6G\beta\text{—}4G + G. \quad (2)$$

Reaction (2) results in the formation of the β-compound, and both give rise to free glucose. It would be expected that small quantities of cellotriose (Gβ—4Gβ—4G) would also be formed at this stage together with traces of Gβ—3Gβ—4G, but the great predominance of 1:6-transfers results in their concentration being too low to detect under the present conditions except with high concentrations of cellobiose (25–30 %). There then appears a small amount of a compound with a chromatographic mobility compatible with cellotriose.

The free glucose liberated in reactions (1) and (2) can also act as acceptor and, since it rapidly reaches high concentrations, transfers to all positions except C-1 are demonstrable, with 1:6-transfers again predominating. In this way the disaccharides gentiobiose, more cellobiose, laminaribiose and sophorose are formed by transfers to C-6, C-4, C-3, and C-2 respectively. All these products of transfer can themselves act as acceptors. Then from the β-compound

$$G\beta\text{—}4G + G\beta\text{—}6G\beta\text{—}4G \rightarrow G\beta\text{—}6G\beta\text{—}6G\beta\text{—}4G + G, \quad (3)$$

and from gentiobiose

$$G\beta\text{—}4G + G\beta\text{—}6G \rightarrow G\beta\text{—}6G\beta\text{—}6G + G. \quad (4)$$

The products of reactions (3) and (4) are respectively the δ- and γ-compounds. Again, trisaccharides resulting from transfer to laminaribiose and sophorose would be expected, but are below the limits of detection. That they can be formed, however, is shown by the experiments in which laminaribiose was the starting material. Here a Gβ—6Gβ—3G trisaccharide is formed. When glucose oxidase is added to the reaction mixture to remove the glucose as it is formed, new disaccharides and hence γ-compound are no longer formed. Transfers to sugars and sugar derivatives other than glucose are also possible. Methyl β-glucoside, salicin, D-galactose and D-xylose have been shown to be acceptors during the present experiments.

Thus complex series of oligosaccharides are built up and the chromatographic picture obtained depends on three factors: (i) the great preponderance of transfers to C-6; (ii) the linkage in the starting disaccharide; (iii) the initial concentration of the starting material. The first ensures that chains of 1:6-links predominate. The second determines the linkage at the reducing end of the chains. However, there is a complication, since the presence of free glucose together with factor (i) ensures that gentiobiose is always present and therefore that there are always chains which end in 1:6-linkages, whatever the linkage in the primary disaccharide. The third factor determines whether there are appreciable amounts of linkages other than 1:6 in parts of the chain distant from the terminal reducing-end linkage. Thus it is only with high concentrations

of starting material that the intermediates reach sufficient concentrations for the second link to be other than 1:6, and as 1:4 is the next most probable by a good margin this is the most likely configuration for that linkage. The probability that linkages other than 1:6 would occur further away than one from the terminal linkage is small, even with high initial concentrations (25–30 %) of disaccharide.

Alternative routes are also possible. Thus some gentiobiose undoubtedly arises from glucose in strong sugar solutions, but the following considerations show that free glucose plays a minor role in the other syntheses. The β-compound is not formed from glucose alone in the presence of the enzyme (Fig. 5*c*), and the addition of glucose in the presence of cellobiose actually diminishes formation of β-compound, simultaneously increasing that of gentiobiose (Fig. 4), i.e. glucose competes successfully for the transferred radicals. Moreover, when cellobiose is the substrate, glucose continually increases in concentration throughout the course of the reaction, whereas the oligosaccharides early reach maxima which are many times their final equilibrium concentrations (Figs. 3, 4 and 6). Further, as is shown in Fig. 6, the initial rate of formation of α-compound from cellobiose is 20–30 times as high as from an equivalent concentration of glucose.

Thus it seems probable that the first route pictured above is the most important for the formation of β-compound. It is, however, possible that higher saccharides are formed by transfer of more than one residue at a time, e.g. gentiotriose could be formed from β-compound by transfer of a gentiobiosyl residue to glucose (eqn. 5):

$$G\beta\text{—}6G\beta\text{—}4G + G \rightarrow G\beta\text{—}6G\beta\text{—}6G + G. \quad (5)$$

Gentiobiose might arise by a similar transfer to water. This would be analogous to the *D*-enzyme of potato, which has been shown to transfer two or more glucose residues of a maltodextrin substrate to a suitable acceptor (Peat, Whelan & Rees, 1956). No evidence for or against such transfers can be adduced from the present experiments. The upper limit of chain length for acceptors is not known, but oligosaccharides of at least six units have been detected in these experiments. In addition to a diminishing donor concentration the acceptor-chain length may be a factor in determining the point at which the hydrolytic aspect of the reaction becomes overwhelming.

In the preceding discussion it has been assumed that water was an acceptor competing with sugar for the transferred glucosyl radicals, and this assumption has been shown to be compatible with the observed reactions. However, no evidence is available to show whether or not this competition is a result of the action of two different enzymes or a series of enzymes, or whether hydrolysis and transfer are both catalysed by a single enzyme. Although the latter is the simpler picture and appears to be true for β-glucuronidase (Fishman & Green, 1956), the identity of hydrolytic and transfer enzymes in our unpurified preparations must rest on indirect evidence (cf. Doudoroff, Barker & Hassid, 1947; Bealing & Bacon, 1953; Takano & Miwa, 1950, 1953). Studies of the pH–activity relationships and the results of fractionation of the β-glucosidase of *A. niger* (Stone, 1954) suggest that two components with β-glucosidase activity are present and that each fraction possesses both hydrolytic and transfer activity, although the ratio of these activities is different. This may be compared with the finding of Durell & Fruton (1954) that although trypsin and papain both catalyse transamidation reactions with the same substrates the efficiency of the transfer is different. However, there is no doubt that transfer is kinetically favoured compared with hydrolysis, since in a 5 % cellobiose solution the molar concentration of water is 400 times that of the sugar.

Similar difficulties arise in trying to decide whether a given enzyme is specific for the transfer from one particular carbon atom to another, or whether a single enzyme can catalyse all of the transfers demonstrated in this work. The simplest assumption would be that it can, and this would not be incompatible with any of the suggested reaction mechanisms. However, caution is necessary, since the discovery of trehalase in *A. niger* (Bourquelot, 1893) and its differentiation from the maltase of the same organism, by their different heat stabilities, suggests at least that enzymes with varying preferences may exist even if the specificities are not absolute. Moreover, Bealing & Bacon (1953) and Edelman (1954) have evidence that yeast and mould invertases, both fructosidases, produce a qualitatively different mixture of oligosaccharides. Also Barker *et al.* (1955) find with their resting-cell preparations of *A. niger* (strain 152) that laminaribiose appears before gentiobiose, whereas the opposite is true in the present experiments.

In the light of the present observations, it is possibly not without significance that a number of naturally occurring oligosaccharides, e.g. raffinose, gentianose, stachyose and verbascose, have structures in which one or more monosaccharide residues are attached to the C-6 hydroxyl group of sucrose. It is possible that gentianose arises from sucrose by the activity of the β-transglucosylase which, as the present work shows, occurs in gentian roots. Raffinose, stachyose and verbascose form a homologous series of α-1:6-galactosides of sucrose and have been found to occur together in *Verbascum thapsiforme* Schrad. (Hérissey, Fleury, Wickström, Courtois & Le Dizet, 1954; see also French, 1954)

and may be synthesized by enzymes with similar specificities.

The ability of alcohols to act as acceptors in the presence of β-glucosidases (Rabaté, 1935; Takano & Miwa, 1950; Jermyn & Thomas, 1953) also suggests that many of the heterosidic glycosides occurring in plants may be formed by transfer, especially as they are often accompanied by enzymes able to hydrolyse them. Thus Miller (1943) was able to show that *o*-chlorophenol and ethylene chlorohydrin, both potential glucose acceptors, were able to induce the formation of *o*-chlorophenyl β-gentiobioside and 2-chloroethyl β-glucoside in gladiolus corms, although these do not normally contain gentiobiose.

The study of transfer reactions involving β-glucosides naturally raises the question whether β-linked polysaccharides are synthesized by this route. This would imply an enzyme with an affinity for oligosaccharide acceptors that is higher than those studied in this work. Further, the degree of homogeneity of the linkages will depend on the transfer specificity of the enzyme. Thus enzymes with the degree of transfer specificity demonstrated in the present work would account for the formation of polysaccharides such as lutean, which is reported to contain 1:6-, 1:3- and 1:4-linkages (Lloyd, Pon & Stacey, 1956). On the other hand, from what we know of the structure of cellulose, its synthesis, if it is by a transfer mechanism, would require an enzyme with an absolute transfer specificity.

SUMMARY

1. During the hydrolysis of cellobiose to glucose by an enzyme preparation from *Aspergillus niger*, there is a transient formation of a number of glucose oligosaccharides.

2. Three disaccharides and two trisaccharides have been isolated and identified. A third trisaccharide and a tetrasaccharide were also observed in chromatograms of the reaction mixture.

3. Only the disaccharides were detected when the *A. niger* preparation was incubated with glucose under the same conditions.

4. Oligosaccharides were also produced during the incubation of the *A. niger* preparation with laminaribiose, gentiobiose, methyl β-glucoside and salicin.

5. The effects of cellobiose concentration, and of the presence of glucose, galactose or xylose, and glucose oxidase on the formation and nature of the oligosaccharides were investigated.

6. Enzyme preparations from other microfungi and from *Helix pomatia*, sweet almonds and gentian roots showed a qualitatively similar formation of oligosaccharides during cellobiose hydrolysis.

7. The formation of oligosaccharides is interpreted in terms of an enzyme-catalysed transference of glucosyl residues from cellobiose and other β-glucosides to suitable acceptors. Hydrolysis results when water is the acceptor.

8. The factors influencing the nature and amounts of oligosaccharides formed and the general significance of transferase reactions are discussed.

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The Isolation of a Ketonic Dihydroxy Kober Chromogen from the Urine of Pregnant Women

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In 1943 Pincus & Pearlman briefly referred in a review article to evidence obtained by them 'for the presence of an oestrogen in human pregnancy urine which is ketonic and contains a hydroxyl group other than the usual phenolic one'. Experimental details of this work were not published, but subsequently Huffman & Grollman (1947) suggested that this ketonic oestrogen might be 16-oxo-oestradiol-17β (oestra-1:3:5-triene-3:17β-diol-16-one), which previously had been prepared from oestrone by Huffman (1942) (cf. Huffman & Lott, 1948); it was also suggested by these authors that 16-oxooestradiol-17β might be an intermediate in the metabolic conversion of oestrone (oestra-1:3:5-trien-3-ol-17-one) into oestriol (oestra-1:3:5-triene-3:16α:17β-triol). More recently Migeon (1953) has reported the detection in extracts of acid-hydrolysed human urine of a H_2SO_4–fluorogen which behaved like 16-oxooestradiol-17β on counter-current distribution in two different solvent