UNIVERSITY OF EDINBURGH

INVESTIGATIONS ON FRUCTOSANS.

A

THESIS

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-by-

ROBERT G. J. TELFER, B.SC., A.R.I.C.

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## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td><strong>Part I. A Fructosan from Dactylic glomerata.</strong></td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>22</td>
</tr>
<tr>
<td>Experimental</td>
<td>42</td>
</tr>
<tr>
<td>Summary</td>
<td>83</td>
</tr>
<tr>
<td><strong>Part II. Investigations on Inulin.</strong></td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>85</td>
</tr>
<tr>
<td>Experimental</td>
<td>92</td>
</tr>
<tr>
<td>Summary</td>
<td>107</td>
</tr>
<tr>
<td><strong>Part III. Investigations on the fructosan from Perennial Rye Grass (Lolium perenne)</strong></td>
<td></td>
</tr>
<tr>
<td>Discussion</td>
<td>108</td>
</tr>
<tr>
<td>Experimental</td>
<td>119</td>
</tr>
<tr>
<td>Summary</td>
<td>141</td>
</tr>
<tr>
<td><strong>General Discussion</strong></td>
<td>142</td>
</tr>
<tr>
<td><strong>Bibliography</strong></td>
<td>146</td>
</tr>
<tr>
<td><strong>Acknowledgments</strong></td>
<td>150</td>
</tr>
</tbody>
</table>
INTRODUCTION

Despite the fact that many naturally occurring fructose polymers have been isolated from widely different plant sources\(^1\) over the last hundred years, it is only within the last quarter of a century that this group of polysaccharides has been subjected to detailed structural investigations.

Polyfructosans, although found in leaves and stems, appear to play the rôle of reserve carbohydrates, and their distribution in the various parts of the plant shows considerable seasonal fluctuation\(^2\). It was pointed out by Schlubach and Sinh\(^3\) that the naturally-occurring polymers of fructose could be divided into two classes:

(a) The inulin type, where the second or reducing carbon atom of one fructose residue is linked to the first carbon atom of the adjacent residue. On complete methylation and hydrolysis, polysaccharides of the inulin group yield 3:4:6-trimethyl fructose and form acetates which are laevo-rotatory.

(b) The phlein type, in which the linkage is between the reducing carbon atom of one fructose residue and the sixth carbon atom of the adjacent fructose residue. The fructosans of this group form dextro-rotatory acetates and on methylation and hydrolysis yield 1:3:4-trimethyl fructose.

The Inulin Group of Polyfructosans.

Inulin:

Inulin/
Inulin was the first of the fructosans to be isolated and has been investigated more than any of the others. It was discovered by Rose(4) who separated it from an extract of artichoke tubers. It also occurs in large quantities in dahlia tubers, where it is the main reserve polysaccharide of the plant, and also in chicory and dandelion roots.

The first structural investigations on the polysaccharide were those of Irvine and Steele(5) who methylated inulin by treatment with sodium hydroxide and dimethyl sulphate, followed by treatment with Purdie's reagents, methyl iodide and silver oxide, this being one of the first examples of the application of the methylation technique in the polysaccharide field. On hydrolysis with 1% oxalic acid, the methylated polysaccharide yielded a trimethyl fructose which was characterised by further methylation to tetramethyl fructose, which was identical with that isolated from methylated sucrose.

Tetramethyl fructose was obtained in 95% yield of the total and these workers concluded that inulin is an aggregate of fructose units, each molecule having lost two hydroxyl groups.

Haworth and Learner(6) followed up this work, using the new methods for determining monosaccharide structures, developed by Haworth about this time. Inulin was methylated by one treatment with dimethyl sulphate and sodium hydroxide, followed by three treatments with Purdie's reagents/
reagents. The resulting trimethyl inulin on hydrolysis, gave 3:4:6-trimethyl fructofuranose, which was characterised by the following series of reactions:

\[
\begin{align*}
\text{CH}_2\text{OH} & \quad \text{COOH} \\
\text{MeO} - \text{C} - \text{H} & \quad \text{MeO} - \text{C} - \text{H} \\
\text{H} - \text{C} - \text{OMe} & \quad \text{H} - \text{C} - \text{OMe} \\
\text{CH}_2\text{OMe} & \quad \text{CH}_2\text{OMe}
\end{align*}
\]

The D-trimethyl-\(\gamma\)-arabonolactone(1) can only come from 3:4:6-trimethyl fructofuranose, which necessitates \(C_1 - C_2\) linkages in the inulin molecule. Haworth and Learner, therefore, advanced the following formula for inulin.

Using Haworth's end-group assay method, Haworth, Hirst and Percival(7) obtained 3.7% of tetramethyl-D-fructofuranose from fully methylated inulin. They therefore concluded that inulin is composed of a chain of about 30 \(D\)-fructofuranose residues and has a molecular weight/
weight of about 5,000. Molecular weight determinations by osmotic pressure measurements on acetylated and methylated inulin, gave values of 8,820 and 6,210. Using the ebullioscopic method, in aqueous solution, Drew and Haworth determined the molecular weight to be not less than 3,200 or 3,600. These authors also failed to find a sample of inulin, which did not show a slight and progressive reducing action on boiling Fehling's solution, and claimed that this was due to the presence of reducing groups at the ends of open chain molecules and subsequent progressive hydrolysis.

Inulin is very readily hydrolysed even in the presence of mere traces of acid, to yield fructose, glucose and difructose anhydrides. The difructose anhydrides occur to the extent of about 5% in an inulin hydrolysate and are much more resistant to acid hydrolysis than inulin itself. By the process of fractional precipitation, Jackson and McDonald have shown these products to be three different compounds. Haworth and Streight proved difructose anhydride I to be 1, 2; 2, 1'-di-D-fructofuranose anhydride. Difructose anhydride II was shown to be 2, 1'; 4, 2'-di-D-fructofuranose anhydride while the other was found to be the 1, 2'; 2, 3' isomer.

![Difructose Anhydride I](image1)

![Difructose Anhydride II](image2)

![Difructose Anhydride III](image3)
A further three di-D-fructose dianhydrides have been produced by the action of heat or hydrochloric acid on fructose solutions. These are distinguished from the dianhydrides produced on hydrolysis of inulin, by the presence of a pyranose ring in the molecule. They have been named diheterolevulosan I (di-D-fructopyranose 1, 2'; 2, 1'-dianhydride) diheterolevulosan II (D-fructopyranose -fructofuranose 1, 2'; 2, 1'-dianhydride) and diheterolevulosan III (anomer of diheterolevulosan II or di-D-fructopyranose 1, 2'; 2, 3'-dianhydride).

It is now quite certain that difructose anhydrides are not present preformed in the fructosan molecules, but arise by side syntheses on hydrolysis of the polysaccharides. Recently, Bell and Palmer have pointed out that the formation of methylated difructose anhydrides, on hydrolysis of the methylated polyfructosans, can complicate the gravimetric chromatographic analysis of the partially methylated fructoses.

Many workers have noted the presence of glucose in inulin hydrolysates. Hirst, McGilvray and Percival estimated the glucose produced by hydrolysis of dahlia inulin (Blue Danube variety) - as 5.7% using the method of quantitative paper chromatography. By determining the total quantities of tetramethyl and trimethyl glucose produced from methylated inulin, using alkaline hypiodite oxidation, a value of 5.7% calculated as glucose, was again obtained. From their investigations, the authors suggest as a possible structure for the fructosan, a chain of about 35/
35 fructofuranose residues, with the potentially reducing fructose residue linked to a glucopyranose unit by a sucrose-type linkage and with a second glucopyranose residue linked through C_1 and C_2 at some undetermined position in the chain as indicated below:

Bell and Palmer, investigating the structure of inulin from *Inula helenium*, found that on mild acid hydrolysis, the fructosan liberated 2.9 moles of D-glucose per 100 hexose radicals. This value was calculated from the oxygen uptake of samples of the hydrolysate in the presence of notatin and catalase. On hydrolysis of the methylated inulin/
inulin about 50% of the estimated glucose in the original inulin was obtained as a mixture of all three trimethyl glucoses, unsubstituted at position 5, with traces of 2:3:4:6-tetramethyl glucose. The authors claim however, that the glucose might well have arisen from terminal non-reducing radicals, linked as in sucrose. Assuming that the polysaccharide molecule is terminated by a glucose moiety of a sucrose radical, the molecular weight of the polysaccharide would be about 5,600 and the chain length 35. From the ratio of tetramethyl- and trimethyl-fructose, produced on hydrolysis of the methylated inulin, a chain length of 36 was calculated, which is the same as that determined by Hirst, McGilvray and Percival\(^{20}\) for dahlia inulin.

**Asparagosin:**

First isolated from asparagus roots by Tanret\(^{22}\), asparagosin was structurally investigated by Schlubach and Böel\(^{23}\). Their product had a molecular weight of 1,635 when determined by the cryoscopic method, corresponding to 10 fructose residues. Methylation of the polysaccharide acetate, and hydrolysis of the fully methylated material yielded dimethyl-trimethyl- and tetramethyl-\(^D\)-fructose in the ratio 1:8:1 (calculated as D-fructose). The trimethyl fraction was identified as 3:4:6-trimethyl-\(^D\)-fructose by preparation of its crystalline osazone.

**Sinistrin:**

The polyfructosan sinistrin, obtained by Schlubach and Loop\(^{24}\) from the sea onion (\textit{Scilla maritima}) was acetylated, methylated, hydrolysed and the components of the hydrolysate/
hydrolysate separated by high vacuum distillation of the benzoyl derivatives. Dimethyl-trimethyl- and tetramethyl-D-fructose were obtained in the ratio 1:3:1 or 2:5:2. The trimethyl fraction was identified as 3:4:6-trimethyl D-fructose by conversion to the osazone. The greater part of the osazone however, could not be crystallised and this, together with the value the above authors obtained for the specific rotation of the trimethyl fraction, suggested that it was a mixture. Molecular weight determinations gave a value of 2,526 for sinistrin, corresponding to 15-16 fructose units.

**Gruminin.**

Schlubach and Koenig (25) isolated a fructosan from rye and named it gruminin. Molecular weight determinations indicated that gruminin was built up from 9-10 D-fructose units. Hydrolysis of the methylated polysaccharide gave a trimethyl fraction, which appeared to be 3:4:6-trimethyl fructose, although definite proof of this was not obtained. Because the dimethyl fructose underwent decomposition on distillation, it was not possible to determine the exact ratio of the partially methylated fructose.

**Kritesin.**

The fructosan kritesin was isolated from ears of barley (Ackermaenne isaria) by Schlubach and Rathje (26). Hydrolysis of the methylated polysaccharide and separation of the partially methylated sugars by fractional distillation of the benzoyl derivatives, in high vacuum, gave tetramethyl-trimethyl- and dimethyl-D-fructose in the ratio 1:12:2:1.
Again, however, the trimethyl fraction was found to be a mixture, as it gave two osazones, one containing 3 methoxyl groups and apparently identical with that of 3:4:6-trimethyl-D-fructose, and the other containing 1 methoxyl group.

As may be seen from the above, much of the work of Schlubach and his collaborators was incomplete and most of his results await confirmation.

The Phlein Group of Polyfructosans.

Secalin:

Secalin, the name given to the fructosan isolated from the stems of unripe barley by Belval, was investigated by Schlubach and Bandmann. After hydrolysis of methylated secalin, the products of hydrolysis were separated by fractional distillation of the \( \beta \)-naphthyl derivatives. 1:3:4:6-tetramethyl fructofuranose, 1:3:4-trimethyl fructose and an unidentified dimethyl fructose, were obtained in the ratio 1:2:1.

Pyrosin:

The structure of the fructosan pyrosin, isolated from wheat stalks, was investigated by Schlubach and Huchtig, who showed it to be similar to secalin. Hydrolysis of methylated pyrosin and separation of the components by benzoylation, followed by fractional distillation in high vacuum, yielded 1:3:4:6-tetramethyl D-fructose, 1:3:4-trimethyl D-fructose and a dimethyl fructose in the ratio 1:4:3:1.

Both secalin and pyrosin showed the presence of small amounts of unidentified aldose by the method of Auerbach-Bodländer.

Grass/
Grass Levan:

Phlein, the fructosan considered by Schlubach as the parent of the polyfructosans in which the D-fructose residues are linked through carbon atoms 2 and 6, was constitutionally investigated by Schlubach and Sinn. The polysaccharide was isolated from the roots of Phleum pratense and on methylation, followed by hydrolysis, gave 1:3:4-trimethyl fructose with a small amount of dimethyl fructose (1.9%), which was attributed to undermethylation. As 1:3:4-trimethyl fructose alone was produced, the authors claimed that phlein consists of a closed ring of 15-16 fructose units, this number being deduced from cryoscopic determinations of the molecular weight.

Challinor, Haworth and Hirst isolated from rough-stalked meadow grass (Poa trivialis), a fructosan giving only fructose on hydrolysis. Its physical and chemical properties were shown to be identical to those of the levan produced by the action of E. mesentericus on sucrose. The bacterial levan on methylation, hydrolysis and fractional distillation, gave tetra-methyl D-fructofuranose, in amount corresponding to a chain of 10-12 D-fructose units. The authors ascribed the following structure to the levan.
Bell and Palmer\textsuperscript{(33)} using a column of silica gel\textsuperscript{(34)} to separate the partially methylated sugars obtained from the hydrolysis of methylated levans from Italian rye grass \textit{(Lolium italicum)} and leafy cocksfoot grass \textit{(Dactyliis glomerata)}, showed that the former gave 1:3:4:6 tetramethyl D-fructose, 1:3:4-trimethyl D-fructose and dimethyl fructose in the ratio 1:12:1 and the latter in the ratio 1:11:1.7. These findings call for a chain length of 14 units, although the molecular weights, determined by physical methods (ultracentrifuge), show molecules containing about 30 units, thus implying the presence of branch points in the molecule.

Palmer\textsuperscript{(21)} by estimating the glucose produced on hydrolysis of different samples of leafy cocksfoot levan, and assuming one glucose residue per chain, put forward values of 29 and 37 for the chain lengths.

In a more detailed analysis of the hydrolysis products of methylated levans from leafy cocksfoot grass and Italian rye grass, Bell and Palmer\textsuperscript{(19)} reported the production of a little methylated difructose anhydride with small quantities of trimethyl glucose, the 2:4:6 isomer predominating, as well as traces of 2:3:4:6 tetramethyl glucose. A chain length of 23-24 units was reported for a sample of cocksfoot grass levan, precipitated from 62% ethanol, the value being based on end-group assay.

Laidlaw and Reid\textsuperscript{(35)} isolated a fructosan from perennial rye grass \textit{(Lolium perenne)} and showed the glucose content to be 2% by quantitative paper chromatography. They obtained 4% 1:3:4:6-tetramethyl D-fructofuranose from a hydrolysate/
hydrolysate of the methylated fructosan and concluded that the molecule had a chain length of 25-30 units. Evidence was found for the production of a little aldose (9%) in the tetramethyl fraction, as well as traces of trimethyl- and dimethyl aldoses. The above authors suggested a structure (II) for the polysaccharide with a terminal sucrose residue.

![Chemical structure](image)

Recently, Schlubach and Holzer\(^{36}\) claimed to have isolated a levan from Lolium perenne which yielded only fructose on hydrolysis.

**Other Polyfructosans.**

Three fructosans have been investigated, and appear to fall outside the above two general groups. These are asphodelin, irisin and triticin.

**Asphodelin:**

Isolated from asphodelus tubers, asphodelin was first intensively investigated by Colin and Neyron\(^{37}\) who described it as a non-reducing polysaccharide yielding fructose (6 parts) and glucose (1 part) on hydrolysis. This indicated that asphodelin was either a glucofructosan or an intimate mixture of a glucosan and fructosan.

Schlubach and Lendzian\(^{38}\) hydrolysed methylated asphodelin and obtained tetramethyl-, trimethyl- and dimethyl-fructose in the ratio 1.1 : 8.32 : 1.65. The trimethyl/
trimethyl fructose fraction contained an appreciable methylated glucose content. The authors stated that the glucose probably arose from a glucosan impurity.

Irisin:

An investigation of the structure of irisin, the fructosan isolated from the roots of Iris pseudacorus was made by Schlubach, Knoop and Liu. The hydrolysis of methylated irisin, tetramethyl-D-fructofuranose and a dimethyl fructose, which could not be conclusively identified, were obtained in equal amounts. More recently, Bell and Palmer have made an investigation of methylated irisin, using partition chromatography. These authors obtained 1:3:4:6 tetramethyl D-fructose (7 parts) and a dimethyl fructose (7 parts), which was identified as the 3:4 isomer. In addition, an appreciable trimethyl fraction was obtained, consisting of 1:3:4-trimethyl-D-fructose (2 parts) and (probably) 3:4:6-trimethyl-D-fructose (1 part).

Triticin:

Triticin the polyfructosan from Triticum repens L. was structurally investigated by Schlubach and Peitzner. The fructosan was acetylated, methylated, hydrolysed and the components of the hydrolysate separated by high vacuum distillation to yield tetramethyl-, trimethyl- and dimethyl-fructose in the ratio 3:1:3. The tetramethyl fraction was shown to be 1:3:4:6 tetramethyl-D-fructofuranose and the dimethyl fructose was thought to be identical with that obtained by hydrolysis of trimethyl sinistrin. The trimethyl fructose could not be identified. Cryoscopic determinations/
sions of the molecular weight of the polysaccharide gave values of 2,600-2,830, indicating that the molecule is built up from 16 - 17.5 fructose units.

Arni and Percival\(^{(41)}\) investigated a sample of triticin from the rhizomes of Couch grass \((Triticum repens L.)\). From the hydrolysis products of methylated triticin \(1\:3\:4\:6\)-tetramethyl D-fructofuranose (ca. 42\%), \(1\:3\:4\) (ca. 11\%) and \(3\:4\:6\)-trimethyl fructose (ca. 4\%) and \(3\:4\)-dimethyl fructose (ca. 39\%) were separated and identified. The authors suggested a highly branched structure for the polysaccharide, containing about equal numbers of fructofuranose residues linked by 2:1- and 2:6- linkages, and built up from about 30 fructose residues. In addition the authors suggested that the fructofuranose aggregate may be terminated by a glucose residue linked as in sucrose.

The Rôle of Glucose in the Fructosan Molecule.

One of the most interesting present-day problems in fructosan chemistry is the importance of glucose and its function in the molecular structure of the polyfructosans. Tanret\(^{(42)}\) in his investigations on inulin, concluded that there was one glucose residue to every twelve fructose, his deductions being based on the lowering of the rotation on acid hydrolysis. Schlubach and his co-workers, found only very small amounts of aldose after hydrolysis of several different fructosans, and concluded that the polysaccharides were composed entirely of fructose residues, the low rotation values being ascribed to destruction of fructose on hydrolysis.

Hydrolysis/
Hydrolysis Products of Fructosanes
(Schlubach et. al. 1929-37)

<table>
<thead>
<tr>
<th>Source</th>
<th>Name</th>
<th>% yield on hydrolysis (Acid)</th>
<th>Fructose</th>
<th>Aldose</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agropyron repens</td>
<td>Triticin</td>
<td>94.6</td>
<td>0.7</td>
<td></td>
<td>95.3</td>
</tr>
<tr>
<td>Rye</td>
<td>Graminin</td>
<td>88.8</td>
<td>0.59</td>
<td></td>
<td>89.4</td>
</tr>
<tr>
<td>Scilla maritima</td>
<td>Sinistrin B.</td>
<td>94.4</td>
<td>1.8</td>
<td></td>
<td>96.2</td>
</tr>
<tr>
<td>Asparagus</td>
<td>Asparagosin</td>
<td>92.2</td>
<td>-</td>
<td></td>
<td>92.2</td>
</tr>
<tr>
<td>Asphodel</td>
<td>Asphodelin</td>
<td>88.9</td>
<td>11.1</td>
<td></td>
<td>100.0</td>
</tr>
</tbody>
</table>

In the case of asphodelin, Schlubach believed the high aldose value to originate from a glucosan impurity (see back).

It should be noted that evidence for the presence of glucose based on the lowering of rotation on acid hydrolysis, is far from conclusive, for the formation of difructose anhydrides could also cause a similar effect, since these three side products are all dextro-rotatory. Schlubach and Klamer(43) isolated $\alpha$-pentacetyl glucose from inulin after acetylation, hydrolysis and further acetylation, and also directly, by treating triacetyl inulin with acetic anhydride and sulphuric acid. In addition, by estimations of the reducing power of the fructosan, they decided that the polysaccharide contained some 8% of glucose. A control experiment, to discover whether the rearrangement of fructose to glucose took place on acid hydrolysis or not, gave a negative result. It is noteworthy that inulin has been found to yield glucose when treated with the enzyme inulase. Pringsheim and Ohlenmeyer, using this enzyme, estimated the/
the glucose content as 1.5\% by differential titration with Fehling's solution and hypochlorite solution. Generally, the aldose content of the fructosans has been found to be lower when estimation has been made by an enzymatic method rather than a purely chemical one. Thus, Adams, Richtmeyer and Hudson\(^{[45]}\) estimated 1.7\% glucose in an enzymatic hydrolysis of inulin.

Earlier workers in the realms of fructosan chemistry considered glucose more likely to be an isomerisation product from fructose, or a hydrolysis product from an accompanying glucosan impurity, than an integral part of the fructosan molecule.

Irvine and Montgomery\(^{[46]}\) claimed to have isolated 3:4:6 trimethyl glucose after hydrolysis of methylated inulin but pointed out that this product was derived from 3:4:6-trimethyl fructofuranose, for control experiments indicated that the following change took place:

\[
\begin{align*}
\text{3:4:6 trimethyl fructofuranose} & \quad \text{3:4:6 trimethyl glucopyranose.}
\end{align*}
\]

These findings, however, have not since been verified.

The rate of hydrolysis of the fructosans under very mild conditions, to yield glucose and fructose, shows that the glucose, if present as an accompanying glucosan impurity, must be formed at the same rate as sucrose, and consequently/
consequently, the glucose polysaccharide must be very different from those so far examined, in that they are completely hydrolysed under very much milder conditions.

Other objections to the "glucosan impurity" theory include the fact that many samples of fructosans, isolated from wide sources, have been found to contain approximately the same amount of glucose.

Further, hydrolysis of some methylated fructosans has given rise to small amounts of partially methylated glucose, where the ratios of trimethyl glucose to tetramethyl glucose, do not favour the concept of a glucosan impurity.

With the advent of filter paper partition chromatography and its application to the quantitative analysis of sugars, much more accurate assays could be made of the glucose and partially methylated glucose obtained from the fructosans and methylated fructosans respectively. Schubach and his collaborators favour the concept of a closed loop for the fructosan molecules rather than an open chain configuration, and one of the main facts supporting this idea, is that the polyfructosans are virtually non-reducing, thus excluding a structure with a free reducing end-group. Bell, Manners and Palmer have recently verified the non-reducing action of fructosans by showing that several of these polysaccharides failed to reduce alkaline 3,5-dinitrosalicylate.

More recently however, another explanation has been put forward for the non-reducing properties of the polyfructosans/
polyfructosans. This involves the concept of an open-chain configuration for the fructosan molecules, terminated by a glucopyranose unit linked as in sucrose. A considerable amount of evidence has been brought forward to support this theory. This includes the production of tetramethyl glucopyranose on hydrolysis of the methylated fructosans from dahlia tubers[20] couch grass rhizomes[41] and perennial rye grass[35](see back). By boiling aqueous solutions of the fructosans from perennial rye grass and couch grass rhizomes, a sugar which travelled on the paper chromatogram at the same rate as a sucrose standard, was produced. In the case of the polyfructosan from rye grass, this sugar was found chromatographically, to give only glucose and fructose, on hydrolysis, thereby providing further evidence for the suggestion that it might actually have been sucrose. Palmer[21] has carried out an extensive investigation into the glucose content of a large number of fructosans, using notatin and catalase in conjunction with Warburg manometers and has put forward the theory that in the polyfructosan group of carbohydrates each fructosan molecule contains only one glucose residue. The same author points out that Tanret, in 1893, found the glucose content of fructosan fractions, isolated from tubers of Helianthus tuberosus, increased as the fructosans became more soluble in alcohol, presumably as the molecular weight decreased. These findings were verified for samples of fractionated grass levans, for the glucose content was found to increase as the solubility
in alcohol increased. For samples of unfractionated grass levans, Elmer found the molecular weights from the glucose content, and the results were in good agreement with those determined by physical measurements of sedimentation and diffusion constants.

Lastly a considerable amount of evidence favouring the concept of a sucrose end-group, has resulted from work on the biosynthesis of the fructosans.

Bacon and Edelman\(^{52}\) examined the carbohydrates of the Jerusalem Artichoke (\textit{Helianthus tuberosus} L.) employing qualitative and quantitative paper partition chromatography. They were able to demonstrate the existence of at least seven non-reducing substances, the fastest-moving travelling on the paper chromatogram at the same rate as sucrose, and the slowest remaining at the starting-line. All these oligosaccharides were found to consist mainly of fructofuranose residues, but all gave glucose on hydrolysis, the amounts of the latter decreasing with decreasing velocity on the paper chromatogram.

Similar results were independently obtained by Dedonder\(^{53}(54)\) in his investigations on the carbohydrates of the roots of the Jerusalem Artichoke.

Edelman and Bacon\(^{55}\) have suggested a possible means of biosynthesis of glucosidofructosans in the plant. These authors examined the carbohydrates in extracts of Jerusalem Artichoke tubers before and after addition of sucrose and found that their observations were best explained/
explained by assuming that transference of fructofuranose residues - "transfructosidation" - had taken place from higher oligosaccharides to sucrose, to give a trisaccharide. Their hypothesis was supported by treating mixtures of inulin and sucrose with carbohydrate-free enzyme preparations when paper partition chromatography showed the formation of a trisaccharide and other higher saccharides in smaller amounts. Continuing their work, Edelman and Bacon(56) demonstrated the existence in tuber extracts from Helianthus tuberosus L., of a hydrolytic enzyme system which liberated fructose from inulin and related substances, but which had relatively little action on sucrose. Similar researches into the fructose-containing carbohydrates of barley(57) likewise demonstrated the presence of a series of fructose oligosaccharides with sucrose as the lowest member, all apparently giving rise to glucose on hydrolysis, the amount of which diminished with increasing chain length. Employing radioactive glucose, Edelman and Bealing(58) further elucidated the reactions involved in the process of transfructosidation. Using mould invertase preparations, radioactive glucose and inactive sucrose in an acetate buffer, incubating them at 20°, followed by chromatography on paper sheets, they determined the activity of the sugar spots with a Geiger-Muller counter. In this way, results were obtained which strongly supported the hypothesis that the enzyme transfers fructose residues to suitable acceptors including glucose and sucrose. By the action of yeast invertase/
invertase preparation on a concentrated sucrose solution, Alton, Bell, Blanchard, Gross and Rundell (59) synthesized a trisaccharide which they named kestose. Methylation studies showed this sugar to be $6 - (\beta - D$-fructofuranosido) $\beta - D$-fructofuranosido $\alpha - D$-gluco-pyranoside, II

This trisaccharide may be regarded as the first product in the biosynthesis of a levan-type fructosan from sucrose. Similarly, by the action of a mould extract on sucrose, Pazur (60) has reported the synthesis of a trisaccharide, which might likewise be regarded as the first product in the synthesis of an inulin-type fructosan from sucrose. No rigorous proof was obtained in this case however, of the trisaccharide's structure.
PART I.

A FRUCTOSAN FROM DACYLLIS GLOMERATA

DISCUSSION.

The fructosan from mid-season cocksfoot grass (*Dactylis glomerata*), was extracted from the milled, oven-dried grass, employing the method used by Laidlaw and Reid (35) for the extraction of the levan from perennial rye grass. Ether extraction removed most of the chlorophyll and other pigments, while extraction with 80% aqueous methanol removed the free sugars present in the grass, together with some colouring matter. As the fructosan was water-soluble, it was removed from the grass residue by shaking with cold water for several hours. During all the extractions, care was taken to ensure that the medium never at any stage became acidic, by extracting in the presence of barium carbonate, a little toluene also being added to prevent bacterial action. The aqueous extract was deproteinised by a modification of Doak's method (61) due to Laidlaw and Reid (62) cadmium sulphate and sodium hydroxide solutions being added to the hot grass extract, simultaneously, with vigorous stirring. Any inorganic ions left in solution were removed by passage through columns of Amberlite resins (I.R.-100 and I.R.-4B). The last traces of protein were then removed by the method of Sevag, Lackmann and Smollens (63) which consists of shaking the aqueous extract with chloroform (0.25 vol.) and n-butanol (0.1 vol.), the proteins being thereby removed as a gelatinous layer. The polyfructosan was precipitated from/
from the concentrated aqueous solution with methanol to give a product, which had \( [\alpha]_D \), -40.4; very small ash and nitrogen contents and which slowly reduced boiling Fehling's solution. The slow reduction of Fehling's solution could be attributed to partial degradation of the fructosan molecule, on prolonged boiling of the solution, with exposure of reducing groups, for the same observations were made with a sample of pure sucrose.

In order to test the purity of the fructosan it was acetylated with acetic anhydride in pyridine solution, the solution in pyridine being effected according to the method of Pacsu and Mullen.\(^{64}\) The purified acetate \( [\alpha]_D \), + 22° in chloroform, was deacetylated with sodium methoxide in anhydrous methanol according to Zemplén and Pacsu\(^{65}\) to give a product showing \( [\alpha]_D \), -40.2°. This value for the specific rotation of the regenerated fructosan, being almost identical with that of the starting material, suggests that the fructosan preparation was pure.

**The Hydrolysis of the Polysaccharide.**

The fructosan was very readily hydrolysed with 1% oxalic acid, the final value of \( [\alpha]_D \) being -84°, which is 91% of the equilibrium value of \( [\alpha]_D \) for pure D-fructose.

Chromatographic examination of the hydrolysate indicated the presence of a little glucose with fructose. A sample of the hydrolysate of the fructosan regenerated from the polysaccharide acetate was likewise found to contain a little glucose along with the fructose.

Hydrolysis of the fructosan also occurred by heating
an aqueous solution under reflux, on a boiling water-bath. Such a hydrolysis has been termed "autohydrolysis" and has been observed with the fructosan tritici\(\text{\textsuperscript{41}}\) (from the rhizomes of couch grass) and the levan from perennial rye grass.\(\text{\textsuperscript{35}}\) On heating for some 50 hours, the specific rotation was found to change continually and the solution became more and more acidic as shown by a gradual fall in p.H. The development of acidity in the solution was not due to uronic acids for the p-anisidine hydrochloride\(\text{\textsuperscript{46}}\) and naphthoresorcinol tests for the detection of uronic acids proved negative. Acidic materials were detected however, in samples of the solution run on paper chromatograms in n-butanol saturated with ammonia (1.5 N.) as solvent.\(\text{\textsuperscript{67}}\) Several acid-alkali developing sprays were used and showed the presence of three acids on the paper chromatogram, very near the starting-line, the fastest-moving travelling at the same speed as a lactic acid standard.

Examination of the solution on the paper chromatogram from time to time, showed a progressive breakdown of the fructosan, to give first, higher oligosaccharides, followed by lower oligosaccharides and monosaccharides. Naphthoresorcinol and hydrochloric acid spray\(\text{\textsuperscript{68}}\)(\(\text{\textsuperscript{69}}\)) showed material moving slightly faster than fructose on the paper chromatogram and seen as a faint red "streak" below the red fructose spot. This fast-moving material could not be detected with aniline oxalate spray\(\text{\textsuperscript{70}}\) and might/
might have been a mixture of difructose anhydrides.

One of the oligosaccharides produced during the heating process travelled at the same rate as a sucrose standard. It was eluted from the paper and hydrolysed to give both glucose and fructose, suggesting that the oligosaccharide might indeed have been sucrose.

**Quantitative Estimation of Glucose and Fructose by Paper Chromatography.**

The glucose and fructose produced on hydrolysis of the polysaccharide with 0.1 N oxalic acid were estimated by the technique of quantitative paper chromatography, the sugars being extracted from the paper strips with cold water, using the apparatus described by Laidlaw and Reid.\(^{(71)}\)

The fructose was estimated by titration of the formic acid liberated on oxidation with sodium metaperiodate\(^{(72)}\) and the glucose by the Nelson colorimetric method.\(^{(73)}(74)\)

In order to enable the accurate estimation of a small amount of glucose in the presence of a large excess of fructose, the solution mixture was spotted on a number of chromatograms by means of a micro-pipette, the same weight of sugar being added to each paper. The glucose strips from six paper chromatograms were eluted with cold water, and the glucose present in the combined eluates estimated. The weight of glucose on one paper was thus found and compared with the weight of fructose per paper.

Using ribose as a reference sugar, the fructose gave a recovery of 102%. A synthetic mixture of glucose (2.9%) and fructose (97.1%) gave a recovery of glucose 3.1% and fructose/
fructose 96.8%.

The following average result was obtained for the percentages of glucose and fructose in the polysaccharide:

- Glucose = 3.2% (by colorimetric assay).
- Fructose = 96.8% (by periodate oxidation).

Studies on the Methylated Polysaccharide.

The fructose from cocksfoot grass was methylated directly using a procedure similar to that employed by Hirst, McGilvray and Percival (20) for the methylation of inulin. The polysaccharide was methylated by the simultaneous addition of methyl sulphate and sodium hydroxide in an atmosphere of nitrogen. In the trial methylation, the reaction was carried out at a temperature of 50°C, the partially methylated material isolated and the Purdie reagents, methyl iodide and silver oxide finally employed to give a product with a methoxyl content of 44.2%. The large-scale methylation was carried out at room temperature and a product with a methoxyl content of 44.6% was obtained, without making use of Purdie's reagents.

The methylated fructose from the large-scale methylation was fractionated by refluxing with chloroform-light petroleum mixtures of varying composition. The largest fraction thus obtained, was hydrolysed with a solution of oxalic acid in 80% aqueous methanol, as described by Haworth, Hirst and Percival (7) for the hydrolysis of trimethyl inulin. After removing a little insoluble material, the methanol was removed from the solution, replaced with water and hydrolysis continued with aqueous oxalic/
oxalic acid, to convert any glycosides to free sugars.

The partially methylated sugars in the hydrolysate of the methylated fructosan were separated by partition chromatography, this method having recently replaced that of high vacuum fractional distillation. Bell and Palmer have obtained a quantitative separation of tetramethyl D-fructofuranose, 1:3:4-trimethyl D-fructose and 3:4-dimethyl D-fructose on a column of silica gel. A method of separation that has been widely used is that employing a column of powdered cellulose, and this method was used to separate the components of the hydrolysate of the methylated fructosan.

Chromatographic examination of a small portion of the hydrolysate of the main fraction above, indicated the presence of trimethyl fructose with a little tetramethyl fructose and tetramethyl glucose and traces of a dimethyl fructose.

The same observations were made for the portion of methylated polysaccharide, which was insoluble in the above hydrolysis medium but which dissolved in aqueous ethanolic sulphuric acid. Urea oxalate, and naphthoresorcinol and hydrochloric acid sprays were used to detect the methylated fructoses, the former giving blue-black colorations and the latter deep red. Aniline oxalate spray was employed to detect any methylated aldoses present in the hydrolysate, and showed the presence of one only. This was exhibited as a deep pink spot on the paper chromatogram, travelled at the same rate as tetramethyl/
tetramethyl D-fructofuranose, and appeared to be tetra-
methyl glucose. The mixture of methylated sugars from
the main hydrolysis was added to a column of powdered cell-
sulose, which had been subjected to preliminary washings.
The eluting solvent used was a mixture of n-butanol (30%) and
light-petroleum (70%) saturated with water.\(^{(75)}\)

The eluate was collected at an automatic turntable
and the course of the separation followed by a series of
paper chromatograms run in duplicate, one being sprayed
with aniline oxalate to detect methylated glucoses and the
other with urea oxalate to show the presence of methylated
fructoses.

Two main fractions, referred to below as fraction A
and fraction B respectively, were obtained. Fraction A
travelled at the same rate on the paper chromatogram as
tetramethyl fructose and fraction B at the same rate as
1:3:4 trimethyl fructose. A very small third fraction
(7mg.) of dimethyl fructose was also obtained, but appear-
sed to have no structural significance. The tetramethyl
fraction gave a pink colour with aniline oxalate, indicat-
ing the presence of tetramethyl glucose, but no such pink
spot was obtained with the trimethyl fraction.

**Colorimetric Determination of Tetramethyl
Fructofuranose.**

It has been pointed out by Bell and Palmer\(^{(33)}\) that
1:3:4:6-tetramethyl-D-fructofuranose is volatile, a pure
sample undergoing a regular decrease in weight on drying
in a vacuum desiccator.

It was therefore decided to estimate the tetramethyl
fructofuranose/
fructofuranose colorimetrically in dilute solution, by a modification of Roe's procedure based on the Seliwanoff colour reaction, \(^{(76)}(41)\) employing resorcinol and hydrochloric acid. Standard solutions of chromatographically pure tetramethyl-D-fructofuranose in saturated aqueous benzoic acid solution were employed over the concentration range 0.05 to 0.2mg./c.c., to give a standard curve which showed a linear relationship between \(\log \frac{I}{I_0}\) and concentration. An aliquot of fraction A was saturated with benzoic acid and the tetramethyl-D-fructose estimated colorimetrically, using the standard graph. The fraction was thus found to contain 0.110g. tetramethyl D-fructose.

**Examination of Fraction A.**

The aldose content of this fraction was estimated by oxidation with alkaline hypoiodite, using a modification of the method described by Hirst, Rough and Jones.\(^{(77)}\) Under the conditions described by these workers, aldoses are oxidised quantitatively by alkaline hypoiodite, whereas, theoretically ketoses are unattacked. The stability of tetramethyl fructofuranose to alkaline hypoiodite was demonstrated by Hirst, McGilvray and Percival.\(^{(20)}\) Palmer\(^{(21)}\) however, has indicated that alkaline hypoiodite is not specific for aldoses and has pointed out that several investigators have obtained reduction of hypoiodide with ketoses. In the present investigations, authentic samples of tetramethyl- and 1:3:4-trimethyl-D-fructose were employed with this reagent, when the amount of reduction was found to be very small and may have arisen/
arisen from impurities in the sample and/or experimental error. Laidlaw and Reid(33) using a sample of pure 1:3:4-trimethyl-D-fructose also observed a very small, practically negligible iodine uptake. When a sample of fraction A was oxidised by alkaline hypoiodite, the aldose content was estimated as 13% of the fraction, corresponding to 17mg.

The tetramethyl-D-fructofuranose in fraction A was characterised by conversion to the crystalline tetramethyl-D-fructofuranamide. This was achieved by oxidation of the syrup with nitric acid followed by esterification with methanolic hydrogen chloride then methylation with methyl iodide and silver oxide. The amide was finally produced by the action of methanolic ammonia on the methyl ester as described by Avery, Haworth and Hirst.(75) The series of reactions are represented below.
The low methoxyl content determined for the crystalline amide (46.8%; theory 49.8%) may be due to a tendency for the glycosidic methoxyl group to be liberated as methanol immediately on heating, and this may be partly lost by distillation.

After extraction with light petroleum, the remainder of fraction A was found to crystallise partially on seeding with tetramethyl-D-glucose. The crystals appeared to be tetramethyl glucose, their melting point being undepressed when mixed with some authentic crystalline tetramethyl-D-glucopyranose.

Examination of Fraction B.

This fraction (2.211g.) crystallised completely and was shown by alkaline hypobromite oxidation to be free from aldoses. Periodic acid oxidation by Reeves' method gave formaldehyde, corresponding to a yield of 80% of theory, assuming one mole of formaldehyde per mole of trimethyl fructose. Of the four possible trimethyl fructoses, the 3:4:6- and 1:3:4-isomers will liberate formaldehyde, whereas 1:4:6- and 1:3:6-trimethyl fructoses will not.
The physical constants of this fraction however, showed that it was the 1:3:4- and not the 3:4:6- isomer.

The first methylation studies on the polyfructosan therefore indicated that the polysaccharide was composed of fructofuranose residues linked through the 2-6 positions, the relative amounts of tetra- and trimethyl fructofuranose suggesting a chain length of some 22 anhydrofructose units.

The small amount of methylated glucose accounted for, however, in the hydrolysate of the methylated fructosan, was an undesirable feature of the above investigation and it was decided to attempt a quantitative separation of tetramethyl glucose from tetramethyl fructose.

Investigation of the Methylated Polysaccharide from the Trial Methylation

Hirst, McGilvray and Percival(20) reported that tetramethyl methyl fructofuranoside and tetramethyl methyl glucopyranoside can be separated by preferential extraction of the furanoside with light petroleum in a liquid extractor. To verify this, the methylated polysaccharide from the trial methylation was hydrolysed, and the products of hydrolysis converted to the corresponding glycosides by treatment with methanolic hydrogen chloride. The methyl glycosides thus prepared were fractionated in all glass apparatus as described by Brown and Jones.(80) Extraction was continued for four periods of 4, 5, 6 and 7 hours respectively and each extract was taken to dryness. As the fourth extract amounted to a few mg. only, it was disregarded/
disregarded and the other three hydrolysed and examined on the paper chromatogram. All three extracts were found to contain both tri- and tetramethyl fructose, the latter being in very low concentration in the third extract. The glycosidic material remaining in the extraction apparatus was separated and hydrolysed. Chromatographic examination of the hydrolysate showed the presence of trimethyl fructose in large excess, tetramethyl fructose, tetramethyl glucose and a little dimethyl fructose.

**Investigation of a Second Fraction of Methylated Polysaccharide from the Large-scale Methylation.**

As the above investigation showed that light petrol-leum effects a partial separation of methyl tetramethyl glycoside and methyl tetramethyl fructoside, it was decided to investigate a second fraction of methylated fructosan employing this method of separation.

The remainder of the material from the methylation was treated with Purdie's reagents, to give a product with a methoxyl content of 45.4%. This was hydrolysed with methanolic oxalic acid as described above, the products of hydrolysis separated and converted to the corresponding glycosides with methanolic hydrogen chloride. The aqueous solution of glycosides was extracted with purified light petroleum in the presence of a little barium carbonate in a liquid extractor for three periods of 10, 12 and 16 hours respectively. Each extract was hydrolysed with N/5 sulphuric acid and the hydrolysates examined on the paper chromatogram. In this way, the first extract was seen to/
to contain only ketose, but the second and third extracts
contained small amounts of tetramethyl aldose also. The
hydrolysed extracts were combined to give fraction (a).
The glycosidic material remaining in the extractor was
separated and hydrolysed, to give fraction (b), shown on
chromatographic examination to contain a high proportion
of trimethyl fructose, with small amounts of tetramethyl
fructose and tetramethyl glucose.

**Examination of fraction (a).**

The sugars in fraction (a) were separated into two
fractions, a₁ and a₂ on a cellulose column with light pet-
:roleum-δ-butanol solvent, as before. Fraction a₁ con-
tained 60mg. tetramethyl fructofuranose, estimated by the
colorimetric method described above (p.28) and 13.4mg.
tetramethyl glucose, estimated by alkaline hypoiodite oxida-
tion. Fraction a₂(0.228g.) crystallised completely.

Hypoiodite oxidation indicated the presence of a small
quantity of aldoses, but aldoses could not be detected on
a paper chromatogram run in benzene-ethanol-water(167:45:15)
{61} it having been shown that 1:3:4-trimethyl fructose and
under these conditions.

No dimethyl sugars were detected in fraction (a).

**Examination of fraction (b).**

Separation of this fraction into its components was
attempted by elution through a cellulose column with ben-
zene-ethanol-water solvent, this having been found cap-
:able of separating tetramethyl glucose from tetramethyl
fructose/
fructose on the paper chromatogram. Three main fractions, \( b_1, b_2 \text{ and } b_3 \) were collected. Fraction \( b_1 \) contained tetramethyl glucose, tetramethyl fructose and a little trimethyl fructose. These were separated on sheets of Whatman 3M filter paper, using the same solvent. The tetramethyl fructose, estimated colorimetrically (p. 28) was found to amount to 30.6m.g., whilst alkaline hypoiodite showed the presence of 29.0m.g. of tetramethyl glucose. The trimethyl fructose amounted to 22m.g.

The tetramethyl glucose thus obtained from fraction \( b_1 \) was identified by its melting point, which was not depressed on admixture with an authentic specimen of 2:3:4:6-tetramethyl D-glucose, and its specific rotation \( ([\alpha]_D^o = +96^\circ \rightarrow +84^\circ \text{ in water}) \).

Fraction \( b_2 \) (1.862g.) was twice recrystallised from carbon tetrachloride-light petroleum and the supernatant liquor from each crystallisation combined and examined on the paper chromatogram, when a small quantity of trimethyl aldose was observed. This amounted to about 1 mg. as determined by alkaline hypoiodite oxidation.

Fraction \( b_3 \) was a mixture of trimethyl fructose and dimethyl fructose, with a trace of trimethyl glucose. These were separated on a cellulose column, elution with (a) light petroleum-\(n\)-butanol (7:3) saturated with water, as eluant and then with (b) light petroleum-\(n\)-butanol(1:1), yielding trimethyl fructose (16.5m.g.).

Hydrolysis of the second fraction of methylated fructose
fructosan has therefore given 1:3:4:6-tetramethyl D-fructose (4%), 2:3:4:6-tetramethyl D-glucose (1.8%), 1:3:4-trimethyl D-fructose (93.5%) and a dimethyl D-fructose (0.7%). The polysaccharide would therefore appear to possess a molecular structure comprising a straight chain of some 25, 2-6 linked fructofuranose residues, terminated by a glucopyranose residue, linked as in sucrose.

**Molecular Weight Determination on the Methylated and Acetylated Polysaccharide.**

Farger's isopiestic method\(^{(32)}\) for the determination of molecular weights was originally confined to compounds of low molecular weight, but Caesar, Gruenhut and Cushing\(^{(33)}\) found it possible to examine polymers of molecular weight 1,000 - 100,000 by this technique.

The main fraction of methylated polysaccharide from the large-scale methylation was investigated by this method, and the molecular weight was found to lie in the region 3,440-5,160, corresponding to a chain length of 17-25 units. In order to reduce the above range, the concentration of the fructosan solution was increased, but no reproducible results could be obtained, all the drops of methylated fructosan increasing in size. The same observations were made with solutions of the polysaccharide acetate - a phenomenon which has also been noted by Arni and Percival\(^{(41)}\) for methylated triticiin and by Laidlaw and Reid\(^{(35)}\) for the methylated fructosan from Lolium perenne.

**Oxidation of the Fructosan by the Periodate Ion.**

A/
A series of investigations commenced by Malaprade\(^{(84)}\) and continued by other workers\(^{(85)}\) showed that α-glycols, α-hydroxy ketones and α-hydroxy aldehydes are attacked by periodic acid with cleavage of the intermediate C - C bonds.

\[
R'\text{CHOHCHOH}R + \text{HIO}_4 \rightarrow R'\text{CHO} + R\text{CHO} + \text{HIO}_3 + \text{H}_2\text{O}
\]

\[
R'\text{CO}\text{CHOH}R + \text{HIO}_4 \rightarrow R'\text{COOH} + R\text{CHO} + \text{HIO}_3
\]

\[
R'\text{CHOHCHO} + \text{HIO}_4 \rightarrow R'\text{CHO} + R\text{COOH} + \text{HIO}_3
\]

In the majority of cases, this reaction occurs quantitatively, and this factor, together with the specificity of the reagent for such groups, makes it a useful tool in carbohydrate chemistry. One molecule of periodate is consumed for each C - C bond split, and when more than two adjacent hydroxyl groups are present, oxidation proceeds through this portion of the molecule, liberating formic acid from -CHOH groups and formaldehyde from -CH\(_2\)OH groups. Both of these may be readily estimated.\(^{(79})(86)(87)(88)}\)

Oxidation by the periodate ion provides a convenient method of estimating the chain length of a polysaccharide molecule, for if the reducing end-group contains three adjacent hydroxyl groups, formic acid will be liberated. Provided that the non-reducing end-group and the non-terminal residues do not produce formic acid on periodate oxidation, then the estimation of the formic acid produced provides a direct measurement of the number of reducing end-groups.

From the experimental evidence, the fructosan from \textit{Dactylis glomerata} contains 2-6 linked fructofuranose residues/
residues, and such a chain would be oxidised as follows:

\[
\begin{align*}
\text{Non-reducing end-group} & \quad \text{Non-terminal residues: Uptake reducing end group.} \\
\text{Uptake: 1 mole;} & \quad \text{1 mole; no formic acid.}
\end{align*}
\]

When the periodate uptake of the fructosan from *Dactylis glomerata* was determined, a constant value corresponding to a periodate uptake of 1.02 moles per fructose residue was obtained, in agreement with the concept of a 2-6 linked polyfructosan.

We might now consider the oxidation of the fructosan end-group on the assumption that it is either a fructofuranose residue linked through C₆ or a glucopyranose residue linked through C₁.

By analogy with fructose, a fructofuranose reducing end-group linked through C₆ is capable of splitting in two different ways on periodate oxidation.
Cleavage by method (i) will produce two moles of formic acid and one mole of glycollic acid (three moles titratable acid), taking up three moles of periodate during the cleavage. Cleavage by method (ii) however, will produce one mole of formaldehyde, one mole of formic acid and one mole of glyoxalic acid (two moles titratable acid), the uptake of periodate again being three moles.

According to Khovine and Arragon(69), fructose splits in both ways, although cleavage by method (ii) appears to occur preferentially. It is reasonable to presume that the reducing end-group under consideration will split in both ways also.

If the polysaccharide is terminated by a non-reducing glucopyranose group linked as in sucrose, the glucopyranose residue will take up two moles of periodate and yield one mole of formic acid on periodate oxidation, as indicated below.
In order to estimate the formic acid liberated, oxidation was performed with potassium periodate, according to Halseall, Mirst and Jones. The sparingly soluble potassium salt is recommended in preference to the soluble sodium salt in order that over-oxidation may be prevented. Experimentally, it was found that one mole of formic acid was liberated for every twenty anhydrofructose residues. Since a fructofuranose reducing end-group produces two or three moles of titratable acid, depending on the method of oxidation, the yield of formic acid could only agree with a "chain length" of 40-60 units, if the chain is indeed terminated by such a group. This value is considerably in excess of the "chain length" determined from methylation studies. If, however, the fructofuranose chains are terminated by a non-reducing glucopyranose residue, then the experimental results would call for a "chain length" of some twenty units. This figure, however, appears to be somewhat low and it may be that the polyfructosean preparation contains both types of chain - (a) those terminated by a fructofuranose end-group, and (b) those terminated by a non-reducing glucopyranose end-group.

Formaldehyde could not be detected in the solution of periodate-oxidised fructosean by the sensitive potassium ferricyanide-phenylhydrazine hydrochloride test. Although...
Although a reducing fructofuranose end-group should give rise to formaldehyde on periodate oxidation, it is quite possible that the formaldehyde may have been produced, but in insufficient quantity for detection.

The polyfructosan would thus appear to consist of a chain of some twenty-five fructofuranose residues linked through C2-C6 positions and terminated by a glucopyranose end-group, linked as in sucrose, indicated below.
A Fructosan from Dactylis glomerata.

The Preparation of the Polysaccharide.

The polyfructosan was extracted from leafy cocksfoot grass (*Dactylis glomerata*), which was oven-dried, milled material, prepared from grass cut on 26th May 1949, at the Jellott’s Hill Agricultural Research Station. Portions (100g.) of the milled material (moisture content 9.8%) were extracted with ether (1 l.) for 5 hours and the residue filtered and dried. The solvent was changed to 80% aqueous methanol and extraction continued for a further 5 hours, after which the grass was filtered and extracted for 3 hours with fresh 80% aqueous methanol (1 l.). The residue was removed, dried in air and extracted with distilled water (1.3 l.) by shaking for 12 hours at room temperature. The grass was removed by filtration, washed with water and the extract and washings (ca. 300 c.c.) combined. The solution was heated to 93°, cadmium sulphate solution (40 c.c.; 10%) and sodium hydroxide solution (20 c.c.; 0.5N.) added, and the whole kept at 95° for 3 minutes. After cooling, the cadmium hydroxide-protein complex was removed by filtration through "Filter cel". The clear filtrate was deionised on columns of Amberlite resins (1.R-100 and 1.R-43) and the last traces of protein removed by shaking with chloroform (0.25 vol.) and n-butanol (0.1 vol.) (63). Evaporation of the neutral solution to 100 c.c. gave a light amber coloured solution from which the fructosan was precipitated.
ated by pouring into methanol. The white precipitate was removed at the centrifuge, washed twice with acetone and dried in a vacuum desiccator over phosphoric oxide and paraffin wax. The polysaccharide was thus obtained as a fine white powder. The above process was repeated until some 30g. fructosan had been prepared and the combined products were well shaken to ensure homogeneity. The average yield of polysaccharide was ca. 3% of the dry weight of the grass. The fructosan had [\(\alpha\)]\(_D\) -40.4° (c, 1.7 in water) (Found: ash, 0.37%; as sulphate, 0.45%; N = 0.05%).

### Reducing Action of the Fructosan

A portion of the fructosan feebly reduced Fehling's solution on boiling for 10 minutes. The same observations were made with samples of Analar sucrose, triticin and inulin. It would appear that the polysaccharide is non-reducing, but is partially degraded on prolonged heating, with the exposure of free reducing groups.

### The Acetylation of the Fructosan

A specimen of the polysaccharide (1.5g.) was dissolved in dry pyridine according to the method of Passu and Mullen. Acetic anhydride (20c.c.) was added with stirring over 7 hours and the solution left for 2 days. The acetylated fructosan was precipitated with water (1 l.), washed with water, and the product dried in a vacuum desiccator over calcium chloride, giving a white powder. After reprecipitation from chloroform, with light petrolëum (b.p. 60-80°) a fine white powder was obtained (1.8g.;
$[\alpha]_D^{10} + 22^0$ in chloroform, c, 1.1; $\eta^{24} / c'$, 1.09 where $c'$ is the concentration in g. mols. of the unit $C_{12}H_{16}O_6$ per l.; Found: CH$_3$ CO, 43.1%)

**The Deacetylation of the Acetylated Fructosan.**

The acetyl derivative (0.5g.) was dissolved in chloroform (2.5c.c.), the solution cooled in a freezing mixture, and a solution of sodium (0.25g.) in absolute methanol (1 c.c.) added. The mixture was shaken for 5 hours and ice water (1 c.c.) added, followed by acetic acid (0.5c.c.; 10%). Water (4c.c.) was then added and the solution left to separate overnight. The regenerated fructosan was precipitated from the aqueous layer with methanol. The product (0.22g.) showed $[\alpha]_D^{10} = -40.2^0$ (c. = 1.1 in water).

**The Hydrolysis of the Fructosan.**

A sample of the fructosan (0.149g.) was hydrolysed by heating at 70° with oxalic acid solution (10c.c.; 0.1M) for 1 hour.

$[\alpha]_D^{10} = -40.3^0$ (zero)$\rightarrow -46.3^0$(10 minutes)$\rightarrow -67.1^0$ (20 minutes)$\rightarrow -78.5^0$(30 minutes)$\rightarrow -83.9^0$(40 minutes) constant.

The hydrolysate was neutralised with calcium carbonate, filtered, and inorganic ions removed from the solution by shaking with Amberlite resin. The solution was evaporated to a syrup under reduced pressure and examined on the paper chromatogram. Chromatograms run in butanol-ethanol-water and ethyl acetate - acetic acid - water and developed with aniline oxalate and naphthoresorcinol and hydrochloric acid showed the presence of
fructose, with only a little glucose.

A sample of the fructosan, regenerated from the polysaccharide acetate, hydrolysed in the same way, was likewise found to yield only fructose and a small amount of glucose.

Degradation of the Polysaccharide on Boiling in Aqueous Solution.

Preliminary Experiment:

The polysaccharide (0.1g.) was dissolved in distilled water (10c.c.) and heated on the boiling water-bath for 8 hours, when the specific rotation was found to fall from -40° to -36°. Samples of the solution were examined on the paper chromatogram, employing the solvent benzene-butanol-pyridine-water = 1:5:3:3; (91) and developing with aniline oxalate and naphthoresorcincol and hydrochloric acid sprays. It was thus seen that the fructosan had been degraded to about seven different products, including a sugar travelling at the same rate on the chromatogram as a fructose standard. An intense "trail" extending two centimetres below the starting-line could also be seen.
Detailed Examination I.

The polyfructosan (0.6446 g.) was dissolved in distilled water (50 c.c.) and heated on the boiling water-bath. At regular intervals, the pH and specific rotation of the solution were determined and samples of the solution examined on the paper chromatogram as above.

<table>
<thead>
<tr>
<th>Time</th>
<th>$[\alpha]_D^\circ$</th>
<th>pH</th>
<th>Colour of Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hrs.</td>
<td>-40.3°</td>
<td>6.09</td>
<td>Colourless</td>
</tr>
<tr>
<td>4 hrs.</td>
<td>-38.8</td>
<td>5.23</td>
<td>Colourless</td>
</tr>
<tr>
<td>8 hrs.</td>
<td>-35.6</td>
<td>4.70</td>
<td>Colourless</td>
</tr>
<tr>
<td>12 hrs.</td>
<td>-39.7</td>
<td>4.13</td>
<td>Faint yellow</td>
</tr>
<tr>
<td>16 hrs.</td>
<td>-45.8</td>
<td>3.90</td>
<td>Faint yellow</td>
</tr>
<tr>
<td>20 hrs.</td>
<td>-49.0</td>
<td>3.71</td>
<td>Yellow</td>
</tr>
<tr>
<td>24 hrs.</td>
<td>-52.7</td>
<td>3.57</td>
<td>Yellow</td>
</tr>
<tr>
<td>30 hrs.</td>
<td>-52.2</td>
<td>3.28</td>
<td>Light brown</td>
</tr>
<tr>
<td>36 hrs.</td>
<td>-58.8</td>
<td>3.28</td>
<td>Light brown</td>
</tr>
<tr>
<td>51 hrs.</td>
<td>-37.2</td>
<td>3.14</td>
<td>Light brown</td>
</tr>
</tbody>
</table>

Examination of the solution from time to time on paper chromatograms run in benzene-butanol-pyridine-water and ethyl acetate-acetic acid-water solvents and developed with urea oxalate, aniline oxalate and naphthoresorcinol and hydrochloric acid sprays, gave the following results:

Samples of the solution collected up to 16 hours showed the presence of some 6-9 different products. Glucose was present in the sample collected after 16 hours heating but appeared to be absent in the 8 hour sample. After 4 hours refluxing, the fructosan was seen to have been degraded.
graded only slightly, the solution at this stage showing only a very faint trail about 3 centimetres below the starting line on the paper chromatogram. Material moving more rapidly than fructose on the paper chromatogram, was observed in all samples collected after 16 hours, and was exhibited as a long faint streak extending from the fructose spot over a distance of about 4 centimetres. This material was only observed with the naphthoresorcinol spray and may have been a mixture of difructose anhydrides in small concentration.

Aniline oxalate showed the presence of traces of arabinose in samples collected after 16 hours, together with a second pink spot corresponding to a sugar travelling a little slower than sucrose on the paper chromatogram.

The 20 and 24 hour samples contained only fructose, glucose and a sugar travelling at the same rate as a sucrose standard, while the 30 and 39 hour samples had fructose and glucose only. The portion of the solution collected after 51 hours contained fructose, glucose and two products, one travelling slightly faster than sucrose and one a little slower than sucrose.

A saturated solution of p-anisidine hydrochloride in n-butanol used as developing spray, failed to show the presence of uronic acids. As a further test, to the final solution (5 c.c.), naphthoresorcinol solution (1 c.c.; 1% in alcohol) and an equal volume of concentrated hydrochloric acid were added, and the solution heated to boiling over a small flame. After keeping at the/
the boiling point for one minute, the solution was set aside for 4 minutes and cooled in a stream of cold water. An equal volume of ether was then added and the solution shaken vigorously for a few minutes. No blue coloration could be seen in the ether layer, which assumed a brown-red colour. It would thus appear that uronic acids were absent from the solution.

**Detailed Examination II.**

The polyfructosan (0.184g.) was dissolved in water (250 c.c.) and the solution heated at 100° as above, the following rotation changes being observed:

\[
[\alpha]_D = -40.1^\circ \text{(zero)} \rightarrow -37.5^\circ (4.5 \text{ hours}) \rightarrow -36.7^\circ (9 \text{ hrs.}) \\
\rightarrow -34.5^\circ (14 \text{ hours}) \rightarrow -42.4^\circ (19 \text{ hours}) \rightarrow -48.9^\circ \\
\rightarrow -34.4^\circ (29 \text{ hours}) \rightarrow -36.2^\circ (35 \text{ hours}).
\]

After heating for 22 hours, a sample (15 c.c.) of the solution was examined on the paper chromatogram, and showed the presence of fructose, glucose, a sugar travelling at the same rate as a sucrose standard and two slower-moving oligosaccharides in smaller concentration. The entire sample, after concentrating to small volume, was run on six paper chromatograms employing ethyl acetate-acetic acid-water = 3:1:3 solvent. Side strips of each paper were developed with aniline oxalate and the "sucrose areas" cut from the central portions of the paper chromatograms and eluted with cold water. The eluates were combined and after concentration to small volume, were hydrolysed with dilute oxalic acid as detailed above.

The hydrolysate was neutralised, filtered and the filtrate deionised/
deionised with Amberlite resins to give a solution containing both glucose and fructose, as observed on the paper chromatogram.

In view of the fact that the tests for uronic acids had proved negative, it was decided to search chromatographically for other acidic material, using the method employed for fatty acids. As solvent, m-butanol saturated with ammonia (115N.) was used. The paper chromatograms were run for 48 hours and developed with bromocresol purple, methyl red-methylene blue, thymol blue and bromo-thymol blue sprays. It was thus seen that samples of the solution collected on and after 14 hours heating, showed the presence of three acidic substances, travelling distances 0 cm., 1.4 cm., and 3.8 cm., respectively, when a lactic acid standard moved 3.9 cm. from the starting line.

The Estimation of the Fructose and Glucose in the Polysaccharide.

A portion of the fructosan (0.293 g.) was dissolved in water (25c.c.) and oxalic acid (0.16 g.) added, to give a solution decinormal with respect to oxalic acid. The solution was heated at 70° to constant rotation, the following observations being made:

\[ [\alpha]_D = -41.9^\circ \text{(zero)} \]; -65.0\(^\circ\) (20 minutes); -84.2\(^\circ\) (30 minutes); -85.1\(^\circ\) (40 minutes) constant.

The hydrolysate was treated as described above and concentrated to 4 c.c. under reduced pressure. Samples of the solution were run on six quantitative paper chromatograms in ethyl acetate-acetic acid-water solvent, the solution being added to the papers with a micro-pipette. In this way/
way, the same amount of sugar solution was added to each paper and the concentration of the sugar "spots" on the side strips was kept the same as those on the central area of each paper chromatogram. The papers were run for 33 hours at 20° and the side-strips developed with aniline oxalate spray, to show the presence of fructose in high concentration, and glucose in much lower concentration. The sugar areas from the central portions of the paper chromatograms were cut off along with paper strips of the same size, containing no sugars.

**Estimation of Fructose**

The fructose-containing paper strips and two paper blanks, were freed from acetic acid, prior to aqueous extraction, by evacuating in a desiccator over water for some 10 hours. The fructose was extracted from the papers by cold water elution, as described by Laidlaw and Reid, by paper blank strips being extracted alongside. Sodium metaperiodate solution (1c.c.; 0.25M) was added to each solution, and the solutions oxidised by heating on a boiling water bath for 30 minutes. After cooling in a stream of running water, ethylene glycol (0.3c.c.) was added and the liberated formic acid titrated against 0.01N. sodium hydroxide solution, using methyl red-methylene blue indicator.

**Results:**

| Normality of sodium hydroxide | 0.0124 N. |
| Paper blank titre I | 0.065 c.c. |
| Paper blank titre II | 0.100 c.c. |
| Average paper blank titre | 0.093 c.c. |
| Fructose titre I | 0.870 c.c. |
| Fructose/ |
Fructose titre II = 0.830 c.c.
Average fructose titre = 0.850 c.c.
Corrected for blank, titre = 0.757 c.c.
Weight fructose per paper = \( \frac{0.757 \times 60 \times 0.0124}{1000} \) g.
= 0.555 mg.

**Estimation of Glucose:**

The glucose was estimated by the Nelson colorimetric method.\(^{(73)(74)}\) A standard glucose solution (0.01%) was prepared and into four boiling tubes 1, 2, 3 and 4 c.c. portions of this were pipetted. One tube was employed for a paper blank and one for a water blank. A seventh tube was used with the glucose eluted from the six chromatogram papers. The solutions were placed in a boiling water bath for 15 minutes in order to equalise the amount of oxygen in each, and after cooling for 10 minutes in cold water, copper reagent (2 c.c.) was added to each (25A: 1E), where

\[
A = \text{Anhydrous sodium carbonate (25g.), Rochelle salt (25g.), sodium bicarbonate (20g.) and anhydrous sodium sulphate (200g.), dissolved in water (800c.c.)}
\]

and diluted to 1 l.

\[
B = \text{Copper sulphate pentahydrate (15%) containing one or two drops concentrated sulphuric acid, per 100c.c.}
\]

The solutions were heated at 100° for 20 minutes and cooled for 10 minutes in cold water. During each boiling process, the tubes were fitted with close-fitting glass bulbs to minimise evaporation losses. When cooled, arsenomolybdate reagent (2 c.c.) was added to each tube. This/
This reagent was prepared by dissolving ammonium molybdate (25g.) in water (450c.c.), adding concentrated sulphuric acid (21c.c.), mixing well and adding to this solution, a solution of disodium hydrogen arsenate septahydrate (3g.) dissolved in water (25c.c.). The solutions in each tube were then diluted to 25c.c. (calculated by weight) and estimated colorimetrically by means of the Spekker Absorptiometer (1cm. cells and green 604 filters). The standard solutions were examined against the water blank and the readings plotted graphically. The glucose from the fructosan was examined against the paper blank.

Results:

<table>
<thead>
<tr>
<th>Concentration of glucose (mg./c.c.)</th>
<th>Drum Readings $\log_{10} I_1/I_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.168</td>
</tr>
<tr>
<td>0.2</td>
<td>0.350</td>
</tr>
<tr>
<td>0.3</td>
<td>0.516</td>
</tr>
<tr>
<td>0.4</td>
<td>0.700</td>
</tr>
</tbody>
</table>

Drum reading for fructosan glucose = 0.170

Concentration of standard glucose solution = 0.0099%.

Weight of glucose per paper = \( \frac{0.1}{6} \times \frac{0.0099}{0.010} \) mg.

= 0.017 mg.

Ratio Glucose:Fructose = 0.017 : 0.563

= 1 : 33.1

On repeating the above hydrolysis and estimations, a ratio of Glucose:Fructose = 1 : 28.5 was obtained.

The Estimation of Glucose and Fructose, using Ribose as Reference Sugar.

The estimation of the glucose and fructose produced on/
on hydrolysis of another portion of the fructosan was made, this time an accurately weighed sample of recrystallised ribose being added to the solution immediately after hydrolysis. The fructose and ribose were estimated by peridate oxidation and the glucose by the Nelson colorimetric method.

**Results:**

- Weight of fructosan employed = 70.4 mg.
- Weight ribose added = 63.4 mg.
- Weight of fructose per paper = 0.663 mg.
- Weight of ribose per paper = 0.543 mg.
- Weight of glucose per paper = 0.023 mg.

Total weight glucose : fructose per paper = 0.686 mg.

Assuming ribose recovery 100%,

Recovery of fructosan = \(\frac{63.4 \times 0.663 \times 162}{0.543 \times 70.4 \times 180} \times 100\) = 102%

Ratio Glucose : Fructose = 0.023 : 0.663 = 1 : 28.8

Percentage Fructose = \(\frac{0.663}{0.686} \times 100\) = 97.8%

Percentage Glucose = \(\frac{0.023}{0.686} \times 100\) = 3.4%

**Control Experiment.**

A synthetic glucose - fructose mixture was prepared in approximately the same ratio as obtained in the above estimations. The sugars were then estimated as described above.

**Results:**

- Weight of glucose used = 36.6 mg.
- Weight of fructose used = 1.2300 g.
- Volume of solution = 25.0 c.c.

Percentage glucose = \(\frac{0.0366/1.2666 \times 100}{1.2300/1.2666 \times 100}\) = 2.9%

Percentage fructose = \(\frac{1.2300/1.2666 \times 100}{25.0}\) = 97.1%
Weight fructose per paper = 0.972 mg.
Weight glucose per paper = 0.031 mg.

Percentage glucose = \( \frac{0.031}{1.003} \times 100 = 3.1\% \)
Percentage fructose = \( \frac{0.972}{1.003} \times 100 = 96.9\% \)

**Studies on the Methylated Polysaccharide:**

**Trial Methylation:**

A portion of the polyfructosan (5g.) was dissolved in sodium hydroxide solution (65c.c.; 35%) and dimethyl sulphate (30c.c.) added dropwise over 4 hours, with vigorous stirring. The reaction flask was immersed in cold water and the reaction carried out in an atmosphere of nitrogen. After three such methylations, the solution was heated to 100° and filtered hot, to give a cream-coloured residue which was washed thoroughly with boiling water.

The partially methylated fructosan was dispersed in acetone (125c.c.) and sodium hydroxide solution (100 c.c.; 35%) and dimethyl sulphate (45c.c.) added dropwise over 6 hours, the reaction flask being immersed in a water-bath at 50°. On attempting to distil off the acetone under reduced pressure, the partially methylated polysaccharide turned oily and the solution darkened, when nearly all the acetone had been removed from the solution. The distillation was stopped, the solution filtered and the sticky residue boiled with water and filtered. The product thus obtained was dried at the pump, dissolved in chloroform (100c.c.) and the solution left overnight over anhydrous sodium sulphate. The methylated polysaccharide was precipitated from solution with light petroleum (b.p. 60-80°)
to give a fine white powder (4g.; OMe, 42.2%; theory 45.6%). The product was dispersed in acetone (125c.c.) and sodium hydroxide solution (150c.c.; 35%) and dimethyl sulphate (60c.c.) added over 6 hours at 50°, in an atmosphere of nitrogen.

The acetone was removed from the solution and the methylated material filtered off and washed with boiling water until free from sulphate. The product was dried, reprecipitated from chloroform as above, and refluxed with methyl iodide (60c.c.) for 2 days, silver oxide (40g.) being added portionwise, at intervals. After filtration, methylated material was recovered from the silver oxide by repeated extraction with boiling chloroform and the filtrate and combined extracts dried over anhydrous sodium sulphate. After concentration, the methylated polysaccharide was precipitated with light petroleum, yielding a fine white solid (2g.; OMe, 44.2%)

Test Hydrolysis of the Methylated Fructosan.

A specimen of the methylated fructosan (ca. 10mg.) was dissolved in methanolic oxalic acid (5c.c.) prepared by dissolving oxalic acid (1.35g.) in water (33c.c.) and adding to methanol (100 c.c.). The solution was heated at 80° for 24 hours, water (10c.c.) added, and the solution distilled under reduced pressure at 40°, to a volume of 5c.c. Heating was continued at 80° under reflux, for a further 4 hours and the solution cooled, neutralised with calcium carbonate and filtered. The concentrated filtrate was examined on the paper chromatogram using n-butanol-ethanol-water solvent, and developed with a saturated solution/
solution of urea oxalate in water. This indicated the presence of tetramethyl fructofuranose \((R_g = 1.0)\) and a trimethyl fructose \((R_g = 0.84)\). There was no evidence for the presence of dimethyl fructose, monomethyl fructose or free sugar.

**Large Scale Methylation of the Fructosan.**

The polysaccharide \((10g.)\) was dissolved in sodium hydroxide solution \((150c.c.; 30\%)\) and subjected to methylation with sodium hydroxide solution \((850c.c.; 30\%)\) and dimethyl sulphate \((300c.c.)\) over 3 days in the cold, in an atmosphere of nitrogen, with vigorous stirring.

The partially methylated polysaccharide was filtered off, washed with boiling water and dispersed in acetone \((250c.c.)\). Sodium hydroxide solution \((450c.c.; 30\%)\) and dimethyl sulphate \((150c.c.)\) were added to the acetone solution over 2 days in the cold in an atmosphere of nitrogen.

The acetone was removed under reduced pressure and the methylation in acetone repeated twice more in the cold.

Acetone was finally distilled off, the solution heated to boiling and the methylated polysaccharide filtered and washed thoroughly with boiling water. After drying at the pump, the methylated fructosan was dissolved in chloroform and precipitated with light petroleum \((b.p. 60-80^\circ)\) to yield a white powder \((9g.; OMe, 44.6\%)\).

**Fractionation of the Methylated Polysaccharide.**

Fractionation of the methylated fructosan was effected by refluxing with chloroform-light petroleum \((b.p. 40-60^\circ)\) mixtures of varying composition. The following
results were obtained:

<table>
<thead>
<tr>
<th>Fraction</th>
<th>% soluble in CHCl₃</th>
<th>[α]ᵤ⁰</th>
<th>C₆H₁₂O₆</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27%</td>
<td>-54°</td>
<td>45.3</td>
<td>0.4 g.</td>
</tr>
<tr>
<td>2</td>
<td>30%</td>
<td>-54°</td>
<td>45.3</td>
<td>0.6 g.</td>
</tr>
<tr>
<td>3</td>
<td>32.5%</td>
<td>-56</td>
<td>45.2</td>
<td>4.5 g.</td>
</tr>
<tr>
<td>4</td>
<td>35%</td>
<td>-56</td>
<td>45.5</td>
<td>1.6 g.</td>
</tr>
</tbody>
</table>

Test Hydrolysis of Fraction 3.

A sample of fraction 3 (ca. 500mg.) was hydrolysed with methanolic oxalic acid followed by aqueous oxalic acid as described above. The hydrolysate was neutralised with calcium carbonate, filtered and the filtrate examined chromatographically. The observations made were identical to those for the paper chromatograms run with samples of the hydrolysate from the hydrolysis of the material from the trial methylation, i.e. a large spot corresponding to a trimethyl fructose (Rf, 0.84) and a fainter, smaller spot corresponding to tetramethyl fructofuranose (Rf, 1.0).

Large Scale Hydrolysis of Fraction 3.

To a portion of fraction 3 (3.209g.), methanol (100c.c.) and water (34c.c.) containing oxalic acid (1.35g.), were added and the solution refluxed at 80° for 24 hours. A little insoluble material (0.187g.) was filtered off and recovered by extraction with hot chloroform, followed by precipitation in light petroleum (b.p. 60-80°). Water (ca. 300c.c.) was added portionwise and the methanol-water mixture distilled at 40° under reduced pressure. The volume of the solution was reduced to ca. 150c.c. and the solution heated at 80° for 5 hours. After neutralisation and/
and filtration, the solution was reduced to very small volume at 35° and the mobile syrup extracted several times with boiling chloroform. The combined extracts were dried over anhydrous sodium sulphate and reduced to small volume.

Examination of the resultant pale yellow syrup on the paper chromatogram with n-butanol-ethanol-water solvent, and several developing sprays gave the following results:

**Urea Oxalate:** An intense spot (black-blue-grey) corresponding to a trimethyl fructose (Rg, 0.84), a weaker spot of tetramethyl fructofuranose (Rg, 1.00) and a faint trace of a dimethyl fructose (Rg, 0.62).

**Aniline Oxalate:** A large light-brown spot (Rg, 0.84) with a heavy pink spot (Rg, 1.00) and a very light brown spot (Rg, 0.62).

**Naphthoresorcinol and hydrochloric acid.** A very deep red spot of trimethyl fructose, a lighter red spot of tetramethyl fructofuranose and a faint pink spot corresponding to dimethyl fructose.

**Inspection of Insoluble Material from the Hydrolysis.**

The insoluble material from the above hydrolysis gave a positive Molisch test and showed \( \left[ \alpha \right]_D^\circ = -56^\circ (c, 1.1 \text{ in chloroform}). \) The material (100mg.) was dissolved in hot ethanol (ca. 1c.c.; 96%) and sulphuric acid (ca. 1.5c.c.; 0.05N) added slowly and the solution heated under reflux at 100° for 8 hours. The ethanol was removed, replacement made with water and the solution refluxed for 18 hours at 100°. The solution was neutralised with barium carbonate.
carbonate and filtered to give a filtrate, which, when concentrated and examined on the paper chromatogram gave results identical to those recorded above for the three different developing sprays, except for the presence of a small spot just below the starting line on the paper chromatogram. The latter probably arose from a little partially hydrolysed material.

The Separation of the Methylated Sugars in the Hydrolysate from Fraction 3.

The sugars arising from the hydrolysis of fraction 3 were separated on a column of powdered cellulose (70 x 3cm.) using the technique developed by Hough, Jones and Wadman. Light petroleum (b.p. 100-110°)-a-butanol (7:3) saturated with water was used as eluant.

The light petroleum employed was purified by shaking with concentrated sulphuric acid (1/3 vol.), followed by washing with water and sodium bicarbonate solution and distillation. The butanol was refluxed for 10 hours with sodium hydroxide (10g. per l.) and distilled, the fraction b.p. 117-118.5° being collected.

The column was washed with water, butanol saturated with water and finally with the solvent to be used as eluant.

The hydrolysate of the methylated polysaccharide was added to the column and after two hours, elution was commenced. The first 100c.c. eluate collected was found chromatographically to contain no sugars. The column was then placed on an automatic turntable, the receiving tube being changed every 4 minutes by a timing device.
Every tenth tube was evaporated to dryness on the boiling water-bath, 2 drops of water added to each and the solutions spotted on paper chromatograms. Each chromatogram was duplicated, one being developed with urea oxalate in order to detect methylated fructoses and the other with aniline oxalate to show the presence of methylated aldoses. Fractionation occurred as follows:

<table>
<thead>
<tr>
<th>Tubes</th>
<th>Rg. value of Syrup</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>75-105</td>
<td>1.01</td>
<td>A (tetramethyl)</td>
</tr>
<tr>
<td>110-250</td>
<td>0.88</td>
<td>B (trimethyl)</td>
</tr>
<tr>
<td>640-740</td>
<td>0.62</td>
<td>C (dimethyl)</td>
</tr>
</tbody>
</table>

The tetramethyl sugar fraction, in addition to giving a blue-black coloration with urea oxalate, gave a pink coloration with aniline oxalate, whereas pure tetramethyl fructofuranose gave only a faint yellow-brown coloration with aniline oxalate. Fraction A therefore appeared to contain some tetramethyl glucopyranose (Rg, 1.00) together with the tetramethyl fructofuranose (Rg, 1.01). There was no evidence for the presence of trimethyl or dimethyl fructose in the aniline oxalate series of paper chromatograms.

Investigations of the Fractions from Column I.

Fraction A:

The tetramethyl fructofuranose in this fraction was estimated colorimetrically by a modification of Roe's procedure.

Construction of Standard Curve for Tetramethyl-D-fructofuranose.

Reagents:

**Acid Reagent:** 130 g. glycerol B.P.
100 c.c. conc. HCl (A.R.) containing
45 mg. Cu SO₄, 5 H₂O/l.
50 c.c. H₂O

Reagent: 0.45% in H₂O

Stock tetramethyl fructose solution: ca. 5 mg./c.c. in
saturated benzoic acid solution.

The tetramethyl fructose employed in preparing the stock
solution was chromatographically pure.

Standard tetramethyl fructose solutions:
(Prepared by dilution of the stock
solution).

<table>
<thead>
<tr>
<th>Concentration (mg./c.c.)</th>
<th>Spekker Reading (log 10 X₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.045</td>
<td>0.235</td>
</tr>
<tr>
<td>0.075</td>
<td>0.365</td>
</tr>
<tr>
<td>0.119</td>
<td>0.505</td>
</tr>
<tr>
<td>0.159</td>
<td>0.660</td>
</tr>
<tr>
<td>0.170</td>
<td>0.735</td>
</tr>
<tr>
<td>0.198</td>
<td>0.830</td>
</tr>
</tbody>
</table>
glass joints and phosphate buffer (2c.c.) added to each, followed by iodine (1c.c.; 0.1N.) and the stoppers sealed with a little potassium iodide solution (10%). Two water blanks were treated identically. The tubes were allowed to stand in the dark at room temperature for 3 hours, sulphuric acid (2c.c.; 4N.) added, and the iodine titrated with sodium thiosulphate (0.0115N.) using starch indicator.

Results:
Water blank titre (average) = 3.721 c.c.
Sugar solution titre (average) = 7.741 c.c.

Volume N. thiosulphate used = (3.721 - 7.741) x 0.0115c.c. = 0.0112 c.c.

Weight tetramethyl glucose = (236/3000) x 0.112/102 = 1.33mg.

Weight of tetramethyl glucose in fraction A = 13.3 x 10^8 = 17 mg.

Fraction A therefore contains 87% tetramethyl fructose
13% tetramethyl glucose

Fraction B.

The "trimethyl fraction" was estimated gravimetrically, the purification (dewaxing) process being identical to that described previously for fraction A.

Weight of trimethyl fructose = 2.311 g.

Estimation of the Aldose Content of Fraction B.

A portion of this fraction (52.2mg.) was dissolved in water (10c.c.) and samples (2c.c.) of this solution taken for the estimation. The aldose content was estimated by alkaline hypoiodite oxidation, using the procedure described above.

Results (A)
Average/
Average water blank titre = 8.721 c.c.
Average sugar solution titre = 8.614 c.c.
Titration difference = 0.107 c.c.

Another sample of fraction B (88.0mg.) was dissolved in water (20 c.c.) and portions (2 c.c.) used as above. Water blanks and a standard solution of 1:3:4-trimethyl fructose-furanose of concentration 8.3mg./c.c. was also employed, 1 c.c. samples being used.

Results (B)

Average water blank titre = 8.707 c.c.
Standard sugar solution titre (average) = 8.644 c.c.
Fraction B solution titre (average) = 8.610 c.c.

The differences in the above titrations are considered sufficiently small to be attributed to experimental error and/or impurities in the samples used.

Fraction C.

This fraction was estimated gravimetrically and amounted to 7 mg. It was chromatographically pure with \( R_g \) value 0.62 in butanol-ethanol-water solvent.

Identification of the Partially Methylated Sugars from the Cellulose Column.

Fraction A:

The remainder of the aqueous solution of fraction A left after the above estimations had been carried out was taken to dryness at 35° to give a pale yellow mobile syrup which showed \( n^\circ_o = 1.4500 \) \([\alpha]_o^\circ = +39^\circ (d = 1.0 \text{ in water}) \) and OMe, 49.4% (theory 52.5%).

Investigation into the Structure of the Tetramethyl Fructose.

The tetramethyl fructose was proved to be 1:3:4:6-
tetramethyl fructofuranose, by conversion to the crystalline tetramethyl fructofuranamide, the method employed being similar to that described by Avery, Haworth and Hirst.

Fraction A (ca. 40mg.) was oxidised with concentrated nitric acid (1c.c.; d. 1.42) in a water-bath, the temperature of which was slowly raised. The reaction appeared to commence at a bath temperature of about 60° and heating was maintained up to the boiling point, where it was kept for two hours. The cooled solution was diluted with water and distilled at 40° to remove the nitric acid. This process was repeated some 10 times, when methanol was used in place of water and finally, anhydrous methanol.

The resulting syrup was dissolved in methanolic hydrogen chloride (3c.c.; 4%) and gently refluxed overnight, neutralised with silver carbonate and the filtered solution evaporated to a syrup which was methylated with the Purdie reagents, methyl iodide (5c.c.) and silver oxide (1.5g.) for 8 hours. The material was removed by extraction in chloroform and after evaporation of the solvent, the syrup was treated with methanol saturated with ammonia (3c.c.) and the solution allowed to stand at 0° for 3 days. Removal of the methanol gave a crystalline solid which was recrystallised 3 times from ether-light petroleum (b.p.40-60°), to give long needle-shaped crystals (11mg. of crude product).

The crystals showed m.p. 99-101° (not depressed on admixture with an authentic specimen of tetramethyl fructofuranamido).
Found: C, 48.9%; H, 3.4%; OMe, 40.6%.

Calculated for: \( \text{C}_9\text{H}_{18}\text{O}_5 \): C, 48.6%; H, 3.2%; OMe, 41.9%.

**The Isolation of Tetramethyl Glucose from Fraction A.**

The remainder of the syrup from fraction A was extracted with light petroleum (b.p. 35°) and both the extract and syrupy residue taken to dryness. A speck of authentic crystalline tetramethyl glucose was added to each of the syrups, which were kept in a vacuum desiccator over phosphoric oxide and paraffin wax in a refrigerator for 3 weeks. The "extract" remained as a syrup, but the "residue" crystallised partially. The crystals separated on porous tiles had m.p. 32°, not depressed on admixture with an authentic specimen of tetramethyl glucose.

**Fraction B.**

The trimethyl fraction crystallised completely when kept at 0°, and was seen to be chromatographically pure. Aniline oxalate failed to show the presence of any aldose material on the paper chromatogram. The sugar showed m.p. 73-75° after two recrystallisations from carbon tetrachloride-light petroleum, not depressed on admixture with an authentic specimen of 1:3:4-trimethyl-D-fructofuranose.

\[ [\alpha]_D = -27.4° (4 minutes); -47.3° (20 minutes); -56.6° (60 minutes); -58.8° (5 hours); -60.6° (20 hours); -61.1° (66 hours, constant). \] (c, 1.4 in water)

\( \eta^\infty \) (fused crystals), 1.4662.

Found: C, 48.9; H, 3.4; OMe, 40.6%

Calculated for: \( \text{C}_9\text{H}_{18}\text{O}_5 \): C, 48.6; H, 3.2; OMe, 41.9%

**Oxidation by Periodic Acid.**

The trimethyl fructose (17.4mg.) was oxidised and...
the formaldehyde liberated estimated as the formaldehyde-dimedon compound, using the method of Reeves (79).  

The sugar was dissolved in water (2c.c.), sodium bicarbonate solution (2c.c.; 1 N.) added, followed by periodic acid (2c.c.; 0.3 M.) and the solution allowed to stand overnight at room temperature. Excess periodate and iodate were destroyed by the addition of hydrochloric acid (3c.c.; 1 N.), followed by sodium arsenite solution (2c.c.; 1.2 N.). When the yellow colour had completely disappeared, sodium acetate (2c.c.; 1 M) was added, followed by dimedon reagent (2c.c.; 35mg./c.c. in 95% ethanol) and the precipitation allowed to occur overnight.

Yield of formaldehyde-dimedon compound = 16.2 mg.  
This corresponds to a yield of 79.6% of theory, assuming 1 mole of formaldehyde per mole of trimethyl fructose; m.p. 186-187° not depressed on admixture with an authentic specimen of formaldehyde-dimedon compound.

A glucose control solution oxidised by this method gave "F-B" compound (m.p. 186-187°) in 94% yield, and a formalin control gave a 96.5% yield.
THE PRODUCTS OF HYDROLYSIS OF THE
FULLY METHYLATED POLYSACCHARIDE.

[SUMMARY]

Methylated Fructosan

[3.022 g; OMe = 45.2%]

Hydrolyse with aqueous methanolic oxalic acid, followed by aqueous oxalic acid.

Methylated Sugars.

Cellulose column.

Fr. A.

Tetramethyl fructose [0.110 g] estimated colorimetrically and Tetramethyl glucose [19 mg] estimated by alkaline hypohypochlorite oxidation.

Fr. B.

Trimethyl fructose [2.211 g] estimated gravimetrically.

Fr. C.

Dimethyl fructose [4 mg].

No trimethyl glucose.
Investigation of the Methylated Polysaccharide from the trial Methylation.

A portion of methylated fructosan, (1.002g.) obtained from the trial methylation was hydrolysed, the partially methylated sugars converted to the corresponding glycosides and an attempt made to separate tetramethyl methyl glucoside from tetramethyl methyl fructofuranoside by solvent extraction.

The methylated polysaccharide was hydrolysed with methanolic oxalic acid as detailed above, the solution neutralised with calcium carbonate, filtered and evaporated to a mobile syrup, which was extracted with chloroform in the presence of anhydrous sodium sulphate, and the combined extracts (25.150 c.c.) taken to dryness. The pale yellow syrup (1.001g.) was dissolved in methanolic hydrogen chloride (50 c.c.; 0.3%) and allowed to stand at room temperature for 6 hours. The solution, after neutralisation with barium carbonate and filtration, was taken to dryness, extracted with chloroform and the combined extracts taken to dryness, to give a non-reducing syrup (0.918 g.)

The methyl glycosides thus obtained were fractionated in all glass apparatus as described by Brown and Jones. The solvent used was contained in a flask above which were mounted two extractors and a reflux condenser. The methyl glycosides were dissolved in water (50 c.c.) and the solution placed in the upper extractor. The lower contained water (50 c.c.) and a little barium carbonate was introduced into each extractor.
The extraction was performed with light petroleum (b.p. 38-40°), used in 200 c.c. portions. Each extract was hydrolysed at 80° for 2½ hours with 2.5% oxalic acid solution, and the neutralised hydrolysates examined on the paper chromatogram in conjunction with standards of 1:3:4-trimethyl fructose, tetramethyl fructofuranose and tetramethyl glucose. Aniline oxalate, urea oxalate and naphthoresorcinol and hydrochloric acid, were used as developing sprays. The following observations were made:

<table>
<thead>
<tr>
<th>Duration of Extraction (hours)</th>
<th>Weight (mg.)</th>
<th>Chromatogram Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>42</td>
<td>Tri- and tetramethyl fructose.</td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>Tri- and tetramethyl fructose</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>Tri- and tetramethyl fructose, the latter in low concentration.</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>-</td>
</tr>
</tbody>
</table>

No trace of tetramethyl glucose could be seen in the above chromatographic examinations.

The aqueous solution remaining in the extractors was evaporated to dryness and the residual syrup extracted with chloroform in the presence of anhydrous sodium sulphate. The extracts were taken to dryness, giving a colourless syrup (0.714g.) a portion of which was hydrolysed with oxalic acid solution (10 c.c.; 2.5%) and the hydrolysate examined as before. The syrup was thus found to consist of a large excess of trimethyl fructose with a little di- and tetramethyl fructose. Aniline oxalate gave a pink spot corresponding to tetramethyl glucose.

It thus appears that light petroleum (b.p. 38-40°) effects a partial separation of tetramethyl methyl fructofuranoside.
fructofuranoside from tetramethyl methyl glucoside.

Investigation of a Second Fraction of Methylated Poly saccharide from the large-scale methylation.

Fractions 1, 2, 4 and some unfractiated material from the large-scale methylation were combined to give fraction X (3.073g.). This was dissolved in methyl iodide (20c.c.), the solution refluxed gently on the water-bath and silver oxide (10g.) added portionwise over 6 hours. After the addition of methanol, (15c.c.), the solution was filtered and the residue extracted with boiling chloroform. The filtrate and combined extracts were concentrated to small volume and the product recovered by precipitation from light petroleum (b.p. 40-60°). The methylated material, after separating and drying showed, 

\[ [\alpha]_D^{\text{H}} = -54.4^\circ (c, 1.2 \text{ in chloroform}) \]

Found: OMe, 45.4%.

The hydrolysis of fraction X.

A portion of fraction X (2.512g.) was hydrolysed with methanolic oxalic acid, the solution neutralised with calcium carbonate and filtered. The filtrate was concentrated under reduced pressure at 35°, with constant addition of methanol, which was finally replaced by anhydrous methanol. The volume of the solution was reduced to 5c.c. and methanolic hydrogen chloride (50c.c.; 0.3%) added and the contents of the flask well shaken. The solution was set aside at room temperature for 3 hours, neutralised, filtered and methanol removed as before. The aqueous solution (50c.c.) was extracted in the presence of a little barium carbonate, with purified light petroleum (b.p./
(b.p.38-40°) in a liquid extractor for 3 periods of 10, 12 and 16 hours respectively. The three extracts were concentrated almost to dryness and hydrolysed separately with sulphuric acid (15 cc.; H/5) for 6 hours at 80°. The first contained only ketose, but the second and third also contained small quantities of tetramethyl aldose.

The hydrolysed extracts were combined to give fraction a. The aqueous solution in the extractor was filtered and hydrolysis carried out with sulphuric acid (250 cc.; H/5) for 6 hours at 80°, to give fraction b. Chromatographic examination of the products of this second fraction showed a high proportion of trimethyl fructose, with small amounts of tetramethyl fructose and tetramethyl glucose.

Separation of fraction a.

The sugars in this fraction were separated into two fractions a₁ and a₂, on a cellulose column (60 x 1.8 cm.) using light petroleum (b.p.100-120°)-n-butanol (7:3) saturated with water, as eluent. After tube 580 had been collected from the automatic turntable, the eluent was changed to a 1:1 mixture of the above two solvents, saturated with water. Fraction a₁, obtained by combining the contents of tubes 20-36 was examined on the paper chromatogram and found to contain both tetramethyl glucose and tetramethyl fructose. The contents of tubes 37-130 contained only trimethyl fructose and were combined to give fraction a₂. No dimethyl sugars could be detected on further elution.

Estimation of the tetramethyl fructose in fraction a₁

The/
(pH 11.4) and iodine (1c.c.; N/10). After leaving in the dark for 3 hours, sulphuric acid (2c.c.; 4N) was added to each and the iodine titrated with sodium thiosulphate solution (0.01220 N.).

Weight of tetramethyl glucose found = 13.4 mg.

**Control Alkaline Hypoiodite Oxidation.**

In order to discover if tetramethyl fructose was attacked by alkaline hypoiodite, the following control experiment was performed:

Chromatographically pure tetramethyl fructofuranose (15.3 mg.) was dissolved in water (5c.c.) and portions (1c.c.) subjected to the action of alkaline hypoiodite as detailed above. Two water blanks were run at the same time.

**Results:**

- Normality of thiosulphate solution = 0.01220 N.
- Water blank titre I = 7.856 c.c.
- Water blank titre II = 7.836 c.c.
- Sugar titre I = 7.822 c.c.
- Sugar titre II = 7.744 c.c.

From the above results, it appears that tetramethyl fructofuranose is not attacked by alkaline hypoiodite under the conditions employed, or is attacked to an insignificant extent only.

**Examination of fraction a**

The remaining aqueous solution of this fraction was concentrated to a syrup, which, on thorough drying showed $[\alpha]_D^{20} +35.4^\circ$ (c, 0.6 in water) $n_2^\circ$, 1.4512.

(Found: $\text{C}_8\text{H}_{20}\text{O}_3$, 51.4%)

Gravimetric/
Gravimetric Examination of Fraction e2

The contents of the turntable tubes 37-130 were de-waxed and the solution concentrated to a pale yellow syrup (0.228 g.). This crystallised completely when kept at 0° and showed m.p. 74-75° after recrystallisation twice from carbon tetrachloride-light petroleum. The melting point was not depressed on admixture with an authentic specimen of 1:3:4-trimethyl fructose. \( \mu^0, 1.4652 \) (fused crystals) \([\alpha]_b^0, -60° \) (equilibrium value) (c, 1.2 in water)

(Found: OMe, 41.2%)

Examination of fraction e2 for the presence of aldose

Hypobromite oxidation of samples of this fraction indicated the presence of trimethyl aldose (3 mg.). Aldoses however could not be detected on a paper chromatogram run in benzene-ethanol-water (167:45:15), it having been shown that 1:3:4-trimethyl fructose separated from 2:3:4- 2:4:6- and 2:3:6-trimethyl glucose under these conditions. The small amount of oxidation observed would thus appear to have arisen from impurities in the samples used.

Separation of fraction e2

Attempted separation of this fraction into its components was made by elution through a cellulose column (66 x 2.2 cm.) with benzene-ethanol-water solvent, this having been found capable of separating tetramethyl glucose from tetramethyl fructose on the paper chromatogram. Three main fractions e2, e3 and e3 were collected.

Fraction e1 contained tetramethyl glucose, tetra-
methyl fructose and a little trimethyl fructose. These were separated on 4 sheets 3 M.M. filter paper, by the above-mentioned solvent and extracted from the paper with hot alcohol and water. The tetramethyl fructose was estimated colorimetrically (Acid Reagent-Resorcinol method) and amounted to 30.6 mg. Alkaline hypoiodite showed the presence of 29.0 mg. tetramethyl glucose in this fraction, and the trimethyl fructose amounted to 22 mg. as determined by direct weighing. Fraction b₂ (1.862 g.) was twice recrystallised from carbon tetrachloride-light petroleum and the supernatant liquors from each recrystallisation combined and examined on the paper chromatogram, when a small quantity of trimethyl aldose was observed (ca. 1 mg. as determined by alkaline hypoiodite oxidation).

Fraction b₃ (55.8 mg.) was a mixture of tri- and dimethyl fructose, with a trace of trimethyl aldose. The methylated fructoses were separated on a column of cellulose (56 x 1.6 cm.), elution first with light petroleum-n-butanol (7:3) saturated with water and then with those solvents in the ratio 1:1, saturated with water, yielding trimethyl fructose (36.0 mg.) and dimethyl fructose (16.4 mg.)

Identification of tetramethyl glucose.
The aldose material from fraction b₁, after two recrystallisations from ether-light petroleum (b.p. 40-60°) had m.p. 85°, not depressed on admixture with an authentic specimen of 2,3,4,6-tetramethyl-D-glucose and showed \[ [\alpha]_d^{15}, +96° \rightarrow +84° \] (c, 0.5 in water)

(Found: OMe, 51.4%)

Examination/
Examination of dimethyl fructose.

The dimethyl fructose from fraction b3 had Rg, 0.61
in butanol-ethanol-water = (40:10:50) and showed ν°, 1.4784 and OMe, 27.2°.
MOLeCULAR WIGHT DETERMINATIONS.

(a) Molecular Weight of the Methylated Polysaccharide.

The molecular weight of the fractionated methylated polysaccharide (fraction 3) was determined using Rangar’s isopiestic method (82)(83).

A chloroform solution of known concentration of the polysaccharide (1.032%) was compared with a series of chloroform solutions of sucrose octacetate of known molar concentration. For this purpose, a series of drops taken alternately from the methylated polysaccharide solution and one of the standard solutions were introduced into a capillary and the ends sealed. A series of capillaries were made in this way, to cover a molar concentration of 0.001M - 0.006M for the standard material. The capillaries were immersed in water in a constant-temperature room and the size of drops measured with a travelling microscope, having a vernier accurate to 0.02m.m. Readings were taken after 1 hour and again after 20 hours.

Results:

Concentration of methylated fructosan solution = 1.032%

<table>
<thead>
<tr>
<th>Molarity of sucrose octacetate drops</th>
<th>Change in size of drops (10⁻³ m.m.)</th>
<th>Sum of changes w.r.t. methylated fructosan</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>6 -6 10 -4 16</td>
<td>46</td>
</tr>
<tr>
<td>0.002</td>
<td>3 30 -5 0 27</td>
<td>5</td>
</tr>
<tr>
<td>0.003</td>
<td>-6 15 -2 1 12</td>
<td>-12</td>
</tr>
<tr>
<td>0.004</td>
<td>0 13 -8 14 -1</td>
<td>-36</td>
</tr>
<tr>
<td>0.005</td>
<td>-7 15 -6 5 -4</td>
<td>-37</td>
</tr>
<tr>
<td>0.006</td>
<td>-9 13 -10 8 -2</td>
<td>-42</td>
</tr>
</tbody>
</table>

F = Methylated fructosan
S = Sucrose octacetate

From the above table, it appears that the isopiestic condition lies between 0.002M and 0.003M, whence

Molecular/
Molecular Weight - \[1.032 \times 10^{-5} \text{ to } 1.032 \times 10^{-5}\]

Since the molecular weight of a methylated anhydrofructose unit is 204, the molecular size is 17-25 units.

The estimation was repeated using a fresh set of standards over the range 0.002M - 0.007M and a 2% solution of the methylated polysaccharide. In every case, however, it was found that the drops of the methylated fructosan solution expanded at the expense of the acetate solution drops.

(b) Molecular Weight of the Acetylated Fructosan.

Attempts were made to determine the molecular weight of the fructosan acetate by the method detailed above. Using a 4% solution in chloroform with sucrose octacetate standards over the range 0.001M - 0.005M, the drops of the former were found to expand in every capillary. The determination was repeated first with a 2% solution of the polysaccharide acetate over the sucrose octacetate range 0.001M - 0.007M, followed by a 1% solution over the range 0.0005M - 0.005M, but in each case the polysaccharide acetate solutions were found to expand at the expense of the solutions of sucrose octacetate.

Oxidation of the Fructosan by the Periodate Ion.

Estimation of the Uptake of Periodate

To a solution of the polysaccharide (ca. 300mg., accurately weighed) in water (35c.c.) was added sodium metaperiodate solution (15c.c.; 0.3M) and the solution allowed to stand in the dark at room temperature. A blank/
blank experiment omitting only the polysaccharide, was run concurrently. At regular intervals, portions (5 c.c.) were removed by pipette and the periodate uptake determined by the addition of excess sodium arsenite solution (M/10) and potassium iodide and titration of the excess arsenite with standard iodine.

**Results:**

Weight of polysaccharide = 0.2864g. in 50 c.c.

<table>
<thead>
<tr>
<th>Duration (Hours)</th>
<th>Titre of 0.0399 Iodine</th>
<th>Periodate uptake in mole/fructose residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank</td>
<td>Test</td>
</tr>
<tr>
<td>1</td>
<td>3.13 c.c.</td>
<td>6.55 c.c.</td>
</tr>
<tr>
<td>2</td>
<td>3.26 c.c.</td>
<td>6.66 c.c.</td>
</tr>
<tr>
<td>3</td>
<td>3.21 c.c.</td>
<td>6.91 c.c.</td>
</tr>
<tr>
<td>4</td>
<td>3.23 c.c.</td>
<td>7.00 c.c.</td>
</tr>
<tr>
<td>6</td>
<td>3.21 c.c.</td>
<td>7.07 c.c.</td>
</tr>
<tr>
<td>22</td>
<td>3.23 c.c.</td>
<td>7.18 c.c.</td>
</tr>
<tr>
<td>30</td>
<td>5.19 c.c.</td>
<td>7.14 c.c.</td>
</tr>
</tbody>
</table>

**Estimation of liberated formic acid.**

The polyfructosan was subjected to oxidation by potassium periodate, according to Halsall, Hirst and Jones. The polysaccharide (ca. 300mg., accurately weighed) was dissolved in water (35 c.c.) and potassium chloride (1 g.) and sodium metaperiodate solution (15 c.c.; 0.3M) added, and the mixture shaken in the dark at room temperature. After 3-4 days, samples (5 c.c.) were withdrawn at intervals. After the potassium periodate had completely separated, ethylene glycol (0.3 c.c.) was added to destroy excess periodate and the formic acid titrated against standard sodium hydroxide (0.0127N.) using methyl red as indicator. A blank experiment, omitting only the polysaccharide, was run concurrently and treated in exactly the same way as the test solution. In addition, a solution of the fructosan/
fructosan in water, of the same concentration as the polysaccharide solution submitted to oxidation, was titrated directly against the standard sodium hydroxide.

Results:

Fructosan blank Titre = 0.005 c.c. 0.0127 N. NaOH for 30 mg. fructosan.

Weight of polysaccharide = 0.2762 in 50 c.c.

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>Alkali titre after correction for blank of formic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>73</td>
<td>0.588 c.c.</td>
</tr>
<tr>
<td>100</td>
<td>0.627 c.c.</td>
</tr>
<tr>
<td>126</td>
<td>0.660 c.c.</td>
</tr>
<tr>
<td>171</td>
<td>0.688 c.c.</td>
</tr>
<tr>
<td>264</td>
<td>0.761 c.c.</td>
</tr>
</tbody>
</table>

Examination for the presence of formaldehyde

To the solution of periodate oxidised polysaccharide (5 c.c.) was added hydrochloric acid (5 c.c.; 1N) and sodium arsenite solution (2 c.c.; 1.2N) to destroy excess periodate, followed by phenylhydrazine hydrochloride solution (2 c.c.; 1%) and potassium ferrocyanide solution (1 c.c. 5%). On addition of a few drops of concentrated hydrochloric acid, no port wine coloration was obtained, whereas a wine colour was given with a formalin standard solution containing 0.001 mg. formaldehyde.

Treatment with Dimedon solution:

To the solution of periodate oxidised fructosan (5 c.c.) (containing 27.32 mg. polysaccharide) were added hydrochloric acid (2 c.c.; 2N) and sodium arsenite solution (2 c.c.; 1.2N). After the yellow colour had completely disappeared, sodium acetate solution (2 c.c.; 1M) and dimedon/
dimedon solution (2c.c.; 85mg./c.c. in 95% alcohol) were added, and the solution allowed to stand at room temperature. A blank experiment was performed simultaneously, by the addition of the above reagents to 5 c.c. of a solution of sodium metaperiodate and potassium chloride, in water.

After standing for 48 hours, the solutions were seen to have acquired a faint turbidity, but filtration failed to yield an appreciable residue.
A polyfructosan has been extracted from mid-season cocksfoot grass (*Dactylis glomerata*) after the removal of free sugars, chlorophyll and other pigments. Protein impurities were removed from the fructosan giving a product which showed the same specific rotation as the material regenerated from the polysaccharide acetate.

The products of hydrolysis of the fructosan by 1% oxalic acid and by boiling water were studied. The glucose and fructose liberated on acid hydrolysis were estimated by quantitative paper chromatography giving:

- Fructose **99.97%** and Glucose **0.03%**.

The polysaccharide was methylated with sodium hydroxide and methyl sulphate in an inert atmosphere in the cold, and fractionated.

A portion of the methylated fructosan was hydrolysed and the products of hydrolysis converted to the corresponding glycosides. Fractionation by extraction with light petroleum led to a partial separation of tetramethyl methyl fructofuranoside and tetramethyl methyl glucoside. The free sugars obtained on hydrolysis of the glycosides were separated on columns of cellulose to give 1:3:4:6-tetramethyl D-fructose (4%), 2:3:4:6-tetramethyl D-glucose (1.8%), 1:3:4-trimethyl D-fructose (93.3%) and a dimethyl D-fructose (0.7%).

The molecular weight of the methylated fructosan was determined by Barger's method. A value of 3,440/
3,440-5,160 was obtained for the methylated polysacchar-ide, corresponding to a molecular size of 17 - 25 units.

(6) Oxidation of the polysaccharide by the periodate ion was studied. The uptake of periodate was estimated as 1.02 moles per fructose residue and 1 mole of formic acid was liberated for every 19-20 residues. No form-alddehyde was detected in the solution of periodate-oxidised fructosan.

(7) The above results indicate that the greater part of the polysaccharide possesses a molecular structure comprising a straight chain of ca. 25 2-6-linked fructofuranose residues terminated by a glucopyranose residue linked as in sucrose.
The inulin used in the present investigations was prepared by extracting the roots of dahlia tubers (Crimson Flag variety) with hot water. The aqueous extracts were heated and lime water added to p.H.8 and the precipitate filtered. Dilute oxalic acid was then added to the filtrate to p.H.7, followed by decolorising charcoal, and the solution heated and filtered. On standing at 0°, inulin was deposited from the solution.

The glucose-fructose ratio of the polysaccharide was determined as described above (p. 29) by quantitative paper chromatography, the fructose being estimated by periodate oxidation and the glucose by the Nelson Colorimetric method. The results showed glucose present to the extent of 25%, the fructose amounting to 97.2%. The first batch of inulin to be deposited from the aqueous solution contained 2.4% glucose and 97.6% fructose. These figures are in agreement with Palmer's theory that each fructosan molecule contains one glucose residue, for the least soluble inulin fraction would be expected to have the longest chain material, with relatively less glucose than the more soluble fractions.

When an aqueous solution of the fructosan was refluxed for a prolonged period, its specific rotation was found to change continuously, and chromatographic examination of the solution showed that the polysaccharide was gradually broken down during the heating process, with
the successive production of higher oligosaccharides, lower oligosaccharides and monosaccharides.

Aniline oxalate, and naphthoresorcinol and hydrochloric acid developing sprays were used to detect the degradation products, and one of the samples of the solution thus examined, was seen to have an oligosaccharide, which travelled on the paper chromatogram at the same rate as a sucrose standard. This oligosaccharide was separated on paper chromatograms, eluted with cold water and hydrolysed with dilute oxalic acid. Chromatographic examination of the hydrolysate showed the presence of glucose and fructose, thus suggesting that the oligosaccharide might have been sucrose.

A control experiment was performed to see if any higher saccharides were produced on prolonged boiling of an aqueous solution of glucose with a large excess of fructoee. Paper chromatographic examination showed the presence of glucose and of fructose in high concentration with a trace of what appeared to be a pentose sugar, travelling faster than fructose on the paper chromatogram and giving a pink colour with aniline oxalate. There was no indication, however, of any other products in the solution.

The Hydrolysis of Periodate-Oxidised Inulin.

Hirst, McGilvray and Percival(20) investigated inulin from the Blue Danube variety of dahlia tubers and suggested that, in addition to a terminal glucopyranose residue linked as in sucrose, the fructoæan molecule might contain a second glucose residue linked through C1 and C3. The presence/
presence of this second glucose residue, linked within the chain, was indicated by the isolation of a significant amount of 2:4:6-trimethyl-D-glucose on hydrolysis of the methylated polysaccharide (p. 6). Bell and Palmer (19) likewise found that inulin from *Inula helena*um, on methylation and hydrolysis gave mixtures of all the trimethyl compounds of D-glucose, with the 2:4:6-isomer predominating. In addition, these authors found traces of 2:3:4:6-tetramethyl glucose, but suggested that all the methylated glucose originated from terminal non-reducing radicals linked as in sucrose.

A glucose residue within the fructosan chain linked through carbon atoms C1 and C5, would possess no adjacent hydroxyl groups and hence should be resistant to oxidation by the periodate ion. (84) (85) It was therefore decided to test for the presence of such a glucose unit by oxidising a portion of the Crimson Flag inulin with sodium metaperiodate and hydrolysing the oxidised product to discover if any glucose was present in the hydrolysate.

The solid, periodate-oxidised inulin was deposited from the aqueous solution as oxidation proceeded, and after several days it was separated at the centrifuge and excess periodate destroyed by addition of ethylene glycol. The oxidised polysaccharide was soluble in a mixture of dilute mineral acid and alcohol, and portions were hydrolysed under different conditions, but chromatographic examination of the hydrolysates failed to show the presence of any sugars.

After/
After separating the oxidised fructosan at the centri-
fuge, the supernatant liquid was neutralised and sulphur
dioxide passed into the solution to destroy excess period-
ate. The solution was then made 0.5N. w.r.t. sulphuric
acid and heated at 100° for 4 hours. Chromatographic
examination of the neutralised solution failed to show the
presence of any sugars.

In order to discover if glucose is destroyed under
the conditions employed in the hydrolysis of the oxidised
inulin, a control experiment was performed. About 1mg.
of chromatographically pure glucose was dissolved in normal
hydrochloric acid and the solution neutralised with silver
carbonate and examined on the paper chromatogram. Using
aniline oxalate spray, an extremely faint pink spot was
observed corresponding to a trace of arabinose, together
with a very heavy brown glucose spot, which showed that
under the conditions of the experiment, glucose was virtu-
ously preserved. It would appear therefore that, as
-glucose could not be detected in the hydrolysates of peri-
odate oxidised inulin, no C\textsubscript{1} - C\textsubscript{3} linked glucose residues
are present in the variety of inulin (Crimson Flag)
employed in the present investigations.

The Degradation of Inulin in a Hot 3\% Aqueous Solution.

A 3\% aqueous solution of inulin was heated at 100°
and the p.H. and specific rotation of the solution deter-
mined from time to time. In addition, samples of the
carbohydrate solution were examined periodically on the
paper/
paper chromatogram. The specific rotation of the solution was found to change continuously until after 25 hours a value of $\alpha = -84^\circ$ had been obtained. (Original value, $\alpha = -40^\circ$). Chromatographic examination of the fructosan solution showed a progressive breakdown of the polysaccharide with the production of higher and lower oligosaccharides, which broke down further to give fructose and glucose. In addition, naphthoresorcinol and hydrochloric acid spray showed the presence of materials moving faster than fructose on the paper chromatogram and at the same rate as three substances which had been obtained from a preliminary experiment involving the prolonged refluxing of an aqueous inulin solution. These products gave only fructose on acid hydrolysis and could not be detected with aniline oxalate. They gave a deep red colour with naphthoresorcinol and hydrochloric acid, were dextro-rotatory and may have been a mixture of difructose anhydrides. The oligosaccharides present in a sample of the fructosan solution after 12 hours heating were separated on paper chromatograms, eluted from the papers with cold water and hydrolysed. Chromatographic examination of the hydrolysis products showed that each had given both glucose and fructose. The intensity of the glucose spot relative to the fructose was seen to diminish with the speed of the oligosaccharide on the paper chromatogram. In all cases however, the fructose spot was much more intense than that of glucose, suggesting that each of the "oligosaccharide areas" eluted did not contain/
contain one oligosaccharide only, with one glucose residue, but rather a mixture of two oligosaccharides, one with a terminal glucose residue and the other composed entirely of fructose residues.

The Heating of a Buffered Aqueous Inulin Solution at 100°.

Inulin was heated at 100° in a phosphate buffer solution (p.H., 6.80) and the solution examined at regular intervals for changes in specific rotation, which was found however to remain constant. Chromatographic examination of the solution from time to time, before and after deionisation, failed to show the presence of any degradation products. The autohydrolysis process is therefore arrested when the p.H. of the solution is maintained at neutrality, so that the process is at least, in part, an acid hydrolysis.

The Heating of an Aqueous Inulin Solution in an Inert Atmosphere.

An aqueous inulin solution was heated at 100° as described previously, but this time a gentle stream of oxygen-free nitrogen was swept over the surface of the solution. Again the solution was examined from time to time for changes in specific rotation and p.H. and samples were examined on the paper chromatogram. Observations, similar to those made when an aqueous inulin solution was heated in air (p.H. 8.8) were made, but it was obvious that the reduced availability of oxygen retarded the breakdown of the polysaccharide. After 75 hours, paper chromatographic examination showed that the solution still contained several oligosaccharides in small concentration. The heating of an aqueous inulin solution/
solution of the same concentration in air, resulted in complete breakdown of the fructosan after some 24 hours.

A fresh sample of inulin was dissolved in water which had been aerated for 20 hours with oxygen-free nitrogen and the solution heated at 100°, a gentle stream of nitrogen being bubbled through the solution. Once again the degradation of the polysaccharide was slowed down, presumably due to the absence of oxygen. It may be therefore, that the degradation process is initiated by some form of oxidative breakdown of the fructosan molecule, with the formation of acidic materials, which bring about complete hydrolysis of the polysaccharide.

Test for the Hydroperoxide Group.

An aqueous inulin solution was heated at 100° and samples of the solution tested at regular intervals for the presence of the hydroperoxide group (93) by the ammonium thiocyanate-ferrous ammonium sulphate colour reaction. No trace of such a grouping could be detected however.

The Degradation of Sucrose on Heating in Aqueous Solution.

When an aqueous solution of chromatographically pure sucrose was heated at 100°, it was found to break down to glucose and fructose, the process being complete after some 30 hours. As degradation of the disaccharide took place, the pH of the solution fell from 6 to 3, due to the formation of 2 acidic materials, detected on the paper chromatogram.
EXPERIMENTAL.

EXTRACTION OF THE POLYSACCHARIDE.

The inulin was prepared by extraction from the roots of dahlia tubers (Crimson Flag variety). After mincing, the roots (3 kg.) were extracted at 60° for 45 minutes with water (5 l.) and the aqueous solution filtered. The residue was extracted further with water (5 l.) at 70° for 1 hour and filtered. The aqueous extracts were heated to 60°, lime water added to pH 8, and the resulting precipitate filtered. Dilute oxalic acid was then added to pH 7, followed by decolorising charcoal, the solution kept at 70° for a few minutes and filtered. Inulin was deposited from the solution on standing at 0°.

The inulin received for the present investigations was made up of several fractions of varying purity. With the exception of fraction 1 (ca. 5 g.), the first inulin to be deposited from the aqueous extract, all the fractions (ca. 130 g.) were combined, dissolved in water (4 l.) at 70° and allowed to stand at 0°, when inulin was deposited. The polysaccharide was separated at the centrifuge, suspended in acetone (2 l.), centrifuged and dried in vacuo over phosphoric oxide and paraffin wax. The aqueous centrifugate was concentrated to 1 l. and left at 0° overnight, when a second fraction of inulin was deposited. This was separated and dried as described above, and the two polysaccharide fractions combined and thoroughly mixed to give the main inulin fraction. Unless otherwise stated, this will be the inulin referred to below. It had \([\alpha]_D^{\infty} = -40.3^\circ\) (c, 2.4 in water) and was obtained as a fine, white powder.
powder (ca. 105g.)

The Glucose-Fructose Ratio of the Inulin.

1. A portion (ca. 0.1g.) of the inulin was hydrolysed at
70° with oxalic acid (10c.c.; 0.1N.) to constant rotation,

\[
[\alpha]_D^0 = -41.0^\circ \text{ (zero); } -47.8^\circ \text{ (10 minutes); } -57.8^\circ \text{ (25 minutes); }
-73.8^\circ \text{ (55 minutes); } -85.1^\circ \text{ (120 minutes) constant.}
\]

The hydrolysate was neutralised, filtered, deionised
and concentrated to small volume. The glucose-fructose
ratio was then determined by quantitative paper chromatography\(^{(71)}\), the fructose being estimated by periodate
oxidation\(^{(72)}\) and the glucose by the Nelson colorimetric
method\(^{(73)}\)(74) as described for the fructosan from Dactilis
glomerata (p. 22). This gave,

Glucose : Fructose = 1 : 34.6

Assuming the polysaccharide preparation to be pure,

Percentage glucose = 2.8%  
Percentage fructose = 97.2%

11. The glucose-fructose ratio of a sample of fraction I
inulin (\(\left[\alpha\right]_D^0 = -38.3\)) was estimated in the same way, the
fructosan (0.25g.) being hydrolysed with oxalic acid
(25c.c.; 0.1N.) at 70° for 2 hours.

\[
[\alpha]_D^0 \text{ was } -40.8^\circ \text{ (zero) } \rightarrow \text{ -83.2° (90 minutes), constant.}
\]

The ratio was found to be,

Glucose : Fructose = 1 : 40.7

Assuming the fructosan to be pure,

Percentage glucose = 2.4%  
Percentage fructose = 97.6%

Degradation/
Degradation of Inulin in Hot Aqueous Solution.

A portion of the inulin (0.4680g.) was dissolved in water (25c.c.) and heated at 100°, on the boiling water-bath. The solution was examined at regular intervals for rotation changes, and samples were run on the paper chromatogram, employing benzene-butanol-pyridine-water solvent(91) and developing sprays of aniline oxalate, and naphthoresorcinol and hydrochloric acid solutions, double papers being run for each solution sample. The main observations are listed below.

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>$[\alpha]_D$</th>
<th>Chromatogram Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-39.8°</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-41.1°</td>
<td>Heavy trail at the starting-line with about 6 faint spots, the most concentrated travelling at the same rate as fructose.</td>
</tr>
<tr>
<td>20</td>
<td>-54.5°</td>
<td>Heavy fructose spot; about 8 slower-moving materials and 2 faster-moving, the latter only observed with naphthoresorcinol spray.</td>
</tr>
<tr>
<td>31</td>
<td>-68.6°</td>
<td>Heavy fructose spot, with 3 slower and 2 faster substances. One of the slower substances travelled at the same rate as a sucrose standard.</td>
</tr>
<tr>
<td>42</td>
<td>-71.6°</td>
<td>As for the 31-hour sample.</td>
</tr>
<tr>
<td>50</td>
<td>-75.6°</td>
<td>As above, but &quot;streaking&quot; effects/</td>
</tr>
</tbody>
</table>
Duration (Hours)  [α]_D Chromatogram Observations
-effects made observations difficult
60  -57.1° Very bad "streaking" effects made observations impossible.

The sample of the solution taken after 31 hours was run on six paper chromatograms by the side-strip technique for 44 hours in benzene-butanol-pyridine-water solvent. The side-strips were developed and the suspected sucrose areas on the main papers cut off and the sugar eluted with cold water. The combined eluates were made 3% w.r.t. oxalic acid and hydrolysed at 80° for 2 hours. After neutralisation, filtration and deionisation with Amberlite resins, the solution was concentrated to ca. 0.5 c.c. and the whole run on a paper chromatogram for 43 hours in ethyl acetate-acetic acid-water solvent, with standards of glucose, fructose and sucrose. On developing the paper with aniline oxalate spray, the oligosaccharide suspected of being sucrose, was seen to have hydrolysed to two sugars, one of which travelled at the same rate as the fructose standard and the other at the same rate as the glucose standard. In addition, a pink spot was noted, a short distance below the starting-line.

**Control Experiment.**

A control experiment was performed to discover if any higher saccharides were produced on heating glucose with a large excess of fructose for a prolonged period at 100°.

Chromatographically/
Chromatographically pure fructose (5g.) and glucose (0.1g.) were dissolved in water (50c.c.) and heated at 100° on the boiling water-bath for 45 hours. The solution was concentrated to small volume and examined on the paper chromatogram in exactly the same way as the solution samples above. In this way, heavy spots of glucose and fructose were seen, with no trace of material moving more slowly on the paper chromatogram. Only on one paper, where the fructose and glucose spots were very intense, was a third spot observed. This latter gave an extremely faint pink coloration with aniline oxalate and was distant 33.5cm. from the starting-line, when a sucrose standard had travelled 20cm. It would appear that this pink spot was produced from a very small trace of pentose, presumably formed from glucose and/or fructose, aniline oxalate giving a brown coloration with hexoses, but a pink colour with pentoses.

The Hydrolysis of Periodate-Oxidised Inulin.

Inulin (ca. 5g.) was dissolved in water (600c.c.) sodium meta-periodate solution (250c.c.; 0.3M.) added, the solution well shaken and left in the dark at room temperature for 4-5 days. As oxidation continued, the oxidised polysaccharide was seen to come out of solution. The solid product of oxidation was separated at the centrifuge, washed well with water containing a little ethylene glycol to destroy excess periodate, and the oxidised inulin thoroughly dried over phosphoric oxide and calcium chloride, yielding a white powder. The centrifugate/
centrifugate was also retained for further examination. The oxidised inulin was insoluble in mineral acids, alkalis and common organic solvents, but was soluble in a mixture of dilute mineral acid and alcohol.

(a) Oxidised inulin (ca. 1g.) was dissolved in aqueous methanolic hydrochloric acid (25c.c. methanol; 25c.c. hydrochloric acid, 2N.) and hydrolysed at 100° for 18 hours. Chromatographic examination of the solution after neutralisation, deionisation and concentration, showed that no sugars had been produced on hydrolysis of the oxidised polysaccharide.

(b) A sample (ca. 0.5g.) was dissolved in aqueous methanolic sulphuric acid (10c.c. methanol; 10c.c. sulphuric acid, 0.5N.) and refluxed on the boiling water-bath for 4 hours. The methanol was then removed from the solution with constant addition of water, the solution made 0.5N. w.r.t. sulphuric acid and refluxed for 2 hours. After neutralisation with barium carbonate, filtration and deionisation, the solution was concentrated to small volume and examined on the paper chromatogram, when again, no sugars could be detected.

(c) A further sample (ca. 0.5g.) was dissolved in methanolic hydrogen chloride (20c.c.; 1.5%) and refluxed for 20 hours. The methanol was replaced by water in the usual manner, and the solution refluxed for 2 hours. On treating the solution as detailed in (a) above, no sugars could be detected on the paper chromatogram.

Examination of the Centrifugate.

After/
After separating the solid, periodate-oxidised inulin at the centrifuge, the supernatant liquid was neutralised with barium carbonate, filtered and sulphur dioxide passed into the filtrate, to destroy excess periodate. The solution was then made 0.5N. w.r.t. sulphuric acid and heated at 100° for 4 hours. After cooling, the solution was neutralised with barium carbonate, filtered, deionised and taken to dryness, giving a tarry mass which was extracted with boiling chloroform. The residue and concentrated extracts were examined chromatographically but no sugars could be detected.

Control Experiment.

In order to discover if glucose is destroyed under the conditions employed in the hydrolysis of the oxidised inulin, a control experiment was performed.

Chromatographically pure glucose (ca. 1mg.) was dissolved in hydrochloric acid (25c.c.; 1N.) and refluxed at 100° for 6 hours. The acid solution was then treated as detailed in (a) above, and examined on the paper chromatogram. Using aniline oxalate spray, a very faint pink spot was seen together with a very heavy brown glucose spot. The faint pink coloration was approximately the same distance from the starting-line as a fructose standard and may have been due to a trace of arabinose, produced from glucose during the treatment with hydrochloric acid.

Degradation Studies of Inulin in Hot Aqueous Solution.

(a) The Heating of a 3% Aqueous Inulin Solution.

Inulin/
Inulin (1.503g.) was dissolved in water (50c.c.), heated to 75° to effect complete solution and cooled. The specific rotation and p.H. of the solution were found and the solution heated on the boiling water-bath. Examination of the solution was made from time to time, for changes in p.H. and specific rotation. At the same time, samples of the carbohydrate solution were taken for examination on the paper chromatogram. The following observations were made:

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>$[\alpha]_D$</th>
<th>p.H.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-40.3°</td>
<td>6.28</td>
</tr>
<tr>
<td>4</td>
<td>-39.3</td>
<td>5.14</td>
</tr>
<tr>
<td>8</td>
<td>-45.9</td>
<td>4.40</td>
</tr>
<tr>
<td>12</td>
<td>-60.9</td>
<td>3.93</td>
</tr>
<tr>
<td>16½</td>
<td>-79.1</td>
<td>3.70</td>
</tr>
<tr>
<td>21</td>
<td>-74.8</td>
<td>3.49</td>
</tr>
<tr>
<td>25</td>
<td>-84.2</td>
<td>3.35</td>
</tr>
</tbody>
</table>

In the chromatographic examinations, double papers were run, with glucose, fructose and sucrose standards, one paper being developed with aniline oxalate and the other with naphthoresorcincinol and hydrochloric acid. Aniline oxalate gave brown spots with glucose, fructose and the oligosaccharides produced during the heating process, while naphthoresorcincinol and hydrochloric acid gave red spots with fructose and fructose-containing oligosaccharides. In all of the samples, naphthoresorcincinol spray also indicated the presence of materials moving faster than fructose on the paper chromatogram and at the same rate as three substances, which had been obtained from an aqueous/
aqueous inulin solution after boiling 35 hours. These products gave only fructose on hydrolysis, were dextro-rotatory and could not be detected on the paper chromatogram with aniline oxalate spray. They gave a deep red colour with naphthoresorcinol and hydrochloric acid however, and appeared to be difructose anhydrides.

Sample. Chromatogram Observations (Aniline Oxalate Spray)
4 hours Very heavy spot at the starting-line, with "streak" about 2.5 cm. below it. Very faint spots at 9.7 cm. and 4.4 cm. (Sucrose standard 9.5 cm.)
8 hours As for the 4-hour sample but heavier spots at 9.7 cm. and 4.7 cm., and spot corresponding to fructose.
12 hours Concentrated spot corresponding to fructose and faint glucose spot. Sugar travelling at the same rate as the sucrose standard. Three higher saccharides and heavy spot at the starting-line.
16½ hours Fructose in high concentration, little glucose and oligosaccharide travelling at the same rate as the sucrose standard.
24 hours As for the 16½ hour sample but only a trace of the third (oligosaccharide) spot.

Separation and Hydrolysis of Oligosaccharides.
The sample of the solution collected after 12 hours was concentrated to small volume and run on 4 paper chromatograms in benzene-butanol-pyridine-water solvent for 3 days, employing the side-strip technique. On developing the side-strips with aniline oxalate, fructose, glucose, a trail extending 2.5 cm. below the starting-line and/
and oligosaccharides at distances 20.6, 12.1, 7.5 and 4.1cm. from the starting-line were seen. The oligosaccharides were eluted and hydrolysed with oxalic acid (3c.c.; 1%) for 1 hour at 80°. The solutions were neutralised with calcium carbonate, concentrated, microfiltered and run on paper chromatograms in ethyl acetate-acetic acid-water solvent for 48 hours. On developing with aniline oxalate, all four oligosaccharides were seen to give both glucose and fructose on hydrolysis. The intensity of the glucose spot relative to the fructose was seen to diminish with the speed of the oligosaccharide on the paper chromatogram. In all cases the fructose spot was much more intense than that of glucose.

(b) The Heating of a Buffered Aqueous Inulin Solution.

Inulin (1.584g.) was dissolved in a buffer solution (50c.c.; p.H. 6.80) prepared by dissolving potassium dihydrogen phosphate (0.5447g.) in water (30c.c.) and adding to this a solution (70c.c.) of disodium hydrogen phosphate (1.3675g.). The solution was boiled and examined as described above, giving the following results.

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>[α]D</th>
<th>pH</th>
<th>Colour of Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-36.5</td>
<td>6.80</td>
<td>Colourless</td>
</tr>
<tr>
<td>4</td>
<td>-36.8</td>
<td>6.82</td>
<td>Yellow</td>
</tr>
<tr>
<td>8</td>
<td>-36.8</td>
<td>6.82</td>
<td>More yellow</td>
</tr>
<tr>
<td>12</td>
<td>-39.2</td>
<td>6.83</td>
<td>Darker</td>
</tr>
<tr>
<td>16</td>
<td>-39.2</td>
<td>6.76</td>
<td>Darker</td>
</tr>
<tr>
<td>21</td>
<td>-39.2</td>
<td>6.70</td>
<td>Dark Amber</td>
</tr>
<tr>
<td>27</td>
<td>-38.8</td>
<td>6.62</td>
<td>Solution too dark</td>
</tr>
<tr>
<td>32</td>
<td>-40.0</td>
<td>6.48</td>
<td>(for accurate</td>
</tr>
<tr>
<td>37</td>
<td>-</td>
<td>6.40</td>
<td>(polarimeter readings</td>
</tr>
</tbody>
</table>
Chromatographic Examination:

All samples of the solution were evaporated to dryness, giving a white residue which was dissolved in a little water and the solution examined on the paper chromatogram. In every case, aniline oxalate and naphthoresorcinol sprays failed to show the presence of any oligosaccharides or sugars, only a heavy spot being observed at the starting-line. Those samples of the solution collected after 12, 21 and 32 hours, were deionised with Amberlite resins, using both L.R.-100 and L.R.-4B. at the same time, lest the development of acidity might cause partial hydrolysis of the fructosan. Again paper chromatographic examination showed only the presence of unchanged polysaccharide at the starting-line.

(b) The Heating of an Aqueous Inulin Solution in an Inert Atmosphere.

(I) Inulin (1.446g.) was dissolved in water (50c.c.) and the solution heated and examined as previously described. This time however, a gentle stream of oxygen-free nitrogen was swept over the surface of the inulin solution during the heating process. The following observations were made:

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>$\left[\alpha\right]_D$</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-40.1°</td>
<td>6.88</td>
</tr>
<tr>
<td>5</td>
<td>-43.2</td>
<td>6.52</td>
</tr>
<tr>
<td>10</td>
<td>-44.6</td>
<td>6.11</td>
</tr>
<tr>
<td>15</td>
<td>-45.0</td>
<td>5.80</td>
</tr>
<tr>
<td>20</td>
<td>-45.6</td>
<td>5.56</td>
</tr>
<tr>
<td>25</td>
<td>-47.7</td>
<td>5.36</td>
</tr>
<tr>
<td>30</td>
<td>-49.4</td>
<td>5.13</td>
</tr>
<tr>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (hours)</td>
<td>$[\alpha]_D$</td>
<td>$D_{H_2}O$</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>35</td>
<td>-51.5°</td>
<td>5.04</td>
</tr>
<tr>
<td>42</td>
<td>-53.6</td>
<td>4.88</td>
</tr>
<tr>
<td>52</td>
<td>-67.1</td>
<td>4.66</td>
</tr>
<tr>
<td>63</td>
<td>-76.1</td>
<td>4.48</td>
</tr>
<tr>
<td>75</td>
<td>-78.5</td>
<td>4.24</td>
</tr>
</tbody>
</table>

Sample | Chromatogram Observations (Aniline Oxalate Spray) |
---|---|
5 hours | Brown coloration at the starting-line. |
10 hours | As for the 5-hour sample, with a faint trail just below the starting-line. |
15 hours | As for the 10-hour sample; trail now about 3cm. long. |
20 and 25 hours | As above; trail now about 7cm. long. |
30 hours | Heavy brown spot at the starting-line, with a 3.5cm. trail. Three oligosaccharides in low concentration and a little sugar corresponding to fructose. |
42 hours | As for the 30-hour sample, but 5 oligosaccharides now present in moderate concentration. |
52 hours | Spot at the starting-line with a 3cm. tail; 7 oligosaccharides all in approximately equal concentration. Fructose in high concentration and a little glucose. |
63 hours | As for the 52-hour sample, with the start-line spot, tail and slowest-moving oligosaccharides in very low concentration. |
75 hours | Only fructose with a little glucose, an oligosaccharide travelling at the same rate as sucrose and traces of higher saccharides. |
All samples were run on paper chromatograms which were developed with naphthoresorcinol and hydrochloric acid. In this way, the samples collected on and after 42 hours were seen to contain material travelling faster than fructose on the paper chromatogram and at the same rate as the substances referred to above (p. 55) obtained from inulin and believed to be difructose anhydrides.

(II) Inulin (1.508 g.) was dissolved in water (50 c.c.) which had been aerated for 20 hours with oxygen-free nitrogen, and the solution heated at 100° with a gentle stream of nitrogen bubbling through the solution. The process was continued for 60 hours, when a sample of the solution, examined on the paper chromatogram, was seen to contain fructose and about five oligosaccharides, all in high concentration. In addition a heavy trail was observed at the starting-line, showing once again that the degradation of the polysaccharide had been slowed down, presumably due to the absence of oxygen.

It may be therefore, that the degradation process is initiated by some form of oxidative breakdown of the fructosan molecule, with the formation of acidic materials which bring about complete hydrolysis of the polysaccharide.

Test for the Hydroperoxide Group.

Inulin (1.528 g.) was dissolved in water (50 c.c.) and heated at 100°. Samples of the solution were tested after 4, 8, 12, 16 and 20 hours for the presence of hydroperoxide groups. The sensitive ammonium thiocyanate-ferrous/
ferrous ammonium sulphate colour reaction\(^{(93)}\) was employed, but failed to detect the presence of such groups. It would appear therefore, that the breakdown of the fructose cannot be explained by the formation of hydroperoxide groupings.

**The Degradation of Sucrose on heating in Aqueous Solution.**

Chromatographically pure sucrose (1.704g.) was dissolved in water (50c.c.) and the solution heated on the boiling water-bath. The following observations were made.

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>([\alpha]_D)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>65.3°</td>
<td>5.98</td>
</tr>
<tr>
<td>4</td>
<td>61.6°</td>
<td>4.96</td>
</tr>
<tr>
<td>8</td>
<td>50.3°</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>32.9°</td>
<td></td>
</tr>
<tr>
<td>15(\frac{1}{2})</td>
<td>17.5°</td>
<td></td>
</tr>
<tr>
<td>19(\frac{1}{2})</td>
<td>-2.2°</td>
<td>3.81</td>
</tr>
<tr>
<td>23(\frac{1}{2})</td>
<td>-10.6°</td>
<td>3.66</td>
</tr>
<tr>
<td>29</td>
<td>-13.5°</td>
<td>3.46</td>
</tr>
<tr>
<td>36</td>
<td>-12.3°</td>
<td>3.32</td>
</tr>
<tr>
<td>41</td>
<td>-12.3°</td>
<td>3.23</td>
</tr>
</tbody>
</table>

**Chromatographic Examination.**

Examination of portions of the solution from time to time showed the gradual breakdown of sucrose with the formation of glucose and fructose. No sucrose could be detected in the solution after 30 hours heating. All samples of the solution collected, were run on paper chromatograms in butanol-ethanol-water solvent for 46 hours, using a lactic acid standard. Portions of the sucrose/
sucrose solution collected after 4, 8 and 15½ hours, showed a faint acid spot at a distance of 5.3 cm. from the starting-line. (Lactic acid distance, 11.2 cm.). The 19½ and 23½ hour samples showed two faint acid spots distant 5.3 and 8.7 cm. respectively and the 29 and 36-hour samples showed the presence of only one acid at a distance of 8.7 cm. Methyl red-methylene blue indicator was used to detect the acids on the paper chromatograms.

No materials moving faster than fructose on the paper chromatogram could be detected with naphthoresorcinol and hydrochloric acid spray.
Inulin, extracted from dahlia tubers (Crimson Flag variety), on hydrolysis, gave glucose (ca. 3%) and fructose (ca. 97%), estimated by quantitative paper chromatography.

A sample of the inulin was oxidised by the periodate ion and the oxidised material hydrolysed under different conditions. Chromatographic examination of the hydrolysates failed to show the presence of any sugars.

Aqueous inulin solutions have been subjected to prolonged heating on the boiling water-bath in air, in nitrogen and in a medium buffered at pH 6.8. The degradation products produced by these treatments were examined on the paper chromatogram.

An aqueous solution of sucrose was likewise heated on the boiling water-bath, when the sucrose was found to be degraded to glucose and fructose after ca. 30 hours.
PART III.

INVESTIGATIONS ON THE FRUCTOSAN FROM PERENNIAL RYE GRASS (Lolium perenne).

DISCUSSION.

Evidence has been brought forward (p.18) to suggest that the fructosans are terminated by a glucose residue, linked as in sucrose. Thus, Laidlaw and Reid,\(^{(35)}\) from methylation studies, periodate oxidation and "autohydrolysis" of the fructosan from Lolium perenne, suggested that this polysaccharide might consist of a chain of \(\text{C}_2 - \text{C}_6\) linked fructofuranose residues, terminated by a glucopyranose moiety linked as in sucrose. Sucrose, however, has never been isolated from a fructosan in sufficient quantity for identification. It was the purpose of the present investigation to attempt the isolation of some pure sucrose from the "autohydrolysate" of the fructosan from Lolium perenne.

Preparation of the Polysaccharide.

The fructosan was extracted from milled, oven-dried perennial rye grass (Lolium perenne). The method employed was essentially the same as that described (p. 33) for the isolation of the fructosan from Dactylis glomerata, portions of the grass being extracted with ether and 80\% aqueous methanol to remove the colouring matter and sugars present in the grass. Cold water extraction then removed the fructosan. In the present extraction, however, proteins were removed from the aqueous extract by a co-precipitation process, employing cadmium sulphate and barium hydroxide solutions,\(^{(62)}\) thereby lessening the concentration/
concentration of inorganic ions in the aqueous solution of the polysaccharide. After deionisation of the solution with Amberlite resins, the last traces of protein were removed by the method of Sevag, Lackmann and Smolens (p.22), the solution digested with charcoal and filtered. The fructosan was precipitated from the concentrated aqueous solution with methanol, to give a product which had $[\alpha]_D -41.0^\circ$ and on mild acid hydrolysis gave glucose, a large excess of fructose and a trace of arabinose.

**Degradation of the Fructosan on heating in aqueous solution.**

**Small-scale Experiment I:** An aqueous solution of the polysaccharide was heated at $100^\circ$ for 30 hours, when the pH. of the solution was found to fall gradually from 5 to 3 and the specific rotation to change from $-43^\circ$ to $-76^\circ$.

Chromatographic examination of the solution from time to time, showed the progressive breakdown of the fructosan with the formation first of higher oligosaccharides, followed by lower oligosaccharides then glucose and fructose. Degradation of the polysaccharide seemed complete after 24 hours.

All the samples collected were run on paper chromatograms which were developed with naphthoresorcinol and hydrochloric acid. In this way, each portion of the solution was seen to contain material which travelled faster than fructose on the paper chromatogram and appeared as a faint red trail, extending downwards from the fructose spot over about 5cm. As suggested already (p.10)
this may have been due to the presence of small quantities of difructose anhydrides in the solution. In addition, samples of the solution collected on and after 13 hours, showed the presence of an even faster-moving material.

Each sample of the solution was run on a paper chromatogram in butanol-ethanol-water solvent and the papers developed with methyl red-methylene blue indicator. Two acids were thus detected in the samples, one travelling very slowly on the paper chromatogram and the other at the same rate as a lactic acid standard.

**Small-scale Experiment II:** An aqueous solution of the fructosan was heated at 100°C as above, when very similar observations were made, regarding changes in pH, specific rotation and observations on the paper chromatogram.

A sample of the solution collected after 6 hours was run on several paper chromatograms and the substance travelling at the same rate as sucrose was eluted along with the next two fastest moving materials.

It was thought that these three products might represent di-, tri- and tetrasaccharides respectively. On hydrolysis, each gave only glucose and fructose, the intensity of the fructose spot relative to that of glucose, diminishing with increasing speed of the material on the paper chromatogram. The relative intensities of the two sugar spots however, suggested that the three areas eluted did not contain pure di-, tri- and tetrasaccharides respectively, each with a glucose residue, but rather a mixture of two oligosaccharides, one with a glucose residue and the other composed entirely of fructose residues.
Large-scale Degradation of the Fructosan on Heating in Aqueous Solution.

50g. of the fructosan were dissolved in 1.4 l. of water and heated at 100° for 6½ hours. Chromatographic examination of the solution after 6 hours heating indicated the presence of some 10 different products, varying from what appeared to be unchanged or slightly degraded polysaccharide at the starting-line, to a sugar corresponding to fructose.

It has been shown by Whistler and Durso(94) that monosaccharides can be separated from higher saccharides by use of a charcoal column, water elution removing the simple sugars and aqueous alcohol eluting the oligosaccharides from the column. By gradually altering the percentage of alcohol in the aqueous alcoholic eluent, it has been found possible to separate oligosaccharides of different chain-length from one another on the charcoal column.(95) It was decided to attempt a separation of the degradation products, employing a charcoal-hydrostopercel column constructed from equal quantities of decolorising charcoal and hydrostopercel. The volume of the carbohydrate solution was adjusted to 500c.c., concentration of the solution being carried out at 35° under reduced pressure, and the carbohydrate solution added to the charcoal column.

The column was eluted first with water and then with 30% aqueous alcohol followed by 50% aqueous alcohol. It was hoped that water elution would remove monosaccharides only, but in addition, paper chromatographic examination
of the aqueous eluates showed the presence of higher saccharides. The eluate was collected in batches of about 250 c.c., each of which was examined polarimetrically and chromatographically, in order that the course of the separation might be followed. In this way, six fractions were collected. Fraction A (11.181 g.) contained fructose and a little glucose. Fraction B (24.276 g.) likewise had a high fructose content with a little glucose, but in addition, a third sugar was observed on the paper chromatogram travelling more slowly than glucose but faster than sucrose and referred to as sugar \( x \). Fraction C (3.228 g.) contained five products, including what appeared to be fructose, glucose and sucrose. Fraction D (0.513 g.) contained an oligosaccharide (sugar \( y \)) travelling considerably slower than sucrose on the paper chromatogram with traces of lower saccharides. Fraction E (15.438 g.), which contained the majority of the 30% alcoholic eluates was shown chromatographically to contain some 6 oligosaccharides moving more slowly than sucrose. Fraction F (0.196 g.) contained material, which, on chromatographic examination, appeared as a spot at the starting-line.

**Separation of Fraction E.**

This fraction was dissolved in 250 c.c. water and added to a second charcoal-hyflosupercel column. Again the course of the separation was followed by polarimetric and chromatographic examination of the eluates. The water eluates were found to contain fructose with a little glucose and were combined to give Fraction B (1) (17.630 g.)
5% aqueous alcohol was then used as eluant and fraction B (II) (4.422g.) and B (III) (0.546g.) collected. Fraction B (II) contained sugar X in high concentration with traces of fructose and four higher saccharides. In addition, when examined chromatographically, traces of materials moving faster than fructose could be detected with naphthoresorcinol and hydrochloric acid spray. As suggested before (p.100) these might have been a mixture of difructose anhydrides. Fraction B (II) also contained a product moving faster on the paper chromatogram than the suspected difructose anhydrides and observed as a blue spot with the naphthoresorcinol spray.

Fraction B (III) was shown chromatographically to contain sugar X and a sugar corresponding to sucrose, in approximately equal amounts, with traces of three higher saccharides.

Separation of Fraction C.

An attempt was made to isolate pure sucrose from fraction C, employing a cellulose column(75) which was eluted with benzene-butanol-pyridine-water solvent. Eight fractions were thus collected. Fraction C (I) (0.059g.) contained substances moving faster than fructose on the paper chromatogram, undetectable with aniline oxalate but giving a red colour with naphthoresorcinol and hydrochloric acid. In addition, the above-noted material giving a blue coloration with naphthoresorcinol spray was present in fraction C (I). Fraction C (II) (0.092g.) contained fructose and a little glucose, while fraction C (III) (0.442g.) had sugar X and traces of fructose and sucrose. (?) Fraction/
Fraction C (IV) (0.914g.) contained sugar x and a sugar corresponding to sucrose, in approximately equal amounts, together with traces of a third substance moving slightly slower than sucrose on the paper chromatogram. Fraction C (V) (0.242g.) contained sugar x, sucrose and two higher saccharides, the slower moving (sugar y) being in much higher concentration. Fraction C (VI) (0.393g.) contained sugar y in high concentration with faint traces of higher saccharides. Fraction C (VII) (0.445g.) contained sugar y mainly, with a small amount of a second oligosaccharide travelling slightly more slowly on the paper chromatogram and fraction C (VIII) (0.070g.) contained the two oligosaccharides of fraction C (VII) in approximately equal concentration.

**Examination of Fractions.**

**Fraction C (III):** This fraction was virtually chromatographically pure, containing a sugar, referred to above as sugar x, which travelled slightly faster than sucrose on the paper chromatogram. It reduced Fehling's solution and ammoniacal silver nitrate and on hydrolysis with invertase or dilute oxalic acid, gave fructose only. This sugar may have been 6-D-fructofuranosyl-1-D-fructofuranose.

**Fraction C (IV):** This contained the sugar of fraction C (III), a sugar travelling more slowly on the paper chromatogram and corresponding to sucrose and traces of a higher oligosaccharide. The slower-moving of the two main sugars in this fraction did not reduce Fehling's solution or ammoniacal silver nitrate and on hydrolysis, gave glucose and fructose.

Fraction/
Fraction C (V): This fraction contained four oligosaccharides: (a) the sugar of fraction C (III); (b) a sugar corresponding to sucrose; (c) a third oligosaccharide in low concentration and (d) sugar y in high concentration. The speeds of the four oligosaccharides on the paper chromatogram was such that a > b > c > d. On mild acid hydrolysis (a) and (d) gave fructose only, while (b) and (c) gave glucose and fructose in apparently equal amounts. (a), (c) and (d) reduced ammoniacal silver nitrate solution but (b) did not. On treatment with invertase, (a) gave fructose only, (b) glucose and fructose, (c) glucose, fructose and a little unchanged starting material and (d) fructose, sugar x and a little unchanged sugar y.

Fractions C (VII) and C (VIII): These consisted mainly of the oligosaccharide of fraction C (V), together with a slower-moving oligosaccharide. Fraction C (VI) contained the former in large excess, while C (VII) contained approximately equal quantities of both.

The two oligosaccharides were separated on paper chromatograms and the slower-moving (about 60 mg.) examined. It was non-reducing towards Fehling's solution and ammoniacal silver nitrate and gave glucose and fructose on hydrolysis with either invertase or dilute oxalic acid. On mild acid hydrolysis, the oligosaccharide gave glucose (1 part) and fructose (2 parts), thereby suggesting that it was a trisaccharide composed of two fructose residues and a glucose moiety, all three sugars being linked through the glycosidic carbon atoms.

On controlled hydrolysis with Amberlite resin (I.R.100), the oligosaccharide was gradually hydrolysed
to give first sucrose and fructose and then glucose and fructose. The production of sucrose and fructose on graded hydrolysis of the trisaccharide is additional evidence for the suggestion (p.12) that the original fructo-isean was terminated by a glucopyranose unit, linked as in sucrose.

The trisaccharide produced in the present investigations could conceivably have arisen by hydrolysis of the fructo-isean in such a way as to liberate the last two fructose residues in the polysaccharide chain, together with the glucose end-group.

**Examination of Fraction F.**

On hydrolysis with 2% oxalic acid, fraction F was found to give rise to fructose and arabinose in high concentration, a little glucose and traces of xylose and galactose.

**Separation of the Disaccharides.**

Fractions B (III) and C (IV) were combined to give fraction S (1.401g.). A portion (1.112g.) was dissolved in benzene-butanol-pyridine-water solvent and fractionation attempted on a column of cellulose. Eight fractions were thus collected. Fraction S (I) contained those materials referred to above, moving faster than fructose on the paper chromatogram and giving a red coloration with naphthoresorcinol and hydrochloric acid. In addition, the above-mentioned material (p.113) giving a blue coloration with the same developing spray, was also present in this fraction. Fraction S (II) contained fructose and a little glucose and fraction S (III) consisted/
consisted almost entirely of sugar x, and a trace of what appeared to be sucrose. Fractions S (IV) and S (V) contained sugar x and a sugar corresponding to sucrose. Fraction S (VI) had sucrose (?) and a slower-moving oligosaccharide in low concentration. Fraction S (VII) contained the slower-moving oligosaccharide of fraction S (VI) and traces of higher saccharides. Fraction S (VIII) had higher saccharides only.

Separation and Identification of Sucrose.
Fractions S (IV), S (V) and S (VI) were run on paper chromatograms of Whatman 3 M.M. paper, yielding about 300mg. pure sucrose, m.p. 184-185° undepressed on admixture with an authentic specimen of sucrose. The sugar was non-reducing and was hydrolysed to give glucose (1 part) and fructose (1 part). It gave an X-Ray powder photograph identical with that given by authentic sucrose and was further characterised by conversion to the octacetate (m.p. 72-73°, undepressed on admixture with an authentic specimen of sucrose octacetate).

Separation and Examination of a Reducing Glucose-Fructose Disaccharide.
The slower-moving oligosaccharide from fraction S (VI), was separated from sucrose as described above and combined with fraction S (VII) to give fraction R (about 40mg.). This fraction was a dark brown syrup, chromatographic examination of which showed the presence of sucrose and sugar x impurities. The main oligosaccharide of the fraction reduced Fehling's solution and ammoniacal silver nitrate, and gave glucose and fructose on treatment with/
with invertase. The glucose-fructose ratio was 1 : 1.3, determined by the method described above, and the oligo-saccharide reduced alkaline hypoiodite, the extent being 55% of that expected for a disaccharide containing one free aldose reducing group. The low value obtained here may have been due to the impurities present in the fraction. The disaccharide may have been produced by a reversion synthesis during the degradation of the polysaccharide. A control experiment showed that the solvent employed in the present investigations (benzene-butanol-pyridine-water) did not produce any structural changes on samples of pure sucrose and sugar x.
EXPERIMENTAL

Investigations on the Fructosan from Perennial Rye Grass (Lolium perenne).

The fructosan from Lolium perenne has already been structurally investigated by Laidlaw and Reid (35) who, from methylation studies, periodate oxidation and autohydrolysis of the polysaccharide, suggested that the chain of fructofuranose residues might be terminated by a glucose residue, linked as in sucrose. The purpose of the present investigation was to attempt a separation of some pure sucrose from an "autohydrolysate" of the fructosan.

Preparation of the Polysaccharide.

The polysaccharide was extracted from perennial rye grass, which was oven-dried, milled material prepared from grass cut on 26th May 1949 at the Jealott's Hill Agricultural Research Station. Portions (150g.) were extracted with ether, 80% aqueous methanol and finally with water, as described under the preparation of the fructosan from leafy cocksfoot grass (p.42). In the extraction of fructosan from rye grass, however, proteins were removed by a coprecipitation process employing cadmium sulphate solution (33c.c.; 0.36N) and barium hydroxide solution (50c.c.; 0.36N) thereby lessening the concentration of inorganic ions in the aqueous solution (32). After deionisation with Amberlite resins (I.R.-100 and I.R.-4B), and removal of the last traces of protein by shaking with chloroform (0.25 vol.) and n-butanol (0.1 vol.) (63), the solution was stirred with charcoal (ca.10g.) and hyflo-supercel (ca.10g.) and filtered. The fructosan was precipitated/
precipitated from the concentrated aqueous solution with methanol, separated and dried to yield a pure white product which had, \( \left[ \alpha \right]_{D}^0 = -41.0^\circ \) (c, 1.0 in water). The average yield of fructosan was about 7% of the dry weight of the grass.

**Hydrolysis of the Fructosan.**

The fructosan (0.211g.) was hydrolysed with oxalic acid (20c.c.; 1%) at 70°.

\[ \left[ \alpha \right]_{D}^0 = -40.2^\circ \text{(zero)}; \quad -70.5^\circ \text{(10 minutes)}; \quad -86.4^\circ \text{(20 minutes) constant.} \]

Chromatographic examination of the neutralised hydrolysate showed only the presence of fructose, a little glucose and a trace of arabinose.

**Small-Scale Degradation of the Fructosan in Hot Aqueous Solution I.**

The polysaccharide (1.790g.) dissolved in water (50c.c.) was heated on the boiling water-bath, samples of the solution being examined from time to time for changes in specific rotation and \( \text{p.H.} \). Each sample of the solution collected was also examined on the paper chromatogram in conjunction with standards of fructose, glucose and sucrose. Developing sprays of aniline oxalate \( ^{69} \) and naphthoresorcinol and hydrochloric acid \( ^{67}(68) \) were employed. The following results were obtained:

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>( [\alpha]_{D} )</th>
<th>p.H.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-42.8(^\circ)</td>
<td>5.01</td>
</tr>
<tr>
<td>4</td>
<td>-36.3(^\circ)</td>
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<td>8</td>
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<tr>
<td>11</td>
<td>-69.3(^\circ)</td>
<td>3.86</td>
</tr>
<tr>
<td>13</td>
<td>-73.7(^\circ)</td>
<td>3.72</td>
</tr>
<tr>
<td>Duration (hours)</td>
<td>[α]d</td>
<td>D.H.</td>
</tr>
<tr>
<td>-----------------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>16</td>
<td>-72.6°</td>
<td>3.54</td>
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<tr>
<td>20</td>
<td>-72.6</td>
<td>3.59</td>
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<tr>
<td>24</td>
<td>-79.6</td>
<td>3.20</td>
</tr>
<tr>
<td>28</td>
<td>-79.6</td>
<td>3.11</td>
</tr>
<tr>
<td>30</td>
<td>-76.1</td>
<td>3.07</td>
</tr>
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</table>

**Sample Chromatogram Observations (Aniline Oxalate)**

4 hours  Long streak from the starting-line extending to a point alongside the fructose standard.

8 hours  8-10 spots extending from the start-line, the fastest corresponding to fructose.

11 hours  As for the 8-hour sample but much more concentrated fructose spot and fainter oligosaccharide spots, especially near the starting-line.

13 hours  Fructose, glucose a disaccharide travelling at the same rate as the sucrose standard, but slower than glucose. This latter substance will be referred to as sugar X.

16 and 19  Fructose, glucose and a faint trail extending a few centimetres behind the glucose spot.

24, 28 and 30 hours  Glucose and fructose only.

All the samples collected were run on paper chromatograms, which were developed with naphthoresorcinol and hydrochloric acid. In this way, each portion of the solution was seen to contain material which travelled faster than fructose on the paper chromatogram and appeared as a faint red trail extending downwards from the fructose spot over a distance of about 5cms. In addition, the/
the samples collected on and after 13 hours showed the presence of another material, distant 30.5 cm, from the starting-line (fructose standard 12 cm; "red trail" below fructose, 13.6-19.2 cm.)

Each sample was run in butanol-ethanol-water solvent (66) and the paper chromatograms developed with methyl red-methylene blue indicator (92). The samples of the solution were thus seen to contain two acidic materials, one just below the starting-line on the paper chromatogram and one at a distance of 8.1 cm. (Levulinic acid standard, 13 cm.)

Small-Scale Degradation of the Fructosan in Hot Aqueous Solution II.

The fructosan (1.801 g.) was dissolved in water (50 c.c.) and heated on the boiling water bath as above. The following changes were observed:

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>(\alpha)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>4</td>
<td>-36.9</td>
<td>4.52</td>
</tr>
<tr>
<td>6</td>
<td>-40.3</td>
<td>4.39</td>
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<td>12</td>
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<td>-81.9</td>
<td>3.69</td>
</tr>
<tr>
<td>18</td>
<td>-84.7</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>-72.2</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-86.3</td>
<td></td>
</tr>
</tbody>
</table>

Sample Chromatogram Observations (Aniline Oxalate)

4 hours Long trail from the starting-line, extending downwards, as far as the fructose standard.

6 hours 8 distinct spots and trail 2.5 cm, below the starting-line.
9 hours  As for the 6-hour sample but heavier fructose spot.
12 hours  As for the 9-hour sample but more fructose and
          fainter oligosaccharide spots, especially just
          below the starting-line.
15 hours  Fructose, glucose and traces of 5 oligosaccharides.
18 hours  Fructose and a little glucose. Trace of a third
          sugar travelling at the same rate as the sucrose
          standard.
21 and 24 Hours  Glucose and fructose only.

Naphthoresorcimol and hydrochloric acid spray indicated
the presence of material moving faster than fructose
and exhibited as a faint red trail from the fructose spot
over a distance of about 5cms.

Again methyl red-methylene blue spray showed the presence
of two acids, one observed just below the starting-
line, the other travelling at the same rate as a lactic
acid standard and approximately half as fast as a
levulinic acid standard.

Examination of Oligosaccharides.

A sample of the solution (Experiment II) collected
after 6 hours, was run on 4 paper chromatograms by the
side-strip technique. The paper strips were developed
with aniline oxalate and the substance travelling at the
same rate as sucrose on the paper chromatogram was eluted
from the main papers along with the next two fastest-
moving materials. It was thought that these three prod-
ucts might represent di-, tri- and tetrasaccharides
respectively. On hydrolysis, each was seen to give only
glucose/
glucose and fructose, the intensity of the fructose spot relative to the glucose diminishing with increasing speed of the material on the paper chromatogram. The relative intensities of the fructose and glucose spots, however, suggested that each of the three areas eluted did not contain pure di-, tri- and tetrascaccharides respectively, but rather a mixture of two oligosaccharides, one with a glucose residue and the other composed entirely of fructose residues.

Large-Scale Degradation of the Fructosan in Hot Aqueous Solution.

The polysaccharide (50g.) was dissolved in water (1.4 l.) and heated on the boiling water-bath. The following observations were made.

<table>
<thead>
<tr>
<th>Duration (hours)</th>
<th>$[\alpha]_D$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-41.1°</td>
<td>4.40</td>
</tr>
<tr>
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<td>4.19</td>
</tr>
<tr>
<td>6</td>
<td>-43.6</td>
<td>4.08</td>
</tr>
</tbody>
</table>

The solution was heated for a further 30 minutes and allowed to cool. After neutralisation with barium carbonate and filtration, the clear filtrate was concentrated to a syrup at 35° under reduced pressure.

Chromatographic Examination of the Solution.

A portion of the solution, collected after 6 hours heating, was concentrated and examined on the paper chromatogram. Benzene-butanol-pyridine-water solvent was employed, the papers being run for 63 hours and developed with aniline oxalate and napthoresorcinol and hydrochloric acid sprays.
The following spots and their distances from the starting-line were observed (see diagram above).

**Solution:** 0 cm.; 1.1 cm.; 2.9 cm.; 5.2 cm.; 9.7 cm.; 13.9 cm.; 18 cm.; 20.1 cm.; 23.9 cm.;

**Standards:** Fructose, 26.8 cm.
Glucose, 23.4 cm.
Sucrose, 16.1 cm.

**Preparation of Charcoal Column**

A charcoal-hyllosupercel column (38.5 cm. x 4.6 cm.) was constructed from decolorising charcoal (120 g.) and hyllosupercel (120 g.). Before attempting a separation of sugars and oligosaccharides, the column was washed with water (5 l.)

**Attempted Fractionation of the Degradation Products.**

The syrup from the above solution was dissolved in water (500 c.c.) and added to the charcoal column, allowed to penetrate completely and eluted, first with water and then with 30% aqueous alcohol, followed by 50% aqueous alcohol. The eluates were collected in batches of ca. 250 c.c. and portions examined in a 1 dm. polarimeter tube for changes in rotation. The following observations were made.

**Aqueous Eluates**

<table>
<thead>
<tr>
<th>Eluate Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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</thead>
<tbody>
<tr>
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<tr>
<td>9</td>
<td>10</td>
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30% Alcohol Eluates

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<th>17</th>
<th>18</th>
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<tbody>
<tr>
<td>Polarimeter reading</td>
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<td>-0.02</td>
<td>-0.26</td>
<td>-0.37</td>
<td>-0.51</td>
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50% Alcohol Eluates

<table>
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<th>Eluate Number</th>
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<th>24</th>
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Chromatographic Examination of the Eluates.

Aqueous Eluates

<table>
<thead>
<tr>
<th>Eluate Sample</th>
<th>Chromatogram Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>No sugars.</td>
</tr>
</tbody>
</table>
| 3 and 4       | Large excess of fructose; a little glucose. 3 and 4 were combined and concentrated to a syrup = Fraction A (11.181g.)
|               | High fructose content; sugar travelling faster than sucrose but slower than glucose, referred to as "sugar x". |
| 5             | High concentration of fructose and sugar x. 5, 6 and 7 were combined to give Fraction B (24.276g.), shown on further chromatographic examination to contain fructose, a little glucose, sugar x and a trace of sucrose (?) |
| 6 and 7       | A little fructose, sugar x, sucrose(?) and two higher saccharides. |
| 8 and 9       | Very little fructose, sugar x, sucrose(?) and two higher saccharides. 8-13 were combined to give Fraction C (3.229g.) |
| 10, 11, 12    | Chromatographic examination of this fraction/ |
ion indicated the presence of 5 spots as shown in the diagram below.

![Diagram showing spots and distances](image)

- F.G. = Fructose
- G = Glucose
- S = Sucrose
- Fr. C. = Fraction C

Distant 32.2 cm., 25.3 cm., 21.5 cm., 15.0 cm. and 11.3 cm. (trace) from the starting line. Standards were sucrose (20.8 cm.), fructose (31.5 cm.) and glucose (27.4 cm.). Fractions A, B and C were shown to contain no substances moving faster than fructose on the paper chromatogram and giving a red coloration with naphthoresorcinol and hydrochloric acid.

### 30% Alcohol Eluates

<table>
<thead>
<tr>
<th>Eluate Sample</th>
<th>Chromatogram Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 and 15</td>
<td>Almost pure oligosaccharide travelling considerably slower than sucrose (10.2 cm. and 14.4 cm. respectively from the starting line), in high concentration. This will be referred to as &quot;sugar y&quot;. Traces of fructose, sugar x and sucrose(?)</td>
</tr>
<tr>
<td>16</td>
<td>Sugar y (14.8 cm.) and a second oligosaccharide (6.5 cm.) in high concentration; ca. 4 oligosaccharides between the latter/</td>
</tr>
</tbody>
</table>
latter and the starting-line, in low concentration.

17 and 18 Sugar y and ca.5 oligosaccharides as in 16 but all in approximately equal concentration.

50% Alcohol Eluates.

<table>
<thead>
<tr>
<th>Eluate Sample</th>
<th>Chromatogram Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>As for 18, but all oligosaccharides in small concentration. 16-19 were combined giving Fraction E (15.438g.)</td>
</tr>
<tr>
<td>20</td>
<td>As for 19 but spots very faint except the one at the starting-line.</td>
</tr>
<tr>
<td>21</td>
<td>Trail (3.5cm.) from the starting-line.</td>
</tr>
<tr>
<td>22 and 23</td>
<td>Trace of carbohydrate at the starting-line.</td>
</tr>
<tr>
<td>24 and 25</td>
<td>No carbohydrates. 20-25 were combined to give Fraction E (0.196g.)</td>
</tr>
</tbody>
</table>

Separation of Fraction E.

Fraction E was dissolved in water (250c.c.) and added to a second charcoal column (62 x 3.5cm.) prepared from decolorising charcoal (100g.) and hyflo-supercel (100g.) Portions of eluate (ca.250c.c.) were collected and examined polarimetrically and chromatographically as before.

Aqueous Eluates.

<table>
<thead>
<tr>
<th>Eluate Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarimeter reading</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>-4.45</td>
<td>-4.48</td>
<td>-0.19</td>
<td>-0.02</td>
<td>-0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Chromatographic/
Chromatographic Examination: Eluates 1, 2, 3 and 9 contained no sugars, 4, 5 and 6 contained fructose in high concentration and a little glucose; 7 and 8 contained fructose only, the latter in very low concentration. 4-8 were combined to give Fraction B (1) (17.620g.), which was shown chromatographically to have no materials moving faster than fructose on the paper chromatogram.

5% Alcohol Eluates.

<table>
<thead>
<tr>
<th>Eluate Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarimeter reading</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
<td>0.15</td>
<td>0.12</td>
<td>0.03</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

Eluate Numbers.

1 and 2 Sugar x in high concentration. Traces of materials moving faster than fructose. Intense blue spot observed with naphthoresorcinol and hydrochloric acid (pink-purple in U.V. light), travelling slightly faster on the paper chromatogram than the next fastest-moving substances in eluates 1 and 2.

3 and 4 Sugar x in high concentration. Traces of fructose, four higher saccharides, substances moving faster than fructose, and the above-mentioned "blue spot".

5 and 6 Oligosaccharides in low concentration, consisting mainly of sugar x. Blue spot, with naphthoresorcinol spray. 1-6 were combined, giving Fraction B (11) (4.422g.) Sugar x, sucrose (?) and "blue spot".

8/
Sugar x and sucrose(?) in approximately the same concentration.

Sugar x and sucrose(?) , the latter in higher concentration.

Sucrose(?) only, with traces of higher saccharides.

As for 11, but only traces present.

No sugars or oligosaccharides 7-13 were combined giving Fraction B (111) (0.546g.), which was shown chromatographically to contain sugar x and sucrose(?) in approximately equal amounts with traces of three higher saccharides.

Separation of Fraction C.

Fraction C (3.024g.) was shaken with benzene-butanol-pyridine-water solvent (15c.c.) for several hours, giving a turbid solution, and added to a cellulose column (70 x 3cm.) which was eluted with the same solvent. The first 00c.c. eluate contained no sugars, whilst the contents of turntable tubes 1-170 contained small amounts of three materials moving faster than fructose on the paper chromatogram, and a faster-moving material, observed as a blue spot on the paper with naphthoresorcinol and hydrochloric acid spray. 1-170 were combined, giving fraction C (1) (0.059g.). Tubes 184-237 contained only fructose and a little glucose and were combined to give fraction C (II) (0.292g.)

238-260 had traces of fructose and sugar x, in higher concentration, 270 had no fructose but sugar x and a trace of sucrose(?) 238-274 were combined to give fraction C (111) (0/
275-310 contained sugar x and sucrose(?) only.
320-347 had sugar x with sucrose(?) in higher concentration
and a third, faint spot moving very slightly slower than
sucrose, on the paper chromatogram.
275-347 were combined giving fraction C (IV) (0.014g.)
348-370 had sugar x, sucrose(?) and two higher saccharides,
the slower-moving (sugar y) being in much higher concen-
tration. 348-374 were combined to give fraction C(V) (0.242g.)
Tubes 375-430 contained the slowest-moving oligosaccharide
of fraction C(V) (sugar y) with faint traces of higher
saccharides, and on combination gave fraction C (VI) (0.393g.)
431-538 contained mainly sugar y and a small amount of a
second oligosaccharide travelling slightly more slowly
on the paper chromatogram. The contents of tubes 431-538
combined, gave fraction C(VII) (0.445g.).
539-592 contained the two oligosaccharides of fraction
C (VII) in approximately equal amounts, while 593-640 had
only the slower of the two, but in trace quantities only.
539-640 were combined, giving fraction C(VIII) (0.070g.).
641-720 contained no carbohydrates.

Examination of Fractions.

Fraction C (III)

This fraction was found chromatographically to con-
stain one sugar only, travelling 22.6cm. from the starting-
line when a sucrose standard had travelled 19.2cm. It
reduced Fehling's solution and ammoniacal silver nitrate.
On mild acid hydrolysis it gave fructose only, and after
incubating/
incubating with a 4% solution of Invertase Concentrate (B.D.H.) gave only fructose with a little of the original oligosaccharide. It showed $[\alpha]_2^\prime -20.8^\circ$ (c, 4.0 in water).

**Fraction C (AV)**

This fraction contained sugar $x$, the sugar of fraction C (III) and an oligosaccharide travelling at the same rate as a sucrose standard on the paper chromatogram. In addition there were traces of a higher oligosaccharide.

A sample of the fraction was run on a paper chromatogram, which was developed with ammoniacal silver nitrate, giving one brown spot corresponding to sugar $x$, the second oligosaccharide (sucrose ?) giving no coloration. From a second sample run on a paper chromatogram by the side-strip technique, the two oligosaccharides were eluted and hydrolysed with dilute oxalic acid. Chromatographic examination of the two hydrolysates showed that the reducing sugar had given only fructose, whereas the non-reducing oligosaccharide gave both glucose and fructose in apparently equal amounts. A third sample was chromatographed and the oligosaccharide areas eluted with water, the eluates concentrated to small volume and Fehling's test applied to each. Again the faster-moving sugar gave a positive test, while the second was found to show a very weak reducing action, only on prolonged boiling of the solution.

**Fraction C (V)**

Chromatographic examination of this fraction showed the presence of four oligosaccharides:

(a) sugar $x$, in low concentration,

(b)/
(b) sucrose (?), in higher concentration,
(c) a third oligosaccharide, in low concentration, and
(d) sugar y, in very high concentration.

The speeds of the four on the paper chromatogram were such that a > b > c > d. All four however, tended to run into one another, and to effect a separation it was necessary to run the paper chromatograms for about 3 days. On mild acid hydrolysis (a) and (d) gave fructose only while (b) and (c) gave apparently equal amounts of glucose and fructose. (a), (c) and (d) reduced ammoniacal silver nitrate solution but (b) did not. Each of the four oligosaccharides was treated with 4% (v/v) Invertase Concentrate solution and the products examined on the paper chromatogram. (a) gave fructose only, (b) glucose and fructose, (c) glucose, fructose and a little unchanged starting material, and (d) fructose, sugar x and a little unchanged sugar y.

Fraction C (VI)

This fraction was shown chromatographically to contain one oligosaccharide travelling 15.0cm, in benzene-butanol-pyridine-water solvent, in the time a sucrose standard travelled 21.1cm. It showed $[\alpha]_D^21 = -21.1^\circ$ (c, 318 in water), strongly reduced Fehling's solution and ammoniacal silver nitrate, and on mild acid hydrolysis gave only fructose. When treated with invertase solution it gave the same products as (d) of fraction C (V).

Fractions C (VII) and C (VIII).

These fractions consisted of the oligosaccharide (sugar y) of fraction C (VI), together with a slower-moving...
moving oligosaccharide. Fraction C(VII) contained the former in large excess, while C(VIII) contained approximately equal quantities of both.

The two oligosaccharides were separated chromatographically by running on Whatman 3 M.M. papers, in benzene-butanol-pyridine-water solvent for 5 days and eluting with water.

**Examination of the Slow-moving Oligosaccharide of Fractions C (VII) and C (VIII).**

The slower-moving of the oligosaccharides of fractions C (VII) and C (VIII) (ca. 60 mg.) was found to be non-reducing towards Fehling's solution and ammoniacal silver nitrate. It showed $[\alpha]_D^2 = +24^\circ$ (c, 0.5 in water) and on treatment with invertase, followed by chromatographic examination, was found to give both glucose and fructose, the same observations being made after mild acid hydrolysis.

The glucose-fructose ratio was found by hydrolysing the oligosaccharide with dilute oxalic acid, separating the components of the hydrolysate on a paper chromatogram and estimating the sugars by periodate oxidation. This gave:

Glucose : Fructose = 1 : 2.0.

The oligosaccharide thus appears to be a trisaccharide containing one glucose and two fructose residues.

A portion of the oligosaccharide was dissolved in water (5 c.c.) and Amberlite resin (I.R. -100; ca. 0.5 g.) added. The solution was heated on the boiling water-bath, and samples (1c.c.) withdrawn at intervals, neutralised with Amberlite resin (I.R. -4B), concentrated and examined on/
on the paper chromatogram. The following observations were made:

**Duration** | **Chromatographic Examination.**
--- | ---
10 minutes | Mostly unchanged oligosaccharide with traces of sucrose and fructose.
30 minutes | Sucrose and fructose in high concentration.
1 and 3 hours | Fructose and glucose only.

**Examination of Fraction F.**

This fraction showed \([\alpha]^2_D -37.2^\circ (c, 1.8 \text{ in water})\) and on hydrolysis with oxalic acid (10c.c.; 2%) at 100° for 2 hours, was shown chromatographically to give fructose and arabinose, both in high concentration, a little glucose and traces of xylose and galactose.

**Separation of Sugar x, Sucrose(?), and a Reducing Glucose-fructose disaccharide.**

Fractions B (111) and C (1V) were combined to give fraction 5 (1.401g.), which was purified by digesting with charcoal and filtering through a pad of hyflo supercel. A portion (1.112g.) was dissolved in benzene-butanol-pyridine-water solvent and fractionation attempted on a cellulose column (100 x 1.7cm.) using the same solvent as eluant. The following fractions were collected and examined chromatographically:

**Fraction 3(1) (0.022g.)** contained the above-noted material, giving a blue coloration with naphthoresorcinol and hydrochloric acid, and small amounts of three substances moving more rapidly than fructose on the paper chromatogram.

**Fraction*/
Fraction 3(II) (0.068g.) contained fructose and a little glucose.

Fraction 3(III) (0.249g.) consisted almost entirely of sugar x with a trace of sucrose (?)

Fraction 3(IV) (0.489g.) contained sugar x and sucrose (?) in apparently equal amounts.

Fraction 3(V) (0.064g.) had almost pure sucrose (?) with a trace of sugar x.

Fraction 3(VI) (0.031g.) contained sucrose (?) and a slower-moving oligosaccharide in low concentration.

Fraction 3(VII) (0.031g.) contained the slower-moving oligosaccharide of fraction 3(VI) and traces of higher saccharides.

Fraction 3(VIII) (0.058g.) had higher saccharides only.

Separation of Pure Sucrose:

Fractions 3(IV), 3(V) and 3(VI) were separated into pure components by running on paper chromatograms of Whatman 3H.M. paper for ca. 3 days, in benzene-butanol-pyridine-water solvent, by the side-strip technique.

Thus, pure sucrose (0.287g.), referred to below as fraction N, was obtained. Allowance being made for loss in the side-strips, this accounts for 0.322g. sucrose.

Separation of a Reducing Glucose–Fructose Disaccharide:

The slower-moving oligosaccharide from fraction 3(VI) was separated from sucrose as described above and combined with fraction 3(VII) to give fraction R. This was obtained as a dark-brown syrup (0.042g.) and appeared to be impure.

Identification of Sucrose.
Fraction N crystallised from aqueous ethanol and had m.p. 184-185°, not depressed on admixture with an authentic specimen of sucrose.

**Chromatographic Examination:** A sample of fraction N was examined on paper chromatograms in conjunction with a standard of pure sucrose. Solvents of benzene-butanol-pyridine-water, ethyl acetate-acetic acid-water and butanol-ethanol-water were employed with developing sprays of aniline oxalate, urea oxalate and naphthoresorcino1 and hydrochloric acid. It was thus seen that fraction N consisted virtually of one sugar, travelling at the same rate as the sucrose standard.

**Reducing action:** The material was non-reducing to Fehling's solution and an ammoniacal solution of silver nitrate.

**Specific Colour test:** On application of the diazouracil colour test for a sucrose-type linkage, a weak but positive result was obtained.

**Hydrolysis:**

(a) **Action of Invertase:** A portion of fraction N was treated with a 4% (v/v) Invertase Concentrate solution, when paper chromatography showed the presence of glucose and fructose only.

(b) **Acid Hydrolysis:** A sample of the fraction was hydrolysed with oxalic acid (1%) at the boiling point. 

\[ [\alpha]^{\circ}_{D}, +59^\circ (\text{zero}) \rightarrow -24^\circ (60 \text{ minutes}) \text{ constant}. \]

(c, 1.5 in aqueous oxalic acid)
The Glucose-Fructose Ratio: This was found as described above and gave the following result:

Glucose : Fructose = 1 : 1.05.

X-ray powder photographs were taken of fraction \( N \) and an authentic specimen of sucrose, and found to be identical.

Found: C, 42.2; H, 6.4

Calculated for \( \text{C}_{12}\text{H}_{22}\text{O}_{11} \): C, 42.1; H, 6.4.

Preparation of Sucrose Octacetate

A sample of fraction \( N \) (ca. 180 mg.) was added to pyridine (3c.c.) and acetic anhydride (2c.c.) at \( 0^\circ \) and shaken at room temperature until the sugar had dissolved (ca. 4 days). The solution was poured into ice and water (4c.c.) and the precipitate (ca. 250 mg.) separated and twice recrystallised from ethanol, giving white needle-shaped crystals, m.p. 72-73\(^\circ\), not depressed on admixture with an authentic specimen of sucrose octacetate.

The material showed \( \alpha^2 \) (fused crystals) 1.4602 and \( [\alpha]_\text{D}^2 + 60^\circ \) (c, 1.1 in chloroform)

Found: C, 49.3; H, 5.8; \text{CH}_3\text{CO}, 50.3.

Calculated for \( \text{C}_{28}\text{H}_{38}\text{O}_{19} \): C, 49.5; H, 5.6; \text{CH}_3\text{CO}, 50.7.

Examination of Fraction \( R \).

This fraction was examined on the paper chromatogram and seen to contain small amounts of sucrose and difructose impurities. The main oligosaccharide in the fraction reduced Fehling's solution and ammoniacal silver nitrate, and gave glucose and fructose on treatment with invertase.
The glucose-fructose ratio was found to be 1 : 1.27 by the method described above, and the oligosaccharide reduced alkaline hypoiodite, the extent being 55% of that expected for a disaccharide containing one free aldose reducing group.

Control Experiment.

A little chromatographically pure sugar x and sucrose were dissolved in a few c.c.s. benzene-butanol-pyridine-water solvent and left at room temperature for a week. Chromatographic examination of the two solutions showed that neither sugar had undergone any changes.
(1) A fructosan has been extracted from perennial rye grass (*Lolium perenne*) and gave glucose and fructose on hydrolysis.

(2) Degradation of 50g. of the polysaccharide, was effected by heating in aqueous solution on the boiling water-bath, and the degradation products separated first on charcoal columns and then by partition chromatography on cellulose. A large number of degradation products were obtained, including sucrose, a non-reducing trisaccharide containing one part of glucose to two of fructose, a fructose-glucose disaccharide which reduced alkaline hypoiodite and several oligosaccharides giving only fructose on hydrolysis.

(3) The isolation of sucrose by the above methods would seem to confirm the theory that the fructosan chain is terminated by a glucopyranose residue attached to the chain of fructose residues by a sucrose-type linkage.
As the present work has been largely concerned with investigations into the rôle of glucose in the polyfructosans, we might now briefly consider the evidence which has been obtained, favouring the concept of a sucrose radical terminating the fructofuranose chains.

The fructosan from leafy cocksfoot grass was found to contain 3.2% glucose. This value for the glucose content indicates a chain-length of some 30 units, assuming there is one glucose residue per fructosan chain. Such a chain-length is in reasonable agreement with that deduced for the methylated polysaccharide (about 25).

Methylation and hydrolysis of the fructosan yielded 1:3:4-trimethyl D-fructose (65.5%) and 1:3:4:6-tetramethyl D-fructose (4%), thereby suggesting that the polysaccharide consists of about 25 fructofuranose residues linked through the C₂- C₆ positions. In addition, about 2% 2:3:4:6-tetramethyl D-glucose was obtained with only a trace of trimethyl glucose. Although the amount of tetramethyl glucose obtained from the hydrolysate of the methylated polysaccharide is less than would be expected from the glucose content of the original fructosan, it is probable that some glucose was lost during methylation and that the tetramethyl glucose originated from a non-reducing glucopyranose "end-group", linked as in sucrose. The molecular weight of the methylated polysaccharide was found, employing Barger's isopiestic method, when agreement was obtained with the value 25.
units, calculated by the method of "end-group" assay. A small amount of dimethyl fructose was isolated from the hydrolysate of the methylated fructosan, but in insufficient quantity to be considered of any structural importance.

Oxidation of the polysaccharide consumed 1.02 moles of sodium metaperiodate per C₆H₁₀O₅ residue, in agreement with the postulate of a molecule composed of fructofuranose residues, linked through the 2-6 positions.

The production of formic acid on periodate oxidation was greater (1 mole per 20 C₆H₁₀O₅ residues) than would be expected if all the chains were terminated by a non-reducing glucopyranose residue (1 mole per 25-30 residues) and less than would be expected for a fructosan terminated by a O₆-linked fructofuranose residue (about 1 mole per 12 C₆H₁₀O₅ residues). It may well be however, that scission of some of the polysaccharide chains occurred during the isolation of the polysaccharide so that the fructosan preparation contained some chains terminated by non-reducing glucopyranose residues and some terminated by reducing fructofuranose residues. Such a preparation would explain the observed value for the formic acid release on oxidation of the polysaccharide with the periodate ion. Further evidence for the presence of a sucrose-type end-group in the polyfructosan was obtained by heating an aqueous solution of the polysaccharide on the boiling water-bath. By this treatment, the fructosan was degraded to give, amongst other products, a sugar which travelled at the same rate on the/
the paper chromatogram as sucrose, and gave glucose and fructose on hydrolysis. This sugar may indeed have been sucrose, liberated from the end of the fructosan chain during the degradation.

In view of the reports of other investigators in the polyfructosan field, that hydrolysis of methylated fructosans gave 2:4:6-trimethyl D-glucose, it was decided to test for the presence of a glucose residue linked through the 1-5 positions. As such a residue would be expected to be resistant to periodate oxidation, a sample of inulin from dahlia tubers (Crimson Flag variety) was oxidised by the periodate ion, and the oxidised material hydrolysed, when chromatographic examination of the hydrolysate failed to show the presence of any sugars. It would thus appear that the 2:4:6-trimethyl glucose isolated by the other workers arose from undermethylation and/or demethylation of the fructosan. It is interesting to note that Haworth, on treating sucrose with methyl sulphate and sodium hydride, produced septamethyl sucrose, which on hydrolysis gave molecular amounts of 1:3:4:6-tetramethyl fructose and what appeared to be 2:3:6-trimethyl glucose, indicating that it is more difficult to completely methylate the glucose residue of sucrose than the fructose residue. As a final verification that the fructosans are terminated by sucrose-type moieties, it was decided to degrade the fructosan from perennial rye grass by heating an aqueous solution on the boiling water-bath, and attempt to isolate sucrose from the degradation products.
Starting from 50g. of polysaccharide, the products from the degradative treatment were separated first on charcoal columns and then on cellulose columns, to give glucose and fructose, a mixture of disaccharides and a mixture of higher oligosaccharides.

The disaccharides were further separated on Whatman 3 M.M. sheets. By this means about 300mg. of a disaccharide was isolated giving equal amounts of glucose and fructose on hydrolysis, and showing no depression in melting point when mixed with an authentic specimen of sucrose. In addition, the sugar gave an X-ray powder photograph identical to that of pure sucrose, and was further characterised as sucrose by conversion to the acetate.

A non-reducing trisaccharide giving on hydrolysis two parts of fructose and one part of glucose was also isolated in small quantity. On graded hydrolysis, this material was shown to give rise to sucrose and fructose, together with traces of glucose and a fructose-containing disaccharide. This trisaccharide may very well have been identical to that produced by Albon, Bell, Blanchard, Gross and Rundell by the action of yeast invertase on sucrose.\(^{(59)}\)

A glucose-fructose disaccharide, which was obtained in very small amount and which reduced alkaline hypoiodite, may have been produced by a reversion synthesis during the degradation process, or from the fructose-containing disaccharide by epimerisation.

From the above evidence it would appear that the fructosan polysaccharides contain one glucose residue per chain, this glucose being present as a terminal non-reducing group, linked as in sucrose.
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