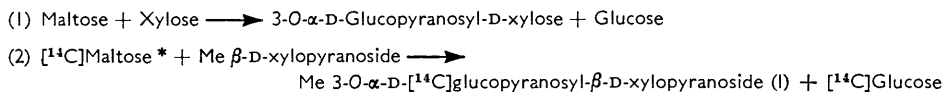


### 778. *Enzymic Synthesis of a Glucoxytan.*

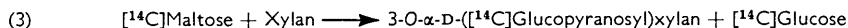
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On use of a xylanase-free transglucosylase from *Penicillium lilacinum*, a glucoxytan was synthesised by transfer of glucosyl units from substrate [<sup>14</sup>C]maltose \* to a receptor esparto grass xylan. Since such a transfer was initially to position 3 of xylose units it could be demonstrated by an increase in the number of periodate-resistant xylose units.

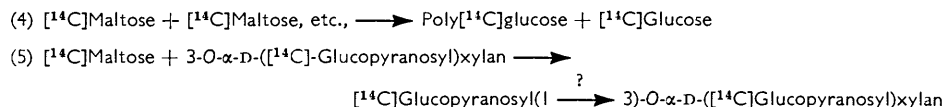
Two model systems<sup>1,2</sup> that we have investigated to demonstrate the specificity of *Penicillium lilacinum* transglucosylase can be summarised by the reactions:



It was thus conceivable that a glucoxytan could be synthesised by transfer of glucosyl units from [<sup>14</sup>C]maltose to position 3 of  $\beta$ -linked xylose units in a xylan:



We now report such a synthesis. The xylan used was obtained from esparto grass, repeated fractional precipitation as its copper complex giving a xylan devoid of arabinose residues.<sup>3</sup> It had been shown<sup>3</sup> that the xylan was a singly branched molecule containing 75 ( $\pm$ 5) 1,4- $\beta$ -linked D-xylopyranose units and that the single branch point was constituted by a 1,3 union. In the synthesis two major problems were encountered. The *Penicillium lilacinum* cell-free extract previously used<sup>1,2</sup> was found to contain xylanase which would have degraded the receptor xylan of reaction (3); however, fractionation of the extract by alcohol yielded a xylanase-free transglucosylase. The second problem was that this enzyme in a control incubation with [<sup>14</sup>C]maltose alone yielded a radioactive polyglucose, indicating that reaction (4) and also probably (5) would also occur during biosynthesis of the glucoxytan:



Xylan is sparingly soluble in water but dissolves in sodium hydroxide and can be retained in solution after neutralisation with hydrochloric acid. Introduction of glucose residues into xylan would increase its solubility in water and so the radioactive polysaccharide obtained from the digest containing [<sup>14</sup>C]maltose, xylan, and transglucosylase was fractionated after removal of the sodium chloride which kept it in solution. As expected, the water-insoluble fraction IB was almost wholly xylose (xylose 93%; glucose 7%). The soluble fraction IA contained 65% of xylose and 35% of glucose and was separated into three components by electrophoresis on cellulose acetate paper in borate buffer. To justify the claim that a glucoxytan was present admixed with polyglucose(s) advantage was taken of the resistance that would be shown to periodate by xylose units substituted at position 3 by glucosyl residues. Further, any glucosyl residues which themselves were substituted at position 3 by further glucosyl residues would also be

\* Isotopic labelling is general, on all carbon atoms, in this and other labelled compounds named in this paper.

<sup>1</sup> Barker, Stacey, and Stroud, *Nature*, 1961, **189**, 138.

<sup>2</sup> Barker, Keith, Stacey, and Stroud, Paper RICC/6, "Uses of Radioisotopes in the Physical Sciences and Industry," Copenhagen, September, 1960.

<sup>3</sup> Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289.

resistant to periodate. Indeed, whereas the original xylan (1.92 mg.) contained only 20  $\mu$ g. of periodate-resistant xylose units, the same weight of glucoxytan fractions IA and IB contained 37 and 48  $\mu$ g., respectively, whilst transglucosylase-treated xylan still contained only 21  $\mu$ g. of such units. These results are consistent with the low degree of branching found previously in the original xylan<sup>3</sup> and could indicate the virtually complete substitution of all the non-reducing terminal xylose units in a 75 unit xylan such as might be present in fraction IB. Analysis also showed that many of the glucose units themselves were periodate-resistant and probably 1,3-linked. The attachment of glucose units to the xylan chain was finally proved by detection of a radioactive disaccharide containing both glucose and xylose in the partial acid hydrolysate of fraction IB. A control experiment showed that this could not have arisen by acid reversion. The more positive specific optical rotations of fractions IA (+10°) and IB (−65°) compared with that of the original xylan (−90°) suggest that the glucosyl units transferred are  $\alpha$ -linked.

#### EXPERIMENTAL

*Isolation of Penicillium lilacinum Transglucosylase.*—Mycelia of *Penicillium lilacinum* were harvested after growth for 5 days at 30° on a medium (300 ml.) containing magnesium sulphate heptahydrate (0.074 g.), ammonium nitrate (0.1 g.), potassium dihydrogen phosphate (0.1394 g.), sodium chloride (0.001 g.), and maltose (50 g.). Washed aqueous suspensions of the cells were shaken intermittently with glass beads for 20 min. in a Mickle tissue disintegrator, then centrifuged, and the cell-free enzyme extract was freeze-dried. A portion (0.25 g.) was dissolved in water, ethanol was added to a concentration (v/v) of 25%, and the mixture was left at 0° for 24 hr. The precipitate (0.006 g.) formed was recovered by centrifugation and freeze-drying, as were further fractions (0.010 g.; 0.038 g.) that were precipitated on addition of ethanol to concentrations of 50% and 75% severally. The final supernatant solution was dialysed and freeze-dried (0.190 g.).

The activity of the four fractions was determined by incorporating them (0.003 g. portions) severally in digests containing (a) maltose (50 mg.) and xylose (25 mg.) in water (1 ml.) and (b) xylan (20 mg.) dissolved in 4% sodium hydroxide and adjusted to pH 7 with 2N-hydrochloric acid (final volume, 1 ml.). Paper chromatography revealed the formation of 3-O- $\alpha$ -glucosylxylose by the 50% and 75% ethanol fractions in digests of type (a) and the degradation of xylan to xylosaccharides by the 25% and 50% ethanol fractions in digests of type (b). No xylanase activity was exhibited by the 75% ethanol fraction, and the supernatant fraction was completely inactive. The 75% ethanol fraction constitutes the *Penicillium lilacinum* transglucosylase used in the enzymic synthesis.

*Properties of the Esparto Grass Xylan used as Receptor.*—Xylan isolated from esparto grass by the method of Chanda, Hirst, Jones, and Percival<sup>3</sup> had  $[\alpha]_D^{19} -90^\circ$  (c 0.5 in N-NaOH) and when hydrolysed with 2N-hydrochloric acid at 100° for 3 hr. yielded only xylose (96%). Xylose was assayed<sup>4</sup> by separation on paper chromatograms irrigated with butanol-ethanol-water (4:1:5), development of the chromatograms with aniline hydrogen phthalate, elution of the coloured spot, and determination of xylose content by measurement of the extinction coefficient of the solution at 360  $\mu$  and comparison with a calibration curve constructed for the range 5–200  $\mu$ g. of monosaccharide. Glucose was assayed in a similar manner by measurement of the extinction coefficient of its chromogen at 390  $\mu$ .

*Enzymic Synthesis of a Glucoxytan.*—[<sup>14</sup>C]Maltose (125 mg.; 0.1 mc) and transglucosylase (4 mg.) were incorporated in a digest (40 ml.) containing xylan (125 mg.) which had been dissolved in N-sodium hydroxide (20 ml.) and neutralised with 2N-hydrochloric acid. After incubation for 5 days at 30° enzyme activity was arrested by boiling. The digest was dialysed for 4 days and freeze-dried (141 mg.; product I).

As a control [<sup>14</sup>C]maltose (12.5 mg.) and transglucosylase (0.4 mg.) in water (4 ml.) were incubated for 5 days at 30°. The solution was then boiled, xylan (12.5 mg.) added, and the whole dialysed for 4 days and freeze-dried (14.5 mg.; product II). Portions of products (I) (1.0 mg.) and (II) (0.2 mg.) were diluted with inactive maltose (ca. 30 mg.) and their <sup>14</sup>C contents

<sup>4</sup> Wilson, *Analyt. Chem.*, 1959, **31**, 1199.

assayed by gas-counting after combustion (results calc. as per g. of carbon). Product (I) showed 35,100 c.p.m., and (II) 101,000 c.p.m. (by reference to a standard compound 9000 c.p.m.  $\equiv 1\mu\text{C/g.}$  of carbon).

Product (I) (140 mg.) was stirred in water (150 ml.) at room temperature for 24 hr., then centrifuged for 6 hr. at 2500 r.p.m., and the filtrate and precipitate were freeze-dried separately, to yield products (IA) (32 mg.),  $[\alpha]_{\text{D}}^{18} + 10^\circ$  ( $c$  0.2 in  $N$ -NaOH) and (IB) (108 mg.),  $[\alpha]_{\text{D}}^{18} - 65^\circ$  ( $c$  0.2 in  $N$ -NaOH). A similar fractionation of the control (II) (14.3 mg.) gave a soluble fraction (IIA) (2 mg.) and an insoluble fraction (IIB) (12.3 mg.). Radioactive assay of portions (0.7 mg.) of the fractions (as above) showed the following activities: IA, 30,080; IB, 4800; IIA, 92,000; IIB, 7000 c.p.m. Electrophoresis on cellulose acetate paper in 0.1M-borate buffer of pH 10 showed that product (IA) contained three components, of which two gave a weak and one a strong reaction with periodate-Schiff reagent. Only one weakly reacting component was detected in product (IIA).

*Sugar Contents of Glucoxylan Fractions.*—Fractions (IA) (0.24 mg.) and (IB) (0.20 mg.) were separately hydrolysed with 2N-hydrochloric acid at  $100^\circ$  for 2 hr. After neutralisation with silver carbonate the filtrates were freeze-dried and analysed by quantitative paper chromatography.<sup>4</sup> Fraction (IA) contained 65% of xylose and 35% of glucose; fraction (IB) contained 93% of xylose and 7% of glucose. Qualitative examination of hydrolysates of products (IIA) and (IIB) showed that the former contained only glucose and the latter contained glucose and xylose.

*Periodate Oxidation of Glucoxylan Fractions.*—The glucoxylan fractions (IA) (1.9 mg.) and (IB) (1.91 mg.), the original receptor xylan (1.92 mg.), and xylan (1.88 mg.) which had been incubated with the transglucosylase alone were oxidised for 8 days severally in the dark with sodium metaperiodate (20 mg.) in water (2 ml.). Ethylene glycol (0.05 ml.) was then added and each solution transferred by micropipette with washing to a dialysis tube. After 3 days' dialysis against running water each tube and its contents were thoroughly extracted and the solutions obtained were freeze-dried. Each periodate-oxidised polysaccharide was hydrolysed with  $N$ -hydrochloric acid (0.2 ml.) at  $90^\circ$  for 2 hr. and neutralised as above and the supernatant liquids obtained on centrifugation were freeze-dried. Assay by quantitative paper chromatography<sup>4</sup> showed that while the original xylan contained only 20  $\mu\text{g.}$  of periodate-resistant xylose units and the enzyme-treated xylan 21  $\mu\text{g.}$  of periodate-resistant xylose units, the yields from fractions (IA and B) were 37 and 48  $\mu\text{g.}$ , respectively. Further, fractions (IA and B) contained 220 and 130  $\mu\text{g.}$  of periodate-resistant glucose units, respectively, which were absent from the original xylan. Quantitative determination<sup>5</sup> of the moles of periodate consumed per 132 g. of polysaccharide showed: IA, 0.85; IB, 0.95; xylan, 1.05.

*Partial Acid Hydrolysis of Periodate-oxidised Glucoxylan (IB).*—A portion (2 mg.) of periodate-oxidised material (IB) was hydrolysed with 0.5N-hydrochloric acid at  $90^\circ$  for 1 hr. After neutralisation the sample was applied in two equal portions to separate chromatograms each carrying as reference compounds maltose, nigerose, glucose, and 3- $O$ - $\alpha$ -D-glucosyl-D-xylose. After irrigation one chromatogram was sprayed with alkaline silver nitrate and showed a component having the same  $R_{\text{F}}$  value as the glucosylxylose together with some glucose, xylose, and oligosaccharides. The various individual components were cut out and assayed for radioactivity by the method of liquid scintillation.<sup>6</sup> The paper disc was placed in a vessel containing a few ml. of liquid scintillator (2,5-diphenyloxazole in toluene) in a refrigerator at  $-10^\circ$ . The scintillations produced by the emission of  $\beta$ -rays were measured by means of a photomultiplier. The activities in c.p.m. were: glucose, 100; glucosylxylose, 50; nigerose, 102; trisaccharide ( $R_{\text{maltose}}$  0.44), 65; together with other components  $R_{\text{maltose}}$  1.8 (40 c.p.m.), 1.3 (20), 1.0 (107), 0.31 (10), 0.16 (20). In a further experiment the glucosylxylose component was again isolated as above and hydrolysed. Glucose and xylose were detected in the hydrolysate.

*Isolation and Properties of the Polyglucose.*— $^{14}\text{C}$ Maltose (500 mg.) and enzyme (5 mg.) in water (20 ml.) were incubated together for 5 days at  $30^\circ$ , during which no precipitation was observed. Enzyme activity was then destroyed by boiling and the mixture freeze-dried. A portion (90 mg.) of the residue was redissolved in water (3 ml.) and passed down a column (2  $\times$  30 cm.) of Sephadex G-25 at 30 ml./hr. Carbohydrate analysis of the 5-ml. fractions collected revealed two components (fractions 9—10, 1.5 mg.; fractions 11—14, 2.5 mg.) in the region expected for molecules with molecular weights  $>4000$ . Periodate oxidation of both

<sup>5</sup> Fleury and Lange, *J. Pharm. Chim.*, 1933, **17**, 107, 196.

<sup>6</sup> Haigh, Proc. UNESCO Radio-isotopes Conf. Paris, 1957, 663.

these fractions and determination of the proportion of resistant glucose units as above revealed 40  $\mu\text{g.}$  per 100  $\mu\text{g.}$  of the first component and 45  $\mu\text{g.}$  per 110  $\mu\text{g.}$  of the second component.

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