469. *Mechanism of the Enzymic Synthesis of a Branched Trisaccharide containing the α-1 : 2-Glucosidic Linkage.*

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A trisaccharide produced during the growth of *B. arabinosaceous* on a [^{14}C]sucrose medium containing lactose has been characterised as *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-{[^{14}C]-α-D-glucopyranosyl-(1 ⟶ 2)}-D-glucose. The distribution of ^{14}C in its three monosaccharide units is in accordance with a mechanism involving the transfer of the glucose residue from sucrose to the reducing moiety of lactose.

It has been shown [1] that certain simple sugars can serve as chain initiators when the dextransucrase of *Leuconostoc mesenteroides* (NRRL B-512) acts on sucrose. Such sugars cause a marked decrease in dextran production, and lead to the synthesis of oligosaccharides. A similar phenomenon was observed with dextransucrase preparations and with growing cultures of *B. arabinosaceous* (Birmingham).[2-5] The oligosaccharides produced in cultures containing sucrose as a substrate, to which isomaltose, maltose, methyl α-D-glucoside, or 3-*O*-methyl-D-glucose had been added as receptors, were formed by the successive transfer of glucosyl units from sucrose molecules to the receptor, with the formation of α-1 : 6-glucosidic linkages. The addition of lactose or cellobiose to a culture led to the synthesis of a trisaccharide in which a glucosyl unit was attached through an α-1 : 2-linkage to the reducing moiety of the disaccharide.[2,4,5] Thus the trisaccharide produced in a lactose–sucrose medium was characterised as *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose, whilst that produced in the presence of cellobiose was analogous, namely, *O*-β-D-glucopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.

These two trisaccharides are of interest for several reasons. They contain the rare α-1 : 2-glucosidic linkage rather than the α-1 : 6-linkage normally synthesised by dextransucrase. They are believed to be the first "branched" trisaccharides obtained by a controlled enzymic synthesis *in vitro*; their formation involves the use of a carbohydrate primer in which a sugar residue, other than the usual non-reducing end unit, has the correct structure to act as a receptor of a transferred sugar residue. The mechanism of their formation could, in fact, be a general one for the production of branched oligosaccharides and, moreover, could represent the first stage in the synthesis of branched polysaccharides, by a route which does not involve prior formation of the linear polymer.[2] It was important therefore to study the synthesis of the branched trisaccharides in greater detail. Although it seemed likely that each was formed by transfer of a glucosyl unit from sucrose to a preformed lactose (or cellobiose) molecule, as follows:

Glu 1——2 Fru + Gal 1——4 Glu ⟶ Gal 1——4 Glu 2——1 Glu + Fru . . (1)

there were other possibilities, such as:

Gal 1——4 Glu + Gal 1——4 Glu ⟶ Gal 1——4 Glu 2——1 Glu + Gal . . (2)

We now report the use of ^{14}C-tracer techniques to elucidate this problem; at the same time confirmatory evidence is presented that the structure previously assigned to the "branched" trisaccharide, derived from lactose, was correct.

A culture of *B. arabinosaceous* (Birmingham), incubated with lactose and [^{14}C]sucrose, produced a [^{14}C]trisaccharide (A), which was isolated from the culture medium by

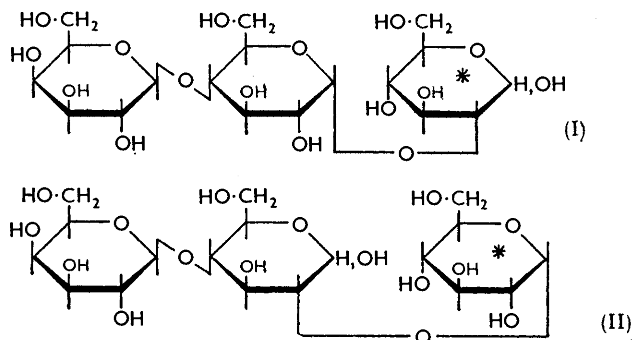
[1] Koepsell, Tsuchiya, Hellmann, Kazenko, Hoffmann, Sharpe, and Jackson, *J. Biol. Chem.*, 1953, **200**, 793.
[2] Bailey, Barker, Bourne, and Stacey, *Nature*, 1955, **175**, 635; 1955, **176**, 1164.
[3] Bailey, Barker, Bourne, and Stacey, *J.*, 1957, 3530, 3536.
[4] Barker, Bourne, Grant, and Stacey, *J.*, 1958, 601; *Nature*, 1956, **178**, 1221.
[5] Bailey, Barker, Bourne, Grant, and Stacey, *J.*, 1958, 1895.

fractionation on a charcoal–" Celite " column.[6] Chromatograms of the trisaccharide indicated that it was pure in both the chemical and the radiochemical sense. The specific radioactivity of the trisaccharide, determined as barium carbonate by the infinitely thick disc method,[7] was 523 μc per g.-atom of carbon, corresponding to 9417 μc per mole of trisaccharide. In a direct determination on the trisaccharide by the infinitely thin film method, the corresponding figures were 519 and 9341. Chromatographic analysis of the culture medium during the incubation period revealed [^{14}C]sucrose, [^{14}C]glucose, [^{14}C]-fructose, the [^{14}C]trisaccharide A, and lactose. This indicated that an enzyme system involving an equilibrium between sucrose, glucose, and lactose was not present.

The trisaccharide A had the same mobility on paper chromatograms and on electrophoretograms as the trisaccharide *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose isolated and characterised by Bailey *et al.*[5] With aniline hydrogen phthalate, it gave a yellowish colour characteristic of a 2-*O*-substituted reducing aldose. It was not revealed by alkaline triphenyltetrazolium chloride which detects all reducing glucosaccharides except those with a 2-*O*-substituent.[8] Its low mobility in borate buffer confirmed the conclusion that the reducing unit was linked through position 2.

Further evidence for the structure of trisaccharide A and qualitative evidence for the distribution of ^{14}C in the three monosaccharide units was obtained by total and partial hydrolysis. The hydrolysates were analysed by paper chromatography. The total hydrolysate was shown to contain galactose and [^{14}C]glucose. The partial hydrolysate contained galactose, lactose, [^{14}C]glucose, and [^{14}C]kojibiose.

The presence of one α- and one β-glycosidic link was confirmed by treatment of trisaccharide A with almond β-glucosidase which produced galactose and [^{14}C]kojibiose.



The evidence presented so far suggests two possible structures (I and II) for trisaccharide A. The first structure could have resulted from the transfer of a lactosyl unit to position 2 of [^{14}C]glucose, derived from [^{14}C]sucrose; the second could have been formed by transfer of a [^{14}C]glucosyl unit from [^{14}C]sucrose to position 2 of the reducing moiety of lactose [eqn. (1)]. Since the radioactivity of the [^{14}C]sucrose employed was roughly 17,000 μc per mole, the radioactivities of structures (I) and (II) should have been approx. 8500 μc per mole, compared with the values 9341 and 9417 actually obtained for trisaccharide A.

The possibility that trisaccharide A arose from two lactose molecules [eqn. (2)] was eliminated when it was shown that no galactose was liberated and that the lactose was inactive throughout the incubation period. Indeed, even if instantaneous equilibration

[6] Whistler and Durso, *J. Amer. Chem. Soc.*, 1950, **72**, 677; Lindberg and Wickberg, *Acta Chem. Scand.*, 1954, **8**, 569.
[7] Skipper, Bryan, White, and Hutchison, *J. Biol. Chem.*, 1948, **173**, 371; Calvin, Heidelberger, Reid, Tolbert, and Yankwich, " Isotopic Carbon," Wiley, New York, 1949; Henriques, Kistiakowsky, Margnetti, and Schneider, *Ind. Eng. Chem. Analyt.*, 1946, **18**, 349.
[8] Feingold, Avigad, and Hestrin, *Biochem. J.*, 1956, **64**, 351.

of the glucose units in the lactose and [^{14}C]sucrose had occurred, the radioactivity of the trisaccharide produced in this way would have been only approx. 2840 μc per mole.

In an attempt to determine quantitatively the distribution of ^{14}C in its monosaccharide units, trisaccharide A was treated with phenylhydrazine, a technique which is known to convert, under suitable conditions, 2-*O*-substituted aldoses into their phenylosazones.[9] Thus compounds (I) or (II) should each have yielded D-[^{14}C]glucosazone and lactosazone. The products were separated on a " Celite " column with a pyridine–benzene–water solvent, in conditions which had been established with artificial mixtures. The specific activity of the " glucosazone " (4203 μc per mole) was much lower than that expected (*ca.* 9400 μc per mole), whereas the " lactosazone," expected to be inactive, had 1184 μc per mole. If specific cleavage of the 1 : 2-linkage had occurred, these values would require the specific radioactivity of the trisaccharide to be only 5387 μc per mole. Clearly, phenylhydrazine under these conditions was rupturing also a percentage of the 1 : 4-linkages, as was confirmed subsequently by treatment of lactose itself.

Conclusive evidence for the distribution of ^{14}C in the monosaccharide units of trisaccharide A was obtained by reduction with potassium borohydride, followed by hydrolysis and radioactivity assay of the products. Paper chromatography of the total hydrolysate of trisaccharide A alcohol revealed the presence of galactose, sorbitol, and [^{14}C]glucose. These three products were isolated by preparative paper chromatography, and their radioactivities determined by the infinitely thin film method.[10] The β-emission measured was shown to bear a linear relation to the amount of compound applied and was calibrated against a standard [^{14}C]glucose sample. The results are shown in the Table. From the

	Counts per min. per μmole	μc per mole		Counts per min. per μmole	μc per mole
D-Glucose	952	9253	Sorbitol	17	165
D-Galactose	6	58	Trisaccharide A	961	9341

ratio of the emission of the glucose to the sum of the emissions of glucose, galactose, and sorbitol, the glucose fragment was calculated to contain 97·6% of the ^{14}C of trisaccharide A alcohol. An alternative calculation was based on a direct comparison of the radioactivities of the trisaccharide A and the glucose fragment of trisaccharide A alcohol. This indicated that 99·1% of the ^{14}C of trisaccharide A was present in the non-reducing glucose unit. These results are conclusive in favour of the " branched " structure (II) for trisaccharide A, rather than (I), which would have given glucose, galactose, and [^{14}C]sorbitol. Moreover, they confirm the structure assigned earlier [2,4,5] on chemical grounds alone, to a trisaccharide prepared in a similar manner.

Thus it is established that the enzymic synthesis of the " branched " trisaccharide *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose proceeds by transfer, directly or indirectly, of the glucosyl residue from sucrose to position 2 of the reducing unit of lactose. When [^{14}C]sucrose is used, as above, the equation becomes:

$$\overset{*}{\text{Glu}}\ 1\text{——}\overset{*}{2}\ \text{Fru} + \text{Gal}\ 1\text{——}4\ \text{Glu} \longrightarrow \text{Gal}\ 1\text{——}4\ \text{Glu}\ 2\text{——}1\ \overset{*}{\text{Glu}} + \overset{*}{\text{Fru}} \quad . \quad (3)$$

where the asterisk denotes a radioactive sugar residue. It remains to be seen whether the transfer is catalysed by dextransucrase itself, or by a closely related enzyme which accompanies it.

Experimental

Materials.—[^{14}C]Sucrose, generally labelled, was obtained from the Radiochemical Centre, Amersham.

Determination of Radioactivity.—(i) *Apparatus.* Radioactivities were determined by means

[9] Brigl and Schinle, *Ber.*, 1929, **62**, 1716; Haworth, Hirst, and Teece, *J.*, 1931, 2858; Rabate, *Bull. Soc. chim. France*, 1940, **7**, 565; Barry, *Nature*, 1943, **152**, 537.
[10] Walker and Whelan, personal communication; Dalgliesh and Dutton, *J.*, 1956, 3784.

of a Geiger–Müller end-window counter. The β-emission of a radioactive specimen was measured for a time sufficient to give a standard counting error of better than ±2% except for samples of specific radioactivity lower than 1·6 μc per g.-atom of carbon when measured by the infinitely thick disc method, or 30 μc per g.-atom of carbon when measured by the infinitely thin film method.

(ii) *Infinitely thick disc method.* Radioactivities were determined after conversion of the compound into carbon dioxide, and thence into barium carbonate.[7] The amount used was sufficient to give a thickness of greater than 20 mg. per cm.^{2}. A sample of poly-{[^{14}C]methyl methacrylate}, supplied by the Radiochemical Centre, Amersham, was used as a standard source of barium [^{14}C]carbonate.

(iii) *Infinitely thin film method.*[10] Three drops of a 0·01% " Teepol " solution were placed on a polished aluminium disc (diam. 2·5 cm.). The disc was flooded with water, and the " Teepol " solution evaporated to dryness in a stream of warm air and under an infrared lamp. An aqueous solution of the compound, containing 30—600 μg., was placed on the disc, and the disc flooded with water in order to spread the compound evenly over the surface. The solution was evaporated to dryness as described above. [^{14}C]Sugars were used as substandards.

Chromatography.—(i) *Solvents.* The solvents used in chromatography were (*a*) butan-1-ol–ethanol–water (4 : 1 : 5) (organic phase); (*b*) benzene–pyridine–water (5 : 4 : 4) (organic phase); (*c*) acetone–water (4 : 1); (*d*) ethyl acetate–acetic acid–saturated aqueous boric acid (9 : 1 : 1); (*e*) butan-1-ol–pyridine–water–saturated aqueous boric acid (6 : 4 : 2 : 1); (*f*) butan-1-ol–ethanol–water–ammonia (4 : 1 : 4·9 : 0·1) (organic phase).

(ii) *Radiochromatograms.* Radiochromatograms were obtained by scanning the chromatograms with a Geiger–Müller end-window counter or exposure to Ilford *X*-ray films (Industrial G) for an appropriate time.

Oligosaccharide Synthesis in a Lactose–[^{14}C]*Sucrose Medium.*—An aqueous medium (100 ml.) containing yeast extract (1%), disodium ammonium phosphate (0·5%), potassium dihydrogen phosphate (0·1%), hydrated magnesium sulphate (0·05%), lactose (10%), and [^{14}C]sucrose (2%, *ca.* 100 μc) was adjusted to pH 7 with sodium hydroxide and sterilised by filtration. The medium was inoculated with *Betacoccus arabinosaceous* (Birmingham) and incubated at 25° for 4 days. Paper chromatography of the culture medium in solvent (*a*) revealed components with R_F values identical with those of lactose (present throughout the incubation period), sucrose (first 24 hr.), fructose (24 hr. only), glucose, and *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.[5] The sugars were detected by acetone–silver nitrate–alcoholic sodium hydroxide.[11] Radiochromatograms showed ^{14}C-activity corresponding to glucose, fructose, sucrose, and *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.

The culture medium was adjusted to pH 7 and heated at 90° for 10 min. The bacterial cells were removed on a centrifuge (30 min. at 4500 r.p.m.). After the addition of ethanol (100 ml.), centrifuging, and removal of the ethanol, the oligosaccharide mixture was fractionated on a charcoal–" Celite " column [6] (40 × 4 cm.). Water (2·5 l.) eluted the monosaccharides and salts: 5% aqueous ethanol (2·5 l.) removed lactose. Trisaccharide A (351 mg.) was obtained by elution with 10% aqueous ethanol (4 l.).

Characterisation of Trisaccharide A.—(i) Paper chromatography of trisaccharide A in solvent (*a*) and of its benzylamine derivative [12] in solvent (*f*) showed that each moved as a single radioactive component with $R_{glucose}$ 0·11 and 0·37, respectively. Paper ionophoresis [13] in borate buffer (pH 10) again showed a single component with M_G 0·33. It was detected with acetone–silver nitrate–alcoholic sodium hydroxide,[11] and with aniline hydrogen phthalate,[14] but not with alkaline triphenyltetrazolium chloride.[8] In all of these tests the behaviour of trisaccharide A was identical with that of authentic *O*-β-D-galactopyranosyl-(1 ⟶ 4)-*O*-[α-D-glucopyranosyl-(1 ⟶ 2)]-D-glucose.[5]

(ii) *Radioactivity of trisaccharide A.* The specific radioactivity of trisaccharide A, determined by the infinitely thick disc method, was 523 μc per g.-atom of carbon, *i.e.*, 9417 μc per mole of trisaccharide.

(iii) *Hydrolysis.* Trisaccharide A (4·7 mg.) was hydrolysed in 1·5N-sulphuric acid (1 ml.) at

[11] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[12] Bayly and Bourne, *ibid.*, 1953, **171**, 385.
[13] Foster, *J.*, 1953, 982.
[14] Partridge, *Nature*, 1949, **164**, 443.

100° for 4 hr. Paper chromatography of the hydrolysate in solvent (*a*) showed components corresponding to glucose and galactose, while radiochromatograms revealed ^{14}C only in the former spot. Paper chromatography of a partial hydrolysate (N-sulphuric acid for 1 hr. at 90°) of trisaccharide A (8·4 mg.) in solvent (*a*) showed components with R_F values identical with those of glucose, galactose, kojibiose, lactose, and trisaccharide A. Radiochromatograms revealed [^{14}C]components corresponding to glucose, kojibiose, and trisaccharide A.

(iv) *Treatment with almond emulsin.* Trisaccharide A (1 mg.) was added to almond β-glycosidase solution [15] (0·1 ml.). Paper chromatography of the digest after 72 hours' incubation at 37° revealed components corresponding to [^{14}C]trisaccharide A (*ca.* 70%), [^{14}C]kojibiose, galactose, and glucose (trace, radioactivity not determined). Under similar conditions lactose was completely hydrolysed and maltose gave a trace of glucose.

(v) *Treatment with phenylhydrazine.* Trisaccharide A (74·7 mg.) in water (1·25 ml.) was treated with phenylhydrazine (165 mg.) in acetic acid (0·165 ml.) at 100° for 2·5 hr. and then left overnight at 0—2° before the mixture of phenylosazones (10 mg.) was isolated. Paper chromatography of the mixture of osazones in solvent (*b*) revealed components corresponding to the phenylosazones of glucose (R_F 0·69) and lactose (R_F 0·38). The osazones were detected by ultraviolet light and by acetone–silver nitrate–alcoholic sodium hydroxide.

The mixture of osazones was fractionated on a " Celite " (40 × 1·7 cm.) column. Dry " Celite " was wetted with benzene-saturated water, and made into a slurry in solvent (*b*), which was also the developing solvent. Paper chromatography of the two isolated fractions, using the same solvent, showed that they moved as single components with R_F values corresponding with those of the phenylosazones of glucose and lactose, respectively. Their specific radioactivities, determined by the infinitely thick disc method, were 4203 μc per mole of monosaccharide and 1184 μc per mole of disaccharide, respectively.

(vi) *Reduction of trisaccharide A.* Trisaccharide A (33·8 mg.) in water (7·3 ml.) was reduced with potassium borohydride (37·5 mg.) at room temperature for 20 hr. The excess of borohydride was destroyed by 3N-sulphuric acid (0·4 ml.), and the volume made up to 15 ml. Part (3 ml.) of the resulting solution was adjusted to pH 7 and passed through a column of Permutit " Biodeminrolit " (15 g.) pretreated with carbon dioxide, and then evaporated to dryness *in vacuo*. Dry methanol (3 × 10 ml.) was added and the whole evaporated to dryness *in vacuo*. The residue was dissolved in water (10 ml.) and freeze-dried. Paper chromatography of the reduced trisaccharide A in solvent (*c*) showed that it moved as a single component with $R_{glucose}$ 0·51. It was detected with acetone–silver nitrate–alcoholic sodium hydroxide [11] and with periodate–permanganate–benzidine,[16] but not with aniline hydrogen phthalate. Radiochromatograms revealed that it moved as a single radioactive compound.

(vii) *Hydrolysis of trisaccharide A alcohol.* Another part (10 ml.) of the solution of the reduced trisaccharide A was adjusted with 3N-sulphuric acid (2·4 ml.) to an acid normality of 0·5N, and heated at 100° for 4 hr., then passed through a column of Permutit " Biodeminrolit " (45 g.), pretreated as above. Paper chromatography of the concentrated solution in solvent (*e*) showed components with R_F values corresponding to glucose, galactose ($R_{glucose}$ 0·85), and sorbitol ($R_{glucose}$ 0·3). Sorbitol did not give a discrete spot, but was well separated from glucose and galactose. Radiochromatograms revealed the presence of ^{14}C in the component corresponding to glucose. Portions of the hydrolysate were fractionated by paper chromatography. A pure glucose fraction was obtained in the above solvent. The eluted aqueous solutions containing glucose and galactose, respectively, were freeze-dried. Dry methanol (3 × 25 ml.) was added to each and the whole evaporated to dryness *in vacuo*. The galactose fraction was further purified in solvent (*a*). A pure sorbitol fraction ($R_{glucose}$ 2·2) was obtained by paper chromatography in solvent (*d*), and was freed from boric acid by the above method.

The concentrations of the aqueous solutions of glucose and galactose were determined by the methods of Somogyi and Nelson.[17] The concentration of sorbitol was determined by oxidation with sodium periodate and determination of the formaldehyde produced with chromotropic acid.[18] The specific radioactivities of glucose, galactose, and sorbitol were determined by the infinitely thin film method, on aliquot portions of the analysed solutions of the compounds. The results obtained are shown in the Table.

[15] Onslow, " Practical Plant Biochemistry," Cambridge Univ. Press, 1929.
[16] Wolfrom and Miller, *Analyt. Chem.*, 1956, **28**, 1037.
[17] Nelson, *J. Biol. Chem.*, 1944, **153**, 375; Somogyi, *ibid.*, 1945, **160**, 61.
[18] Whelan, personal communication.

Compound	Wt. (μg.)	Counts per min.	Counts per min. per μmole	Average counts per min. per μmole	μc per mole
D-Glucose standard	44·19	284	1158	1156	11,236
	66·63	427	1154		
Trisaccharide A	103·3	194	947		
	221·0	431	984		
	419·0	795	957	961	9,341
	508·0	965	958		
	607·0	1154	959		
D-Glucose	32·83	168	922		9,253
	52·02	276	956	952	
	58·83	319	977		
D-Galactose	32·12	1	6	6	58
D-Glucitol	53·10	5	17	17	165

Phenylhydrazine Treatment of Lactose.—Lactose (68·2 mg.) was dissolved in a solution of acetic acid (1·65 ml.) and water (12·5 ml.). Phenylhydrazine (0·22 g.) was added and the solution was heated at 80° for 2·5 hr. and kept overnight at 0—2°. The solid product obtained was analysed by paper chromatography [solvent (*b*)], which revealed a component with R_F value corresponding to lactose phenylosazone and a trace of a component with R_F value corresponding to a monosaccharide phenylosazone.

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