OLIGOSACCHARIDE SYNTHESIS BY *BETACOCCUS ARABINOSACEOUS*

A THESIS

submitted by

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a candidate for the Degree of

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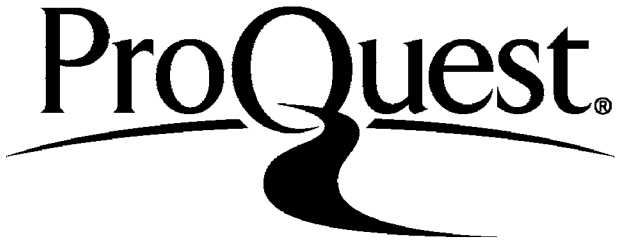

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SUMMARY

A [^{14}C] trisaccharide synthesised by cultures of Betacoccus arabinosaceous in a medium containing lactose and [^{14}C] sucrose has been isolated. This was characterised as the 'branched' structure O-β-D-galactopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucose. The ^{14}C distribution in the trisaccharide was determined by degradation methods. It has been shown that ca. 98% of the ^{14}C activity was located in the non-reducing glucose unit linked to C_2 of the reducing moiety of lactose.

The acceptor specificity of dextransucrase preparations from B. arabinosaceous have been investigated, by incubating certain simple sugars, and related compounds, with the enzyme and sucrose. Chromatography of the digests, and of hydrolysates of the oligosaccharides isolated from them, indicated that D-xylose, D-ribose, L-sorbose, D-mannose, myoinositol, and possibly L-rhamnose, L-fucose, D-arabinose, and methyl β-D-ribopyranoside, acted as acceptors. From consideration of the conformations of these compounds, and of

those already established as acceptors in the dextransucrase-sucrose system a hypothesis has been put forward as to the essential structure for a glucosyl acceptor.

Oligosaccharides synthesised by B. arabinosaceous in a galactose-sucrose medium were isolated and investigated. One of these was characterised as a new non-reducing disaccharide, O-α-D-glucopyranosyl D-galactofuranoside. Evidence has been obtained that a second disaccharide was O-α-D-glucopyranosyl-(1→4)-D-galactose. Possible tri- and tetrasaccharides were also separated from the culture. Total and partial hydrolysis of these sugars suggested that they were synthesised by successive 1:6 additions to a galactose-containing disaccharide.

[^{14}C] Tracer techniques have been used to investigate the reversibility of the dextransucrase reaction. It has been shown that the enzyme does not catalyse the transfer of glucosyl units from dextran, or from the 'branched' trisaccharide, mentioned above, to other acceptors. It has, however, been demonstrated that, in the presence of sucrose and [^{14}C] fructose, the enzyme catalyses the incorporation of [^{14}C] fructose into sucrose.

The significance of the transfer of glucosyl units to sugars in the furanose form has been discussed.

Contents

INTRODUCTION

Organisms of the Leuconostoc series, L. mesenteroides, (Betacoccus arabinosaceous), and L. dextranicum (1), and the related organisms, Streptococcus bovis (2), Streptococcus DS 50 (3), and Betabacterium vermiforme (4) synthesise the polysaccharide, dextran, from the specific substrate sucrose. A similar polysaccharide is produced from amylo-dextrins by Acetobacter viscosum and A. capsulatum (5).

Dextran is a polyglucosan in which the principal linkage between the anhydro-glucose units, of the main chain, is α-1:6-. The fine structure of dextran depends on the organism which synthesises it. The polymer may be essentially unbranched (6), or branched, principally, at C_3 (7,8,9,10) or C_4 (9) of the anhydro-glucose units. There is also evidence that 1:2- branch points may occur in some dextrans (11).

Low molecular weight dextran is important as a blood plasma substitute. Many investigations into the structure of the polysaccharide, and the mechanism of the enzymic reactions producing it, have been reported. This work has been extensively reviewed (12,13,14,15,16). As the present work is concerned with oligosaccharide synthesis

by the dextran-producing organism, B. arabinosaceous, and with the mechanism of the enzymic reactions as revealed by this synthesis, only the relevant literature will be discussed in detail.

In 1941 Hehre (17) showed that cell free extracts of L. mesenteroides contained an enzyme capable of synthesising dextran from sucrose. This enzyme was given the name dextransucrase by Hestrin (18). Later it was shown that sterile extracts of S. salvanicus, as well as L. mesenteroides produced dextran (19). A partially purified enzyme extract was obtained by Tsuchiya et al. (20) from a L. mesenteroides NRRL B512 culture, and Bailey et al. (21) isolated an enzyme preparation from B. arabinosaceous grown in a sucrose medium. Both these enzymes, which were heavily contaminated with dextran, synthesised branched polysaccharides, similar to those obtained from cultures of the organisms.

The dextran synthesised by B. arabinosaceous was highly branched, the branches being linked to position 3 of the anhydro-glucose units (8). When grown on a magnesium deficient medium, however, a much less branched dextran was obtained (22), indicating that a second

enzyme, not dextransucrase, might be responsible for the branching. Some confirmation of this theory was provided by Bailey *et al.* (21), who showed that a highly branched dextran was synthesised by a freshly precipitated enzyme, whereas the same preparation, after storage at 0-2° for several months, synthesised an almost unbranched polymer. Bovey (23) has recently found evidence that a branching enzyme, other than dextransucrase, is present in an extract from *L. mesenteroides* NRRL B512.

Attempts have been made to obtain a purer enzyme, and Bailey *et al.* (24) isolated an enzyme preparation, containing only 7.5% carbohydrate, from a maltose-sucrose culture of *B. arabinosaceous*, but this enzyme was easily denatured, and it seemed that the presence of carbohydrate had a stabilising effect.

In the present work enzyme extracts, prepared according to the method of Bailey *et al.* (21), have been used. Because of the uncertainty as to the purity of these extracts, it cannot be said definitely that reactions catalysed by them were due to dextransucrase.

The overall reaction catalysed by the enzyme

preparations is given by the equation:

$$n\ \text{Sucrose} \longrightarrow \text{Dextran} + n\ \text{Fructose} \quad \text{.....(i)}$$

Hehre has found it to be essentially irreversible (25) and has shown that the reaction does not proceed through a phosphorylated sugar (26). Dextransucrase belongs to the general class of transglycosylases (14), enzymes which operate by transferring a glycosyl group from a donor to a suitable acceptor.

It is generally held that the enzymic synthesis of dextran proceeds through several stages. In 1953 Barker and Bourne (15) proposed the following mechanism:

$$G\text{-}F + E \rightleftharpoons G\text{-}E + F \quad \text{.....(ii)}$$

$$G\text{-}E + A \rightleftharpoons G\text{-}A + E \quad \text{.....(iii)}$$

where G represents a glucose unit,

F " fructose ",

G-F " sucrose,

E " enzyme,

A " acceptor.

For the chain initiation step the acceptor could be a second sucrose molecule, one of the products of the breakdown of sucrose, or some 'primer' molecule already present in the medium. The production of oligosaccharides,

during the synthesis of a polysaccharide, can provide evidence as to the nature of the first step in the build-up of the polymer. Feingold et al. (27) have demonstrated the presence of the trisaccharide, O-β-D-fructofuranosyl-(2→6)-O-β-D-fructofuranosyl-α-D-glucopyranoside, during the synthesis of levan by the levansucrase of Aerobacter levanicum, suggesting that the first step in the synthesis of levan was the transfer of a fructofuranosyl unit to C_6 of the fructosyl moiety of sucrose. Walker and co-workers (28,29) have shown that cellobiose and cellotriose are present during the synthesis of cellulose from glucose by strains of Acetobacter acetiginum and A. xylinum. These oligosaccharides were absent when non-cellulose producing strains of these organisms were grown on glucose, suggesting that they were intermediates in the polysaccharide synthesis.

During the enzymic synthesis of dextran, only leucrose (30,31) and traces of a fructose containing disaccharide, probably isomaltulose (31), have been detected. Leucrose, identified by Stodola et al. (31) as O-α-D-glucopyranosyl-(1→5)-D-fructopyranose, seemed to be synthesised by glucosyl transfer to C_5 of fructopyranose. It is unlikely that leucrose was an intermediate

in the synthesis of dextran as it did not act as a good glucosyl acceptor (32). Also the leucrose production was increased in the presence of fructose, which depressed the rate of dextran synthesis (52).

If sucrose itself acts as acceptor, as well as donor, in the chain initiation step of dextran synthesis, a trisaccharide, O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl β-D-fructofuranoside, might be expected as the product of this step. Such a trisaccharide has been isolated and characterised (33), and Bailey et al. (34) showed that it was a good acceptor for glucosyl units transferred by dextransucrase preparations. The initial rate of reaction was increased by addition of this sugar, and a series of oligosaccharides, produced by successive transfers to C_6 of the terminal glucosyl unit, was present in the product.

Dextran molecules, built up after an initial transfer to a sucrose molecule, would carry a terminal fructose unit. The high molecular weight of the dextrans synthesised by organisms of the Leuconostoc series, and the presence of a levan synthesising enzyme, associated with dextransucrase (35), make it difficult to detect

fructose as a terminal unit of dextran chains (36). The short chain dextrans synthesised by Streptococcus DS 50 have been shown, by Hehre (3), to carry a terminal β-D-fructofuranosyl unit, indicating that sucrose acts as the initial acceptor in this system.

These results suggested that sucrose was probably the acceptor for the chain initiation step in the synthesis of dextran by dextransucrase. However, no conclusive evidence for the mechanism of the step has been reported. It is possible that primer molecules, such as pre-formed dextran, or glucose, produced by the breakdown of sucrose, play a part in the chain initiation.

A number of investigations have been carried out in order to determine the structural features which enable an added molecule to act as an acceptor for glucosyl units transferred from sucrose by dextransucrase. In 1953, Hehre (37) reported that short chain dextrans, added to cultures of L. mesenteroides NRRL B512 containing sucrose, caused an increase in the initial rate of dextran synthesis, estimated by measuring the fructose produced. However, the dextran synthesised was of a lower molecular weight than that produced in cultures containing no added short chain dextrans. Koepsell et al. (32), working with cell

free extracts of the same organism, showed that a number of simple sugars affected the course of the reaction. The addition of isomaltose, maltose, D-glucose, or methyl α-D-glucoside, to enzyme-sucrose digests, increased the initial rate of reaction, gave rise to oligosaccharide production, and lowered the final yield of dextran. Fructose and melibiose depressed the initial rate, but also produced oligosaccharides. D-galactose had little effect on the reaction rate, but again oligosaccharides were produced. These authors found that D-xylose, D- and L-arabinose, L-rhamnose, D-mannose, L-sorbose, cellobiose, trehalose, lactose, melezitose, raffinose, inulin, inositol, mannitol, sorbitol, gluconate, 2-ketogluconate, and glycerol did not affect the reaction.

Bailey et al. (21,24) working with cultures, and enzyme extracts, of B. arabinosaceous, showed that isomaltose, maltose, and methyl α-D-glucoside increased the initial rate of reaction and gave rise to oligosaccharide production. D-galactose, lactose, and cellobiose had little effect on the reaction rate, but produced oligosaccharides.

The oligosaccharides synthesised by L. mesenteroides and B. arabinosaceous in a sucrose medium containing added

maltose (38), isomaltose (21), glucose (21), or methyl α-D-glucoside (24) were shown to arise from successive transfers of a glucosyl unit to C_6 of a free sugar, or to C_6 of a non-reducing, terminal glucoside unit. It has since been shown that the oligosaccharides produced in the presence of 3-O-methyl-D-glucose (39), and of the series of sugars produced by 1:6- addition of glucosyl units to maltose and isomaltose (40), arise in the same way.

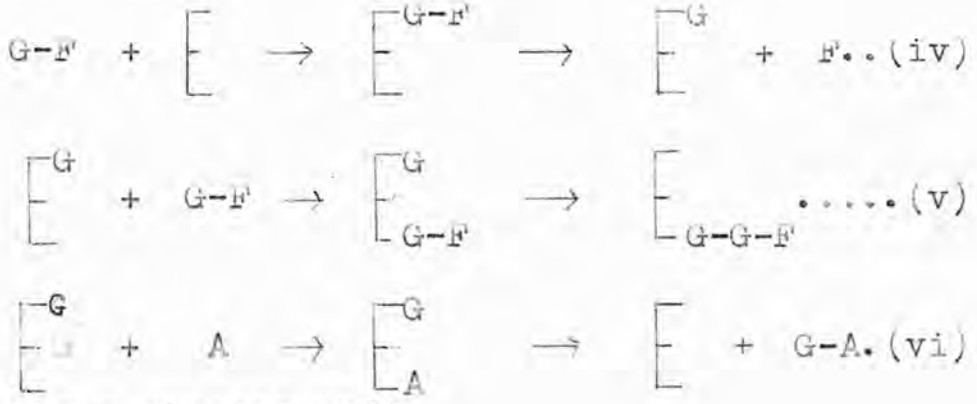
The addition of lactose, or cellobiose, as well as sucrose to cultures of B. arabinosaceous, or to digests of the enzyme from this organism, led to the synthesis of trisaccharides, although the added sugar had little effect on the rate of the reaction. The trisaccharides have been charactised as O-β-D-galacto-pyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucose and O-β-D-glucopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucose (41).

On the basis of these results, Bailey (42) postulated that an α-glucosyl, or an α-galactosyl structure was necessary for an acceptor.

Neely (43) has recently produced some evidence that a 'branched' tetrasaccharide is synthesised by an enzyme preparation from L. mesenteroides NRRL B512, in the presence of sucrose and raffinose. The evidence suggested that the tetrasaccharide was synthesised by glucosyl transfer to C_2 of the glucosyl unit of raffinose.

The production of oligosaccharides in the presence of added sugars, which are capable of acting as glucosyl acceptors, indicates that under these conditions a multi-chain reaction occurs. The absence of oligosaccharide intermediates in the synthesis of dextran from sucrose alone, can also be explained on a multichain mechanism. Sucrose itself is a poor acceptor (44,45), whereas the trisaccharide, postulated as the product of the initial transfer (54), has been shown to be a good acceptor. Therefore it is possible that the initial transfer to a sucrose molecule, is the rate controlling step, which is followed by a rapid build-up to a high molecular weight polymer. Alternatively the synthesis of dextran from sucrose alone could be a single chain mechanism, in which a single enzyme molecule presides over the transfer of a large number of glucosyl units in rapid succession.

Tsuchiya et al. (20) have postulated such a mechanism in order to account for the molecular weight distribution in dextrans produced under different conditions. Bovey (46) has suggested, on similar grounds, that a single chain mechanism occurs in the presence of sucrose alone, whereas in the presence of an added acceptor there is competition between a multichain and a single chain reaction. He proposes the following type of reaction scheme:



where G represents a glucose unit,

F " fructose " ,

G-F " sucrose " ,

A " added acceptor,

┌donor site
└acceptor site represents the enzyme with donor and acceptor sites as indicated.

The reactions shown in equation (v) would lead to a single chain synthesis, and those in equation (vi) to a multichain synthesis of dextran.

With respect to the glucosyl donor, dextransucrase shows a high specificity for sucrose, and it may be that the energy required for the transfer is supplied by the 'high energy' sucrose molecule (47). Neither raffinose (21) nor α-D-galactopyranosyl β-D-fructofuranoside (48) act as substrates, indicating that an unsubstituted glucosyl unit is required. It is likely, in view of this high substrate specificity, that the enzyme associates with the whole sucrose molecule as a preliminary to the glucosyl transfer.

In the present work oligosaccharide syntheses by B. arabinosaceous, and by dextransucrase extracts from this organism, have been investigated, in order to obtain information about the mechanism of the enzymic reactions. The synthesis of the 'branched' trisaccharide in the presence of sucrose and lactose has been reinvestigated using [^{14}C] tracer techniques. A preliminary survey of a number of possible glucosyl acceptors has been made. After consideration of the conformations of the acceptor molecules, a hypothesis has been put forward as to the structural features necessary for association with the acceptor site of the enzyme.

The effect of changes in the acceptor, on the position to which glucosyl residues are transferred, has been studied, using galactose as an acceptor. A number of sugars have been isolated and their structures determined as far as possible.

[^{14}C] Tracer techniques have been used in order to investigate the reversibility of different stages of the dextransucrase reaction, and the possible synthesis of oligosaccharide intermediates.

SECTION I

ISOLATION AND CHARACTERISATION OF A [^{14}C] TRISACCHARIDE SYNTHESISED BY BETACOCCUS ARABINOSACEOUS IN A LACTOSE-SUCROSE MEDIUM.

In lactose and cellobiose the non-reducing moiety has a β-configuration, and it seems that glucosyl or galactosyl units in this configuration cannot accept glucosyl residues transferred from sucrose by dextran-sucrase. Bailey et al. (41) found that trisaccharides were synthesised by B. arabinosaceous in sucrose media containing lactose, or cellobiose. These trisaccharides had a 'branched' structure, a glucosyl unit being linked to the reducing moiety of the parent disaccharide. It appeared that glucosyl transfer to a sugar residue other than the usual non-reducing terminal unit, occurred when the latter had an unfavourable configuration.

This was believed to be the first controlled, in vitro, synthesis of a 'branched' trisaccharide by an enzyme system. The mechanism could be a general one for the synthesis of a 'branched' polysaccharide, by a route which did not involve glycosyl transfer to a preformed linear polymer. These trisaccharides were also of interest in that they contained the rare α-1:2- linkage,

rather than the α-1:6-linkage, normally synthesised by dextransucrase.

It seemed likely that each was produced by glucosyl transfer, from sucrose, to a lactose, or cellobiose, molecule, as shown below:

$$G_1\text{-}_2F + Ga_1\text{-}_4G \longrightarrow Ga_1\text{-}_4G_2\text{-}_1G + F \quad \text{.....(vii)}$$

where $G_1\text{-}_2F$ represents sucrose,

$Ga_1\text{-}_4G$ " lactose,

$Ga_1\text{-}_4G_2\text{-}_1G$ " trisaccharide produced in the presence of lactose,

F " fructose.

However there were other possibilities, such as:

$$Ga_1\text{-}_4G + Ga_1\text{-}_4G \longrightarrow Ga_1\text{-}_4G_2\text{-}_1G + Ga \quad \text{....(viii)}$$

It was decided to investigate this problem, using $[^{14}C]$ tracer techniques. At the same time evidence was obtained which confirmed the structure, O-β-D-galactopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucose, assigned by Bailey *et al.*, to the trisaccharide derived from lactose.

A culture of *B. arabinosaceous* incubated with lactose (10%), and $[^{14}C]$ sucrose (2%, *ca* 100 μC), produced a $[^{14}C]$ trisaccharide, (trisaccharide A) (Expt. 11). The trisaccharide was isolated from the culture medium by

fractionation on a charcoal-'Celite' column (49, 50), and shown to be a pure, radioactive, compound by radiochromatography (Expt. 12). The yield of trisaccharide A corresponded to the transfer, to lactose, of 12% of the glucose available from the sucrose.

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. These results indicated that no incorporation of [^{14}C]glucose, from sucrose, into lactose occurred.

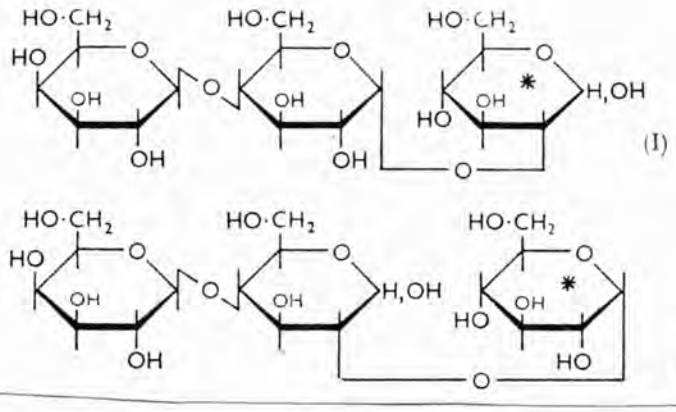
The mobility of trisaccharide A on electrophoretograms (51), and of the trisaccharide and its benzylamine complex (52) on paper chromatograms was the same as that of the authentic O-β-D-galactopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucose of Bailey *et al.* (41). With aniline hydrogen phthalate spray, trisaccharide A gave a yellowish colour, typical of a 2-O-substituted reducing aldose (53). It could not be detected by alkaline triphenyl tetrazolium chloride spray, which reveals reducing glucosaccharides which are unsubstituted at C_2 (54). Its low mobility on electrophoresis in borate buffer confirmed

the presence of a 2-O-substituent (Expt. 12).

Confirmation of the identity of trisaccharide A, with the trisaccharide isolated by Bailey *et al.* from a sucrose-lactose medium, was obtained by treatment of trisaccharide A with almond β-glycosidase. Galactose and [^{14}C]kojibiose were produced indicating the presence of one α- and one β- link (Expt. 15).

Qualitative evidence for the ^{14}C distribution in the three monosaccharide units of trisaccharide A, and further evidence for its structure was obtained by total and partial hydrolysis (Expt. 14). The hydrolysates were analysed by radiochromatography (Expt. 14). 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, in addition to unhydrolysed [^{14}C]trisaccharide A.

Fig. 1.



The evidence presented so far suggested two possible structures, (Fig. 1, I and II) for trisaccharide A.

Transfer of a lactosyl unit to C_2 of [^{14}C] glucose, derived from [^{14}C] sucrose, would give structure 1. Structure II would have been produced by transfer of a [^{14}C] glucosyl unit, from [^{14}C] sucrose, to C_2 of the reducing moiety of lactose (eqn. vii).

The specific radioactivity of the [^{14}C] sucrose was approximately 17,000 μC per mole. Therefore the radioactivities of structures I and II should have been approximately 8,500 μC per mole. Estimation of the specific radioactivity of trisaccharide A by the infinitely thick disc method (55,56,57) gave a value of 523 μC per g.-atom of carbon, corresponding to 9417 μC per mole of the trisaccharide (Expt. 13).

Since no galactose was liberated, and the lactose remained inactive, throughout the incubation period, trisaccharide A could not have arisen from two molecules of lactose (eqn. viii). Even if instantaneous equilibration of the glucose units in the lactose and [^{14}C]-sucrose had occurred, the radioactivity of a trisaccharide produced in this way would have been only ca. 2840 μC

per mole.

In order to determine, quantitatively, the distribution of ^{14}C in trisaccharide A, an attempt was made to degrade the trisaccharide into lactose and glucose containing derivatives. Under certain conditions 2-O-substituted aldoses can be converted into the corresponding phenyl osazones by treatment with phenyl hydrazine (58,59,60,61). It was thought that treatment of trisaccharide A with phenyl hydrazine under the same conditions might result in specific cleavage of the 1:2- link, to give lactose and glucose phenyl osazones.

It was necessary to develope a method to separate lactose and glucose phenyl osazones on a small scale. A solvent, containing pyridine, benzene, and water, was developed, for the separation of mono- and disaccharide osazones on paper chromatograms (Expt. 16). Chromatographic separation could also be achieved by using a 'Celite' column to support the aqueous stationary phase (62), and eluting with the pyridine-benzene-water solvent (63). Artificial mixtures of glucose and lactose phenyl osazones were separated in this way (Expt. 17).

The products of the phenyl hydrazine treatment of trisaccharide A (Expt. 18) were separated on a 'Celite' column, and their radioactivities determined by the infinitely thick disc method (Expt. 19). The specific activity of the 'glucosazone' (4203 μC per mole) was much lower than expected (ca. 9400 μC per mole), whereas the 'lactosazone', expected to be inactive, had 1184 μC per mole. Assuming specific cleavage of the 1:2- link these values corresponded to a specific activity of 5387 μC per mole for trisaccharide A. It is clear that, under these conditions, phenyl hydrazine was also breaking a percentage of the 1:4- links. This was confirmed by treatment of lactose with phenyl hydrazine under the same conditions (Expt. 20). Paper chromatography of the product showed that some monosaccharide osazone was present, as well as the expected disaccharide osazone.

Conclusive evidence for the distribution of ^{14}C in the monosaccharide units of trisaccharide A was obtained by reduction of the trisaccharide A, followed by total hydrolysis and assay of the radioactivity of the products. Structure I would be expected to give galactose, glucose, and [^{14}C] sorbitol. From structure II

galactose, sorbitol, and [^{14}C] glucose would be expected.

Trisaccharide A was reduced with potassium borohydride (Expt. 22). The product was completely hydrolysed, and the hydrolysate was fractionated by preparative paper chromatography (Expt. 23). A solvent, containing butanol, pyridine, and aqueous boric acid, was developed and used for this separation (Expt. 21).

Radiochromatograms revealed the presence of galactose, sorbitol, and [^{14}C] glucose in the hydrolysate of the trisaccharide A alcohol. The specific activities of these three products were determined by the infinitely thin film method (64,65) (Expt. 24). The β-emission was shown to bear a linear relation to the amount of the compound applied, and was calibrated against a standard [^{14}C] glucose sample (Expt. 25). The results are shown in Table I.

TABLE I

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

The direct comparison of the radioactivities of the glucose fragment of the trisaccharide A alcohol, and of trisaccharide A, indicated that 99·1% of the ^{14}C of trisaccharide A was in the non-reducing glucose unit. The glucose fragment was calculated to contain 97·6% of the ^{14}C of the trisaccharide A alcohol, from the ratio of the emission of glucose to the sum of the emissions of glucose, galactose, and sorbitol.

These results show conclusively that trisaccharide A had the 'branched' structure, II. Structure I would have given glucose, galactose, and [^{14}C]sorbitol. Thus they confirm the structure assigned earlier, on chemical grounds, to a trisaccharide prepared in a similar manner (41).

The present work has 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 unit from sucrose, to C_2 of the reducing moiety of lactose. When [^{14}C]sucrose is used the equation can be written:

$$G^*\text{-}F^* + Ga\text{-}G \longrightarrow Ga\text{-}G\text{-}G^* + F^* \quad \text{.....(xi)}$$

where the asterisk denotes a radioactive sugar residue.

Bailey *et al.* (21) have shown that this transfer is catalysed by enzyme extracts from *B. arabinosaceous*. It is not certain whether the synthesis of the α-1:2-link is catalysed by dextransucrase. The unfavourable β-configuration of the non-reducing terminal unit, and the presence of a bulky substituent at C_4 of the reducing unit, might hinder glucosyl transfer to C_6 of either sugar residue, leading to the formation of an α-1:2-link. Alternatively, the branching enzyme or some other enzyme, closely associated with dextransucrase, could be responsible.

SECTION II

INVESTIGATION OF THE ACCEPTOR SPECIFICITY OF DEXTRANSUCRASE PREPARATIONS.

D-Glucose, D-galactose, D-fructopyranose, and sugars containing α-linked, non-reducing, chain terminal glucosyl, or galactosyl, units have been shown to act as acceptors of glucosyl units transferred from sucrose by dextransucrase preparations (21,32,34,39,40). Methyl β-D-glucoside did not act as an acceptor (21), while lactose and cellobiose only accepted at the reducing end of the molecule (41,63). These results suggested that an α-configuration was necessary for an acceptor in the dextransucrase reaction.

The present work describes preliminary investigations into the ability of a number of sugars, and related compounds, to act as acceptors of glucosyl units transferred by dextransucrase preparations. The enzyme preparations used in this investigation were extracted from cultures of *B. arabinosaceous* by the method of Bailey *et al.* (21).

The acceptor abilities of different compounds were tested by incubating the compound to be investigated (8%), with the enzyme, in the presence of sucrose (2%, Expt. 27).

Oligosaccharide production was detected by paper chromatographic analysis, after incubation of the digest at 25° for 23 hours. The digests were analysed again by paper chromatography, after treatment with yeast invertase. This ensured that the presence of new disaccharides was not masked by the sucrose remaining at the end of the incubation period. In every case control experiments showed that no reaction took place in the absence of sucrose.

Di- and oligosaccharides, produced during the incubation period, were isolated by preparative paper chromatography, hydrolysed, and the hydrolysates analysed by paper chromatography (Expt. 28).

The results obtained with different added compounds are summarised in Tables VII and VIII (pp. 110 and 112). Chromatographic evidence indicated that new compounds were obtained in the presence of D-galactose, D-mannose, L-rhamnose, L-fucose, L-sorbose, D-arabinose, D-xylose, and D-ribose. Chromatography of the hydrolysates of these new compounds suggested that those produced in the presence of D-mannose, L-sorbose, D-xylose, and D-ribose contained a glucose unit linked to the added compound. As a more detailed study of the galactose

oligosaccharides was planned, these sugars were not investigated at this stage.

No oligosaccharide production could be detected in the presence of added myoinositol. Hydrolysis of the slow-running myoinositol fraction, however, gave glucose and myoinositol, revealed by paper chromatography, indicating that glucosyl transfer to myoinositol had taken place.

Confirmation of previous work, on the acceptor specificity of the enzyme, was obtained by adding methyl α- and β-D-glucosides, and sorbitol, to enzyme digests containing sucrose. Oligosaccharide production was detected by paper chromatography in the digest containing methyl α-D-glucoside. No glucosyl transfer to methyl β-D-glucoside, or sorbitol, could be detected.

These results confirmed the ability of methyl α-D glucoside and D-galactose to act as glucosyl acceptors in the dextransucrase-sucrose reaction. They also indicatedthat D-mannose, L-sorbose, D-ribose, D-xylose, myoinositol, and possibly L-rhamnose, L-fucose, and D-arabinose, previously thought to have no effect on the dextransucrase reaction, can act as glucosyl acceptors,

giving rise to oligosaccharides. It is appreciated that chromotographic evidence for the production of oligosacharides cannot be regarded as conclusive, particularly in the cases of rhamnose, fucose, and arabinose; the presence of the added sugar could not be detected, with any certainty, in hydrolysates of the oligosaccharides from enzyme digests to which these sugars had been added. However, it was thought that on the basis of the results reported here, and previously, an attempt could be made to deduce the essential structure required for an acceptor of glucosyl units transferred from sucrose by dextransucrase.

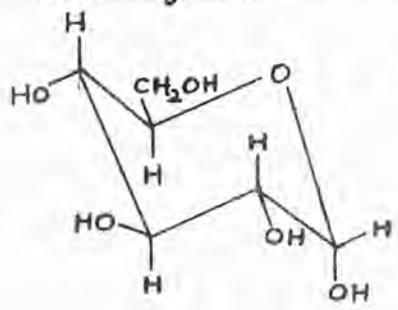
In 1950 Gottschalk (46) put forward the theory that a multipoint contact is established between the enzyme and the substrate as the first step in a transglycosylation reaction. In order to make such a contact a certain arrangement of groups in the cis position is required, cis referring to the mean plane of the pyranose or furanose ring. It seems likely that a similar association of the acceptor molecule with the enzyme must take place before it can receive a transferred glycosyl unit. It is generally held that the acceptor molecule must have certain structural features whch enable it be be closely

associated with the acceptor site of the enzyme, although the specificity requirements of an acceptor are usually less rigid than those of a substrate, or donor, molecule.

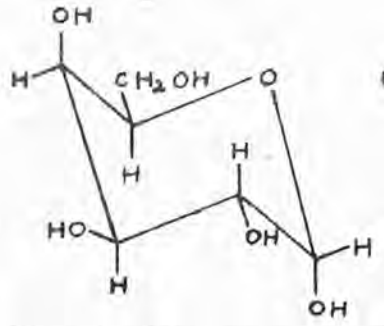
In the case of dextransucrase, no glucosyl transfer to sorbitol could be detected, indicating that a ring structure was necessary for an acceptor. The principles of conformational analysis have been applied in order to find the structural features common to the molecules which accept glucosyl units transferred by dextransucrase. The most favourable pyranose conformations of the monosaccharide acceptors, as assigned by Reeves (67) and Bentley (68), are shown in Fig. 2. Compounds I - IX, with the configuration of the anomeric carbon atom as given in Fig. 2 (both anomers are present in solutions of the free sugars) have an axial hydroxyl group, at the anomeric carbon, and two axial hydrogens on the same side of the mean plane of the pyranose ring. The acceptor ability is retained when the axial hydroxyl group is substituted as shown by the results obtained with methyl α-D-glucoside (XII). In methyl β-D-glucoside (XIII), and the non-reducing units of cellobiose and lactose, which do not act as acceptors, this combination of axial substituents is absent. Therefore it is

Fig. 2

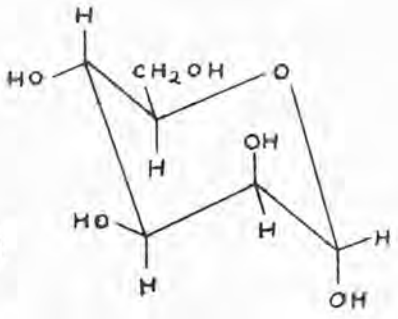
I α-D-glucose



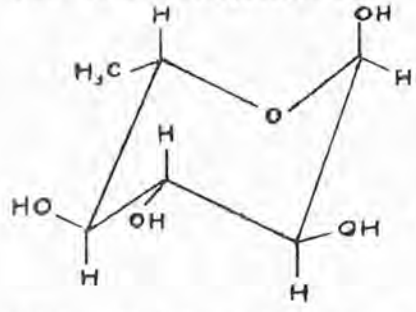
II α-D-galactose



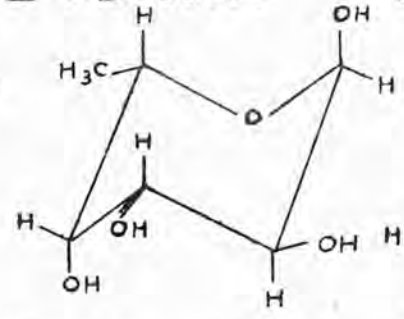
III α-D-mannose



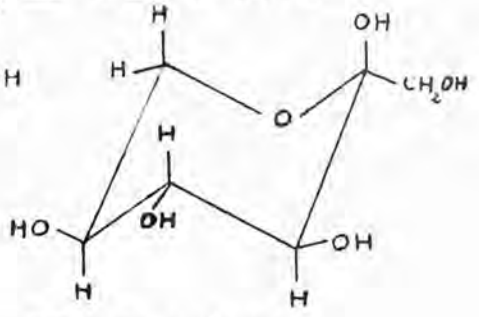
IV α-L-rhamnose



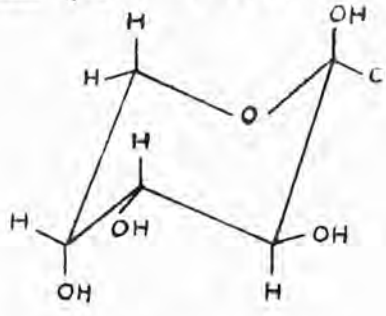
V α-L-fucose



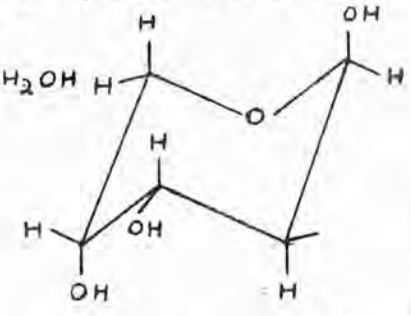
VI α-L-sorbose



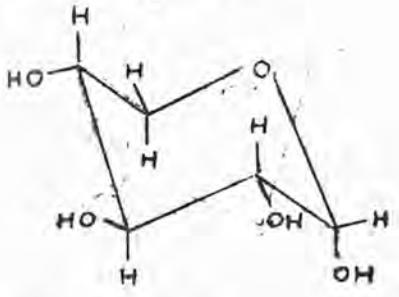
VII β-D-fructose



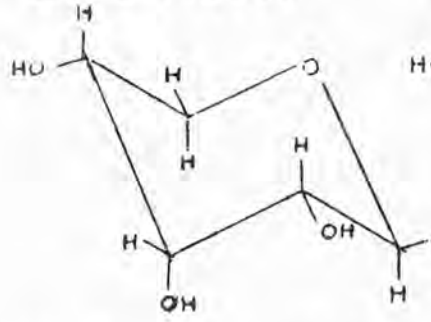
VIII β-D-arabinose



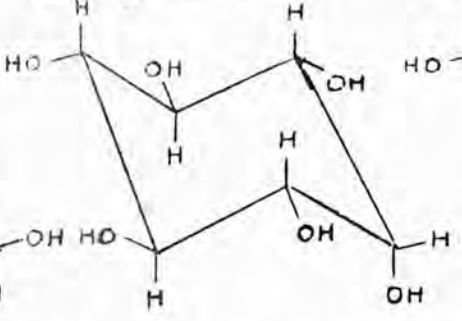
IX α-D-xylose



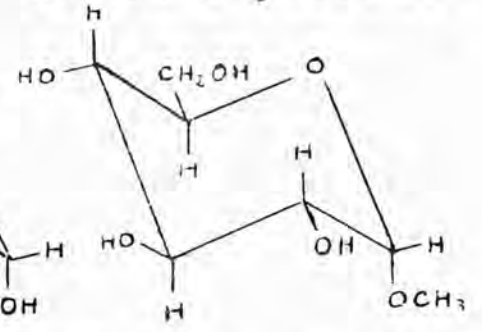
X β-D-ribose



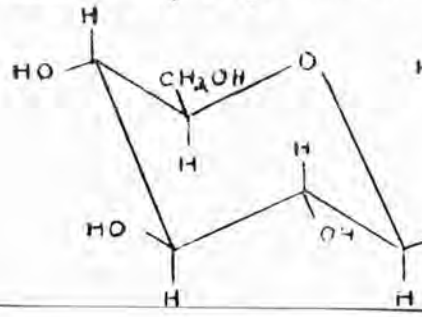
XI myo-inositol



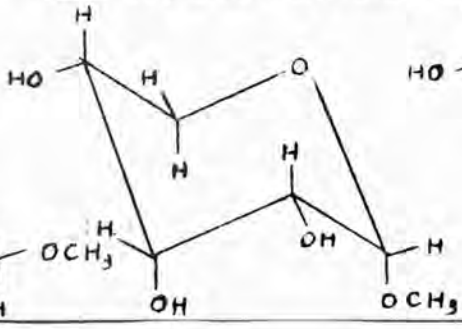
XII Me α-D-glucoside



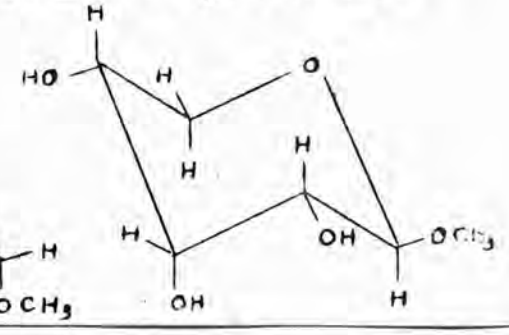
XIII Me β-D-glucoside



XIV Me α-D-riboside



XV Me β-D-riboside



suggested that one axial -OR (where R may be hydrogen, a methyl group, or some other substituent) group is necessary for association with the acceptor site of the enzyme, and possibly two axial hydrogens, on the same side of the mean plane of the pyranose ring.

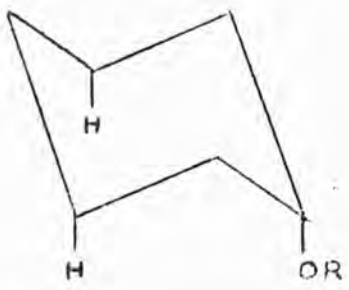
β-D-ribose (X) has this structure, but the axial hydroxyl group is on C_3. On the other hand α-D-ribose has two axial hydroxyl groups, one of which is on the anomeric carbon. An attempt was made to determine which of these configurations accepted transferred glucosyl units, by testing the acceptor abilities of methyl α- and β-D-ribopyranosides. If the acceptor requirements are one axial -OR group, and two axial hydrogens, then the β-ribopyranoside (XV) would be expected to accept. If an acceptor required an axial -OR group at the anomeric carbon, then it was possible that the α-ribopyranoside (XIV) would accept. Chromatographic analyses of enzyme digests containing methyl α- or β-D-ribopyranoside (Expt. 29) showed no definite oligosaccharide production. It was suspected that oligosaccharides synthesised by glucosyl transfer to the methyl ribopyranosides might have mobilities similar to glucose or fructose. Hydrolysis of the sugars running in this region, from

the digest containing methyl β-D-ribopyranoside, gave traces of ribose. No ribose could be detected in hydrolysates of sugars from the digests containing methyl α-D-ribopyranoside. Thus it seemed that only methyl β-D-ribopyranoside acted as an acceptor. This suggested that one axial -OR group and two axial hydrogens were necessary for an acceptor, and that an axial -OR group at the anomeric carbon atom was not essential. The evidence that myoinositol (XI) accepts transferred glucosyl units confirms the suggestion that an axial -OR group at the anomeric carbon was not necessary for an acceptor. This evidence also indicated that the ring oxygen was not essential for association with the acceptor site of the enzyme.

On the basis of the above considerations it is suggested that a molecule may require one axial -OR group, and two axial hydrogens *cis* to the mean plane of the pyranose ring, in order to associate with the enzyme at the acceptor site. Such a structure is shown in Fig. 3.

There is some doubt as to the conformation of the non-reducing moieties in α-linked diglucoses (69). Bentley (70) has recently suggested that a skew

Fig. 3



conformation between the B1 and 3B forms may be the preferred conformation. Such a conformation is shown in Fig. 4.

Fig. 4

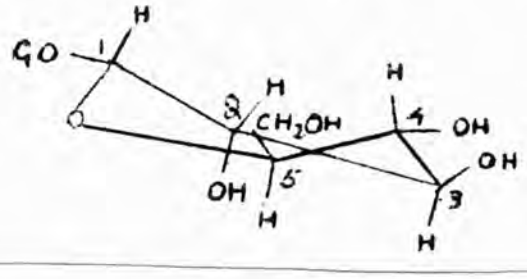


Fig. 4, C_1, C_2, C_3, and C_5 all lie in the same plane.

It can be seen that in this conformation the non-reducing moiety of maltose retains one hydroxyl group, and two hydrogens in positions approximately axial with the mean plane of the pyranose ring. A structure of

this kind might associate with the acceptor site of the enzyme in the same way as the structure, in the chair conformation, shown in Fig. 3.

The present work was intended as a preliminary survey of the acceptor abilities of thesugars, and related compounds, described above. A more detailed study is necessary, in order to confirm that they do in fact accept transferred glucosyl units. It is possible that interesting di- and oligosaccharides would be isolated during the course of such an investigation. Further investigation is also necessary in order to show whether oligosaccharide synthesis of this type is catalysed by dextransucrase, or by some other closely associated enzyme.

More evidence is needed to confirm that the structure proposed above, containing one axial -OR group and two axial hydrogens, enables a molecule to associate with the acceptor site of the enzyme. The acceptor abilities of sugar derivatives, in which the configurations of the anomeric carbon are fixed, would be of particular interest in this respect.

SECTION III

SYNTHESIS OF OLIGOSACCHARIDES BY BETACOCCUS ARABINOSACEOUS IN MEDIA CONTAINING SUCROSE AND GALACTOSE.

The ability of a molecule to act as an acceptor of transferred glucosyl units, in the dextransucrase reaction, may be affected by two factors; the affinity of the molecule for the acceptor site of the enzyme, and the availability of hydroxyl groups in positions suitable for acceptance.

Normally dextransucrase catalyses glucosyl transfer to the primary alcohol group at C_6 of a non-reducing, terminal, α-glucosyl residue (21,24, 38,39, 40). Free glucose is also a good acceptor, increasing the initial rate of reaction, and giving rise to a series of α-1:6-linked oligosaccharides (21). Xylose, on the other hand, only accepts to a small extent, and has little effect on the reaction rate (21). Since xylose has the same disposition of hydroxyl groups, about the pyranose ring, as glucose, it would be expected to have a similar affinity for the acceptor site of the enzyme. Thus it appears that, in this case, the controlling factor is the availability of a primary carbinol group at C_6.

In the presence of added xylose, spray reagents (Expt. 27) suggested that glucosyl transfer to C_2 took place. It has already been established (41,63) that glucosyl transfer to C_2, of the reducing moiety of the acceptor, occurs in the presence of lactose, or cellobiose. The synthesis of the 1:2- link, when these disaccharides were added to enzyme digests containing sucrose, could have been caused by the presence of a bulky substituent at C_4 of the reducing glucose unit, hindering transfer to C_6 of the reducing moieties of the disaccharides. These results suggested that the hydroxyl group at C_2 could act as an alternative acceptor when the synthesis of a 1:6- link was hindered.

It was thought that the presence of an axial, rather than an equatorial, hydroxyl group at C_4, as in galactose, might also affect the ease of transfer to C_6. Preliminary experiments by Grant (71), indicating that more than one disaccharide was produced in the presence of added galactose, supported this view.

Further investigation of di- and oligosaccharide synthesis in the presence of added galactose was of interest for several reasons. The effect of an axial

hydroxyl group at C_4, on glucosyl transfer could be demonstrated; the nature of the disaccharides produced would provide evidence about the acceptor abilities of hydroxyl groups other than the primary carbinol at C_6; and the production of oligosaccharides would indicate that galactose was capable of acting as an acceptor for the first step in the build-up of a dextran-like polymer.

The present work describes the isolation and investigation of di- and oligosaccharides produced by B. arabinosaceous in media containing sucrose and galactose.

Preliminary experiments (Expt. 30) showed that the same oligosaccharides, detected by paper chromatography, were produced by cultures of B. arabinosaceous in media containing sucrose and galactose, as in digests of the dextransucrase preparation containing the same sugars. A culture of B. arabinosaceous, grown in a medium containing galactose (20%), and sucrose, produced three sugars running in the disaccharide region on paper chromatograms, and two higher oligosaccharides (Expt. 31). In order to increase the probability of transfer to galactose, the sucrose concentration was kept low, sucrose being added at intervals throughout the incubation

period. The total sucrose added was equivalent to 2% of the final medium.

Preliminary fractionation of the culture medium was achieved by chromatography on a charcoal-'Celite' column (49, 50). A fraction eluted with aqueous ethanol (5%) contained sugars which were assumed to be disaccharides, from the method of elution, and from their R_f values. Possible tri- and tetrasaccharides were eluted with aqueous ethanol (10% and 15%, respectively). The disaccharides were isolated (Expt. 32) by chromatography on a charcoal-'Celite' column washed with borate buffer (72), followed by preparative paper chromatography. Chromatographically pure disaccharides, A, B, and C, were obtained, with R_g values, 0.39, 0.46, and 0.36, respectively. The M_g values, on electrophoresis in borate buffer (51), were 0.60, 0.44, and 0.54, respectively.

INVESTIGATION OF DISACCHARIDE A.

Disaccharide A was detected on paper chromatograms with acetone silver nitrate-alcoholic sodium hydroxide (73), with aniline hydrogen phthalate (53), and with triphenyl tetrazolium chloride (54), indicating that a reducing sugar, in which C_2 was unsubstituted, was present. With diphenylamine-aniline spray a greyish

colour was obtained, suggesting that a 1:4- link was not present (74).

Acid hydrolysis of disaccharide A (Expt. 33) gave glucose, galactose, and a trace of fructose, indicating that this component was, in fact, a mixture of at least two disaccharides. The relative quantities of the three monosaccharides in the hydrolysate, estimated by inspection of the chromatograms, suggested that a mixture of a glucose-galactose disaccharide, and a glucose-fructose disaccharide, might be present in disaccharide A.

'Disaccharide' A, which contained 88·6% carbohydrate by the anthrone method (75) (Expt. 34), had a reducing power of 32·8% of the theoretical value for a disaccharide, estimated with alkaline hypoiodite (76) (Expt. 35), and 61% of the theoretical value, estimated with the alkaline copper reagent of Somogyi and Nelson (77,78) (Expt. 36).

Reduction of 'disaccharide' A with potassium borohydride, followed by hydrolysis (Expt. 37), gave only glucose and a hexitol fraction, revealed by paper chromatography. It was concluded that glucose was the non-reducing moiety in any disaccharides present in

'disaccharide' A.

On treatment with yeast α-glucosidase (Expt. 44), 'disaccharide' A was hydrolysed to glucose, galactose, and fructose. No hydrolysis took place on treatment with almond β-glycosidase. These results showed that the non-reducing glucosyl residues had an α-configuration.

The results, discussed above, indicated that 'disaccharide' A was a mixture of an α-glucosyl-galactose, and an α-glucosyl-fructose. The values obtained for the reducing powers, with alkaline hypoiodite, and with the alkaline copper reagent, could be explained if 'disaccharide' A was a mixture of ca. 60% of a glucosyl-fructose, and ca. 30% of an α-1:2-glucosyl-galactose. On the other hand, the comparatively high M_g value, and the low R_g value, suggest that a 1:6-glucosyl-galactose was present. Owing to the difficulty of resolving the small amounts of the 'disaccharide' A mixture available, no further characterisation of the disaccharides present in this mixture was attempted.

INVESTIGATION OF DISACCHARIDE B

Chromatographically pure disaccharide B could be detected, on paper chromatograms, with acetone silver

nitrate-alcoholic sodium hydroxide, but not with sprays specific for reducing sugars (Expt. 32). The non-reducing nature of this disaccharide was also indicated by the slow development of the colour due to the silver nitrate spray.

Acid hydrolysis of disaccharide B gave components chromatographically identical with glucose and galactose (Expt. 33).

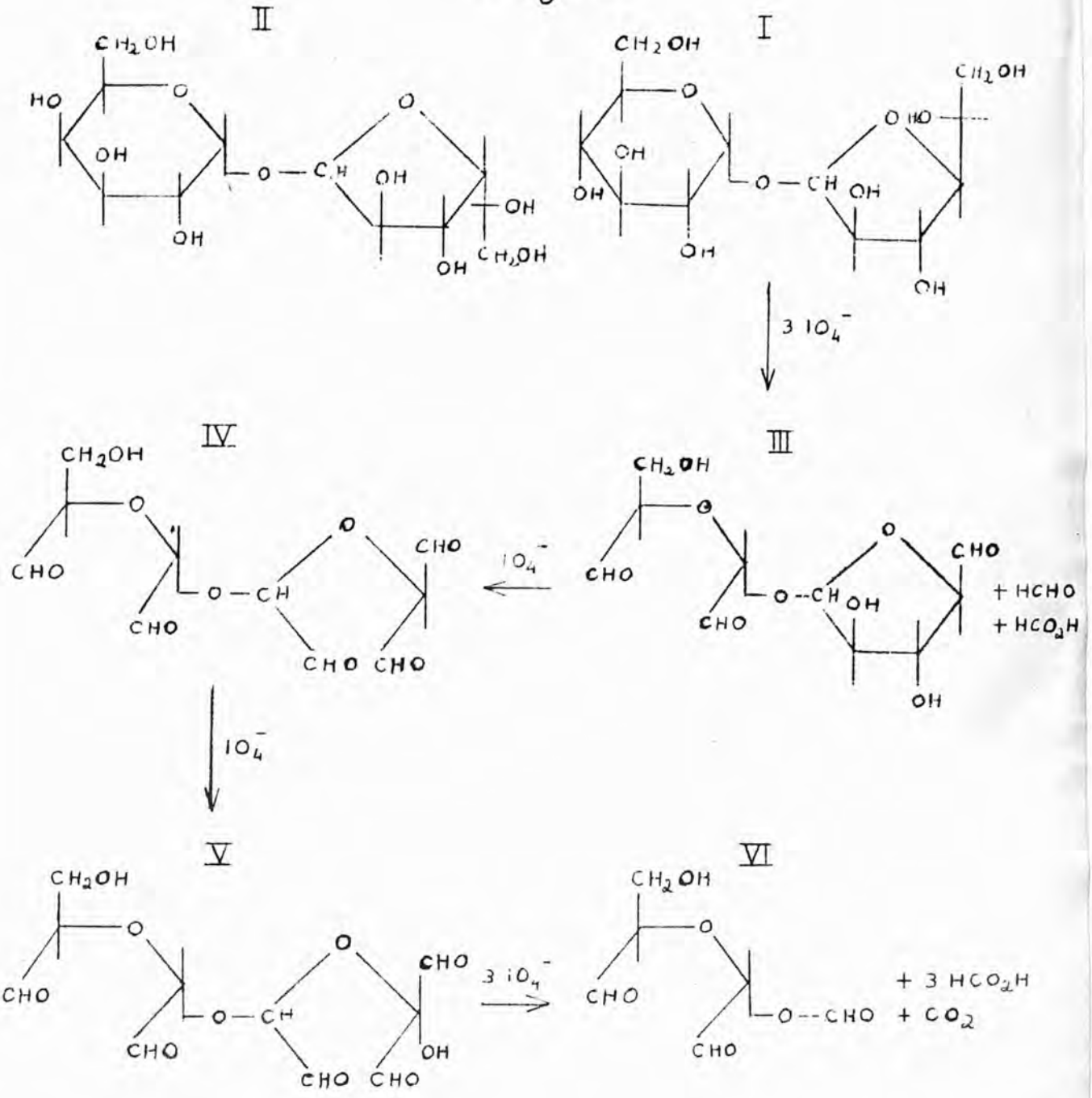
The disaccharide was shown to contain 96·1% of the theoretical carbohydrate content by the anthrone method (Expt. 34). Evidence for the non-reducing nature of disaccharide B was obtained by attempting to oxidise it with alkaline hypoiodite, and with the alkaline copper reagent of Somogyi and Nelson (Expt. 35,36). The non-reducing nature of the disaccharide was confirmed when it was shown that it was not reduced by potassium boro-hydride (Expt. 37). Paper chromatography of the product revealed a single product, identical with disaccharide B. This product was immobile on electrophoresis in molybdate buffer (79), as would be expected of an unreduced disaccharide. Acid hydrolysis of the product revealed only glucose and galactose.

A comparison of the rates of acid hydrolysis of disaccharide B, α,α-trehalose, and sucrose (Expt. 39), showed that, under conditions that completely hydrolysed sucrose, ca. 90% of disaccharide B, but only ca. 10% of the α,α-trehalose, was hydrolysed. This suggested that the glycosidic link between the D-glucosyl and the D-galactosyl units in disaccharide B was weakened by the presence of a furanosyl ring.

When treated with 5 mol. of periodate (Expt. 38), disaccharide B consumed 3·7 mol. periodate in 800 min., with the production of 1·45 mol. of formic acid, and 0·83 mol. formaldehyde. The production of ca. 1 mol. formaldehyde, under these conditions, could only be explained on the assumption that disaccharide B was D-glucopyranosyl D-galactofuranoside (Fig. 5, I) or D-galactopyranosyl D-glucofuranoside (Fig. 5, II).

Measurement of the rate of oxidation of disaccharide B showed that there was an immediate consumption of 1 mol. of periodate, followed by the consumption of a further 2 mol. during the first 260 min. Uptake of periodate after 260 min. proceeded very slowly, the total consumption of periodate, after 800 min. being 3·7 mol. After 24 hours all the periodate had been reduced.

Fig. 5



At the time the periodate oxidation was carried out it was not suspected that a furanoside structure was present in disaccharide B, and it was thought that 5 mol. of periodate would be sufficient to oxidise the disaccharide. A glucopyranosyl galactopyranoside would have consumed 4 mol. of periodate, and produced 2 mol. formic acid and no formaldehyde; no over oxidation would have been expected. Control experiments with α,α-trehalose, showed that a structure of this type consumed 5·6 mol. of periodate, and produced 1·4 mol. formic acid, and no formaldehyde, in 540 min. No further oxidation could be detected after 24 hours. Thus the consumption of more than 4 mol. of periodate in 24 hours, showing that over oxidation was taking place, is confirmatory evidence for the presence of a furanose ring in disaccharide B.

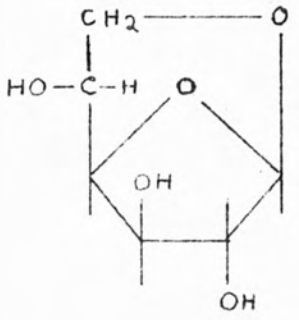
Either of the structures proposed for disaccharide B would be expected to consume 4 mol. of periodate initially, with the scission of the bonds between adjacent carbons carrying hydroxyl groups, giving structure IV (Fig. 5). On prolonged oxidation, 'over oxidation', the uptake of a further 4 mol. of periodate, with the production of VI, 3 mol. of formic acid, and 1 mol. of carbon dioxide, would be expected.

The values actually obtained during the periodate oxidation of disaccharide B can be explained by the proposal that the rate controlling step, in the oxidation of I, or II, to VI, is the oxidation of III to IV, rather than the oxidation of IV to V. Then disaccharide B would consume 3 mol. of periodate, with the production of 1 mol. of formaldehyde, and 1 mol, of formic acid, to give III, and the uptake of a further 5 mol. of periodate to give VI, 3 mol. of formic acid, and 1 mol. of carbon dioxide would proceed at a much lower rate.

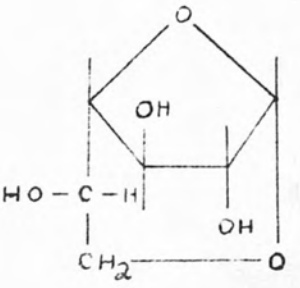
The resistance of III to oxidation could be due to the formation of a cyclic hemi-acetal in the furanosyl residue. Molecular models, however, have shown that this is not possible. On the other hand, it is known that 1:2- diols, in which the hydroxyl groups are firmly locked in the <u>trans</u> position, as in 1:6- anhydro -β- D-glucofuranose, Fig. 6, I, or 1:6- anhydro-β-D- galactofuranose, II, are not oxidised by periodate (80, 81). L-Threitan, III, in which the <u>trans</u> hydroxyl groups are less securely locked, reacts with periodate, but at a slower rate than the corresponding <u>cis</u> compound erythritan, IV, (82). On the basis of these facts it is believed that the rate determining step, in the oxidation

Fig. 6.

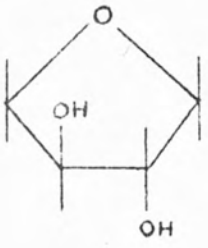
I



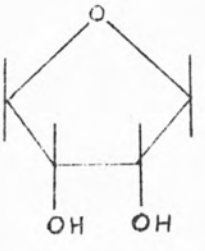
II



III



IV



of disaccharide B is the oxidation of III, in which the adjacent hydroxyl groups are in the trans position. In the later stages, when the oxidation of I, or II, to III has been allowed to go to completion, the disaccharide B will have consumed (3 + 5x) mol. of periodate, and produced (1 + 3x) mol. of formic acid. The consumption of 3·7 mol. of periodate, should, on this basis, be accompanied by the production of 1·42 mol. formic acid. The experimental results are in good agreement with these values. At the same time they provide conclusive evidence for the pyranosyl furanoside structure of disaccharide B.

The product of the periodate oxidation of disaccharide B was hydrolysed. Only a component, with the R_f value of glycerose, could be detected in the hydrolysate. This would be expected from the proposed structure for the disaccharide. The other components that would be obtained from the hydrolysis of IV, (glyoxal and malondialdehyde), V (glyoxal and mesoxalic dialdehyde), and VI (glyoxal and formic acid), would not have been detected by the chromatographic methods used.

Oxidation of disaccharide B with 1 mol. of periodate

(Expt. 40), resulted in the liberation of 0.58 mol. of formaldehyde, showing that the initial attack by periodate was, in fact, at C_5 and C_6 of the furanosyl residue. The oxidation product was reduced with potassium borohydride, and hydrolysed. Chromatographic analysis of the hydrolysate revealed the presence of components corresponding to D-glucose and L-arabinose. This showed that in disaccharide B the glucose moiety was in the pyranose form, as would be expected from the method of synthesis. D-galactopyranosyl D-glucofuranoside (II), would have given D-galactose and D-xylose.

Evidence for the configuration of the glycosidic links was obtained by treatment of disaccharide B with hydrolytic enzymes (Expt. 44). Disaccharide B was hydrolysed by a mixture of yeast α-glucosidase and α- galactosidase, but not by the same mixture when the α-glucosidase activity was inhibited by the addition of D-glucono-δ-lactone (83). Disaccharide B was not hydrolysed by almond β-glucosidase. It was therefore concluded that the D-glucopyranosyl residue had an α-configuration. There was not sufficient evidence to assign a configuration to the D-galactofuranosyl residue, as it is likely that the α-galactosidase was specific

for galactopyranosides.

The evidence presented characterises disaccharide B as α-D-glucopyranosyl D-galactofuranoside (I).

INVESTIGATION OF DISACCHARIDE C

Chromatographically pure disaccharide C could be detected, on paper chromatograms, with acetone silver nitrate-alcoholic sodium hydroxide (73), aniline hydrogen phthalate (53), and triphenyl tetrazolium chloride (54) sprays, indicating that it was α reducing sugar, unsubstituted at C_2 (Expt. 32). With the aniline-diphenylamine reagent (74), a blue colour was obtained, in contrast to the greyish colours given by disaccharides A and B.

Hydrolysis of disaccharide C (Expt. 33) gave components with the same R_F values as D-glucose and D-galactose.

Disaccharide C contained 85·4% of the theoretical carbohydrate content of a disaccharide, measured by the anthrone method (Expt. 34). The reducing power estimated by alkaline hypoiodite (Expt. 35), was 102·4%, and with the alkaline copper reagent of Somogyi and Nelson (Expt. 36) 106·3%, of the theoretical value for a disaccharide. These results showed clearly, that C was

a reducing disaccharide unsubstituted at C_2 of the reducing moiety.

Reduction of disaccharide C with potassium borohydride (Expt. 37), followed by hydrolysis, gave components identical with D-glucose and a hexitol, on paper chromatography. This confirmed the reducing nature of disaccharide C and showed that it was a glucosyl-galactose.

An α-configuration was assigned to the glucosyl residue on the evidence of its behaviour towards hydrolytic enzymes (Expt. 44). Disaccharide C was hydrolysed to glucose and galactose when treated with yeast α-glucosidase. No hydrolysis took place in the presence of almond β-glucosidase.

Oxidation of disaccharide C with 5 mol. periodate, resulted in the consumption of 2·9 mol. of periodate, in 430 min., with the production of 1·05 mol. of formic acid, and 0·17 mol. of formaldehye. The theoretical values for a disaccharide with a 1:3-link are 3, 1, 0 mol. respectively, with a 1:4- or a 1:5- link, 4, 2, 0 mol. respectively, and with a 1:6- link, 5, 3, 0, mol. respectively. The results obtained with disaccharide C

suggested that the linkage between the glucose and galactose units was 1:3-.

Other evidence, however, pointed to a 1:4- or possibly a 1:5- link. Hydrolysis of the products of periodate oxidation, gave components with the R_f values of glycerose, and threose, which would be expected from a 1:4-, or a 1:5- linked disaccharide. A 1:3- linked disaccharide would be expected to yield a pentose, and glycerose, detectable by paper chromatography, and a 1:6- linked disaccharide to yield glycerose, and fragments with not more than three carbon atoms, which would be difficult to detect on paper chromatograms.

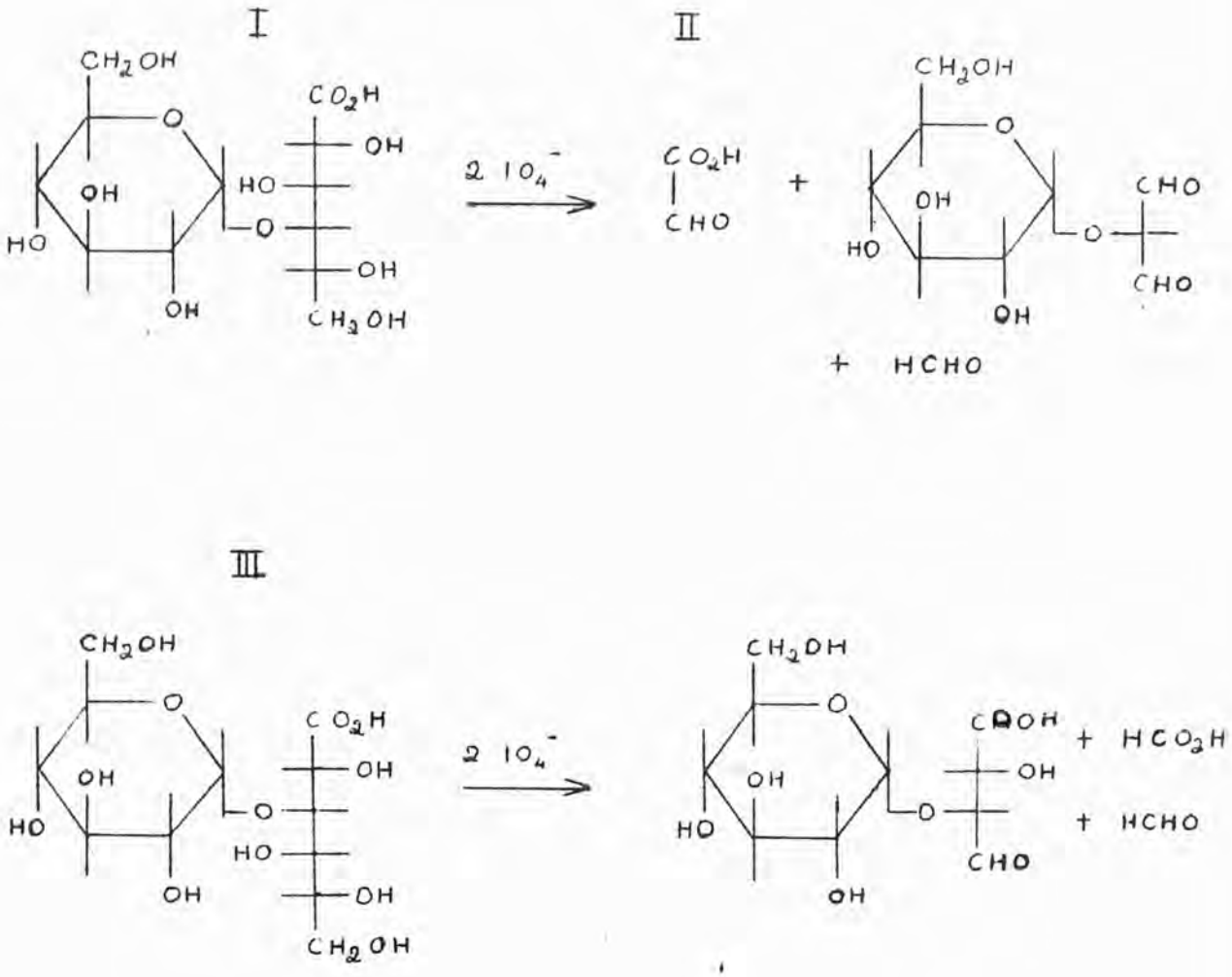
A 1:3-, 1:5-, or 1:6- linked disaccharide would probably give a methyl furanoside on treatment with methanolic hydrogen chloride (84). No such reaction occurred with disaccharide C (Expt. 42). Under the same conditions methyl furanoside production could be detected, on paper chromatograms, in the presence of nigerose, but not in the presence of maltose.

Disaccharide C was oxidised with bromine water (Expt. 43) and the resulting aldobionic acid was separated by preparative paper chromatography. Whelan

and Clancy (85) have shown that, under certain conditions of periodate oxidation, an aldobionic acid is not oxidised in the non-reducing glycosyl moiety, and oxidation of the α-hydroxy acid group is very slow. Under these conditions a 1:4- linked glucosyl-galactonic acid (Fig. 7, I) would be expected to yield glyoxylic acid (II); glyoxylic acid would also be expected from a 1:5- or a 1:6- linked glucosyl-galactonic acid. No glyoxylic acid would be expected from a 1:2- or 1:3- linked glucosyl-galactonic acid, (III). The aldobionic acid from disaccharide C was treated with periodate under these conditions, and the product gave a positive test for glyoxylic acid. This suggested that the reducing moiety of disaccharide C was not substituted as C_2 or C_3.

The blue colour produced by disaccharide C, on paper chromatograms, when treated with the diphenyl-amine-aniline reagent, also indicated that a 1:4- link was present. This reagent has been used to detect 1:4- links in the glucose oligosaccharides. There is no reported evidence that the same blue colour is obtained with 1:4- linked galactose oligosaccharides. However, the contrast between the blue colour given by disaccharide C, and the greyish colours given by disaccharides A and B,

Fig. 7.



suggests that the reagent distinguishes between 1:4- and other links in the galactose series, and that a 1:4- link was present in disaccharide C.

On the evidence of the diphenylamine-aniline spray, the absence of methyl furanoside production, the oxidation of the aldobionic acid, and the hydrolysis of the products of periodate oxidation, it seemed likely that disaccharide C was O-α-D-glucopyranosyl-(1→4)-D-galactose. The low M_g value (0·34), obtained on electrophoresis of disaccharide C in borate buffer, was in agreement with a 1:4- linked structure of this type.

The results obtained on oxidising disaccharide C with 5 mol. of periodate, could be explained, if disaccharide C was O-α-D-glucopyranosyl-(1→4)-D-galactose, on the assumption that the oxidation was incomplete, and that the periodate attack between C_2 and C_3 of the non-reducing unit was hindered. Then the uptake of 5 mol. of periodate and the production of 1 mol. of formic acid would proceed at a faster rate than the uptake of a fourth mol. of periodate and the production of a second mol. of formic acid. There is evidence (86) that a large substituent, such as a

phenyl group, at C_1, may retard the rate of oxidation between C_2 and C_3. Ferrier and Overend (87) have suggested that this is due to the partial locking of the conformation at C_2 and C_3, which would hinder periodate attack. It seems possible that this may occur in the non-reducing unit of a disaccharide. Manners and Archibald (88) have found that formic acid production lags behind periodate consumption, during the oxidation of maltose. This could be partially accounted for if the periodate attack between C_2 and C_3 of the non-reducing moiety was hindered.

If the oxidation of a 1:4- linked glucosyl-galactose follows the course suggested, then hydrolysis of the products of incomplete oxidation would yield principally threose, glycerose, hydroxy malondialdehyde, and formic acid. The chromatographic analysis of the disaccharide C product(which would not have revealed hydroxy malondialdehyde or formic acid) was in agreement with this.

Disaccharide C could be completely characterised by the use of methylation techniques. Valuable evidence for the position of the glycosidic link could also be obtained by periodate oxidation of disaccharide C alcohol under

conditions which avoid oxidation of the glucoside moiety (85).

INVESTIGATION OF HIGHER OLIGOSACCHARIDES.

Chromatographically pure oligosaccharides, B and C isolated from the culture medium by charcoal column chromatography, had R_g values of 0·18 and 0·16 respectively. (Expt. 45). These values, and the composition of the solvent needed to elute them from the charcoal column, (aqueous ethanol, 10% and 15%) suggested that oligosaccharides B and C were a tri- and tetrasaccharide respectively. Total hydrolysis of oligosaccharides B and C gave components with the R_f values of glucose and galactose. The glucose:galactose ratios, estimated by inspection of the chromatograms were 2:1 and 3:1. Partial hydrolysis gave components corresponding to glucose, galactose, and isomaltose, on paper chromatography. These results suggested that oligosaccharides B and C were produced by successive 1:6- additions of glucosyl units to a galactose-containing acceptor.

CONCLUSION.

In the present work it has been shown that at least three glucose-galactose disaccharides were syn-

thesised by B. arabinosaceous in media containing sucrose and galactose. One of these disaccharides has been characterised as α-D-glucopyranosyl D-galactofuranoside. Evidence has been obtained that a second disaccharide was O-α-D-glucopyranosyl-(1→4)-D-galactose, and the third disaccharide may have been O-α-D-glucopyranosyl-(1→6)-D-galactose. Chromatographic evidence suggested that the same disaccharides were synthesised by an enzyme preparation in the presence of galactose and sucrose.

These disaccharides may have been synthesised by enzymes other than dextransucrase. In order to account for all the oligosaccharides synthesised by enzyme preparations from B. arabinosaceous, it could be suggested that enzymes producing 1:1-, 1:2-, and possibly 1:4- links are associated with dextransucrase and the branching enzyme. These enzymes would not normally be required by the organism, and a simpler explanation is that dextransucrase is capable of transferring glucosyl units from sucrose to positions other than C_6 of a non-reducing, terminal α-D-glucosyl unit.

It appears from the results described in this section, that an axial hydroxyl group at C_4 hinders glucosyl transfer to C_6, and can itself act as an

acceptor. In D-fructopyranose (Fig 3, VII) there is an axial hydroxyl group at C_5, in the same position, relative to the structural features proposed as essential for association with the acceptor site of the enzyme, as the axial hydroxyl group at C_4 of galactose. Thus the synthesis of O-α-D-glucopyranosyl-(1→4)-D-galactose, disaccharide C, would be analogous to the synthesis of leucrose. It may be that an axial hydroxyl group, 'trans' to the groups making contact with the enzyme acceptor site, can compete with the primary carbinol group at C_6, for transferred glucosyl units. Possibly a sugar accepts at C_2 when transfer to C_6 is hindered and no axial hydroxyl group in a suitable position is present in the molecule.

The synthesis of higher oligosaccharides containing galactose indicated that galactose was capable of acting as the acceptor in a chain initiation step leading to the synthesis of dextran-like polymers. There was no definite evidence showing which of the galactose-glucose disaccharides acted as an acceptor for the build-up of higher oligosaccharides. The absence of any of the galactose containing disaccharides in the partial hydrolysates of oligosaccharides B and C, however, suggests that the link between the glucose and galactose

units in these sugars was acid labile, and they may have been synthesised by glucosyl transfer to disaccharide B.

The production of the glucopyranosyl galactofuranoside, disaccharide B, is of considerable interst as it is believed to be the first bacterial synthesis of a disaccharide containing a galactofuranose unit to be reported. The significance of this disaccharide in the dextransucrase reaction will be discussed in a later section.

SECTION IV

INVESTIGATION OF THE REVERSIBILITY OF THE DEXTRAN-SUCRASE-SUCROSE REACTION.

The overall reaction catalysed by dextransucrase (eqn. i) has been shown by Hehre (25) to be essentially irreversible. It is generally accepted (15, 46) that the reaction proceeds through several stages, involving association of the enzyme with donor and acceptor molecules. In the present work an investigation into the reversibility of different stages of the reaction, using [^{14}C] tracer techniques, is described.

If the synthesis of α-1:6- links is reversible, dextransucrase should catalyse the transfer of glucosyl units from dextran to some other acceptor. An enzyme preparation was incubated with [^{14}C]dextran (L. mesenteroides NRRL B512) and methyl α-D-glucoside, which is known to be a good acceptor of glucosyl units transferred from sucrose (24), in the presence and absence of fructose (Expt. 46). Radiochromatography of the digest showed that no [^{14}C]glucosyl residues had been transferred from the dextran to methyl α-D-glucoside. Radiochromatography of enzyme digests containing [^{14}C]trisaccharide A and

methyl α-D-glucoside, with and without fructose, showed that no [^{14}C]glucosyl residues were transferred from the trisaccharide to methyl α-D-glucoside. No sucrose synthesis was detected in enzyme digests containing trisaccharide A and fructose alone, and there was no incorporation of ^{14}C into sucrose when this sugar was incubated with the enzyme and trisaccharide A. From these results it was concluded that the synthesis of α-1:6- and α-1:2- links was irreversible.

When sucrose was incubated with a dextransucrase preparation in the presence of added [^{14}C]fructose, radiochromatograms showed that ^{14}C was incorporated into a component with the same R_f value as sucrose (Expt. 47). This phenomenon was observed with different batches of enzyme from B. arabinosaceous, and also with a dextransucrase extract from Streptoccus bovis. Under the same conditions, in the absence of any enzyme preparation, no interchange of ^{14}C between sucrose and [^{14}C]-fructose occurred.

These results indicated that a reversible transfer of glucosyl units from sucrose to fructose was taking place, and a more detailed investigation was carried out. An enzyme preparation from B. arabinosaceous was incubated

with sucrose (1%) and [^{14}C]fructose (10%); the specific radioactivity of the fructose, measured by the infinitely thick disc method (Expt. 48), was 65·3 ± 2·6 mC/gm. mole of fructose. Samples of the digest were fractionated by preparative paper chromatography at intervals over a period of 8 hours (Expt. 49). [^{14}C] Components with the R_f values of fructose, sucrose, leucrose, and possible tri- and tetrasaccharides were isolated from each sample. The total radioactivity of the leucrose fractions, estimated by inspection of the radiochromatograms, increased throughout the incubation period. The tri- and tetrasaccharides appeared to be present in greater concentration during the early stages of the incubation.

That the component with the R_f value of sucrose (disaccharide D) was, in fact, sucrose was demonstrated by the carrier dilution analysis (89). Addition of inactive sucrose to a sample of disaccharide D gave [^{14}C]sucrose with specific radioactivities of 51·1 ± 2·0 μC/gm. mole of sucrose and 53·4 ± 2·1 μC/gm. mole of sucrose on successive crystallisations (Expt. 50). From these values the specific radioactivity of the sucrose in the digest was calculated to be 60·8 ± 2·4 mC/gm. mole of sucrose. This value was in good agreement with the value of 58.6 ± 3.5 mC/gm. mole of sucrose obtained by

estimating the activity directly, by the infinitely thin film method.

Further evidence for the identity of disaccharide D with authentic sucrose was provided by chromatography in several solvents, by mild acid hydrolysis, and by hydrolysis with invertase. In all these tests its behaviour was identical with that of sucrose. Radiochromatography of the hydrolysates revealed only glucose and [^{14}C]fructose.

The specific radioactivities of the [^{14}C]sucrose, and the [^{14}C]fructose, isolated from the digest at intervals throughout the incubation period, were measured by the infinitely thin film method . The specific radioactivity of the [^{14}C]fructose was 65·3 ± 2·6 mC/gm. mole of fructose initially, measured by the infinitely thick disc method (Expt. 48). After 8 hours incubation the specific radioactivity of the [^{14}C]fructose was 60·3 ± 3.6 mC/gm. mole measured by the infinitely thin film method(Expt.51). The specific radioactivity of the [^{14}C]sucrose measured by the infinitely thin film method was 9·5 ± 0.59 mC/mole ofsucrose after 15 min., and 58·6 ± 3.5 mC/gm. mole of sucrose after 8 hours incubation, measured by the infinitely thin film method (Expt. 50). By the method of carrier dilution and

estimation of the radioactivity by the infinitely thick disc method the specific radioactivity of the [^{14}C]sucrose, after 8 hours incubation was estimated to be 60·8 ± 2.4 mC/gm. mole of sucrose. When equilibration of the free [^{14}C]fructose and the fructose moiety of the sucrose was complete, then the specific radioactivity per mole of both fructose and sucrose should have been the same. This activity, calculated on the basis of the quantities of fructose and sucrose initially present, was 59·3 ± 2·4 mC/gm. mole of the sugar. Thus the experimental results showed that a measurable amount (ca. 16%) of [^{14}C]fructose interchange had taken place after 15 min. incubation, and that the equilibration was complete within 8 hours.

This investigation has shown that dextransucrase preparations are capable of transferring glucosyl units reversibly from sucrose to free fructose. It has been shown previously (21,39) that the addition of fructose to dextran-sucrase-sucrose digests depresses the initial rate of dextran synthesis, estimated by measuring the fructose produced. The transfer of glucosyl units to free fructose, with the synthesis of sucrose, as demonstrated in the present work, may account for this inhibitory effect.

If the sucrose synthesising enzyme is, in fact, dextransucrase, then the formation of a glucosyl-enzyme complex and fructose from sucrose and the enzyme is a reversible process. The synthesis of a 1:6- or a 1:2-link appears to be irreversible.

This is in accordance with the views of Hestrin *et al.* (47) on the synthesis of polysaccharides from sucrose by transfructosylation. These authors put forward the theory that the transfer of fructosyl residues proceeded through a fructosyl-enzyme complex, and that the formation of this complex, in the case of levansucrase and mould invertase, was reversible, involving a very small change in free energy. Then the transfer of the fructosyl unit from the complex to the primary carbinol at C_1 or C_6 of a fructofuranosyl acceptor was essentially irreversible, with a comparatively large change in free energy.

Using the notation of these authors, it is suggested that the synthesis of dextran could proceed according to the following scheme:

$$G\sim F + E \rightleftharpoons E\sim G\sim F \rightleftharpoons E\sim G + F \quad \text{.....(x)}$$

$$E\sim G + A \longrightarrow G<A + E \quad \text{.....(xi)}$$

where $\sim$ represents a 'high energy' bond as in sucrose

$<$ represents a 'low energy' bond such as the 1:6-

link.

G represents glucose,

F represents fructose,

G~F represents sucrose,

A represents a molecule accepting at a carbinol site.

E represents the enzyme.

The synthesis of sucrose from glucose and short chain levans, in the presence of levansucrase, has been demonstrated (90,91). This reversion of the normal synthetic reaction only took place to a very small extent, under special conditions, e.g. short chain levans, removal of sucrose by enzymic reactions, and long periods of incubation. Possibly under similar conditions the synthesis of α-1:6- links by dextransucrase could be reversed. The rate of such a reaction, however, would probably be so slow that synthesis of α-1:6- links as shown in eqn. (xi) can be regarded as essentially irreversible.

A $[^{14}C]$component of the enzyme digest running behind sucrose on paper chromatograms (disaccharide E) was identical with leucrose on chromatography in several

solvents, and on treatment with hydrolytic reagents (Expt. 53). No hydrolysis occured under mild acid conditions, or on treatment with invertase. More vigorous acid hydrolysis yielded glucose and a trace of [^{14}C]fructose, detected by radiochromatography. The conditions of this hydrolysis were such that most of the fructose would have been converted to 5-hydroxymethyl-2-furfuraldehyde, which would not have been detected under the conditions of chromatographic analysis used. It was therefore concluded that disaccharide E was, in fact, [^{14}C]leucrose.

The specific activity of the combined [^{14}C]leucroae fraction was 59.00 $\pm$ 3.5 mC/gm. mole leucrose, measured by the infinitely thin film method. A specific activity of this order suggested that the leucrose was synthesised by glucosyl transfer to free fructose, which had specific activities ranging from 65.3 $\pm$ 2.6 to 60.3 $\pm$ 3.6 mC/gm. mole of fructose, throughtout the incubation period. Leucrose synthesised by glucosyl transfer to a [^{14}C]fructose unit from the [^{14}C]sucrose(specific activity ranging from 0 to 58.63 mC/gm. mole of sucrose throughout the incubation period) would have had a lower specific activity.

The suspected tri- and tetrasaccharide fractions (oligosaccharides D and E) isolated from the enzyme digest contained ^{14}C, showing that at least one of the monosaccharide units, in each sugar was fructose. This was confirmed by total hydrolysis (Expt. 54) which gave components corresponding to glucose and [^{14}C]fructose. Partial hydrolysis of both oligosaccharides yielded components with the same R_f values as fructose, glucose, and isomaltose. This suggested that these sugars contained at least two glucosyl residues, joined by α-1:6-links.

Oligosaccharides D and E were present in higher concentrations during the earlier stages of the incubation period than at the end of this period. This suggested that they were not produced by glucosyl transfer to leucrose, which was present in increasing concentrations throughout the incubation period. The presence of a fructosyl unit in the oligosaccharides, and the absence of any fructose-containing sugars in the partial hydrolysates, suggested that the link between the fructosyl residue and the glucosyl residues was acid labile. Thus it is possible that oligosaccharides D and E were produced by successive glucosyl transfers to sucrose, in each case the glucosyl unit being transferred to C_6 of

the terminal, non-reducing, ∝-D-glucosyl unit. Such oligosaccharides may be intermediates in the synthesis of dextran.

In view of the present uncertainty as to the nature of the chain initiation step in the synthesis of dextran, a large scale isolation of oligosaccharides produced during the early stages of the dextransucrase-sucrose reaction, and characterisation of these sugars would be of great interest. The production of oligosaccharide intermediates points to a multichain rather than a single chain mechanism of chain propagation. Therefore it is also important in the elucidation of the chain propagating reactions, to provide confirmatory evidence for the production of such intermediates.

CONCLUSION

A preliminary survey of a number of possible acceptors for the dextransucrase-sucrose reaction has been described in the present work. Evidence has been obtained that cultures of B. arabinosaceous, or dextransucrase preparations from this organism, are capable of synthesising oligosaccharides by glucosyl transfer to D-mannose, L-sorbose, D-xylose, D-ribose, myoinositol, and possibly L-rhamnose, L-fucose, D-arabinose, and methyl β-D-ribopyranoside. Sorbitol and methyl α-D-ribopyranoside did not act as acceptors. Earlier workers (21, 32, 34, 40) have shown that D-glucose, D-galactose, and chain terminal α-D-glucopyranosyl and α-D-galactopyranosyl residues acted as acceptors, whereas chain terminal β-D-glucopyranosyl and β-D-galactopyranosyl residues did not accept.

Consideration of the conformations of the acceptors and non-acceptors has shown that all the acceptors have in common the structural features shown in Fig. 3, i.e. one axial -OR group and two axial hydrogens on the same side of the mean plane of the pyranose ring. This combination of features is not present in the non-acceptors. It has, therefore, been suggested that these

structural features may be essential for an acceptor. It is generally held that an acceptor must associate with an enzyme at a specific acceptor site, before glycosyl groups can be transferred to it from the donor. Possibly a structure such as that shown in Fig. 3 is necessary for a molecule to associate with dextransucrase at the acceptor site.

The production of oligosaccharides by enzymic transglycosylation also indicates that the acceptor has a hydroxyl group available, in such a position that a glycosyl residue can be transferred to it. A more detailed study of the oligosaccharides produced in the presence of added acceptors gave some information about the positions to which glucosyl transfer, by dextransucrase preparations, took place. It has been shown previously (21, 24, 38, 39, 40) that dextransucrase normally synthesises α-1:6- links by glucosyl transfer to a chain terminal α-D-glucopyranosyl residue. Lactose and cellobiose, in which the non-reducing moiety has an unfavourable β-configuration, acted as acceptors in the reducing moiety. In the presence of these sugars transfer to C_2 of the reducing unit took place (41, 63). In the present work [^{14}C] tracer techniques have been used, and

have shown that the trisaccharide produced in the presence of lactose is synthesised by glucosyl transfer to a preformed lactose molecule. The structure previously assigned to this trisaccharide (41) was also confirmed.

It was suspected that the presence of a bulky substituent at C_4 of the reducing unit of lactose, or cellobiose, hindered glucosyl transfer to the C_6 position of this residue, and it seemed that the hydroxyl group at C_2 could accept glucosyl units under these conditions.

The effect of an axial hydroxyl group at C_4, as in galactose, was investigated. Three disaccharides were isolated from a culture of B. arabinosaceous in a medium containing sucrose and galactose. Structural investigations of ~~two~~ these two disaccharides suggested that they were α-1:4-, and possibly α-1:6-, linked glucosyl-galactoses. Thus the presence of an axial hydroxyl group at C_4 prevented the exclusive transfer of glucosyl residues, from sucrose, to C_6 of the acceptor, and it seemed that this axial hydroxyl group competed with the primary hydroxyl group at C_6 for transferred glucosyl units.

The third disaccharide produced in the medium

containing sucrose and galactose, was shown to be α-D-glucopyranosyl D-galactofuranoside. This is believed to be the first bacterial synthesis of a disaccharide containing a galactofuranose residue to be reported. The synthesis of this disaccharide was of particular interest as it suggested that dextransucrase was capable of transferring glucosyl units to the anomeric carbon of a sugar in the furanose form. [^{14}C] Tracer techniques have been used to show that dextransucrase preparations also transferred glucosyl units to C_2 of fructofuranose. In the presence of sucrose and [^{14}C] fructose, enzyme preparations from B. arabinosaceous and S. bovis catalysed the reversible transfer of glucosyl units from sucrose to fructofuranose. [^{14}C] Sucrose, labelled in the fructose moiety, was synthesised in this way.

It has been suggested that the apparent irreversibility of the dextransucrase-sucrose reaction could be accounted for by the rapid conversion of the liberated fructofuranose into the more stable pyranose form. However, solutions of free fructose probably contain ca. 22% furanose, at 25° (92). Solutions of galactose also contain a high proportion of furanose, as shown by the products of acetylation(92). This work has shown that dextransucrase preparations can catalyse

the transfer of glucosyl units to the anomeric carbon atoms of the furanose forms of both fructose and galactose. In the synthesis of dextran, from sucrose, it seems that the formation of a glucosyl-enzyme complex and fructose, from sucrose and the enzyme, is a reversible process. Evidence has been put forward, in this work, that the transfer of glucosyl units, from the glucosyl-enzyme complex, to C_2 or C_6 of an acceptor, is not reversible.

Hestrin and Avigad (93, 94) and Peaud-Lenoel (95) have recently shown that levansucrase catalyses the reversible transfer of β-D-fructofuranosyl units from sucrose to the C_1 position of several aldoses. It may be that the synthesis of sucrose, and of α-D-glucopyranosyl D-galactofuranoside, by dextransucrase are examples of a general reaction of this type. It would be interesting to investigate oligosaccharides produced in the presence of other sugars which exist, to an appreciable extent, in the furanose form when in solution It also seems possible that the synthesis of glucopyranosyl-furanoses of this type may be reversible, and that these sugars could act as substrates for dextransucrase.

The presence of oligosaccharides during the early

stages of the synthesis of dextran by dextransucrase preparations, has been indicated during the present work. A more detailed investigation of such oligosaccharides could provide information about the mechanism of the chain initiation and the chain propagation in dextran synthesis.

GENERAL METHODS

Expt. 1. Growth and maintenance of cultures of Betacoccus arabinosaceous (Birmingham)

A culture of the organism was supplied by Glaxo Laboratories Ltd., and was stated to be of the strain used by Bailey, Barker, Bourne, and Stacey (21). Its viability was maintained by subculturing, every 4-6 weeks, on agar slopes containing yeast extract (1%), peptone (1%), sodium ammonium hydrogen phosphate (0·5%), potassium dihydrogen phosphate (0·1%), sucrose (10%), and agar (2%). After inoculation the cultures were incubated at 25° for 3 days and then stored at 0-2°.

For preparative purposes the organism was grown in a medium containing yeast extract (1%), microcosmic salt (0·5%), potassium dihydrogen phosphate (0·1%), magnesium sulphate (0·05%), and sucrose (as described in later experiments). Other sugars were added to the medium as described in the appropriate experiments. After the addition of sucrose the medium was adjusted to pH 7·00 and steam sterilized at 15 lb. pressure for 15 min.

Expt. 2. Extraction of the enzyme, dextransucrase (21).

The standard preparative medium (Expt. 1) (10-15 ml.), containing sucrose (10%), was inoculated, and incubated

at 25° for 48 hours, in order to obtain a strongly growing culture. The same medium (1 l.) was inoculated from this culture (5 ml.), and incubated at 25° for 17 hours (Batches I, III, and IV), or 48 hours (Batch II). At the end of the incubation period the culture was cooled to 0°, and the temperature was kept at 0-2° throughout the following operations.

Ethanol (540 ml.) was added slowly, with stirring, to the culture medium. After standing for 1 hour a dark, gummy, precipitate was recovered by centrifuging (2,500 r.p.m., 30 min.), dissolved in 0.1M-citrate buffer (125 ml., pH 6.0), and centrifuged (5,000 r.p.m., 30 min.), in order to obtain a cell-free enzyme extract. The supernatant liquid was diluted with 0.1M-citrate buffer (625 ml. pH 6.0), and ethanol (250 ml.) was added. After standing overnight, the enzyme was isolated by centrifuging (2,500 r.p.m., 30 min.), dissolved in water (50 ml.), clarified by centrifugation (5,000 r.p.m., 30 min.), and freeze dried. The enzyme was obtained as a brownish powder.

Expt. 3. Measurement of the activity of the enzyme extract (21)

The enzyme (ca. 30 mg., accurately weighed) was

dissolved in 0.05M-acetate buffer (10 ml., pH 5.0). A portion (2 ml.) was pipetted into a 25 ml. standard flask, and a freshly prepared solution (3 ml.) containing sucrose (10%), in 0.05M-acetate buffer (pH 5.0), was added. This solution was incubated at 25° for 3 hours, together with blank digests, containing respectively (a) enzyme solution (2 ml.), and 0.05M-acetate buffer (3 ml., pH 5.0), and (b) sucrose solution (3 ml.) and 0.05M-acetate buffer (2 ml., pH 5.0).

At the end of the incubation period, the solutions were neutralised with 0.25N-sodium hydroxide (phenol red). The aldoses were destroyed with alkaline hypoiodite solution. Iodine solution (2 ml., containing iodine, 8.7 gm., and potassium iodide, 16.7 g., per l.) was added, followed by 0.175N-sodium hydroxide (1 ml.) The flasks were stoppered, shaken, and allowed to stand at room temperature for 10 min. After acidification with 0.25N-sulphuric acid (1 ml.) the excess iodine was removed with 0.04N-sodium sulphite, using starch glycollate as indicator. The solutions were neutralised with 0.25N-sodium hydroxide, and diluted to 25 ml.

The fructose present in a portion (5 ml.) of each solution was estimated by the Shaffer and Hartmann method (96). To each solution the alkaline copper

reagent (5 ml.) was added. The solutions were heated under standard conditions, acidified with N-sulphuric acid (5 ml.), and the liberated iodine was titrated with 0.005N-sodium thiosulphate, using starch glycollate as indicator. The reagents were standardised against standard solutions of fructose.

The results were expressed as units of dextransucrase activity, a unit being defined as the activity required for the complete conversion of 1 mg. of sucrose into dextran and fructose, in 1 hour, at 25^{0}, provided that not more than half the sucrose is used up.

Expt. 4. Chromatographic analysis.

Sugars and sugar derivatives were analysed by paper partition chromatography on sheets of Whatman No. 1 filter paper. The solvents used to develope the chromatograms are listed below:

(a) n-Butanol-ethanol-water (4:1:5), organic phase, developing time: 2-4 days.

(b) Benzene-pyridine-water (5:4:4), organic phase, developing time: 2-4 hours.

(c) Acetone-water (4:1), developing time: 4-5 hours.

(d) Ethyl acetate-acetic acid-saturated aqueous boric acid (9:1:1), developing time: 2 days.

(e) n-Butanol-pyridine-water-saturated aqueous boric acid (6:4:2:1), developing time: 2 days.

(f) n-Butanol-ethanol-water-ammonia (S.G. 0.880) (40:10:49:1), developing time: 18 hours.

(g) Ethyl acetate-acetic acid-water (9:1:1), developing time: 18 hours.

(h) Ethyl acetate-pyridine-water-acetone (10:5:10:2), to which ethyl acetate was added until two layers separated, organic phase used, developing time: 8 hours.

(i) n-Butanol-benzene-pyridine-water (5:1:3:2), developing time: 48 hours.

(j) n-Butanol-acetic acid-water (4:1:5), organic phase, developing time: 48 hours.

(k) Water-saturated methyl-ethyl ketone, developing time: 4 hours.

The sprays used to detect sugars and sugar derivatives are listed below:

(a) Acetone silver nitrate-alcoholic sodium hydroxide, detected all sugars and sugar derivatives (73).

(b) Aniline hydrogen phthalate, detected all reducing sugars (53).

(c) p-Anisidine hydrochloride, detected all reducing sugars (97).

(d) Sodium periodate-potassium permanganate-benzidine, detected all sugars and sugar derivatives with adjacent hydroxyl groups (98).

(e) Alkaline triphenyl tetrazolium chloride, detected reducing sugars, except those with a 2-O-substituent (54).

(f) Diphenylamine-aniline, gave a blue colour with 1:4-linked glucosaccharides, and a grey or yellow colour with other reducing sugars (74).

Expt. 5. Electrophoretic analysis

Sugars were analysed by electrophoresis on Whatman No. 3 filter paper, in 0.1M-borate buffer (pH 10) (51). The electrophoresis was carried out at 1000 v. for 3 hours.

Sugar alcohols were analysed in molybdate solution (pH 5) at 200 v. for 2 hours (79).

Expt. 6. Preparation of charcoal-'Celite' columns.

The technique used was a modification of the method of Whistler and Durso (49, 50).

Equal volumes of B.D.H. Activated charcoal and 'Celite' were mixed together with concentrated hydrochloric acid (2 vol.). After standing for at least 1 hour, the charcoal-'Celite' mixture was filtered, and

washed with water, by decantation and filtration, until the washings were acid-free. The mixture was then allowed to stand in absolute ethanol for at least 1 hour, filtered, and washed as before. Washed 'Celite' was prepared in a similar way.

The base of the column was packed with a layer of cellulose pulp (1-2 cm.), followed by a layer of 'Celite' (2-3 cm.). The charcoal-'Celite' mixture was then added as a thick slurry, in water, and allowed to settle under gravity, until a column of the required length had been obtained. The column was closed with a layer of 'Celite', followed by a layer of cellulose pulp. Care was taken to ensure that the ends of the charcoal-'Celite' layer were as flat as possible.

Expt. 7. Quantitative analysis of sugars and sugar derivatives.

(a) The total carbohydrate content of sugar solutions was estimated by the anthrone method (75).

To each sample (1 ml.) of the solutions, containing 10-100 μg. carbohydrate, anthrone solution (10 ml.), containing anthrone (20-50 mg.) dissolved in a mixture of concentrated sulphuric acid (70 ml.) and water (30 ml.) was added. The solutions were heated under the standard conditions, and the absorption was measured on a Hilger

Uvispek at 625 mμ. Standard solutions of the sugars to be estimated, and a blank containing water only, were analysed at the same time.

(b) Reducing sugars were analysed by the method of Somogyi and Nelson (77, 78).

To each sample (2 ml.) of the solution, containing the reducing sugar (10-100 μg.), the alkaline copper reagent (2 ml.), described by Nelson, was added. After heating under the standard conditions, the arsenate-molybdate reagent (1 ml.) of Somogyi was added. The solutions were diluted to 10 ml., and the absorption was measured on an Eel Absorptiometer at 606 mμ. Standard solutions of the reducing sugars to be estimated were analysed at the same time, and a graph showing the relationship of the sugar concentration to the absorption was plotted.

(c) Aldoses were estimated by oxidation with alkaline hypoiodite (76).

Samples of the sugar (2-5 mg., accurately weighed) were dissolved in water (5 ml.). To these, iodine solution (2 ml., Expt. 3) and 0.175$\underline{N}$-sodium hydroxide (1 ml.) were added. After standing at room temperature for 15 min. the solutions were acidified with 0.25$\underline{N}$-sulphuric acid (2 ml.), and titrated with 0.005$\underline{N}$-sodium

thiosulphate, using starch glycollate as indicator. Pure samples of the sugar to be estimated were used as standards.

(d) Sorbitol was estimated by a modification of the method of Adcock (99).

To each sample of a solution, containing sorbitol (10-50 μg.), a solution (1 ml.), containing equal volumes of freshly prepared 0.015$\underline{\underline{M}}$-sodium metaperiodate in 0.045$\underline{\underline{N}}$-sulphuric acid and of freshly prepared saturated sodium bicarbonate solution, was added. The solution was allowed to stand in the dark, at room temperature, for 2 hours. The excess periodate was destroyed by the addition of sodium sulphite solution (20%, 0.25 ml.). The formaldehyde, obtained by the oxidation of the sorbitol, was estimated by heating the solutions containing the oxidation product with a solution of chromotropic acid (9 ml.) in a boiling water bath for 30 min. The chromotropic acid solution was prepared by dissolving chromotropic acid (0.5 g.) in water (50 ml.), and adding a mixture of concentrated sulphuricacid and water (2:1, 200 ml.). After cooling the formaldehyde solutions a half-saturated solution of thiourea was added, in order to remove the colour due to free iodine.

The absorption was measured at 606 mμ on an Eel Absorbtiometer.

Care was taken to exclude strong light from all solutions containing chromotropic acid, and all absorptions were measured against a blank containing sulphuric acid and water (2:1) only. Standard curves prepared at different times, using different chromotropic acid and sorbitol solutions showed variations of only 2-3%, but for accurate work each solution of chromotropic acid was standardised against solutions of pure sorbitol. The chromotropic acid solutions were stable for 2-3 days if kept away from light.

Expt. 8. Radiochromatographic analysis of [^{14}C] sugars.

Radiochromatograms were prepared by scanning chromatograms, on which [^{14}C] sugars had been separated, with a Geiger-Muller end-window counter, or by exposure to Ilford X-ray film (Industrial G) for an appropriate length of time. The films were developed with Ilford X-ray developer PQX-1 and fixed with Ilford Hypam fixer.

Expt. 9. Measurement of specific radioactivities.

Infinitely thick disc method (55, 56, 57)

The specific radioactivities of [^{14}C] sugars were determined after conversion of the compound into carbon

dioxide, which was precipitated as barium carbonate.

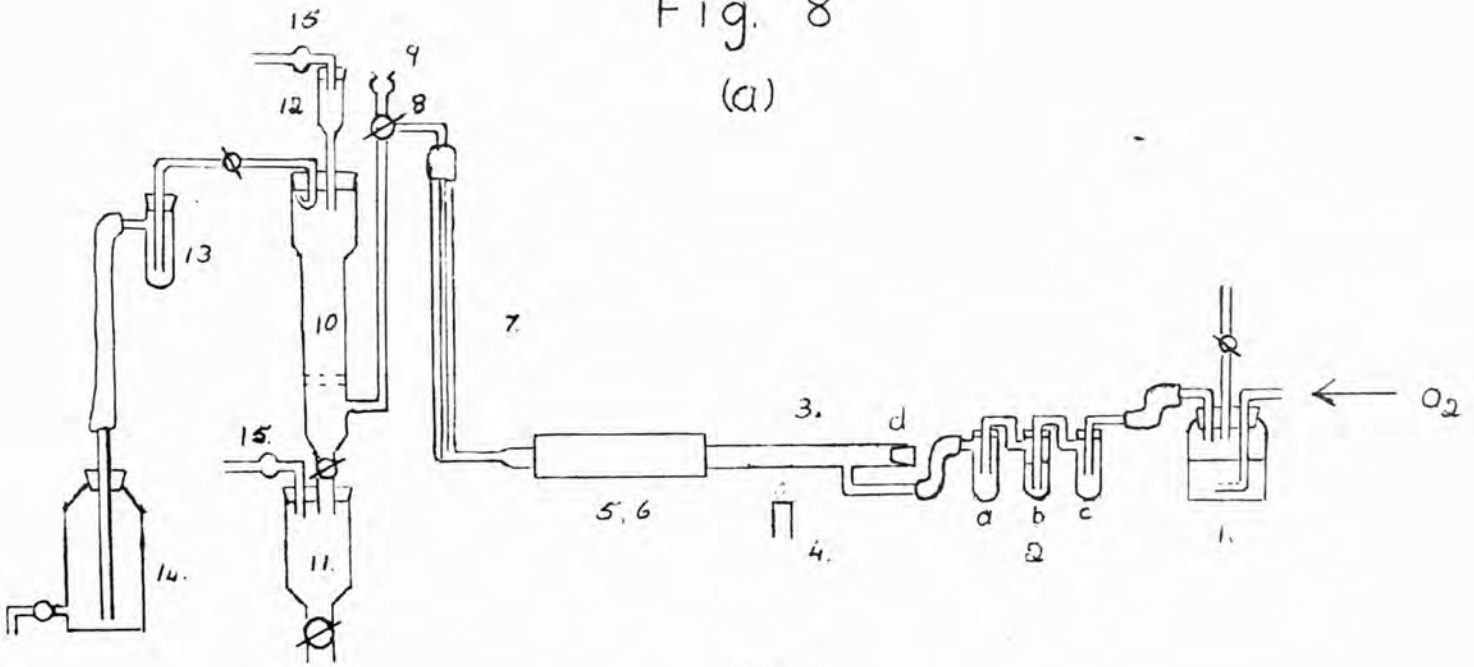
(a) Dry combustion.

The apparatus was of the standard type as shown in Fig. 8a.

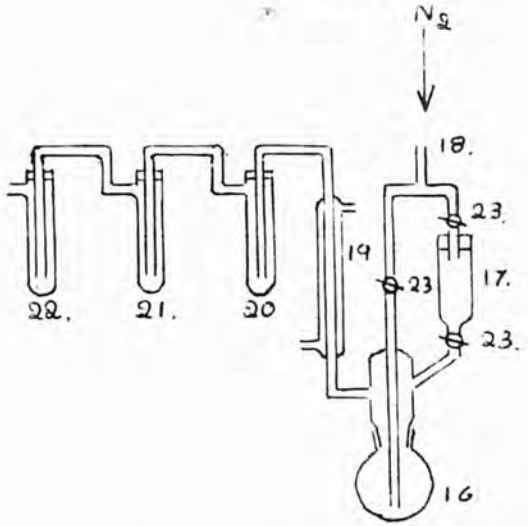
1. 3$\underline{N}$-NaOH (150 ml.)
2. Flow meter. (b) contained barium chloride solution and showed that the gas passed through the apparatus was carbon dioxide free. (a) and (c) were backward and forward suction traps.
3. Glass combustion tube, stoppered at (d).
4. Bunsen burner.
5. Platinum gauze.
6. Electrically heated furnace.
7. Wide bore capillary tube.
8. Three-way tap.
9. Funnel for the addition of ammonium chloride and barium chloride.
10. Absorbtion vessel containing carbonate free $\underline{N}$-sodium hydroxide (5 ml.), prepared by dissolving sodium in carbon dioxide free ethanol and diluting with carbon dioxide free water, and carbon dioxide free water (25 ml.).
11. Vessel for barium carbonate precipitation.
12. Funnel for the addition of sodium hydroxide and water.

Fig. 8

(a)


15
9
12
8
13
10
7
3.
d
15
O_2
5.6
4.
a b c
2
1.
14.
11.

(b)


N_2
18.
23.
19
23
17.
23.
22.
21.
20
16

28

13. Barium solution to check that all carbon dioxide had been absorbed by the sodium hydroxide.
14. Aspirator containing water.
15. Soda lime tubes.

The whole apparatus was swept out with carbon dioxide free oxygen, before starting the combustion, and was kept free of carbon dioxide from the air throughout the experiment.

A sample of the [^{14}C] sugar, sufficient to give a barium carbonate disc of thickness greater than 30 mg. per cm.^{2}, was weighed out in a platinum boat. Vanadium pentoxide (ca. 10 mg.) was added and the sample was introduced into the open end of the combustion tube (d). The sample was heated in a stream of oxygen, at first gently, then more strongly, for about 20 min. The platinum gauze (5) was heated by the electric furnace (6) throughout the combustion. The carbon dioxide evolved was absorbed in the sodium hydroxide solution (10).

After 20 min., the oxygen stream was cut off, by closing the three-way tap (8). The sodium carbonate solution was run into the precipitation vessel (11), and the absorbtion vessel (10) was washed with a little

carbon dioxide free water. M-Ammonium chloride solution (5 ml.) was added through the funnel (9), in order to neutralise the sodium carbonate solution. The carbonate was then precipitated by the addition of M-barium chloride (5 ml.).

The precipitated barium carbonate was allowed to settle for at least 10 min., then collected as a disc by filtration under reduced pressure. The precipitate was dried at 100° under vacuum.

The β-emission of the ^{14}C in the disc was determined using a Geiger-Muller end-window counter. Comparison of this emission with the β-emission from a standard sample of poly-($[^{14}C]$ methyl)-methacrylate enabled the specific radioactivity of the sample to be calculated.

(b) Wet combustion

The combustion tube, heaters, and wide bore capillary tube (3-7) used in the dry combustion were replaced by the standard apparatus shown in Fig. 8b.

16. Combustion flask.
17. Dropping funnel containing combustion fluid (chromic acid prepared by adding syrupy phosphoric acid and fuming sulphuric acid to chromium trioxide).
18. Nitrogen lead.

19. Condenser.

20,22. Traps containing glass wool to absorb sulphur trioxide.

21. Trap containing chromic acid to oxidise sulphur dioxide to the trioxide.

23. Screw clips.

The sample to be combusted, dissolved in carbon dioxide free water (1-3 ml.), was placed in the flask (16). The apparatus was swept out with carbon dioxide free nitrogen, and carbonate free N-sodium hydroxide (5 ml.) was placed in the absorbtion vessel (10) and diluted, as in the dry combustion. The tap of the dropping funnel and the screw clips (23), above the funnel were closed. Combustion fluid (15 ml.) was poured into the dropping funnel, which was then closed to the atmosphere. The screw clip (23) and the tap of the dropping funnel were opened, and the combustion fluid allowed to drop slowly into the flask (16). When all the fluid had been added the mixture was refluxed gently for 20 min. The tap (8) was then closed and the precipitation, filtration, and estimation of the β-emission from the barium carbonate carried out as described for the dry combustion.

Expt. 10. Measurement of specific radioactivities by the infinitely thin film method (64, 65).

Three drops of a 0.01% 'Teepol' solution were placed on a polished aluminum disc (diam. 2.5 cm.). The disc was flooded with water, and the 'Teepol' solution evaporated to dryness under an infra red lamp, in a stream of air. An aqueous solution of the compound under investigation, containing 10-600μg., was placed on the disc, and the disc was flooded with water, in order to spread the compound evenly over the surface. The solution was evaporated to dryness as described above. The β-emission was measured using a Geiger-Muller end-window counter. The specific radioactivity of the sample was estimated by comparison of this β-emission with the emission from [^{14}C] sugars of known specific activity. Preliminary experiments showed that, using quantities of sugars in the range 10-600μg., there was a linear relation between the amount of the [^{14}C] sugar on the disc and the β-emission.

The estimated counting error in any measurement of β-emission was $\pm$ 2%.

SECTION I

EXPERIMENTAL

Isolation and characterisation of a [^{14}C] trisaccharide synthesised by Betacoccus arabinosaceous in a lactose-[^{14}C] sucrose medium.

Expt. 11. Production of a [^{14}C] trisaccharide by B. arabinosaceous in a lactose-[^{14}C] sucrose medium.

[^{14}C] sucrose, generally labelled, was supplied by the Radiochemical Centre, Amersham.

Lactose (10 g.), and [^{14}C] sucrose (2 g., ca. 100 μC.), wereadded to the preparative medium (100 ml., Expt.1), the medium adjusted to pH 7.0, and sterilized by filtration. The medium was inoculated with B. arabinosaceous and incubated at 25° for 4 days.

Paper chromatography of the medium, in solvent (a) revealed components with the R_F values of lactose (present throughout the incubation period), fructose (only present at 24 hours after the start of the incubation), glucose (present after 24 hours incubation), and O-β-D-galactopyranosyl-(1→4)-O-[α-D-glucopyranosyl-(1→2)]-D-glucose, (present after 24 hours incubation). The sugars were detected with spray (a). Radiochromatograms showed ^{14}C activity in the components corresponding

to glucose, fructose, sucrose, and O-β-D-galactopyranosyl-(1→4)-O-[∝-D-glucopyranosyl-(1→2)]-D-glucose.

Expt. 12. Isolation and chromatographic analysis of the [^{14}C] trisaccharide (trisaccharide A).

The culture medium (Expt. 11) was adjusted to pH 7.0 and heated at 90° for 10 min. The bacterial cells were removed by centrifuging (4,500 r.p.m., 30 min.). The dextran was precipitated by the addition of ethanol (100 ml.), and removed by centrifugation (4,500 r.p.m., 30 min.). After removal of the ethanol, the oligosaccharide mixture was adsorbed on to the top of a charcoal-'Celite' column (l. 40 cm., diam. 4 cm., Expt. 6). Washing with water (2.5 l.) eluted the monosaccharides and salts. Elution with 5% aqueous ethanol (2.5 l.) removed lactose. Trisaccharide A (351 mg.) was obtained by elution with 10% aqueous ethanol (4 l.).

Paper chromatography of trisaccharide A, in solvent (a) ($R_{glucose}$ 0.11), and solvent (c), and of its benzylamine complex, in solvent (f), indicated that it was a single compound. Paper electrophoresis in borate buffer (Expt. 5) again showed a single component with M_g 0.33. It could be detected with sprays (a) and (b).

With aniline hydrogen phthalate (b) a yellowish colour was obtained. Trisaccharide A could not be detected with alkaline triphenyl tetrazolium chloride (e). In all of these tests the behaviour of trisaccharide A was identical with that of authentic O-β-D-galactopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→2)]-D-glucose.

Expt. 13. Estimation of the specific radioactivity of trisaccharide A.

The specific radioactivity of trisaccharide A was estimated by the infinitely thick disc method, by dry combustion (Expt. 9). The results were as shown below:

	Counts per min., corrected.	Specific radioactivity μC/g.-atom of carbon.
Standard	3,389	238
Trisaccharide A	7,446	523

Thus the specific radioactivity of trisaccharide A was 523 μC/g.-atom of carbon, or 9,417 μC/mole of trisaccharide.

Expt. 14. Hydrolysis of trisaccharide A.

Trisaccharide A (4.7 mg.) was hydrolysed in 1.5N-sulphuric acid at 100° for 4 hours. Paper chromatography of the hydrolysate, in solvent (a), showed the

presence of components corresponding to glucose and galactose. Radiochromatograms revealed ^{14}C in the glucose spot only.

Partial hydrolysis of trisaccharide A (8.4 mg.), in N-sulphuric acid, at 90°, for 1 hour, gave components corresponding to glucose, galactose, kojibiose, lactose, and trisaccharide A, on chromatography in solvent (a). Radiochromatograms revealed ^{14}C in the glucose, kojibiose, and trisaccharide A components (Fig. 9).

Expt. 15. Action of emulsin on trisaccharide A.

Almond emulsin (β-glycosidase) was prepared from sweet almonds according to the method of Bourquillot (100).

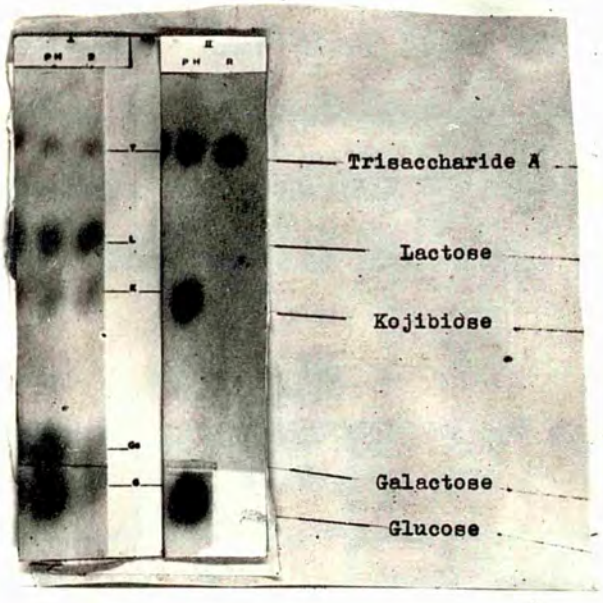
Trisaccharide A (1 mg.) was added to almond β-glycosidase solution (0.1 ml.). Radiochromatographic analysis of the digest, in solvent (a), after incubation at 37°, for 72 hours, revealed components corresponding to [^{14}C] trisaccharide A, (ca. 70%), [^{14}C] kojibiose, galactose and glucose (trace, radioactivity not determined). Under similar conditions lactose was completely hydrolysed, and maltose gave a trace of glucose.

Expt. 16. Development of a solvent for the chromatographic separation of phenyl osazones.

The phenyl osazones of glucose, galactose, and

Fig. 9.

CHROMATOGRAM AND AUTORADIOGRAPH OF THE PARTIAL HYDROLYSATE OF TRISACCHARIDE A.



I Chromatogram sprayed with aniline hydrogen phthalate.
II Autoradiograph.
P.H. Partial hydrolysate.
R. Reference compounds.

lactose were prepared by dissolving the sugar (glucose or galactose, 2 g., lactose, 4g.) in water (10 ml.). The mixture was allowed to stand overnight at 0-2°. The precipitated osazones were filtered, washed with acetic acid (5%), and aqueous ethanol (5%), and dried over phosphorus pentoxide and sodium hydroxide. The products could be recrystallised from aqueous ethanol.

Yields: glucosazone 1 g., m.p. 199-200°.
galactosazone 0.9 g., m.p. 185-187°.
lactosazone 2.8 g., m.p. 194-199°.

The separation of glucosazone and lactosazone, by paper chromatography, in a number of different solvents was investigated. The results are shown in Table II.

TABLE II

Solvent (organic phase used where 2 layers formed).	Developing time, hours.	Result
Acetone-water (4:1)	3	Poor separation.
Pyridine-water-amyl alcohol (1:1:1)	8	Poor separation.
n-Butanol-ethanol-water (4:1:5).	8	Poor separation
Acetone-water (1:1)	3.5	Poor separation, considerable streaking.
Water	2.5	Glucosazone remained on starting line, lactosazone diffused over paper.
Pyridine-amyl alcohol-water-benzene (2:2:2:1).	8	Poor separation.

TABLE II (cont.)

Solvent (Organic phase used where 2 layers formed).	Developing time, hours	Result
Pyridine-water-nitrobenzene (1:1:1)	3	Good separation.
Pyridine-water-nitrobenzene (1:3:4)	3	Lactosazone moved very little, glucosazone diffused over the paper.
Aqueous ethanol (5%)	1	Poor separation, considerable streaking.
Pyridine-water-benzene (1:3:5)	3	Good separation, considerable streaking.
Pyridine-water-benzene (1:1:1)	3.5	Poor separation.
Pyridine-water-benzene (1:1:2)	2	Some separation, considerable streaking.
Pyridine-water-benzene (2:2:3)	1.5	Some separation, some streaking.
Pyridine-water-benzene (4:4:5)	2.5	Good separation, slight streaking.

The solvent pyridine-water-benzene (4:4:5), organic phase, solvent (b), was used for the separation of glucosazone (R_f 0.69) and lactosazone (R_f 0.385), in all later experiments. Glucosazone and galactosazone could not be separated by this solvent.

The osazones could be detected on paper with visible and u.v. light, and with spray (a).

Expt. 17. Separation of glucosazone and lactosazone by column chromatography.

(a) Cellulose column.

An attempt was made to separate glucosazone and lactosazone, by chromatography on a cellulose column (l. 55 cm., diam. 2 cm.). Glucosazone (52.2 mg.) and lactosazone (51.9 mg.), dissolved in boiling ethanol (5 ml.), were adsorbed on dry cellulose. Water (100 ml.) was added to the mixture which was then freeze dried. The resulting powder was slurried in water, and added to the top of the column. The column was eluted with water (6 l.), aqueous acetone (10%, 2 l.) and aqueous acetone (20%, 2 l.). The aqueous eluate contained a component, corresponding to a monosaccharide osazone on paper chromatography. No disaccharide phenylosazone was eluted from the column.

(b) 'Celite' column.

Glucosazone and lactosazone were separated on a 'Celite' column according to the method of Lemieux et al. (62). Dry, acid washed, 'Celite' was wetted with benzene-saturated water, and slurried with solvent (b). A perforated porcelain disc, covered with a filter paper disc of slightly greater diameter than the column, was

placed at the bottom of the column, and the slurry was added, and packed carefully, until the required length was obtained. The column was closed with a disc of filter paper.

Glucosazone (12 mg.) and lactosazone (22 mg.) were dissolved in solvent (b) (3.5 ml.) and added to the top of the column (l. 28 cm., diam. 1.7 cm.). The osazones, which separated as distinct bands, were eluted from the column with solvent (b), and freeze dried.

Expt. 18. Preparation and separation of phenyl osazones from trisaccharide A.

Trisaccharide A (74.7 mg.) was dissolved in water (1.25 ml.) and a solution of phenyl hydrazine (0.165 g.) in glacial acetic acid (0.165 ml.) was added. The solution was heated at 100° for 2.5 hours, during which time crystals of phenyl osazones appeared. After standing overnight at 0-2°, the crystalline product (10 mg.) was filtered, washed with ice-water, and dried over phosphorus pentoxide.

Paper chromatography of the product, and of the mother liquor, in solvent (b), showed the presence of components corresponding to glucosazone and lactosazone. The mother liquor appeared to contain other components,

moving in the glucosazone region.

The crystalline phenyl osazones were dissolved in solvent (b) (2 ml.), and were separated on a 'Celite' column (Expt. 16, 1.40 cm., diam. 1.7 cm.). Chromatographically pure fractions corresponding to mono- and disaccharide phenyl osazones were isolated, and freeze dried. A second fraction of disaccharide phenyl osazone was isolated from the mother liquor by fractionation on a 'Celite' column.

Expt. 19. Estimation of the radioactivities of the mono- and disaccharide phenyl osazones from trisaccharide A.

A sample of the monosaccharide phenyl osazone (1.32 mg.) was diluted with inactive glucose phenyl osazone (7.36 mg.), and the specific radioactivity determined by the infinitely thick disc method, by dry combustion (Expt. 9). The specific radioactivity of a sample of the disaccharide phenyl osazone (0.71 mg.) from trisaccharide A, diluted with inactive lactose phenyl osazone (7.62 mg.), was estimated in the same way. The results were as shown on p. 101.

From these values the specific radioactivity of the monosaccharide phenyl osazone from trisaccharide A was

calculated to be 4203 μC per mole of monosaccharide. The specific radioactivity of the disaccharide phenyl osazone calculated in the same way was 1184 μC per mole of disaccharide.

	Counts per min. corrected.	Specific radioactivity μC per g.-atom of carbon
Standard	5402	238
Monosaccharide phenyl osazone	507.2	35.48
Standard	3362	238
Disaccharide phenyl osazone	65	4.6

Expt. 20. Treatment of lactose with phenyl hydrazine.

Lactose (68.2 mg.) was dissolved in a solution of acetic acid (1.65 ml.) in water (12.5 ml.). Phenyl hydrazine (0.22 g.) was added, and the solution was heated at 80° for 2.5 hours. After standing overnight at 0-2° the solid product was filtered, and analysed by paper chromatography in solvent (b). A component corresponding to lactose phenyl osazone, and a trace of a component corresponding to glucosephenyl osazone were present.

Expt. 21. Separation of glucose, galactose, and sorbitol by paper chromatography.

The separation of glucose, galactose, and sorbitol, by paper chromatography, in a number of different solvents

was investigated. The results are shown in Table III.

TABLE III

Solvent, organic phase used where 2 layers formed	Developing time, hours	Results
isoamyl alcohol-dioxan-water (5:5:2)	72	Glucose and galactose not separated.
Phenol saturated with water	8	Poor separation.
"	18	Some separation.
n-Butanol-ethanol-water (4:1:5).	84	Glucose and sorbitol did not separate.
nButanol-pyridine-water (6:4:3).	18	Poor separation.
Ethyl acetate-acetic acid-saturated aqueous boric acid (9:1:1).	3	No separation of glucose and galactose.
"	18	No separation of glucose and galactose.
Solvent (a) (Expt. 4) followed by solvent (d)	72 3	Separation, but some streaking
Solvent (d), followed by n-butanol-pyridine water (6:4:3).	6 18	Poor separation.
Solvent (d), followed by solvent (a)	18	Some separation, some streaking of sorbitol.
nButanol-pyridine-water-saturated aqueous boric acid (6:4:2:1)	48	Good separation, some streaking of sorbitol.

In n-butanol-pyridine-water-saturated aqueous boric acid (6:4:3:1), solvent (e), which was generally used for the separation of glucose, galactose, and sorbitol, the $R_{glucose}$ values of galactose and sorbitol were 0.85 and 0.50 respectively.

It was found to be difficult to detect the sorbitol, with spray (a) on papers which had been developed with solvents containing boric acid, but it could be detected with spray (d).

Expt. 22. Reduction of trisaccharide A.

Trisaccharide A (55.8 mg.), dissolved in water (7.3 ml.) was reduced with potassium borohydride (37.5 mg.) at room temperature, for 20 hours. The excess borohydride was destroyed by the addition of 3N-sulphuric acid (0.4 ml.) and the solution made up to 15 ml. with water. A portion (5 ml.) of the resulting solution was adjusted to pH 7 and passed through a column of Permutit 'Biodeminrolit' resin (15 g.), pretreated with carbon dioxide. The eluate was evaporated to dryness in vacuo. The residue was distilled with methanol (3 x 10 ml.) in vacuo, in order to remove borate ions, dissolved in water (10 ml.) and freeze dried. Paper chromatography of the reduced trisaccharide A in solvent (c) showed that

it moved as single component ($R_{glucose}$ 0.51). It could be detected with sprays (a) and (d), but not with spray (b). Radiochromatograms showed that it moved as a single radioactive compound.

Expt. 23. Hydrolysis of trisaccharide A alcohol

Another portion (10 ml.) of the solution of the reduced trisaccharide A (Expt. 22) was adjusted, with 3N-sulphuric acid (2.4 ml.) to an acid normality of 0.5N, and heated at 100° for 4 hours. The solution was neutralised by passing it through a column of Permutit 'Biodeminrolit' (45 g.), pretreated with carbon dioxide, and concentrated in vacuo. Paper chromatography of the concentrated solution in solvent (e) revealed components with the R_f values of glucose, galactose, and sorbitol. 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.

The hydrolysate was dissolved in water (0.4 ml.), and a portion (0.2 ml.) was fractionated on Whatman No. 3 paper in solvent (e). A pure glucose fraction was obtained. The eluted solutions containing glucose and galactose, respectively, were freeze dried, and distilled with

methanol (3 x 25 ml.) *in vacuo* The galactose fraction was further purified by preparative paper chromatography in solvent (a). A pure sorbitol fraction was obtained by preparative paper chromatography on Whatman No. 3 paper, in solvent (d) ($R_{glucose}$ 2.2), and was freed from boric acid by the above method.

Expt. 24 *Estimation of the radioactivities of glucose, galactose, and sorbitol from trisaccharide A alcohol.*

The glucose, galactose, and sorbitol fractions (Expt. 23) were separately dissolved in water (5 ml.). The concentrations of glucose and galactose in these solutions were estimated by the method of Somogyi and Nelson (Expt. 7). The concentration of sorbitol was estimated by oxidation with sodium periodate, and determination of the formaldehyde produced with chomotropic acid (Expt. 7). The radioactivities of the glucose, galactose, and sorbitol were determined by the infinitely thin film method (Expt. 10), using aliquot portions of the analysed solutions of these compounds. The results are shown in Table IV.

TABLE IV

Compound	Wt. (μg.)	Counts /min.	Counts/ min./ μ mole.	Mean Counts / min./μ mole.
D-glucose	32.83	168	922	
"	52.02	276	956	952
"	58.83	319	977	
D-galactose	52.12	1	6	6
Sorbitol	53.10	5	17	17

Expt. 25. Estimation of the specific radioactivity of trisaccharide A by the infinitely thin film method.

Trisaccharide A (10.6 mg.) was dissolved in water and the solution diluted to 25 ml. in a standard flask. The radioactivity of the trisaccharide was estimated by the infinitely thin film method, using aliquot portions of this solution.

Standard [^{14}C] glucose (2.79 mg.) of specific radioactivity 11,236 μC/ mole, measured by the infinitely thick disc method, by dry combustion (Expt. 9), was also dissolved in water and the solution made up to 25 ml. in a standard flask. The β-emission from the standard [^{14}C] glucose was determined by the infinitely thin film method as above. The results obtained with

trisaccharide A and the standard [^{14}C] glucose are shown in Table V.

TABLE V

Compound	Wt. (μg.)	Counts/ min.	Counts/ min./ μ mole.	Mean counts/ min./μ mole.
D-glucose (standard)	44.19	284	1158	1156
"	66.63	427	1154	
Trisaccharide A	103.3	194	947	
"	221.0	431	984	
"	419.0	795	957	961
"	508.0	965	958	
"	607.0	1154	959	

The specific radioactivity of trisaccharide A, calculated by comparison of the β-emission from the trisaccharide with that from the standard [^{14}C] glucose was 9341 μC/ mole (cf. Expt. 13) The values for the β-emission obtained from trisaccharide A demonstrate clearly the linear relationship between the amount of sugar on the disc and the β-emission.

SECTION II

EXPERIMENTAL

Investigation of the acceptor specificity of dextransucrase.

Expt. 26. Preparation of dextransucrase.

Active enzyme preparations were extracted from cultures of B. arabinosaceous by alcohol precipitation (Expt. 2). The activities of the enzyme preparations were estimated by measuring the amount of fructose produced in 3 hours, at 25°, in the presence of an excess of sucrose (Expt. 3). Several batches of enzyme were extracted. The yields and activities of the different batches are shown in Table VI.

TABLE VI

	Incubation time, hours.	Yield mg.	Activity units / mg.
Batch I	17	214	0.113
Batch II	48	154	0.128
Batch III	17	8200	0.391
Batch IV	17	400	0.296

Expt. 27. Oligosaccharide production in enzyme digests containing sucrose and added sugars, or related compounds.

Solutions containing enzyme (5 mg.), sucrose (10 mg.), and added sugar, or related compound (40 mg.), in 0.05M-acetate buffer (0.5 ml., pH 5.0) were incubated at 25° for 25 hours. Control digests containing the enzyme and the added sugar, or related compound, were incubated at the same time. Each batch of enzyme was also incubated with sucrose alone.

The digests were examined by paper chromatography, in solvents (a) and (g), and by electrophoresis in borate buffer (Expt. 5). The digests were incubated with invertase (B.D.H. concentrate, 2 drops) for 3 hours at room temperature, in order to hydrolyse any sucrose present at the end of the incubation period, and again examined by paper chromatography, and electrophoresis. The sugars were detected with sprays (a), (b), and (e). The results are summarised in Table VII.

TABLE VII

Added sugar.	Batch No. of enzyme preparation	R_g values of new sugars.	Spray reagents (a)	(b)	(e)
D-galactose	I	0.46	+		+
"	"	0.39	+		?
D-mannose	"	0.41	+		+
"	"	0.48	+		–
L-Rhamnose	"	0.44	+		+
"	"	0.51	+		–
L-Fucose	"	0.43	+		+
"	"	0.52	+		–
L-Sorbose	"	0.36	+		+
"	"	0.45	+		–
D-Arabinose	"	0.43	+		+
"	"	0.62	+	+ (pink)	–
D-Ribose	"	0.54	+		+
"	"	0.72	+	+ (pink)	–
D-Xylose	"	0.47	+		+
"	"	0.71	+	+ (pink)	–
Methyl α-D glucopyranoside	II	0.18	+		
	"	0.28	+		
Methyl β-D-glucopyranoside	"	No new sugars detected.			

TABLE VII (cont.)

Added sugar.	Batch No. of enzyme preparation.	R_g values of new sugars.	Spray reagents		
			(a)	(b)	(c)
Sorbitol	III	No new sugars detected.			
myoinositol	I	No new sugars detected.			

Expt. 28. Separation and hydrolysis of oligosaccharides produced in dextransucrase digests containing sucrose and added sugars, or related compounds.

The oligosaccharides obtained in dextransucrase digests containing sucrose and added sugars, or related compounds (Expt. 27), were separated by preparative paper chromatography on Whatman No. 1 filter paper, in solvent (a). The inositol fraction was separated from the digest to which inositol had been added. The enzyme (Batch III), which was present in the digest to which sorbitol was added, produced oligosaccharides in digests containing sucrose alone. These sugars were also present in the sorbitol digest, and were isolated by preparative paper chromatography. The enzyme batches I and II produced no oligosaccharides in the presence of sucrose alone.

The freeze dried oligosaccharide fractions, and

the inositol fraction, were separately dissolved in water (1 ml.), and hydrolysed by heating in boiling water with Amberlite IR-120 resin (0.1 g.) for 22 hours (101). The hydrolysates were concentrated, and examined by paper chromatography in solvents (a) and (j). The results are shown in Table VIII.

TABLE VIII

Sugar added to dextransucrase-sucrose digest.	Compounds with R_f values identical with those found in oligosaccharide hydrolysate.
D-Mannose	Glucose, mannose.
L-Rhamnose	Glucose, trace of rhamnose and fructose.
L-Fucose	Glucose.
L-Sorbose	Glucose, and sorbose.
D-Arabinose	Glucose, trace of L-arabinose.
D-Ribose	Glucose, ribose.
D-Xylose	Glucose, xylose.
myoInositol	Glucose, myoinositol.
Sorbitol	Glucose, trace of fructose.

Expt. 29. Oligosaccharide production in enzyme digests containing sucrose and methyl α- or β-D-ribopyranoside.

A mixture of methyl α-D-ribopyranoside and methyl

β-D-ribopyranoside was provided by Dr. G. R. Barker of the University of Manchester. The ribopyranosides were separated by preparative paper chromatography in solvent (a).

Enzyme digests containing dextransucrase (Batch III, 1%), sucrose (2%), and methyl α-, or β-D-ribopyranoside (8%) were made up as before (Expt. 27) in 0.05M-acetate buffer (pH 5.0). The digests were incubated at 25° for 24 hours, and analysed by paper chromatography, in solvent (a). The sugars were detected with sprays (a) and (d). The enzyme, Batch III, produced oligosaccharides in a control digest containing sucrose alone, and the same oligosaccharides were produced in the presence of the methyl ribopyranosides. Traces of new sugars running in the same region as glucose and fructose, appeared to be present in the digests containing the added sugars.

The sugars running in the glucose and fructose region were separated from the digests containing methyl α- and β-D-ribopyranosides by preparative paper chromatography. Each fraction was hydrolysed with Amberlite IR-120 resin (Expt. 28). The hydrolysates were analysed by paper chromatography in solvent (i). Traces of ribose appeared to be present in the hydrolysate

from the digest containing methyl β-D-ribopyranoside. No ribose could be detected in the hydrolysate from the methyl α-D-ribopyranoside digest.

SECTION III

EXPERIMENTAL

Investigation of oligosaccharides produced by Betacoccus arabinosaceous in a medium containing sucrose and galactose

Expt. 30. Preliminary investigation of oligosaccharide production by Betacoccus arabinosaceous in media containing sucrose and galactose.

The standard preparative medium (Expt. 1), containing sucrose (2%), and galactose (10%), was inoculated with B. arabinosaceous, and incubated at 25° for 4 days. The medium was examined by paper chromatography at the end of this period, and the oligosaccharides produced were compared with those produced in enzyme digests containing sucrose and galactose (Expt. 27). Oligosaccharides with the same R_f values were produced in the culture and the enzyme digests.

The preparative medium (Expt. 1) containing sucrose (2%) was inoculated with the organism, and incubated at 25° for 60 hours, in order to obtain a high concentration of bacteria. To one portion (1 ml.) of this culture galactose (0.15 g.) and sucrose solution (6%, 0.5 ml.) were added (culture A). To a second portion (1 ml.) galactose (0.15 g.) and sucrose solution (6%, 0.05 ml.)

were added (culture B). The cultures were incubated at 25° for 72 hours. Sterile sucrose solution (6%, 0.05 ml.) was added to culture B at intervals of ca. 7 hours throughout the incubation period. Thus the final volume of this culture was 1.5 ml. A portion of the preparative medium (Expt. 1), containing sucrose (2%) and galactose (10%) was inoculated with the organism and incubated at the same time (culture C).

At the end of the incubation period the cultures were examined by paper chromatography, in solvent (a), the sugars being detected with spray (a). In all the cultures production of two or three new sugars, running in the disaccharide region, had taken place. The relative amounts of these sugars were estimated by inspection of the chromatograms, and by measuring the absorption of light by each chromatogram, with an Eel 'Scanner'. Culture B appeared to contain a higher proportion of disaccharides than culture A and both these cultures contained greater amounts of disaccharides than culture C.

Expt. 31. Isolation of oligosaccharides produced by Betacoccus arabinosaceous in a medium containing sucrose and galactose.

The preparative medium (Expt. 1, 150 ml.) was

inoculated with a strongly growing culture of the organism (0.05 ml.), and incubated at 25° for 60 hours. Galactose (20 g.), which had been purified by charcoal column chromatography (Expt. 6), was added to the culture and the incubation was continued for a further 72 hours. Portions (2.5 ml.) of a sterile sucrose solution (6%) were added to the culture, at intervals of 4 hours, throughout this second incubation period. Paper chromatographic analysis of the culture in solvent (a), showed the presence of glucose, fructose, galactose, sucrose, and oligosaccharides running behind sucrose. Sucrose was still present after incubation at 25° for a further 60 hours. In order to remove sucrose the culture was adjusted to pH 7, by the addition of sodium hydroxide, and treated with invertase (B.D.H. concentrate, 2 ml.). After incubation at room temperature for 1.5 hours the medium was examined by electrophoresis, in borate buffer (Expt. 5, pH 10), and paper chromatography. No sucrose could be detected.

The dextran and bacterial cells were removed from the medium by precipitation with ethanol (200 ml.) and centrifugation (1 hour. 5,000 r.p.m.). The supernatant liquid was filtered through a sintered glass funnel

(No. 4), freed from ethanol, and concentrated under reduced pressure. The concentrated solution (50 ml.) was adsorbed on to the top of a charcoal-'Celite' column (l. 30 cm., diam. 8 cm., Expt. 6). The monosaccharide fraction was eluted with water (4 l.) and the disaccharides with aqueous ethanol (5%, 6 l.), and possible tri- and tetrasaccharides with aqueous ethanol (10%, 6 l.) and aqueous ethanol (15%, 10 l.), respectively. The presence of sugars in fractions from the column was detected by adding portions (0.5 ml.) of selected fractions to a solution of anthrone in concentrated sulphuric acid (0.2%, 0.5 ml.).

The oligosaccharide fractions were concentrated, freeze dried, and examined by paper chromatography in solvents (a) and (g), and by electrophoresis in borate buffer (Expt. 5, pH 10). Paper chromatography showed the presence of one disaccharide, with R_g 0.46, and two disaccharides with R_g ca. 0.38. Electrophoresis showed the presence of three disaccharides (A, B, and C) with M_g values 0.60, 0.44, and 0.34, respectively. Higher sugars, possibly a tri- and tetrasaccharide, with R_g values 0.18 and 0.16 respectively, were also obtained.

Expt. 32. Isolation of disaccharides obtained from a galactose-sucrose culture of Betacoccus arabinosaceous.

A charcoal-'Celite' column (l. 41 cm., diam. 2.5 cm.) was prepared (Expt. 6) and washed with 0.1M-borate buffer (pH 10) until the pH of the washings was the same as that of the buffer (73). The disaccharide fraction (829 mg., Expt. 31) was dissolved in water (8 ml.) and adsorbed on to the top of the column. The column was eluted with 0.1M-borate buffer (2.5 l., pH 10), followed by borate buffer containing ethanol (2.5%, 3 l.). The eluted fractions were neutralised with Amberlite IR-120 resin (H^+), or with Permutit 'Biodeminrolit' resin in the carbonate form. Selected fractions of the eluate were evaporated to dryness and distilled with dry methanol (2 x 5 ml.). These fractions were analysed by paper chromatography in solvent (g), and by electrophoresis in borate buffer (Expt. 5, pH 10). Separation of the disaccharides into fractions containing not more than two chromatographically separate disaccharides was achieved in this way.

The disaccharides were finally separated, as chromatographically pure sugars, by preparative paper

chromatography in solvent (a), on Whatman No. 3 filter paper. The disaccharides were eluted from the paper, freeze dried, and purified by dissolution in dry methanol, filtration, and freeze drying from aqueous solution, after removal of the methanol.

Analysis of the three disaccharides isolated, by paper chromatography in solvent (a), and electrophoresis in borate buffer (Expt. 6, pH 10), gave the following results:

	Yield	Rg value	Mg value
Disaccharide A	32.7 mg.	0.39	0.60
Disaccharide B	47.6 mg.	0.46	0.44
Disaccharide C	40.5 mg.	0.36	0.34

All the sugars could be detected with acetone silver nitrate-alcoholic sodium hydroxide, spray (a), but the spot due to disaccharide B appeared at the same rate as that due to α,α-trehalose, and at a much slower rate than those due to disaccharides A or C, or maltose. Disaccharides A and C, but not disaccharide B, could be detected with aniline hydrogen phthalate, spray (b), and triphenyl tetrazolium chloride, spray (e). Disaccharides A and B gave a faint greyish colour with diphenylamine-aniline, spray (f). Disaccharide C gave a distinct blue colour with this reagent.

Expt. 33. Hydrolysis of disaccharides A, B, and C.

Disaccharides A, B, and C (1 mg. portions) were separately dissolved in N-sulphuric acid (0.5 ml.), and heated at 100° for 4 hours. The solutions were neutralised with Permutit 'Biodeminrolit' resin, in the carbonate form, and analysed by paper chromatography, in solvent (i). The hydrolysates of disaccharides B and C contained components corresponding to glucose and galactose, in approximately equal amounts. Disaccharide A hydrolysate contained components corresponding to glucose and galactose and a trace of a component with the same R_f value as fructose. In this hydrolysate there appeared to be less galactose than glucose.

Expt. 34. Estimation of the carbohydrate content of disaccharides A, B, and C.

'Analar' D-glucose, and D-galactose, (50.1 mg. of each, accurately weighed) were dissolved in water (1 l.). Disaccharides A, B, and C (1-2 mg., accurately weighed) were separately dissolved in water (50 ml.). Portions of these solutions (1 ml.), were treated with a solution of anthrone in sulphuric acid, under the standard conditions (Expt. 7). The carbohydrate content of the disaccharides was calculated by comparison of the

absorption of their solutions, at 625 mμ, with the absorption of the standard solution. The results are shown in Table IX.

TABLE IX

Sugar.	μg./ml.		Uvispek reading.		% Carbohydrate
D-Glucose	50.1	100.2	0.338	0.337	
D-Galactose	50.1		0.336		
Disaccharide A	22.6		0.071		88.6
Disaccharide B	34.1		0.116		96.1
Disaccharide C	24.8		0.075		85.4

Expt. 35. Oxidation of disaccharides A, B, and C with alkaline hypoiodite.

'Analar' D-glucose, and D-galactose (1-4 mg., accurately weighed), dissolved in water (5 ml.), and disaccharides A, B, and C (ca. 2 mg., accurately weighed), dissolved in water (5 ml.), were separately oxidised with alkaline hypoiodite solution (Expt. 7). After acidification of the solutions with 0.25N-sulphuric acid, the liberated iodine was titrated with 0.005N-sodium thiosulphate. A blank (5 ml.), containing no sugar was treated in the same way. The differences between the blank titre, and

and the titre for a reducing sugar, were equivalent to the amount of iodine used up by the reducing sugar. From the standard glucose and galactose solutions a graph relating the aldose to the amount of iodine consumed (thiosulphate equivalent) was plotted. The pure aldose disaccharide equivalents of disaccharides A, B, and C, were calculated from this graph. A second estimation using D-galactose as a standard, and a different thiosulphate solution, was carried out. The results are shown in Table X (a) and (b).

TABLE X (a)

Sugar.	mg.	Thiosulphate titre ml.	Thiosulphate equivalent ml.	Aldose disaccharide equivalent
Blank		25.95		
D-Glucose	3.75	16.87	9.08	
"	2.5	20.45	5.50	
"	1.7	22.02	3.93	
"	0.8	24.03	1.92	
D-Galactose	0.95	23.63	2.26	
"	4.1	16.26	9.63	
Disaccharide A	2.2	25.0	0.95	34.5%
Disaccharide B	2.2	25.77	0.18	5.3%
Disaccharide C	2.95	23.24	3.71	102.4%

TABLE X (b)

Sugar	mg.	Thiosulphate titre ml.	Thiosulphate equivalent ml.	Aldose disaccharide equivalent
Blank		23.42		
D-Galactose	4.3	14.14	9.28	
"	2.9	17.33	6.09	
"	1.45	20.40	3.02	
"	0.95	21.12	2.30	
Disaccharide A	2.05	22.54	0.88	30.8%
Disaccharide B	2.50	23.15	0.27	9.1%
Disaccharide C	2.07	20.83	2.59	110.1%

From the mean values obtained from these two estimations the reducing powers of disaccharides A, B, and C, to alkaline hypoiodite, were calculated to be 32.8%, 7.2%, and 106.3%, respectively.

Expt. 36. Oxidation of disaccharides A, B, and C with an alkaline copper reagent.

Solutions of D-galactose (25-100 μg., in water, 2 ml.) and solutions of disaccharides A, B, and C, (ca. 50 μg. portions, in water, 2 ml.) were heated with the alkaline copper reagent of Nelson (Expt. 7) under the standard conditions. After treatment with the

arsenate-molybdate reagent of Somogyi (Expt. 7) the absorption at 606 mμ was measured on an Eel Absorptiometer. A blank (2 ml.), containing no sugar, was treated in the same way. From the standard galactose solutions a graph relating the absorption to the galactose concentration was plotted. This graph was used to calculate the reducing disaccharide equivalents of disaccharides A B, and C. The results are shown in Table XI.

TABLE XI

Sugar.	μ g.	Absorptiometer reading.	Reducing disaccharide equivalent.
Blank		0.0	
D-Galactose	24.95	11.6	
"	49.90	26.0	
"	74.85	40.5	
"	99.80	53.9	
Disaccharide A	45.2	45.2	61%
"	45.2	45.9	
Disaccharide B	68.2	-0.3	0%
"	68.2	-0.6	
Disaccharide C	49.6	14.8	114%
"	49.6	14.4	

Expt. 37. Reduction of disaccharides A, B, and C, with potassium borohydride.

Disaccharides A, B, and C, (2-3 mg.) were separately dissolved in a solution of potassium borohydride (0.05%, 0.5 ml.). The solutions were allowed to stand overnight at room temperature. The potassium ions were removed by treatment with Amberlite IR-120 resin (H^+). After filtration, the solutions were evaporated to dryness, and distilled with dry methanol (3 x 2 ml.) in order to remove boric acid. The reduction products moved as single components on chromatography in solvent (i). Electrophoresis in molybdate solution (Expt. 5) gave the following results:

Disaccharide A reduction product:- one component, M_S 0.75, trace of component, M_S 0.59.

Disaccharide B reduction product:- one component, M_S 0.0

Disaccharide C reduction product:- one component, M_S 0.62

The reduction products were hydrolysed with 1.5N-sulphuric acid at 100° for 4 hours. The hydrolysates were desalted with Permutit 'Biodeminrolit' resin, in the carbonate form, concentrated, and examined by paper chromatography in solvent (e). The hydrolysates of the reduction products of disaccharides A, and C, contained

components corresponding to glucose and a hexitol. The hydrolysate of the product obtained from disaccharide B contained components corresponding to glucose and galactose.

Expt. 38. Oxidation of disaccharide B with 5 mol. of sodium periodate.

Disaccharide B (10.3 mg., 30.1 μmole) was dissolved in 0.015M-sodium periodate solution (10 ml.) and allowed to stand in the dark at room temperature. Samples (0.4 ml.) were withdrawn at intervals, and diluted to 100 ml. in a standard flask. The periodate uptake was determined by comparing the absorption of this solution, at 223 mμ, measured with a Hilger 'Uvispek', with the absorptions due to 0.06mM-sodium periodate, and 0.06mM-potassium iodate (102). The results are shown in Table XII.

TABLE XII

Time min.	Periodate uptake, moles / mole of disaccharide.
10	1.2
150	2.5
260	2.9
480	3.3
700	3.5
800	3.7

After 24 hours all the periodate had been reduced.

The formic acid produced after 800 min. was estimated by titration of portions (2 ml.) of the solution with 0.01N-sodium hydroxide, after destruction of the periodate ions by adding ethylene glycol (0.04 ml.) and allowing to stand at room temperature for 10 min. It was found that 1.45 mole formic acid/mole disaccharide were produced.

The formaldehyde produced after 800 min. oxidation was estimated with chromotropic acid (Expt. 7) after destruction of the periodate ions with sodium sulphite. It was found that 0.83 mole formaldehyde/mole disaccharide were produced.

As a control experiment, α,α-trehalose dihydrate (10.3 mg., 27.2 μmole) was oxidised under the same conditions. The results are shown in Table XIII.

TABLE XIII

Time min.	Periodate uptake, moles/ mole of disaccharide.
10	0.7
240	3.2
360	3.5
540	3.6
1380	3.6

The formic acid produced after 540 min., was estimated by titration of portions (2 ml.) of the solution with 0.01N-sodium hydroxide, after destruction of the periodate ions by adding ethylene glycol (0.04 ml.) and allowing the solution to stand at room temperature for 10 min. It was found that 1.45 mole formic acid/mole disaccharide were produced.

No formaldehyde could be detected in the oxidation product.

Ethylene glycol (0.04 ml.) was added to the oxidation product of disaccharide B, and the solution was neutralised with 0.01N-sodium hydroxide. The solution was desalted with Permutit 'Biodeminrolit' resin, in the carbonate form, concentrated, and the product was hydrolysed in 1.5N-sulphuric acid (2 ml.) at 100° for 3 hours. The hydrolysate was neutralised with Permutit 'Biodeminrolit' resin, in the carbonate form, concentrated, and examined by paper chromatography in solvents (i) and (k). Sprays (a) and (c) revealed a component corresponding to glycerose.

Expt. 39. Mild acid hydrolysis of Disaccharide B.

Disaccharide B (0.4 mg.) was hydrolysed with 0.25N-sulphuric acid at 100° for 15 min. α,α-Trehalose and

sucrose were hydrolysed under the same conditions as controls. The hydrolysates were desalted and examined by paper chromatography in solvent (i) after neutralisation with barium carbonate. The sugars were detected with spray (a). Disaccharide B had been almost completely hydrolysed (ca. 90%) to glucose and galactose. The sucrose had been completely hydrolysed to glucose and fructose. Only a trace (ca. 10%) of glucose was present in the α,α-trehalose solution (Fig. 10).

Expt. 40. Oxidation of disaccharide B with 1 mole sodium periodate/mole of disaccharide.

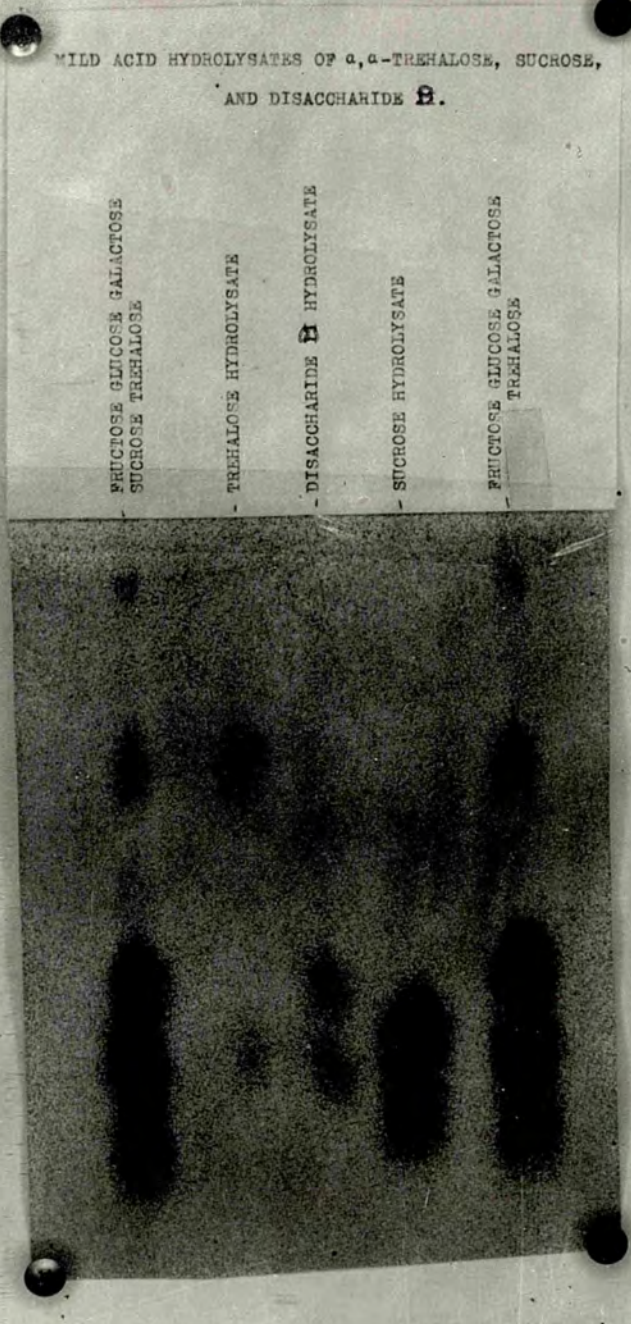
Disaccharide B (0.34 mg.) was dissolved in water (10 ml.) and 0.001M-sodium periodate (1 ml.) was added. The formaldehyde produced was estimated at intervals by the chromotropic acid method (Expt. 7), with results as shown in Table XIV.

TABLE XIV

Time, hours.	Formaldehyde produced moles /mole of disaccharide.
7	0.36
23	0.58
48	0.58

Fig. 10.

MILD ACID HYDROLYSATES OF α,α-TREHALOSE, SUCROSE, AND DISACCHARIDE B.



The solution was desalted with Permutit 'Biodemin-rolit' resin, in the carbonate form, filtered, and reduced with potassium borohydride (0.05 g.) dissolved in water (1 ml.). After standing overnight the potassium ions were removed by the addition of Amberlite IR-120 resin (H^+). The product was filtered, evaporated to dryness, and the boric acid was removed by distillation with dry methanol (3 x 2 ml.). The residue was hydrolysed with 0.25N-sulphuric acid (5 ml.) at 100° for 15 min., desalted with Permutit 'Biodeminrolit' resin, in the carbonate form, concentrated, and analysed by paper chromatography, in solvent (i). The hydrolysate contained components corresponding to D-glucose and L- arabinose (Fig. 11), which could be detected with sprays (a) and (c). Spray (c) gave a pink colour, typical of pentoses with the component corresponding to L-arabinose, and a yellowish colour with the component corresponding to D-glucose.

Expt. 41. Oxidation of disaccharide C with 5 mol. of sodium periodate.

Disaccharide C (10.15 mg., 29.7 μ mole) was dissolved in 0.015M-sodium periodate and allowed to stand at room temperature in the dark. The periodate uptake was measured at intervals by the standard technique (Expt. 38).

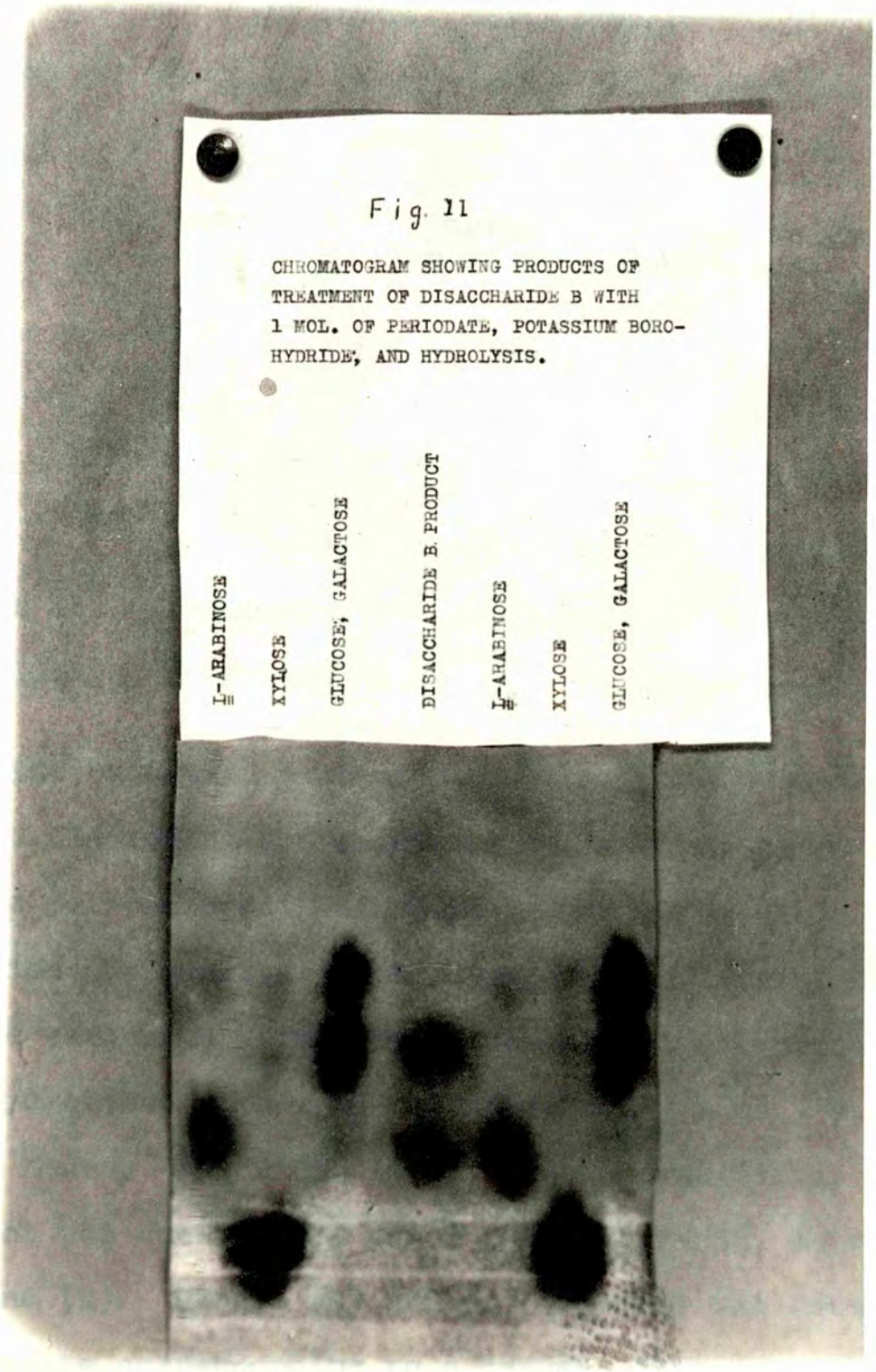


Fig. 11

CHROMATOGRAM SHOWING PRODUCTS OF TREATMENT OF DISACCHARIDE B WITH 1 MOL. OF PERIODATE, POTASSIUM BOROHYDRIDE, AND HYDROLYSIS.

The formic acid and formaldehyde produced, were estimated (Expt. 38) after 430 min. The results are shown in Table XV.

TABLE XV

Time, min.	Periodate uptake, moles/ mole of disaccharide.
10	1.1
150	2.5
250	2.7
430	2.9

Formic acid produced:- 1.05 moles/mole of disaccharide.
Formaldehyde produced:- 0.17 moles/mole of disaccharide.

The oxidation product of disaccharide C was neutralised with 0.01N-sodium hydroxide, after destruction of the excess periodate with ethylene glycol (0.04 ml.). The solution was desalted with Permutit 'Biodeminrolit' resin in the carbonate form, concentrated, and the product was hydrolysed with 1.6N-sulphuric acid (1 ml.) at 100° for 3 hours. After desalting, and concentration, the product was analysed by paper chromatography, in solvents (i) and (k). Sprays (a) and (c) revealed the presence of components corresponding to a hexose, threose, and glycerose.

Expt. 42. Attempted preparation of a methyl furanoside of disaccharide C (84).

Disaccharide C (1 mg.) was dissolved in a solution of hydrogen chloride in dry methanol (4%, 0.5 ml.). The solution was analysed by paper chromatography in solvent (i), and allowed to stand at room temperature for 90 min. At the end of this period the solution was neutralised by the addition of silver carbonate, and again analysed by paper chromatography. No methyl furanoside production could be detected. Control experiments, carried out on a mixture of maltose and nigerose, showed that a methyl furanoside derivative of nigerose, but not of maltose, was produced under these conditions.

Expt. 43. Oxidation of disaccharide C with bromine, followed by oxidation with sodium periodate (85).

Disaccharide C (2 mg.) was dissolved in water (0.5 ml.) and sodium bicarbonate (64.4 mg.) was added. Bromine (0.4 ml.) was added, and the solution was allowed to stand at room temperature, in the dark, for 48 hours. The excess bromine was removed by drawing air through the solution.

The aldobionic acid produced was separated from

the inorganic salts by paper chromatography on Whatman No. 3 paper in solvent (a). The eluted solution of the aldobionic acid was made up to 14.6 ml. with sodium periodate solution, to give a final concentration of 0.4 mM-sodium periodate, and allowed to stand at room temperature, in the dark, overnight.

A sample of the product (1 ml.) was heated to 90° and phenyl hydrazine hydrochloride solution (1%, 5 drops) was added. A pink colour developed. Under the same conditions a pink colour was developed with the bromine-periodate oxidation product of lactose. With laminaribiose the results were not conclusive. A faint pink colour may have been present, but this was much less intense than that due to disaccharide C or lactose.

Expt. 44. Action of enzymes on disaccharides A, B, and C. (a) Action of β-glycosidase. Solutions of disaccharides A, B, and C (1%), were sealed in capillary tubes with equal volumes of almond β-glycosidase (Expt. 15), and incubated at 35° for 3 days. Analysis by paper chromatography in solvent (a), at the end of this period showed that none of the disaccharides had been hydrolysed. Control digests containing the enzyme and lactose, maltose, or cellobiose, were incubated at the same time. After 3

days cellobiose had been completely hydrolysed, and lactose partially hydrolysed. No hydrolysis had taken place in the digest containing maltose.

(b) Action of yeast α-glycosidase.

A yeast α-glycosidase preparation was obtained by grinding fresh baker's yeast (2 oz.) with disodium hydrogen phosphate (9.1 g.) and extracting the mixture with water (200 ml.) (103). The cells were removed by centrifugation (5000 r.p.m., 20 min.). Solutions (1%) of disaccharides A, B, and C were sealed in capillary tubes with equal volumes of the enzyme extract, and incubated at 26° for 48 hours. Paper chromatographic analysis of the digests, at the end of this period, showed that disaccharide B was completely hydrolysed, and that disaccharides A and C were partially hydrolysed. Components corresponding to glucose and galactose were found in all the digests. A component corresponding to fructose was also found in the digest containing disaccharide A. Control digests containing the enzyme and maltose, melibiose, cellobiose, or lactose, were incubated at the same time. Paper chromatography revealed that maltose was completely hydrolysed under these conditions, and that melibiose was partially hydrolysed. Lactose and cellobiose were not hydrolysed.

(c) Action of yeast α-galactosidase. The α-glucosidase activity in the enzyme extract from yeast (Expt. 44 (b)) was suppressed by the addition of glucono-δ-lactone (2%) (83). A solution of disaccharide B (1%) was sealed into a capillary tube with an equal volume of this enzyme preparation, and incubated at 26° for 3 days. Paper chromatography showed that no hydrolysis of disaccharide B had taken place at the end of this period. Control digests containing maltose and melibiose were incubated at the same time. Under these conditions melibiose was partially hydrolysed, but maltose was not hydrolysed.

Expt. 45. Investigation of higher oligosaccharides obtained from a galactose-sucrose culture of Betacoccus arabinosaceous.

Small quantities of higher oligosaccharides were obtained as syrups from the charcoal column chromatography of the galactose-sucrose culture (Expt. 31). Two chromatographically pure oligosaccharides, B and C, (R_g values 0.18 and 0.16, respectively) were separated.

Samples of these oligosaccharides were completely hydrolysed with 1.5$\underline{N}$-sulphuric acid at 100° for 4 hours. After desalting with Permutit 'Biodeminrolit' resin, in the carbonate form, and concentration, the solutions

were analysed by paper chromatography. Oligosaccharide B hydrolysate contained components corresponding to glucose and galactose, in the ratio of *ca.* 2:1. The hydrolysate of oligosaccharide C contained components corresponding to glucose and galactose, in the ratio of *ca.* 3:1.

Oligosaccharides B and C were partially hydrolysed with N-sulphuric acid, at 90° for 1 hour. Paper chromatography of the partial hydrolysates of both oligosaccharides, after desalting and concentration, revealed the presence of components corresponding to glucose, galactose, and *iso*maltose.

SECTION IV

EXPERIMENTAL

Investigation of the reversibility of the dextransucrase -sucrose reaction.

Expt. 46. Incubation of [^{14}C] dextran, and of [^{14}C] trisaccharide A, with dextransucrase, in the presence of added sugars.

Digests were made up containing dextransucrase (Batch II, 1.3 units of activity/ml.), in 0.05M-acetate buffer (pH 5.0), to which [^{14}C] dextran (L. mesenteroides NRRL B512) or [^{14}C] trisaccharide A, were added with other sugars as shown in Table XVI.

TABLE XVI.

Digest No.	Carbohydrate components
1.	[^{14}C] dextran (1%), fructose (1%), methyl ∝-D-glucoside (1%).
2.	[^{14}C] dextran (1%), methyl ∝-D-glucoside(1%)
3.	[^{14}C] trisaccharide A(1%), fructose (1%), methyl ∝-D-glucoside (1%).
4.	[^{14}C] trisaccharide A (1%), methyl ∝-D-glucoside (1%).
5.	[^{14}C] trisaccharide A (1%), fructose (1%).
6.	[^{14}C] trisaccharide A (1%), sucrose (1%).

The digests were analysed by paper chromatography, in solvents (a) and (g), incubated at 25° for 24 hours, and again examined by paper chromatography. Radioautographs were prepared by exposure of the chromatograms to Ilford X-ray film (Industrial G) for 4 weeks. No [^{14}C] compounds, other than [^{14}C] dextran (in digests 1 and 2), and [^{14}C] trisaccharide A (digests 3-6) could be detected.

Expt. 47. Preliminary investigation of the reversibility of the dextransucrase reaction, in the presence of sucrose and [^{14}C] fructose.

An enzyme digest was made up containing dextransucrase (Batch III, 1 mg., 0.39 units), sucrose (2 mg.), and [^{14}C] fructose (20 mg., ca. 30mC/g.-atom C), in 0.05M-acetate buffer (0.1 ml., pH 5.0), and incubated at 25° for 25 hours.

The digest was analysed by paper chromatography, before the addition of the enzyme, and at intervals of 5 hours throughout the incubation period. The chromatograms were developed with solvent (a), and exposed to Ilford X-ray film (Industrial G) for 3 weeks. A control digest, containing the enzyme and sucrose alone, was incubated and analysed at the same time. The sugars

were detected on the chromatograms with spray (a).

The chromatographic analyses indicated that sucrose was present in both digests throughout the incubation period, but that the sucrose concentration decreased. In the digest containing added [^{14}C]fructose no change in fructose could be detected, but in the control digest fructose was present, in increasing concentrations, after 5 hours from the start of the incubation. Both digests contained a sugar with R_f value indicative of a disaccharide but lower than that of sucrose. This sugar was present in increasing concentrations from 5 hours after the start of the incubation.

The radiochromatograms showed that, after 5 hours incubation, the digest to which [^{14}C]fructose had been added, contained [^{14}C]components with the R_f values of sucrose and the second disaccharide.

The experiment was repeated using dextransucrase (Batch IV), and a dextransucrase preparation from Streptoccus bovis, supplied by Dr. R. W. Bailey. Radiochromatograms showed the presence of [^{14}C]sucrose and a second [^{14}C]disaccharide in digests containing these enzymes, sucrose, and [^{14}C]fructose.

In a control experiment sucrose (10 mg.) and [^{14}C]-fructose (100 mg.) were dissolved in 0.05M-acetate buffer (pH 5.0, 1 ml.) and held at 25° for 25 hours. Radiochromatograms showed that no ^{14}C was present in the sucrose at the end of this period.

Expt. 48. Determination of the specific radioactivity of fructose.

[^{14}C]Fructose (ca. 2.5 mg., 50 μC), supplied by the Radiochemical Centre, Amersham, was purified by preparative paper chromatography on Whatman No. 3 paper, in solvent (a). The [^{14}C]fructose was located by radiochromatography, and eluted from the paper.

Inactive fructose (98.75 mg.) was added to the [^{14}C]-fructose solution, which was then diluted to 100 ml. in a standard flask. Aliquot portions (1 ml.) of the standard [^{14}C]fructose solution were added to samples of inactive fructose (17.7 mg. and 16.75 mg.), and freeze dried. The specific activities of the [^{14}C]fructose samples were estimated by the wet combustion technique (Expt. 9), and were found to be 568.4 μC/g.-atom C and 627.2 μC/g.-atom C respectively.

From these results the specific radioactivity of

the [^{14}C]fructose, in the standard solution was calculated to be 10.628 mC/g.-atom C and 11.133 mC/g.-atom C. Hence the mean specific radioactivity of the [^{14}C]fructose standard was 10.880 mC/g.-atom C, or 65.28 mC/mole of fructose.

Expt. 49. Investigation of the reaction of dextransucrase with sucrose and [^{14}C]fructose.

The standard [^{14}C]fructose solution (Expt. 48) was concentrated, transferred to a small test tube, and freeze dried. Sucrose (10 mg.), dissolved in 0.05M-acetate buffer (0.5 ml., pH 5.0), and dextransucrase (Batch IV, 22.12 mg., 8.5 units), dissolved in 0.05M-acetate buffer (0.5 ml., pH 5.0) were added to the [^{14}C]fructose, and the digest was incubated at 25°. The solution was analysed by paper chromatography before the addition of the enzyme. Samples of the digest, measured with an 'Agla' micrometer syringe, were removed at intervals, as shown in Table XVII.

TABLE XVII

Sample No.	Time, hours.	Sample, ml.
1.	0.25	0.1
2.	0.75	0.1
3.	1.50	0.1
4.	2.25	0.1

TABLE XVII (cont.)

Sample No.	Time, hours.	Sample, ml.
5.	3.00	0.1
6.	4.00	0.1
7.	5.00	0.1
8.	6.75	0.05
9.	8.00	0.07

The components of each sample were fractionated on Whatman No. 1 paper, in solvent (a), and the [^{14}C]-components were located by exposing the papers to Ilford X-ray film (Industrial G) for 14 days.

The radiochromatograms revealed the presence of [^{14}C]fructose, and [^{14}C] components, with the same R_f values as sucrose, and leucrose, (disaccharides D and E respectively). Traces of a possible [^{14}C] tri- and [^{14}C]tetrasaccharide (oligosaccharides D and E respectively) were also present. The concentration of disaccharide E appeared to increase throughout the incubation period. Oligosaccharides D and E appeared to be present in higher concentrations during the earlier stages of the incubation than at the end of this period.

Expt. 50. Investigation of disaccharide D from dextran-sucrase digest containing sucrose and [^{14}C]-fructose.

The components of the digest samples (Expt 50) with the same R_f value as sucrose (disaccharide D) were eluted from the papers, and the solutions were diluted to 25 ml. in standard flasks. Samples (1 ml.) of these solutions were analysed for carbohydrate content by the anthrone method (Expt. 7). The specific radioactivity of the disaccharide was estimated by the infinitely thin film method (Expt. 10). The β-emission from the samples of disaccharide D was compared with that from a standard [^{14}C]glucose sample, specific activity 1.1964 mC/g.-atom C, (816 counts/min./μmole). The results are shown in Table XVIII.

TABLE XVIII

Sample No.	Conc. in digest, mg./ml.	Conc. in standard solution, μg./ml.	Counts/ min./ml. standard solution.	Counts/ min./ μ mole.	Specific activity, mC/mole disaccharide.
1.	9.000	36.0	116.8	1109.2	9.544
2.	8.875	35.5	248.8	2396.9	20.623
3.	8.075	32.3	403.2	4271.2	36.750
4.	7.450	29.8	458.8	5267.5	45.322

TABLE XVIII (cont.)

Sample No.	Conc. in digest, mg./ml.	Conc. in standard solution, μg./ml.	Counts/ min./ml. standard solution.	Counts/ min./ μ mole.	Specific activity, mC/mole disaccharide
5.	7.050	28.2	504.0	6109.1	52.562
6.	6.375	25.5	482.2	6463.8	55.614
7.	4.750	9.9	189.4	6542.3	56.290
8.	4.450	8.9	201.2	7766.5	66.703
9.	3.929	11.0	219.4	6813.7	58.625

Dry sucrose (255.7 mg.) was dissolved in a portion (20 ml.) of standard solution 9., containing [^{14}C] disaccharide D (0.22 mg.). The solution was freeze dried, and the sucrose was recrystallised twice from dry methanol and isopropyl alcohol. The specific radioactivity of the sucrose was estimated by dry combustion after each recrystallisation. The results are shown in Table XIX.

TABLE XIX

	Specific activity of sucrose.	
	Recrystallised	In digest
1st recrystallisation	51.12 μC/mole sucrose	59.462 mC/mole sucrose
2nd "	53.40 μC/mole sucrose	62.114 mC/mole sucrose
Mean		60.79 mC/mole sucrose

The standard disaccharide D solutions (1-8) were combined and freeze dried. Disaccharide D was examined by radiochromatography in solvents (a), (g), and (h). In all these solvents disaccharide D moved at the same rate as sucrose.

A sample of disaccharide D was hydrolysed with 0.25 N-sulphuric acid at 100° for 15 min. Another sample of disaccharide D was incubated with invertase (B.D.H. concentrate) at 20° for 1 hour. Radiochromatograms of the products showed that in each case disaccharide D was completely hydrolysed to glucose and [^{14}C] fructose.

Expt. 51. Investigation of [^{14}C] fructose from dextran-sucrose digest containing sucrose and [^{14}C]-fructose.

The [^{14}C] fructose components of the digest samples (Expt. 49) were eluted from the papers and selected eluates were diluted to 250 ml. in standard flasks. As the chromatograms were over loaded with fructose, the elution was not quantitative. The fructose solutions were analysed for carbohydrate content by the anthrone method (Expt. 7). The specific radioactivity of the fructose was estimated by the infinitely thin film method (Expt. 10). The β-emission was compared with the β-emission from a standard [^{14}C] glucose sample, specific

radioactivity 1.1964 mC/g.-atom C (816 counts/min./μmole). The results are shown in Table XX.

TABLE XX

Sample No.	Conc. of fructose μg./ml.	Counts/ min. ml.	μmole/ml.	Counts/ min./ μ mole x 10^4	Specific activity mC/mole fructose.
2.	37.5	1800	0.1097	1.641	70.596
3.	37.1	1639	0.1085	1.511	65.004
4.	36.0	1625	0.1053	1.543	66.378
5.	33.7	1446	0.0985	1.468	63.156
6.	34.9	1471	0.1020	1.442	62.034
9.	24.5	1003	0.0716	1.401	60.270

Expt. 53. Investigation of disaccharide E from the dextransucrase digest containing sucrose and [^{14}C] fructose.

The components of the digest samples (Expt. 49), with the same R_f values as leucrose (disaccharide E) were eluted from the papers, and the solutions were diluted to 10 ml. in standard flasks. Attempts were made to analyse the solutions for carbohydrate content by the anthrone method (Expt. 7) but the concentrations of disaccharide E were too low for accurate results. All

the disaccharide E fractions were combined, freeze dried, and dissolved in water (5 ml.). The carbohydrate content of this solution was estimated by the anthrone method, and the specific radioactivity of disaccharide E was estimated by the infinitely thin film method (Expt. 10) The specific activity of disaccharide E was found to be 59.004 mC/mole disaccharide.

Disaccharide E moved at the same rate as leucrose on paper chromatography in solvents (a), (g), and (h). It was not hydrolysed when heated with 0.25$\underline{N}$-sulphuric acid at 100° for 15 min., or when incubated with invertase (B.D.H. concentrate) at 20° for 1 hour. Disaccharide E was heated with 1.5$\underline{N}$-sulphuric acid at 100° for 4 hours. Radiochromatography of the product revealed the presence of glucose and a trace of [^{14}C]fructose. Under these conditions the bulk of the fructose would have been converted to 5-hydroxymethyl-2-furfuraldehyde which could not have been detected by this method of chromatographic analysis.

Expt. 53. Investigation of oligosaccharides D and E from the dextransucrase digest containing sucrose and [^{14}C] fructose.

Oligosaccharides D and E ($R_{fructose}$ values 0.22, and 0.11 respectively) from the digest samples (Expt.

49) were eluted from the papers and the solutions were diluted to 10 ml. in standard flasks. Attempts were made to analyse these solutions for carbohydrate content by the anthrone method (Expt. 7) but the concentrations of the sugars were too low for accurate results.

The oligosaccharide D fractions were combined, freeze dried, and examined by paper chromatography. The R_f values of this oligosaccharide were the same as those of O-α-D-glucopyranosyl-(1→6)-O-α-D-glucopyranosyl β-D-fructofuranoside in solvents (h) and (i) but not in solvent (a).

Hydrolysis of oligosaccharide D in 1.5N-sulphuric acid at 100° for 4 hours gave a component corresponding to glucose, and a trace of a [^{14}C] component corresponding to fructose, on radiochromatography. Partial hydrolysis of oligosaccharide D in N-sulphuric acid at 90° for 1 hour gave components corresponding to isomaltose, glucose and fructose, on paper chromatography.

The oligosaccharide E fractions were combined and freeze dried. Oligosaccharide E was hydrolysed with 1.5 N-sulphuric acid at 100° for 4 hours. Paper chromatography of the product revealed the presence of components corresponding to glucose and fructose. Radiochromato-

graphy of the product indicated that ^{14}C was present in the fructose.

Partial hydrolysis of oligosaccharide E in N-sulphuric acid at 90° for 1 hour gave components corresponding to isomaltose, glucose and fructose on paper chromatography.

Owing to the small amounts of oligosaccharides D and E separated from the enzyme digest, it was not possible to estimate the relative quantities of the products of hydrolysis on paper chromatograms.

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

Bourne, Hartigan and Weigel, J., 1959, 2332.

b) Biosynthesis of α-D-Glucopyranosyl D-galacto-furanoside and other D-Galactose-containing Saccharides by Betacoccus arabinosaceous.

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