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Studies on Carbohydrate-Metabolizing Enzymes

TRANS- β -GLUCOSYLATION BY BARLEY ENZYMES

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Recent investigations (reviewed by Gottschalk, 1958) have shown that the enzymic hydrolysis of a glycosidic linkage in an oligosaccharide is, in fact, a transglycosylation reaction in which a glycosyl group is transferred from the substrate to a water molecule. The latter thus acts as a glycosyl acceptor:

where R is a hydrogen atom, G-O-X represents the substrate (glycosyl donor) and H-O-R the glycosyl acceptor. Other compounds containing hydroxyl groups (e.g. carbohydrates or alcohols) may function as glycosyl acceptors, and with these the synthesis of a new saccharide or glycoside is observed. In the transglycosylation reaction the configuration of the glycosidic linkage is retained. The hydrolytic and 'transferase' activity of a carbohydrase thus represent two examples of the same enzymic reaction, and the enzyme will show a dual specificity towards the glycosyl donor and the acceptor.

During a study of barley β -glucosidases, Manners (1955a) found that whereas cellobiose in dilute aqueous solution was completely hydrolysed to glucose, in concentrated solution the production of glucose was accompanied by oligosaccharide synthesis. The latter could arise by the enzymic transfer of β -glucosyl radicals to glucose and cellobiose. This paper describes the characterization of these oligosaccharides, and this has enabled the acceptor specificity of barley cellobiase to be determined. For convenience, the enzyme system catalysing reactions (1) and (2) with cellobiose as β -glucosyl donor will be referred to as cellobiase,

although purification has not been attempted and information on the number of active factors is not available.

METHODS AND MATERIALS

Analytical methods

Chromatography. (a) Qualitative. Descending paper chromatograms were prepared at room temperature with Whatman no. 1 paper and either ethyl acetate-pyridine-water (10:4:3, by vol.) (A) or propanol-ethyl acetate-water (6:1:3, by vol.) (B) as solvent. Aniline oxalate or silver nitrate-sodium hydroxide was used as spray reagent. The rate of movement of sugars is expressed relative to D-glucose (R_g values).

(b) Preparative. Charcoal-Celite columns were prepared as previously described (Duncan & Manners, 1958).

Electrophoresis. Separation of oligosaccharides on Whatman no. 1 paper was effected in an apparatus similar to that described by Foster (1952) at 750 v and 10 ma. 0.1N-Borate buffer (pH 10.0) was used.

Acid hydrolysis. Complete acid hydrolysis of oligosaccharides was carried out by heating with 1.5N-sulphuric acid (0.5% solution) at 100° for 3 hr. Reducing sugars were determined with the Somogyi (1952) reagent calibrated against glucose and cellobiose. Partial acid hydrolysis of oligosaccharides and the derived aldonic acids was effected by the methods of Duncan & Manners (1958).

Periodate oxidation. The consumption of periodate, production of formic acid, and formaldehyde were determined by the methods of Fleury & Lange (1933), Halsall, Hirst & Jones (1947) and Hough, Powell & Woods (1956), respectively.

Acetylation of sugars. The oligosaccharide (about 50 mg.) was heated at 135° with sodium acetate (80 mg.) and acetic anhydride (1.5 ml.) until solution was effected, and then for a further 15 min. The acetylated product was separated by pouring into ice-cold water (5-10 ml.), purified by solution in chloroform and recrystallized from methanol.

Enzyme preparation

Flour produced by hammer-milling Spratt-Archer barley (1954 harvest) provided the enzyme source. A sample (300 g.) was extracted with 0.02M-acetate buffer (pH 5.0; 1 l.) for 2 hr. at room temperature with constant stirring; the grain residue was removed and the solution (750 ml.) dialysed for 2 days against running tap water. The solution (780 ml.) was then cooled to 0°, and absolute ethanol (3 vol.) slowly added with constant stirring. The precipitated protein was collected and suspended in M-acetate buffer (pH 3.0; 250 ml.) at 35° for 90 min. to inactivate amylases. The solution was then adjusted to pH 5.0 and insoluble material (*I*) collected by centrifuging, suspended in water and freeze-dried (yield 1.1 g.). The supernatant solution was also freeze-dried (fraction *S*; yield 6.6 g.).

EXPERIMENTAL AND RESULTS

Preliminary experiments

Digests containing cellobiose (0.2 g.), barley fraction *I* or *S* (20 mg.), toluene and 0.04M-acetate buffer (pH 5.0; 1 ml.) were incubated at 35°. Paper-chromatographic analysis (solvent *A*) showed that oligosaccharides were present in both digests within 2 days, with R_f values 0.73 and 0.39 (together with traces of higher oligosaccharides). On continuing the incubation, the cellobiose content decreased and the proportion of oligosaccharides increased. After 18 days, sugars with R_f values 0.73, 0.39, 0.30, 0.25 and 0.13 were present. The R_f values of authentic samples of laminaribiose and gentiobiose were 0.73 and 0.40; cellobiose had R_f 0.53.

A similar digest containing glucose (0.2 g.) was prepared; oligosaccharide synthesis did not take

place. Further control experiments showed that the cellobiose substrate was chromatographically pure (it had previously been chromatographed on a charcoal-Celite column) and was not attacked by heat-inactivated barley fraction *S*.

Preparation and isolation of oligosaccharides

A digest containing cellobiose (27 g.), water (350 ml.) and enzyme solution (150 ml.) was incubated at 35° in the presence of toluene and chloroform. The enzyme solution consisted of a 2% (w/v) solution of barley fraction *S* in 0.04M-acetate buffer (pH 5.0). Samples of the digest were examined chromatographically at intervals; the results were identical with those in the preliminary experiment. After 18 days the digest was heated to inactivate the enzyme and coagulated protein removed by filtration.

The solution was concentrated and the oligosaccharides were separated on a charcoal-Celite column (150 cm. × 5 cm.); details of the separation are given in Table 1.

Characterization of oligosaccharides

With the exception of fraction 6, the oligosaccharides were homogeneous by paper chromatography and electrophoresis. On acid hydrolysis, glucose was the sole product (paper chromatography).

Fraction 1. This sugar had the same R_g , reducing power and specific rotation (+52°) as D-glucose.

Fraction 2. This fraction had the reducing power of a disaccharide, was hydrolysed by almond emulsin to glucose and had the R_g of gentiobiose. A portion (150 mg.) was acetylated; the product

Table 1. Chromatographic separation of oligosaccharides synthesized from cellobiose

Eluent	Vol. of eluent (l.)	R_f value of sugar (solvent <i>A</i>)	Yield (mg.)	Fraction no.	Identity
Water	12.5	1.0	20 000	1	Glucose
	10.0	1.0 + mixture	Trace	—	—
	8.0	—	—	—	—
Ethanol (5%)	3.4	—	—	—	—
	4.0	1.0 + mixture	Trace	—	—
	11.0	0.40	1 500	2	Gentiobiose
	5.0	0.40 + 0.55	Trace	—	—
	7.0	0.55	320	3	Cellobiose
	6.0	—	—	—	—
Ethanol (7%)	12.0	0.75	280	4	Laminaribiose
	6.0	0.75	Trace	—	—
	5.0	—	—	—	—
Ethanol (10%)	10.0	Mixture	Trace	—	—
Ethanol (15%)	8.0	0.15	300	5	Gentiotriose
	10.0	0.25 + mixture	50	6	Celлотriose
	6.0	—	—	—	—
	20.0	0.30	350	7	6 ² -β-Glucosylcellobiose
Ethanol (20%)	10.0	Mixture	Trace	—	—

(160 mg.) had m.p. 192°; $[\alpha]_D^{20} - 5^\circ$ in chloroform (c, 10.0). Gentiobiose β -octa-acetate has m.p. 191–193° and $[\alpha]_D - 5^\circ$ in chloroform (Peat, Whelan & Lawley, 1958).

Fraction 3. This material had the R_g and reducing power of cellobiose. The corresponding acetate (yield 60 mg. from 50 mg.) had m.p. 192–193°, $[\alpha]_D^{20} - 12^\circ$ in chloroform (c, 3.3); an authentic sample of cellobiose β -octa-acetate had m.p. 192°, $[\alpha]_D^{20} - 14^\circ$ in chloroform. On oxidation with sodium metaperiodate buffered at pH 8.0, the fraction (5 mg.) gave formaldehyde (2.05 mol.prop.; theoretical value 2.0).

Fraction 4. This fraction had the same R_g and reducing power as laminaribiose, and was completely hydrolysed to glucose by almond emulsin. The sugar was recrystallized from ethanol and had m.p. 160–163°; $[\alpha]_D^{20} 28^\circ \rightarrow 22^\circ$ in water (c, 1.5) [cf. m.p. 160–163°; $[\alpha]_D^{21} 23^\circ \rightarrow 19^\circ$ in water reported by Connell, Hirst & Percival (1950) for laminaribiose]. The *N*-benzylglycosylamine derivative (cf. Bayly & Bourne, 1953) had the same R_g value as that from an authentic sample of laminaribiose, and the derived osazone had m.p. 194–196° and mixed m.p. 193–194° with laminaribiosazone.

Fraction 5. This sugar had the reducing power of a trisaccharide. Partial acid hydrolysis of both the sugar and the corresponding aldonic acid gave glucose and gentiobiose. On oxidation with sodium metaperiodate at room temperature the initial and final production of formic acid was 4.0 and 5.4 mol.prop. respectively. The theoretical values for gentiotriose are 4.0 and 6.0 mol.prop. Final confirmation of identity was provided by periodate oxidation at pH 8; no formaldehyde could be detected after 2 days (cf. Hough & Perry, 1956).

Fraction 6. The major component of this mixed fraction had the same R_g as cellotriose, kindly supplied by Dr W. J. Whelan (solvents A and B).

Fraction 7. This material had R_g 0.30 (solvent A), the reducing power of a trisaccharide, was hydrolysed by almond emulsin and on partial hydrolysis with acid gave glucose, cellobiose and gentiobiose. After oxidation with hypiodite, a partial hydrolysate contained glucose and gentiobiose. This indicates that fraction 7 is 6²- β -glucosylcellobiose [see Crook & Stone (1957) for definition of nomenclature]. The identity has been confirmed by periodate oxidation. At room temperature, the consumption of periodate was 6.3 and 6.7 mol.prop. after 4 and 24 hr.; the theoretical values for the initial and total primary oxidation are 6.0 and 7.0 mol.prop. respectively. The production of formic acid amounted to 3.9, 4.0 and 4.1 mol.prop. after oxidation for 2, 4 and 5 days (theoretical

value 4.0). On oxidation in phosphate buffer (pH 8.0) at 35°, 1.03 mol.prop. of formaldehyde was liberated (theoretical value 1.0).

Transfer of β -glycosyl radicals to pentoses

Digests were prepared containing cellobiose (100 mg.), D-xylose, D-lyxose or D-arabinose (100 mg. in 2 ml. of water) and barley fraction S (20 mg. in 1 ml. of 0.04M-acetate buffer, pH 5.0) and were incubated at 35°. After incubation for 3 days paper chromatography in solvent A showed glucose, cellobiose and a new component. When xylose was present the new component had R_g 0.86, the same as for authentic 3-O- β -D-glucopyranosyl-D-xylose, the product from lyxose had R_g 0.90 and from arabinose 0.75. After incubation for 5 days the xylose digest contained a second new component with R_g 0.68. All the new sugars stained pink with aniline oxalate and are presumably glucosyl-pentose disaccharides.

DISCUSSION

The present investigation has shown that extracts of ungerminated barley contain carbohydase(s) which can transfer β -glucosyl radicals from cellobiose to glucose and glucodisaccharides, and to various pentoses. However, the barley enzymes are unable to synthesize oligosaccharides from glucose itself, and in this respect differ from the β -glucosidases of almond emulsin (Peat, Whelan & Hinson, 1952) and *Aspergillus niger* (Peat, Whelan & Hinson, 1955; Crook & Stone, 1957). A trans-glycosylation mechanism of the type shown in equations (1) and (2) is therefore indicated.

A solution of cellobiose (5.4%, w/v) was converted into a mixture of glucose (87%), gentiobiose (6.5%), laminaribiose (1.2%), gentiotriose (1.3%), 6²- β -glucosylcellobiose (1.5%) and cellotriose (0.5%). Cellobiose (1.4%) and traces of other oligosaccharides were also present. Since the trisaccharides appear after the disaccharides it is probable that in the initial trans- β -glucosylation reaction part of the cellobiose is hydrolysed to glucose, which can then act as an acceptor molecule. Transfer to the primary alcoholic group (C-6) is predominant. Some of the trisaccharides are then formed by β -glucosyl transfer to the hydroxyl group at C-6 and C-4 of the non-reducing glucose residue of cellobiose or to the similar primary alcohol group of gentiobiose. It seems likely that barley cellobiase is responsible for the observed synthesis of gentiobiose and laminaribiose (glucose acceptor), and of 6²- β -glucosylcellobiose and cellotriose (cellobiose acceptor).

The formation of gentiotriose could be catalysed by the same enzyme, or by a related 'gentiobiase'

(extracts of barley hydrolyse gentiobiose and amygdalin) with gentiobiose rather than cellobiose as the β -glucosyl donor. The possible identity of barley cellobiase and gentiobiase is being investigated.

The metabolism of cellobiose by β -glucosidase preparations from several biological sources has been investigated recently. Barker, Bourne, Hewitt & Stacey (1955) examined the oligosaccharides produced by *Aspergillus niger* (strain 152); a number of di- and tri-saccharides were isolated and their structures indicated transfer to the hydroxyl groups at C-6, C-4, C-3 and C-2 of glucose, and to cellobiose and gentiobiose. In general, the formation of β -1:6-glucosidic linkages was favoured. Similar observations have been made by Crook & Stone (1957) with enzyme preparations from *A. niger* (E19), *Helix pomatia*, sweet almonds and gentian roots. Oligosaccharide formation from cellobiose has also been observed with β -glucosidase preparations from *Aspergillus flavus* (Giri, Nigam & Srinivasan, 1954) and marine algae (W. A. M. Duncan, D. J. Manners & J. L. Thompson, to be published).

Buston & Khan (1956) investigated a related enzyme system in *Chaetomium globosum*; the products from cellobiose included cellotriose and other trisaccharides, laminaribiose, gentiobiose and sophorose. However, this system appears to differ from that in barley since trisaccharides were detected in the initial stages, before the appearance of disaccharides. Indeed, an earlier study by Buston & Jabbar (1954) indicated that cellotriose was the major product, formed by β -glucosyl transfer to cellobiose. The disaccharides may therefore result from trans- β -glucosylation to cellobiose, followed by hydrolysis of the β -1:4-linkage adjacent to the reducing group. Buston & Khan (1956) have, in fact, noted that on prolonged incubation cellotriose disappears from the reaction mixture.

The ability of 'cellobiase' enzymes to catalyse oligosaccharide formation from concentrated solutions of cellobiose thus appears to be a general property with preparations from higher plants, marine algae and certain micro-organisms. However, some differences in acceptor specificity are apparent, and the present study shows that the barley enzyme differs from many others in being unable to effect appreciable transfer to the hydroxyl group at C-2 of glucose. In general, trans- β -glucosylases show a lower specificity towards the acceptor molecule than the corresponding trans- α -glucosylases.

Since the reserve β -glucosan of barley contains only 1:3- and 1:4-linkages (Aspinall & Telfer, 1954), it is unlikely that the transglucosylase activity of barley cellobiase is intrinsically of any

metabolic significance. The observed oligosaccharide synthesis is merely the result of enzyme action in the presence of a low concentration of water. It follows that in the use of β -glucosidases for the structural analysis of polysaccharides (cf. Manners, 1955*b*), enzyme action should take place in dilute solution, and analysis must be confined to the initial stages in order to avoid any possible ambiguity.

It is of interest to note that pentoses may serve as acceptor substrates during trans- β -glucosylation with barley enzymes. This activity is similar to that of enzyme preparations from *Aspergillus niger* (Barker, Bourne, Hewitt & Stacey, 1957) and *Cladophora rupestris* (W. A. M. Duncan, D. J. Manners & J. L. Thompson, to be published). The characterization of the glucosyl-pentoses will be reported later.

SUMMARY

1. Extracts of ungerminated barley catalyse the synthesis of oligosaccharides from cellobiose.
2. The oligosaccharides have been isolated and characterized. They include gentiobiose, laminaribiose, gentiobiose, cellotriose and 6²- β -glucosyl-cellobiose.
3. The acceptor specificity of the barley enzymes and similar enzymes from other biological sources is discussed.

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Progesterone Metabolism: Investigation of the Products of Metabolism with Human Liver *in vitro*

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The metabolism of progesterone *in vivo* in the human subject has been extensively investigated, but only 3 α -hydroxy-5 β -pregnan-20-one (pregnanolone) (Dorfman, Ross & Shipley, 1948), 5 β -pregnane-3 α :20 α -diol (pregnanediol) (Dorfman *et al.* 1948), 3 α -hydroxy-5 α -pregnan-20-one (Dobriner, Lieberman, Rhoads & Taylor, 1948; Dobriner & Lieberman, 1950; Davis & Plotz, 1956; Plotz & Davis, 1956) and 5 α -pregnane-3 α :20 α -diol (*allopregnanediol*) (Kyle & Marrian, 1951) have been shown conclusively to be progesterone metabolites. The metabolism *in vitro* has been investigated in the rat (Taylor, 1954), but progesterone metabolism in the rat appears to be different from that in the human (Wiest, 1956) since the rat does not excrete pregnanediol in the urine (Atherden & Grant, 1957). Taylor (1955) also investigated progesterone metabolism in the rabbit which, like the human, excretes pregnanediol in the urine; but it is well known that steroid metabolism varies with species (see, for example, Brooks & Klyne, 1957).

Thus it seemed desirable that progesterone metabolism *in vitro* in the human should be investigated; Reaven (1955) has shown that $\alpha\beta$ -unsaturated ketonic steroids are transformed into substances which do not absorb at 240 m μ when incubated with human-liver slices; therefore progesterone was incubated with a 'suspension' prepared from post-mortem human liver.

EXPERIMENTAL

Materials

All materials were obtained from British Drug Houses Ltd. unless otherwise stated.

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Steroids. Progesterone (Ciba Laboratories Ltd.), in the form of prisms (' α' -progesterone), had m.p. 127.5–128° [all melting points were determined on a micro-melting-point apparatus (Gallenkamp and Co. Ltd.) and are uncorrected]; 5 α -pregnane-3:20-dione (*allopregnanedione*) (L. Light and Co. Ltd., Colnbrook, Bucks) had m.p. 199–200°; 5 β -pregnane-3:20-dione (*pregnanedione*) (L. Light and Co. Ltd.) had m.p. 122–123°; 3 α -hydroxy-5 α -pregnan-20-one and its acetate (m.p. 134–136°) were supplied by the Medical Research Council Steroid Reference Collection; 3 β -hydroxy-5 α -pregnan-20-one (*allopregnanolone*) (m.p. 193–196°) and pregnanolone (m.p. 149–151°) were samples from Dr W. Taylor, University of Durham; pregnanolone acetate was prepared from pregnanolone and had m.p. 96–100°; pregnanediol (L. Light and Co. Ltd.) had m.p. 235–238°; pregnanediol diacetate was prepared from pregnanediol and had a double m.p. 162° and 181–182°; cholesterol had m.p. 145–146°.

Reagents. Reduced triphosphopyridine nucleotide (TPNH) (Sigma Chemical Co., Mo., U.S.A.), digitonin (AnalaR) and Girard T reagent (Girard & Sandulesco, 1936) were not purified further; alumina (100–200 mesh, Savory and Moore Ltd.) was screened through a no. 90 sieve, the material not passing through being rejected. It was activated to activity II (Brockmann & Schodder, 1941) by heating at 130° for 12 hr.; some was deactivated by exposure to water vapour for 10 days.

Solvents. Acetone was refluxed for 2 hr. with KMnO₄ and NaOH, distilled, refluxed with anhydrous K₂CO₃ and redistilled. Benzene was washed with water, conc. H₂SO₄ and water, dried over CaCl₂ and distilled. CHCl₃ was prepared freshly as required by washing with water, drying over CaCl₂ and distilling. Ether (J. F. MacFarlan and Co.) was treated with FeSO₄ soln., washed with water, dried with Na₂SO₄ and Na wire and distilled. Ethyl acetate (AnalaR) was redistilled. Hexane consisted of the fraction from petroleum boiling at 67–70°; this was washed with conc. H₂SO₄, 2N-NaOH and water, dried over CaCl₂ and distilled. Methanol (AnalaR) was redistilled. Pyridine was dried over BaO, filtered, distilled and the fraction boiling at 112–117° was collected and stored over BaO.