UNIVERSITY OF EDINBURGH

STRUCTURAL STUDIES IN THE POLYSACCHARIDE GROUP

A

THESIS

Presented for the Degree of

DOCTOR OF PHILOSOPHY

by

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CONTENTS

INTRODUCTION

Part I of this thesis deals with an investigation into the molecular structure of inulin. The general structure of this molecule has already been determined, but there are one or two details of fine structure which still require elucidation. One of these is the question of the importance of glucose in the molecule, and this investigation is an attempt to define this.

Inulin was the first of the naturally occuring polyfructosans to be isolated and has received by far the most attention. It was discovered by Rose (1) who seperated it from an extract of artichoke tubers. The name Inulin was first used by Thomson (2) in 1811.

Inulin occurs in large quantities in dahlia tubers, where it is the main reserve carbohydrate of the plant, and also in chicory, Jerusalem artichokes and burdock to a much smaller extent.

The first researches undertaken with a view to establishing the structure of the molecule were those of Irvine and Steele (3), who methylated inulin by treatment with sodium hydroxide and dimethyl sulphate. They report that this only gave them a partially methylated compound which was converted into trimethyl inulin by Purdie's method, using silver oxide and methyl iodide. On hydrolysis with 1% oxalic acid trimethyl inulin gave a trimethyl fructose, which was characterised by further methylation to tetramethyl fructose which was identical with that isolated from sucrose.

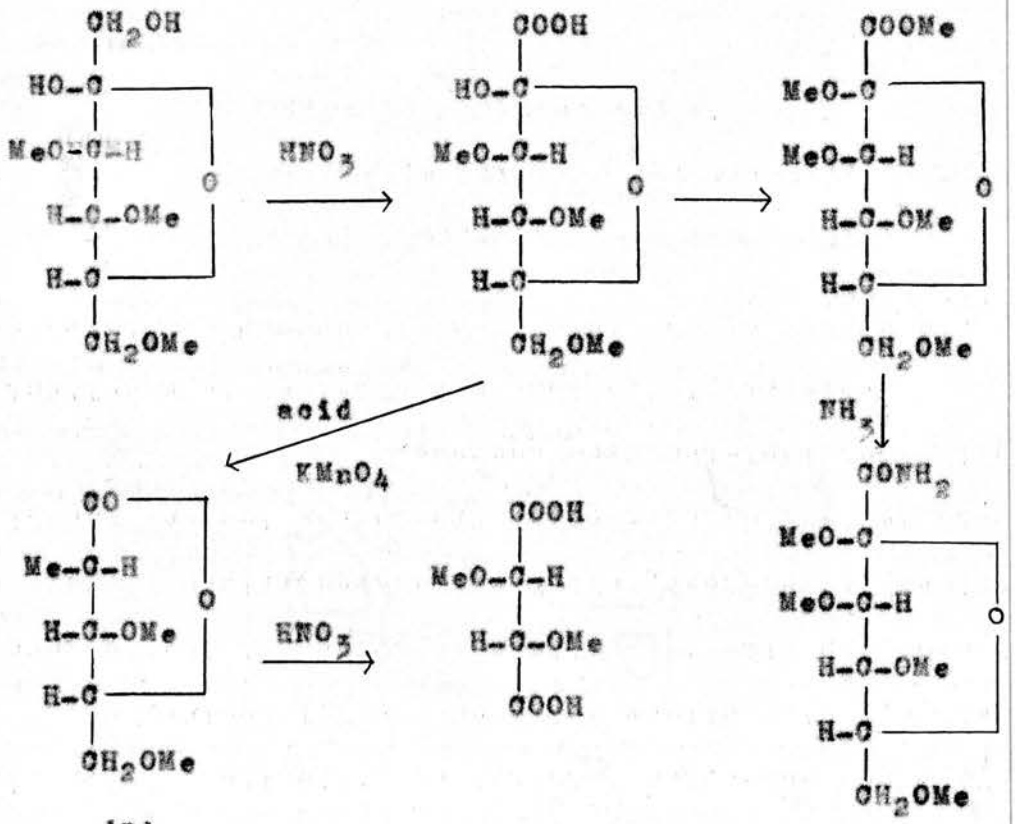
Tetramethyl fructose was obtained in excellent yield of 95% of the total, and these workers therefore

concluded that inulin is an aggregate of fructose units, each molecule having lost two hydroxyl groups in the condensation to polysaccharide.

Irvine and Steele give two interpretations of these facts. Either inulin may be a polymerised anhydro-fructose, the reducing group being eliminated in the condensation, or the fructose residues may be condensed in such a way that each residue loses two hydroxyl groups, one of which is the reducing group.

Based on this latter supposition, and to accord with their evidence, they suggest a structural formula for inulin; however, since knowledge of the structure of the monosaccharides was radically altered by the work of Haworth a few years later, Irvine and Steele's formula now has little but historical interest. Their work is of importance in so far as it is one of the first examples of the application of the methylation technique in the polysaccharide field.

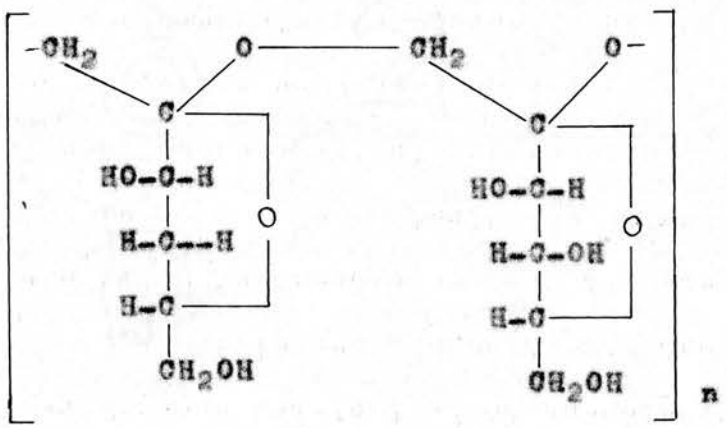
Haworth and Learner (4) followed up this work, using the new methods for determination of monosaccharide structure developed by Haworth about this time. They methylated inulin by one treatment with sodium hydroxide and dimethyl sulphate, followed by three treatments with Purdie's reagents. This gave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



(I)

The D-trimethyl-γ-arabonolactone (I) can only have come from 3:4:6-trimethyl fructofuranose, which can only be obtained if the linkages in inulin are 1-2.

On this evidence Haworth and Learner advanced the following formula for inulin:



Drew and Haworth (5) working on the molecular weight of inulin, concluded, from ebullioscopic measurements, that it was not less than 3200 or 3600, i.e. 20 to 22 anhydro-fructose units. They also failed to find a sample of inulin which did not show a slight and progressive reducing action on boiling Fehlings solution. This, they claim, is due to the presence of reducing groups at the ends of open chain molecules, and subsequent and progressive hydrolysis.

It is on the basis of this hydrolysis of inulin in boiling aqueous solution that they criticised the earlier work of Pringsheim, who had determined the molecular weight of inulin by the cryoscopic method after dissolving his samples in boiling water, which according to Drew and Haworth, rendered his results invalid. Pringsheim replied to this criticism (6) by publishing the results of work on the hydrolysis of inulin and sucrose in boiling water. He found that inulin can be boiled in distilled water, in quartz, or even in ordinary glass vessels, for five hours, without the reducing power amounting to more than 1% of that of fructose. On the basis of the discovery that sucrose reduces boiling Fehling's solution after three minutes, Pringsheim disagrees with Drew and Haworth when they assume that because inulin reduces boiling Fehling's solution it must possess free reducing groups at the ends of the molecular chains.

Pringsheim, Reilly and Donovan (6) give values of 1193 to 2139 for the molecular weight of inulin in aqueous solution, i.e. 7-13 anhydro-fructose residues.

Berner (7), also using the cryoscopic method, gives values of 3500 to 4500 for the molecular weight of inulin from various sources, i.e. chain lengths of 22-28 fructose residues.

These results serve to emphasise the very labile nature of the inulin molecule, but they leave one in doubt as to its exact size, and also as to whether it is an open chain molecule, with a free reducing group at one end, or of some other form, which would be non-reducing.

Two possible structural forms have been proposed for the inulin molecule. Either that inulin is a polymerised aggregate of fructose anhydrides, or that it is a chain of fructose residues.

Evidence for the first type of structure is afforded by the work of Schlubach and Elsner (8) who claimed to have synthesised the basic unit of inulin. They treated fructose with cupric sulphate and acetone and isolated a compound which could be purified free from acetone, and which, on methylation and hydrolysis gave 3:4:6-trimethyl fructose, which was proved identical with that isolated by Haworth and Learner from inulin. Their compound had the properties and molecular weight of a fructose anhydride, and they concluded from these studies that the basic unit of inulin is either a 1:2-fructose anhydride or a 1:2-2:1-difructose anhydride.

This type of polymerised structure for a polysaccharide molecule assumes that the individual units hold together by hydrogen bonding, and this admits the possibility of inconstant molecular weights, and on this point the evidence is conflicting. It must

also be taken into account that the evidence of Schlubach and Elsner is none too sure, as they must have worked with fructopyranose, and from methylation studies inulin has been shown to consist of fructose which is present solely in the furanose form (4).

By far the greater weight of evidence points to a chain structure of chemically linked units, so much so in fact, that the polymerised form of molecular structure is no longer considered.

Evidence for this type of structure comes from the methylation studies of Haworth and Learner (4), and Haworth, Hirst and Percival (9). These latter workers obtained trimethyl inulin from the triacetyl compound by treatment with sodium hydroxide and dimethyl sulphate in acetone solution, a procedure discribed by Haworth and Streight (10). After hydrolysis of the trimethyl inulin, they quantitatively determined the amount of tetramethyl fructose, which, on the chain theory, is derived from the non-reducing end group. From the percentage of tetramethyl fructose isolated, and identified as 1:3:4:6-tetramethyl fructofuranose, these authors concluded that inulin consists of a chain of 30 residues.

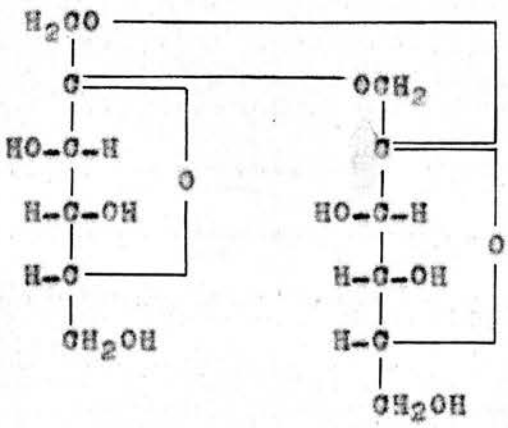
Thus the general structure of the inulin molecule is now known as a result of these methylation studies, but two principle anomalies as to its fine structure have arisen. One of these is the question of the importance of difructose anhydrides in the molecular structure, and the other deals with the role played by glucose in the molecule.

Jackson and Goergen (11) isolated 5% of non-reducing difructose anhydrides from an acid hydrolysate of inulin. They describe these compounds as far more resistant to acid hydrolysis than the remainder of the molecule, and they maintain that this property assures their survival during the orignal hydrolysis. In a continuation of this work, Jackson (12) isolated three distinct compounds by fractional crystallisation. Based on the fact that these compounds occur in inulin irrespective of the natural source of the polysaccharide, and that they cannot be removed by eleven "recrystallisations" of Dahlia inulin, he states that they must be present preformed in the molecule, and as a total of three difructose anhydrides is isolated, the minimum molecular weight of the polysaccharide becomes 18,000.

It would appear that Jackson has here committed an error of reasoning, as it is obvious that the fact of isolating these difructose anhydrides from an inulin hydrolysate, no matter the source or purity, does not preclude the fact that they might be by-products of the hydrolysis, and this is the contention of two separate groups of workers.

Pringsheim and Ohlmeyer (13) isolated an enzyme, inulase, from Aspergillus niger, and they used this enzyme to hydrolyse inulin under very mild conditions. They found that the polysaccharide was 95% hydrolysed and after destruction of the fructose they attempted to isolate Jackson's difructose anhydrides, but all their attempts failed. They are, therefore, inclined to the belief that these compounds are formed during the acid hydrolysis. Haworth and Streight (14) provided

further evidence towards this belief. They prepared Jackson's compounds by his methods, and converted them into the hexamethyl derivatives. On hydrolysis, these gave only 3:4:6-trimethyl fructofuranose, identical with that isolated from the hydrolysate of trimethyl inulin. Haworth and Streight thus gave the following formula to the difructose anhydrides:

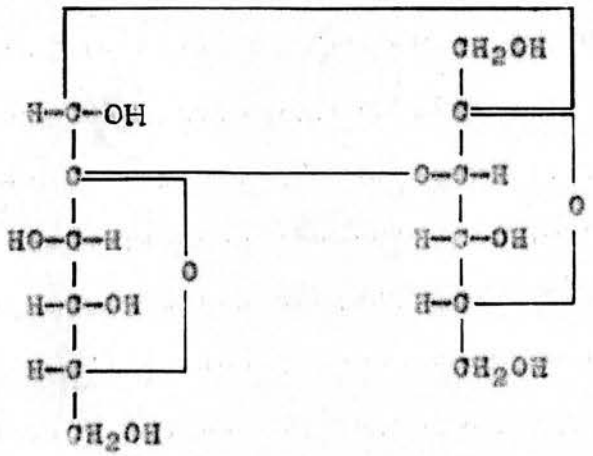


1:2'-2:1'-di-D-fructofuranose anhydride

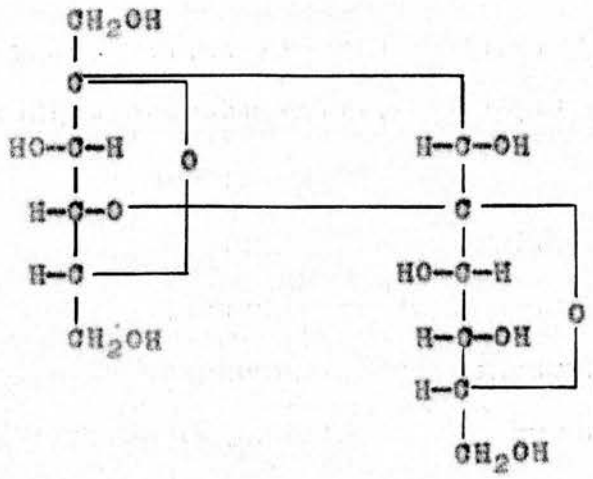
There is here the possibility of three stereoisomeric forms, depending on whether the linkage between the two fructose molecules is $\alpha\alpha$, $\alpha\beta$, or $\beta\beta$. Therefore the isolation by Jackson of three different anhydrides assumes added importance.

Haworth and Streight found that the hexa-acetate of the isolated difructose anhydride was soluble in, and crystallisable from, hot water. They repeatedly extracted triacetyl inulin with hot water but obtained nothing in the extracts. It seems probable then, that these compounds do not exist preformed in inulin, but are formed during the acid hydrolysis. The fact that they are much more resistant to hydrolysis than the remainder of the molecule, cannot therefore refute the theory that inulin is a chain of fructofuranose residues.

The work has been continued in an effort to establish the structure of these three compounds. The structure of difructose anhydride I is that proved by Haworth and Streight, but the other two compounds are not stereoisomers as they suggested. Difructose anhydride III was shown by Jackson and McDonald (15) to be a 1:2'-2:3'-difructofuranose anhydride. These authors also produce evidence to show that difructose anhydride II is a 2:1'-4:2'-difructofuranose anhydride, and this structure has now been definitely established (16).



1:2'-2:3'-di-D-fructofuranose anhydride (III)



2:1'-4:2'-di-D-fructofuranose anhydride (II)

Jackson and McDonald (15) suggest a possible mechanism for the origination of the difructose anhydrides from inulin, as it now seems unlikely that they exist preformed in the polysaccharide molecule. Their theory is that during the hydrolysis the inulin aggregate is ruptured at various points, leaving shortened chains each having a reducing group at one end. In a relatively small number of instances the hydroxyl group of the terminal reducing residue apparently condenses with one of the hydroxyl groups of the penultimate fructose residue, thus forming a difructose anhydride entity which is so stable as to resist further hydrolysis. This condensation of one fructose residue with a closely contiguous one is in keeping with the known tendency of fructose derivatives to polymerise. On the penultimate fructose residue positions 3, 4 and 6 bear hydroxyl groups which are available for this condensation, and the union, through an atom of oxygen, of C_3 with C_2 of the terminal residue, would lead to the formation of difructose anhydride III. Similarly, difructose anhydride II would be formed by union of C_4 with C_2 of the terminal residue. This condensation can occur at any time before the complete resolution of the inulin fragments into individual fructose units. Difructose anhydride I can only be formed by the momentary isolation of a fragment composed of two fructose units, followed by condensation; or by simultaneous hydrolytic splitting of the fragment and condensation to the anhydride.

The question as to the origin of these anhydrides now appears to be answered, but the anomaly exists

that, while glucose has been demonstrated in inulin hydrolysates by many workers, and in a variety of ways its function in the molecular structure has not been determined by the definite isolation of methylated derivatives from a hydrolysate of trimethyl inulin.

Tanret (17) was the first worker to produce evidence for the presence of glucose in inulin. He concluded from the lowering of the rotation on acid hydrolysis, that it contained one glucose to every twelve fructose residues, i.e. 7.7%.

The most conclusive work on this subject to date would appear to be that of Schlubach and Elsner (18) who have demonstrated the presence of glucose in inulin by three different methods. After cautious hydrolysis of triacetyl inulin with an acetyl bromide, acetic acid, hydrogen bromide mixture; debromination with silver carbonate; and acetylation with an acetic anhydride, sulphuric acid mixture, they isolated ∝-pentacetyl glucose. This same product was obtained directly from triacetyl inulin by treatment with an acetic anhydride, sulphuric acid mixture. They also estimated the reducing power of inulin, hydrolysed with .05N sulphuric acid, by the Willstatter-Schudel method; and compared this value with the total reducing power as estimated by Bertrand's method. The results indicated an aldose content of 8%, which is in good aggrement with the value given by Tanret.

Schlubach and Elsner explored the possibility of the rearrangment of some fructose to glucose during the hydrolytic process by a control experiment on sucrose, but they obtained a negative result.

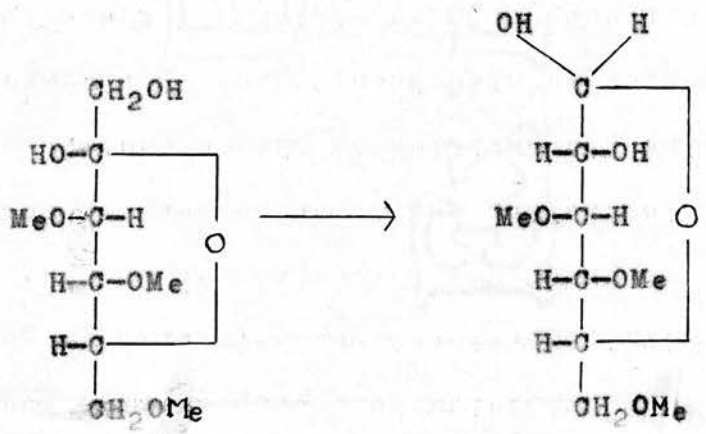
This work might be criticised on the grounds that acetylation, because of the conditions employed, which are drastic for a molecule as labile as that of inulin, might give rise to changes in the structure. In the polysaccharide field a molecule is often methylated by treatment of the triacetyl compound with methylating reagents, but it has been found in the case of starch that the molecule breaks up on acetylation, and the high molecular weight compound is only obtained if starch is methylated directly. In spite of this criticism however, which is countered by the control experiment on sucrose, this work remains the soundest proof for the existance of glucose preformed in inulin; but these authors do not attempt to determine its position in the molecule.

Glucose has also been found after hydrolysis of inulin under very mild conditions by the enzyme inulase, isolated from Aspergillus niger as reported by Pringsheim and Ohlmeyer (18). They estimated 1.5% aldose, which they assumed to be glucose, by differential titration with Fehling's and hypoiodite solutions. As the inulin was only 95% hydrolysed, there exists the possibility that some glucose might be present in the remaining, unhydrolysed, portion.

In further experiments, Ohlmeyer and Pringsheim (19) confirmed their previous value of 1.5% for the glucose content of an enzymatic hydrolysate of inulin. A similar, control, experiment on sucrose, produced exactly the calculated quantities of glucose and fructose; thus providing further evidence that the glucose obtained from inulin is not formed secondarily

from fructose. From the fact that no samples of inulin of $[\alpha]_D$ more negative than -49.2° have ever been prepared, and that increasing purification does not alter the amounts of glucose present, they conclude that the glucose is an integral part of the molecule and not produced from an impurity.

The isolation of a methylated derivative of glucose from a hydrolysate of trimethyl inulin is reported by Irvine and Montgomery (20). These authors, during work which confirmed the results of Haworth, Hirst and Percival, isolated tetramethyl glucose, obtained after the further methylation of a trimethyl glucose present in the acid hydrolysate of trimethyl inulin. They claim that the trimethyl compound is 3:4:6-trimethyl glucose. From control experiments on purified 3:4:6-trimethyl fructofuranose, prepared from trimethyl inulin, they concluded that the glucose derivative had been formed during the hydrolysis as a result of the change:



It must be pointed out here that no similar interconversions of partially methylated sugars in the hydrolysates of methylated polysaccharides have been reported subsequently.

Adams, Richtmeyer and Hudson (21) also mention the presence of glucose in an enzymatic hydrolysate of inulin. They estimated it to occur to the extent of 1.7%, which is in agreement with the value given by Pringsheim and Ohlmeyer.

Our knowledge of the part played by glucose in the structure of inulin in in a somewhat unsatisfactory state. There is a difference of 6.5% between values given for its concentrarion, and the various authors differ as to its mode of origin. The work which forms the subject matter of Part I of this thesis was undertaken in order to reinvestigate the structure of the inulin molecule, with particular reference to the position and importance of glucose. The mildest possible conditions have been used, and full advantage has been taken of the modern technique of paper partition chromatography, which has now assumed a position of major importance among the methods available for the structural elucidation of many naturally occuring polymeric compounds.

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EXPERIMENTAL

The Extraction of Inulin from a Natural Source and its Purification.

Dahlia tubers (3.5 kg.) of "Blue Danube" variety, were minced finely, and the expressed juice filtered through cloth. After an hour the filtrate had solidified and it was then treated with hot water (1500 ml.) milk of lime was added to pH8, and the solids were filtered off. The solution was heated to 60-70° C and dilute aqueous oxalic acid was added to pH7; carbon was added to decolourise the product, and after filtration the solution was chilled when inulin separated. This solid was filtered off and kept in acetone overnight. The product was dried as completely as possible by suction and finally in an oven at 50° C. Fraction Ia (50 g.)

The mother liquor was concentrated to 500 ml. under diminished pressure at 40°, and on chilling a further crop of inulin was obtained, which was dried as before. Fraction Ib (35 g.)

The minced pulp was extracted with water (5 l.) at 60° for 1½ hours. The extract was filtered through cloth and the filtrate treated as before with milk of lime, followed by oxalic acid and carbon. Inulin was deposited on standing, which was filtered off, washed with cold water and dried as before with acetone. Fraction IIa (64 g.)

The mother liquor from this separation was concentrated to 1 l. under diminished pressure, and on chilling a further quantity of inulin separated. Fraction IIb (108 g.)

The pulp was further extracted with water (4 l.)

at 60° for 1 hour. The extract was treated as previously and on concentration and cooling inulin separated, which was dried in acetone. Fraction III (29 g.)

The total yield of inulin is 286 g., as a fine white powder, which represents 9.7% of the weight of tubers taken.

The above fractionation was performed in order to ascertain whether the whole of the inulin contained glucose, or only a particular portion of it. By hydrolysing a sample from each fraction with sulphuric acid ($\frac{n}{60}$), and analysing the neutralised hydrolysates on the paper chromatogram (1), glucose was demonstrated in the hydrolysates of all these fractions.

The ash contents of the various fractions were estimated and found to be negligible.

The specific rotations of these inulin fractions varied from −34° to −38°, while the most negative value given in the literature for pure inulin is −40.2°. In order to purify the inulin a sample of $[\alpha]_D$ −34.7° was dissolved in hot water, and the solution chilled to bring down the polysaccharide, which was filtered off and the process repeated in all seven times. After finally drying in acetone the sample showed $[\alpha]_D$ −40.0°. Glucose was still present in an acid hydrolysate as was shown on the paper chromatogram.

Quantitative Estimation of the Glucose and Fructose liberated on Hydrolysis of Inulin.

The paper chromatogram technique of Flood, Hirst and Jones was employed (2). Inulin (14.32 mg., $[\alpha]_D$ −40.0°) and ribose (7.99 mg.) as reference sugar were weighed

into a small tube, sulphuric acid (0.4 ml. $\frac{n}{60}$) added, the tube sealed and the polysaccharide hydrolysed by immersion of the tube in a boiling water bath for 3 hours. After neutralisation, the hydrolysates were spotted onto the starting lines of the paper chromatograms which were allowed to run for 48 hours. The side strips were cut off and the position of the sugars shown by development with ammoniacal silver nitrate solution. Those strips of the main portions of the chromatograms containing the sugars were extracted with water (5 ml.) and the sugars in the extracts were estimated using the Somogyi copper reagent. 5 ml. samples of standard solutions of glucose, fructose and ribose; and blanks containing an equal volume of distilled water were also treated at the same time. After heating in a boiling water bath for 25 minutes iodine was liberated from the excess reagent by the addition of potassium iodide solution (2.5%), and the acidified solutions were titrated with sodium thiosulphate (approx. $\frac{n}{200}$)

5 ml. ribose solution (35.0 mg./l.) required 0.588 ml. thio.
The ribose from the paper required 2.497 ml. thiosulphate.

0.588 ml. ≡ 0.175 mg. ribose

∴ 2.497 ml. ≡ 0.744 mg. ribose.

5 ml. glucose solution (41.0 mg./l.) required 1.118 ml. thio.
The glucose from the paper required 0.457 ml. thiosulphate.

1.118 ml. ≡ 0.205 mg. glucose

∴ 0.457 ml. ≡ 0.0837 mg. glucose.

5 ml. fructose solution (37.0 mg./l.) required 1.23 ml. thio.
The fructose from the paper required 6.23 ml. thiosulphate.

1.23 ml. ≡ 0.185 mg. fructose

∴ 6.23 ml. ≡ 0.937 mg. fructose.

Estimation of glucose and fructose using ribose as reference sugar:-

0.744 mg. ribose corresponds to the 7.99 mg. weighed in.

∴ 0.0837 mg. glucose corresponds to $\frac{7.99}{0.744} \times 0.0837$ mg.

$= 0.9$ mg. glucose.

and 0.937 mg. fructose corresponds to $\frac{7.99}{0.744} \times 0.937$ mg.

$= 10.06$ mg. fructose.

14.32 mg. inulin gives $\frac{180}{162} \times 14.32$ mg.

$= 15.92$ mg. hexose.

∴ The percentage of the constituent sugars in the sample taken is :-

Glucose: $\frac{0.9}{15.92} \times 100 = 5.6\%$

Fructose: $\frac{10.06}{15.92} \times 100 = 63.2\%$

A similar experiment gave values of Glucose = 4.7%

Fructose = 83.5%

The course of the hydrolysis of inulin by sulphuric acid ($\frac{n}{60}$) was observed polarimetrically. It was found that equilibrium rotation ($[\alpha]_D^{18} = -81^\circ$) was reached in 30 minutes. This experiment was repeated using aqueous oxalic acid (2.25%), and a similar result was obtained.

The quantitative estimations were repeated using this strength of aqueous oxalic acid, and the following results were obtained:

Glucose.	6.8%	7.0%	4.4%	
Fructose	87.0%	94.5%	93.4%	94.5%

The Oxidation of Inulin by the Periodate Ion

a) The Estimation of the Liberated Formic Acid.

In order to obtain an estimate of the chain length of the inulin molecule the polysaccharide was

subjected to oxidation by potassium periodate using the method of Halsall, Hirst and Jones (5). To dried inulin (approx. 500 mg.) was added potassium chloride (1g.) and sodium periodate solution (10 ml.; $\frac{M}{4}$), the volume was made up to 50 ml. The whole was shaken for 8 days and at the end of this time samples were taken out at intervals, centrifuged, 5 ml. portions taken, 0.3 ml. ethylene glycol added to destroy excess periodate, and the formic acid titrated against standard sodium hydroxide (approx. $\frac{n}{200}$), using methyl red indicator. A blank experiment was run concurrently, omitting only the polysaccharide. The oxidation was carried out in the dark and at room temperature.

Sodium hydroxide = 0.010n.

Experiment 1) 320 mg. inulin

Duration of Oxidation	Alkali Titre.	No. of Residues / mole. of Formic
138 hours	0.319 ml.	62
163 "	0.367 ml.	53.7
213 "	0.404 ml.	49
235 "	0.406 ml.	48.6
305 "	0.415 ml.	47.9

Experiment 2) 210 mg. inulin

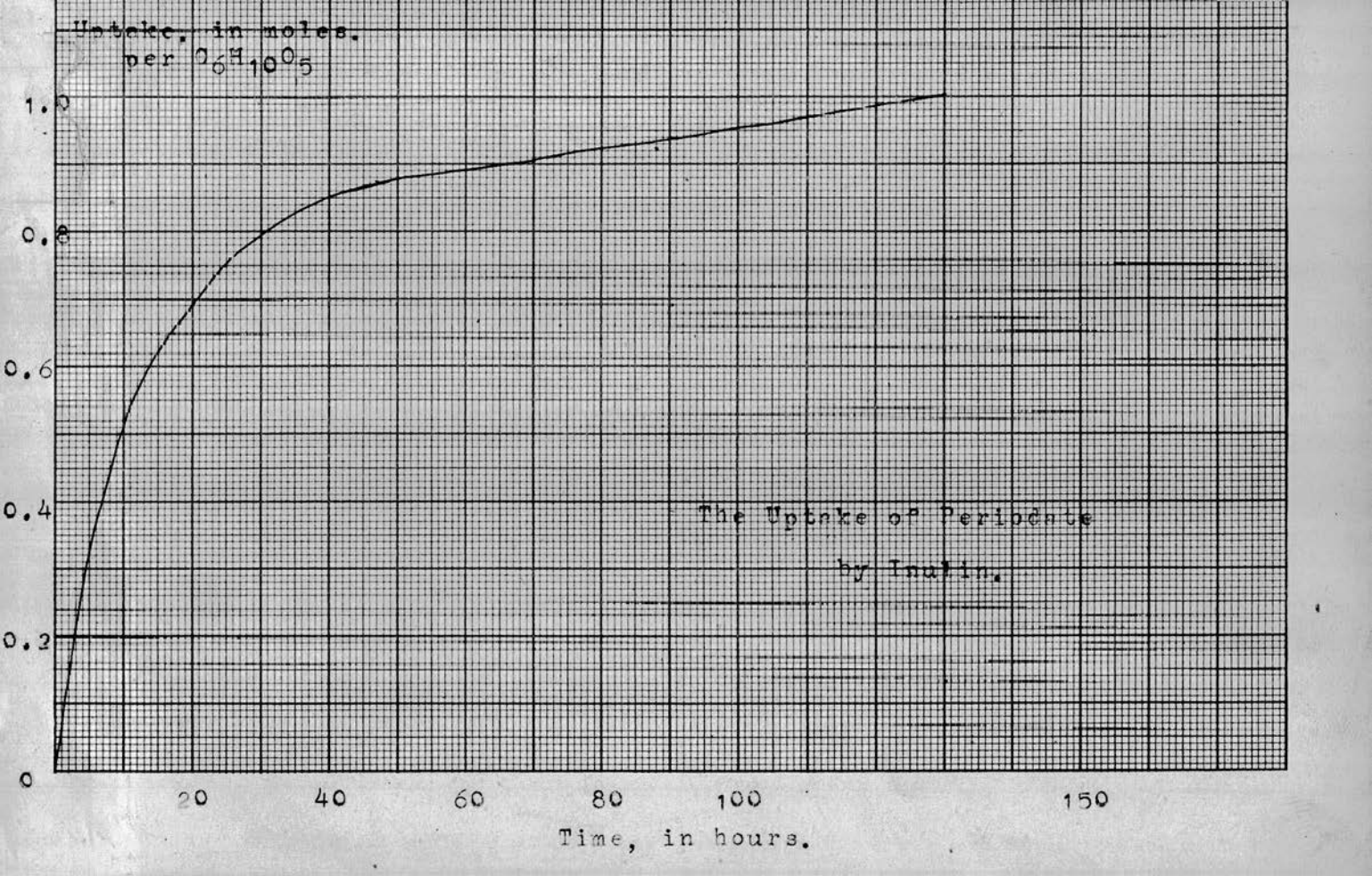
192 hours	0.220 ml.	57
240 "	0.265 ml.	48.8
336 "	0.272 ml.	47.6

b) The Estimation of the Uptake of Periodate.

In the determination of the periodate uptake of inulin dried polysaccharide (approx. 50 mg.) were oxidised with sodium periodate (10 ml.; $\frac{M}{8}$), The samples were

allowed to react in the dark for varying lengths of time. The excess periodate was estimated by the addition of solid potassium iodide and titration of the liberated iodine against sodium arsenite ($\frac{n}{10}$)

Weight of Inulin taken	Duration of oxidation	Uptake in moles/$C_6H_{10}O_5$
59.8 mg.	18 hours	0.665
49.6 mg.	47 "	0.874
47.0 mg.	88 "	0.925
48.4 mg.	130 "	1.03


Uptake, in moles.
per $C_6H_{10}O_5$
1.0
0.8
0.6
0.4
0.2
0
20
40
60
80
100
150
Time, in hours.
The Uptake of Pericarp
by Inulin.

DISCUSSION

One of the outstanding recent advances in chemical technique is the development of filter paper partition chromatography by Consden, Gordon and Martin (13) for the analysis of mixtures of amino acids. The method has been applied to the analysis of sugar mixtures by Partridge (1), who found that the different sugars travelled down the paper chromatogram at different rates, so that the unknown components of a mixture could be identified by comparison of the distance they had moved with the distance travelled by known compounds. Flood, Hirst and Jones (2) have extended the use of the method by making possible the quantitative analysis of a sugar mixture after the various components have been separated on the paper chromatogram. The method has now been further developed by Brown, Hirst Hough, Jones and Wadman (14) to cover the separation of such mixtures of methylated sugars as are obtained by the hydrolysis of methylated polysaccharides.

Thus a mixture of sugars as is present, for instance, in an acid hydrolysate of a polysaccharide can be both qualitatively and quantitatively analysed using the paper chromatogram. Only very small amounts of the polysaccharide are necessary, and a complete analysis can be performed in far less time than was possible by earlier methods.

Inulin has been extracted from Dahlia tubers under mild conditions, by an established method (6), so as to reduce to a minimum the risk of working with a partially degraded compound. The inulin was obtained in good yield, and was purified by repeated

"recrystallisayions" from water, the rotation being taken as the criterion of purity. As the purification progressed the rotation became increasingly negative but remained constant at -40.0°. This corresponds with the most negative value given in the literature (7), and thus this compound would appear to be the same as that obtained by Berner. By use of the paper chromatogram glucose was shown to be present in an acid hydrolysate of this sample, and it is therefore considered probable that the glucose is a constituent of the molecule and not present as an impurity.

Inulin has been hydrolysed with $\frac{n}{60}$ sulphuric acid, and the course of the hydrolysis followed polarimetrically. The polysaccharide was hydrolysed completely in about 30 minutes. The experiment was repeated using 2.25% aqueous oxalic acid and a similar result obtained.

On estimating quantitatively the glucose and fructose present in inulin, the following values were obtained:

	Glucose	Fructose	Conditions
Expt. I	4.7%	95.5%	$\frac{n}{60}$ sulphuric acid 100° C, 2hours.
Expt. II	5.6%	65.2%	As above
Expt. III	6.8%	87.0%	2.25% oxalic acid
		94.5%	100° C., 2 hours.
Expt. IV	7.0%	93.4%	As above.
	4.4%	94.5%	

From these results it will be seen that the percentage of glucose does not vary outside the experimental error of the method (2) when oxalic acid

is used for the hydrolysis instead of sulphuric acid, but it is apparent that the fructose is being destroyed by the mineral acid. Considering the percentage of glucose to remain constant irrespective of the hydrolytic conditions, and that the total sugars estimated amount to 100% of the inulin hydrolysed by the oxalic acid, it may be concluded that the glucose present is preformed in inulin, and not formed secondarily from fructose during the hydrolysis. An average value for the amount of glucose present in inulin is 5.7%. This value is in close agreement with the results of other workers who have estimated the glucose content of inulin (10) (11).

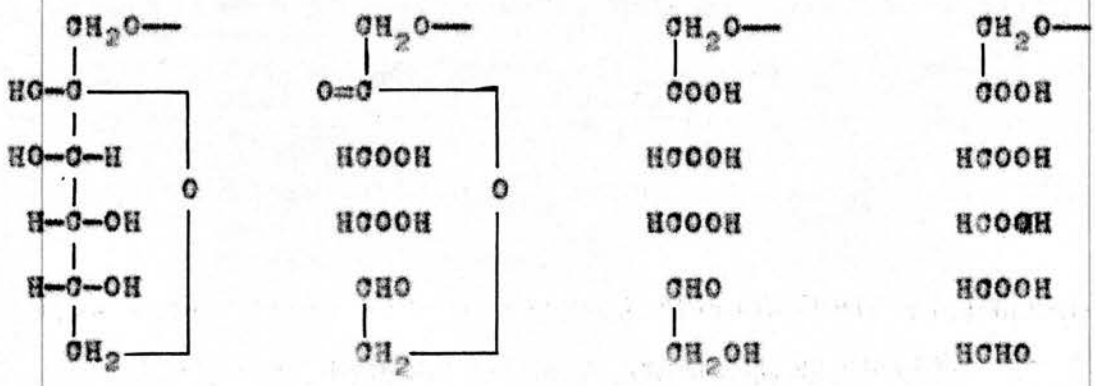
Inulin was subjected to oxidation by the periodate ion; in the first instance the release of formic acid was investigated, and in the second the uptake of periodate was determined. Periodic acid oxidation, first introduced by Malaprade (8), finds many applications in the field of sugar chemistry because of the highly selective nature of the reaction. It is applicable only to compounds having two or more hydroxyl groups or hydroxyl and amino groups (9) attached to adjacent carbon atoms, the C—C bond between these atoms being broken in the reaction. One molecule of periodate is consumed for each C—C bond split, and formic acid is liberated if more than two adjacent hydroxyl groups are present. If the substance examined is not cleaved by periodate it is evident that no adjacent hydroxyl groups are present.

If the terminal residues of a polysaccharide are such that they contain three adjacent hydroxyl

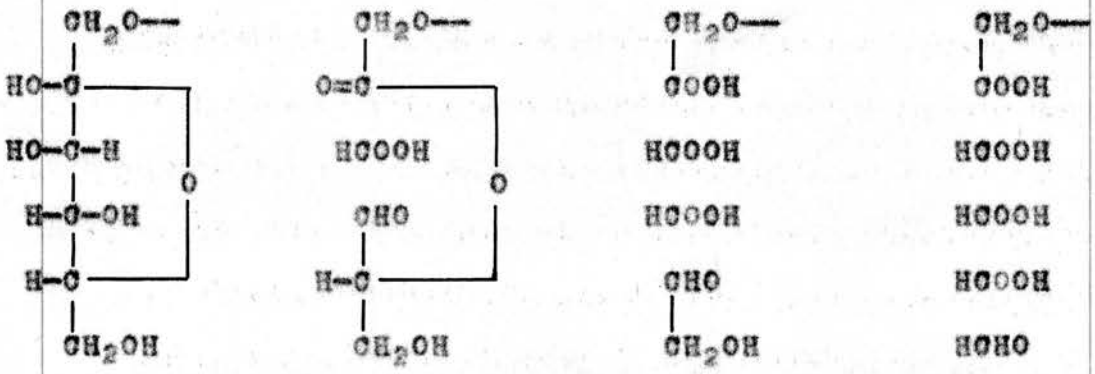
groups, then periodate oxidation affords a possible method of estimating their number, provided that the non-terminal residues are such that they do not yield formic acid. This method should be applicable to any polysaccharide consisting of chains of 1:4- linked hexopyranose residues. Earlier attempts to make use of this procedure encountered difficulty in that the oxidation is not arrested at the stage when 1 mole. of formic acid has been liberated from the terminal group. In order to prevent this over oxidation, Halsall, Hirst and Jones (8) used potassium periodate, which is only slightly soluble in water. Using this salt, and keeping the concentration of the formic acid produced to a low value (ca. 10 mg./100 ml.) they were able to obtain consistant and reliable figures for the amount of formic acid liberated.

If the inulin molecule is a straight chain linked through the 1:2- positions, as was shown by Haworth and Learner (12), each residue, apart from the reducing end group, will take up one mole. of periodate to give a polymeric aldehyde. The graph of the uptake of periodate against time for the inulin molecule shows that there is a falling off in the rate of uptake when a value of 1 mole./ residue is reached. Thus the value expected for a 1:2- linked polyfructosan is obtained in practice.

In the reducing end group of inulin position C_6 is not blocked, so that, in all probability, this residue exists in the pyranose form. On oxidation by the periodate ion each reducing group will give rise to 3 moles. of formic acid.



If the end reducing group, like the remainder of the molecule, exists in the furanose form, the reaction with periodate is somewhat different from the above, but the end products are the same.



Experimentally it was found that 1 mole. of formic acid was liberated for every 48 anhydrofructose residues, but as three molecules are released from each reducing end group, the value for the chain length of the inulin used becomes 144 assuming that the glucose present is part of the polysaccharide molecule, and not present as an associated polysaccharide which cannot be removed by the purification process used.

SUMMARY

1) Inulin has been extracted from Dahlia tubers and purified to constant rotation, $[\alpha]_D$ -40.0°, which is in very close agreement with the most negative value given in the literature, -40.2°, (7).

2) The percentage composition of this inulin has been determined after hydrolysis with aqueous oxalic acid (2.25%), :- Glucose, 5.7%, Fructose, 94.3%.

3) This pure polysaccharide has been oxidised with potassium periodate, and the release of formic acid estimated. The value for the chain length determined by this method is 14 anhydrofructofuranose residues.

4) The uptake of periodate is consistant with the view that inulin consists of a chain of anhydrofructofuranose residues linked through the 1:2- positions.

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EXPERIMENTAL

The Methylation of Inulin.

The method is a modification of that used by Haworth and Learner (1). Pure inulin (20 g. $[\alpha]_D^{15}$ -39.9°) was dissolved in sodium hydroxide solution (240 ml.; 35%) and the flask suspended in a water bath, the temperature of which was kept constant at 35° C throughout the experiment. The solution was stirred mechanically, and dimethyl sulphate (120 ml.) was added as evenly as possible over two days. A total of three such treatments were given.

After the third methylation the temperature of the water bath was brought up to the boiling point and maintained there for 30 minutes, then cooled to 10° while the alkali was neutralised by the addition of sulphuric acid (25%). Half its volume of ethanol was added to the mixture and the precipitated sodium sulphate filtered and washed with chloroform (500 ml.). The aqueous ethanol filtrate was concentrated under diminished pressure to a syrup, which soon solidified. This solid was extracted with chloroform (200 ml.), and the extract, together with the above washings, was distilled under reduced pressure, to leave a friable, pale yellow solid, (16.3 g., $[\alpha]_D^{18}$ -48.4° c = 1.00 in $CHCl_3$).

The Purification of the Crude Methylated Inulin.

The material obtained from the above process was contaminated with sodium methyl sulphate, which was removed by washing with successive quantities of hot water. After three washings the methylated inulin was dried when it had $[\alpha]_D^{18}$ -52.5° and OMe = 43.0%.

This product was subjected to a further methylation

in acetone solution, using the method of Haworth and Streight (2). The temperature was maintained at 50° while sodium hydroxide solution (400 ml.; 33%) and dimethyl sulphate (150 ml.) were added during 9 hours. At the end of the methylation, water (150 ml.) was added, and the acetone distilled. The methylated inulin, which separated from the solution as pellets, was filtered and washed with water, firstly cold, then hot, when the product (13.5 g.) was colourless and the washings free from sulphate.

The Fractionation of the Methylated Inulin.

The methylated inulin (13.5 g.) was dissolved in chloroform (40 ml.) and fractionally precipitated by the addition of light petroleum (40–60°) in 100 ml. portions, the solution being stirred vigorously meanwhile.

Fraction I	400 ml. petroleum	5.1 g.
Fraction II	800 ml. "	7.2 g.
Fraction III	900 ml. "	0.4 g.
Residue after distillation of the solvent		0.5 g.
Total recovery		13.2 g.

The first three fractions were fine white, almost crystalline powders. Their constants were as follows:

Fraction I	$[\alpha]_D^{17}$ −55.1° (c = 1.0 in $CHCl_3$)	OMe = 42.0%
Fraction II	$[\alpha]_D^{17}$ −54.2° (" ")	OMe = 43.6%
Fraction III	$[\alpha]_D^{17}$ −54.0° (" ")	OMe = 43.4%

The rotation of trimethyl inulin in chloroform is given as −54.0° (2)(3).

The Remethylation by Purdie's Method.

The above three fractions were further methylated in 4 g. portions using methyl iodide (70 ml.) and

silver oxide (50 g.) added in 5 g. quantities to the gently refluxing methyl iodide solution every 30 minutes. The product was recovered by extraction of the solids with chloroform, which was partially removed under diminished pressure, and the methylated inulin fractionated by the gradual addition of light petroleum. The results of a typical experiment are given.

Fraction I	1.2 g.	OMe = 44.2%
Fraction II	2.5 g.	OMe = 43.2%
Residue	0.4 g.	

A total of 12 g. of trimethyl inulin was prepared by this method.

The methylation of a further quantity of Inulin.

The method employed was essentially the same as previously except that the treatments with dimethyl sulphate and sodium hydroxide were carried out in an atmosphere of nitrogen. Pure inulin (20 g.; $[\alpha]_D$ -40.0°) was dissolved in sodium hydroxide (280 ml.; 35%) and dimethyl sulphate (120 ml.) added dropwise in 25 ml. portions every 30 minutes. The mixture was maintained in a constant state of agitation. The reaction flask was surrounded by a water bath at 35° . After the addition of all the dimethyl sulphate the reaction mixture was stirred overnight. A further quantity of sodium hydroxide (200 ml.; 35%) was then added followed by dimethyl sulphate (120 ml.) in 25 ml. portions as previously, the addition extending over one day. The solution was neutralised with sulphuric acid (25%), and half its volume of ethanol added. The precipitated sodium sulphate was filtered and washed with chloroform and the washings dried over anhydrous sodium sulphate.

The aqueous ethanol filtrate was taken down to dryness under diminished pressure and the residual solid extracted with chloroform. The two chloroform extracts were combined and distilled to leave a pale yellow solid (17 g.)

This was dissolved in boiling acetone (500 ml.) sodium hydroxide (250 ml.; 30%) added followed by three 25 ml. portions of dimethyl sulphate. The temperature of the surrounding water bath was maintained at 55–60° throughout. At the end of the first treatment, sodium hydroxide (150 ml.; 30%) and two 25 ml. portions of dimethyl sulphate were added dropwise. Five hours after the termination of the addition of the dimethyl sulphate the acetone was distilled, and the pellets of partially methylated inulin filtered and washed with water. They were again dissolved in boiling acetone (400 ml.) and treated as previously with sodium hydroxide (150 ml.; 30%) and dimethyl sulphate (50 ml.). Five hours after the termination of this methylation, further similar quantities of these reagents were added and the product recovered as before. After washing with hot water, the pellets were extracted with chloroform, the extract dried over anhydrous sodium sulphate and distilled under reduced pressure to leave a white solid (18 g.)

This methylated inulin was refluxed with methyl iodide (125 ml.) and silver oxide (100 g.) added in portions over 5 days. The product was recovered by extraction in chloroform, the solution concentrated, and the methylated inulin fractionally precipitated by the addition of light petroleum (40–60°).

Fraction I 1l. petroleum 4.2 g. OMe = 44.9%

Fraction II 2l. petroleum 7.4 g. OMe = 45.5%

The Hydrolysis of Trimethyl Inulin.

The method used was that described by Haworth, Hirst and Percival (5). Trimethyl inulin (7.45 g. OMe = 44.9%, methylated by the first series of reactions) was treated with methanol (225 ml.), water (75 ml.) and crystalline oxalic acid (3.0g.) at 80° for 18 hours. The acid was neutralised with calcium carbonate and the filtered solution taken to dryness at 40°/15 m.m. The residue was extracted with chloroform in the presence of anhydrous sodium sulphate (50 ml.; 4 times) and the extracts taken down under reduced pressure to a brown syrup (7.99 g.)

This syrup was dissolved in methalolic hydrogen chloride (125 ml.; 0.25%) and allowed to stand at 20° for 73 hours. The acid was neutralised with barium carbonate and the unfiltered solution taken down to a syrup which was extracted with chloroform. The filtered extracts were combined and evaporated, in the presence of a little barium carbonate, to a pale brown syrup (8.06 g.) which was non-reducing.

The Fractionation of the Methylfructosides.

The methylfructosides obtained as described above were fractionated by solvent extraction in all-glass apparatus as described by Brown and Jones (4). The solvent used was contained in a flask above which were mounted two extractors and a reflux condenser. The methylfructosides (8.06 g.) were dissolved in water (50 ml.) and this solution placed in the upper of the two extractors; the lower extractor contained

water. A little solid barium carbonate was introduced into each extractor to prevent the development of any local acidity.

In an attempt to isolate the tetramethyl methylfructofuranoside the extraction was first performed using light petroleum (300 ml.; 38–40°) as solvent, and continuing the extraction for varying lengths of time as shown in the table below. A sample of each of the fractions so obtained was hydrolysed with aqueous oxalic acid (0.4 ml.; 2.25%) at 80° for two hours, and the neutralised hydrolysates examined qualitatively on the paper chromatogram (5).

Fraction	Duration of extraction	Weight	η_D^{17}	R_G Values
A	3 hours	603 mg.	1.4558	0.88 1.00
B	8 "	364 mg.	1.4562	As for "A"
C	13 "	574 mg.	1.4580	As for "A"

The aqueous solution was now removed from the upper extractor and concentrated at 40°/15mm.. This was extracted similarly with chloroform (300 ml.) and the fractions hydrolysed and examined on the paper chromatogram as before.

Fraction	Duration of extraction	Weight	η_D^{17}	R_G Values
D	2 hours	5.408 g.	1.4580	0.875 1.00
E	7 "	0.786 g.	1.4573	As for "D"

A total of 7.718 g. syrup was thus obtained by these solvent extractions. No more sugar was recovered on extracting for a further 9 hours with chlorofrom. The aqueous solution was evaporated to a smaller volume,

made 2.25% with respect to oxalic acid and hydrolysed at 80° for 2 hours. No sugars were detected on a paper chromatogram of the neutralised hydrolysate. The total recovery in this fractionation was 96.5%.

The Preparation of Tetramethyl methylfructofuranoside.

The method used was that developed by Menzies (6).

a) Preparation of Methylfructofuranoside.

Dry commercial fructose (7.0 g.) was dissolved in hot dry methanol (300 ml.) and methanolic hydrogen chloride (50 ml.; 3.5%) added. The mixture was allowed to stand until the rotation became constant ($[\alpha]_D^{16}$ +6.0°) after 40 minutes. This is the maximum value noted by Menzies. The solution was neutralised by the careful addition of sodium methoxide, and then evaporated to a very viscous syrup. This syrup was extracted with hot dry ethyl acetate (5 times; 20 ml.). On evaporation a colourless syrup remained (2.7 g. $[\alpha]_D^{16}$ +34.0° c = 1.3 in water)

b) Methylation of the Methylfructofuranoside.

The syrup obtained as above was dissolved in methyl iodide (30 ml.) containing methanol (5 ml.) and was methylated by the addition of silver oxide (20 g.) added over 8 hours. The mixture was refluxed overnight and the product recovered by extraction with hot methanol. Three further methylations were given using pure methyl iodide (25 ml.) as solvent and 20 g. portions of silver oxide. The product was finally recovered by chloroform extraction of the silver oxide, when a colourless mobile syrup was obtained (3.06 g.).

This syrup was distilled at 0.05 mm. when two fractions were obtained.

Fraction I 83–84° (bath temperature) 2.17 g. η_D^{18} 1.4437

Fraction II 90° " " 0.51 g. η_D^{18} 1.4444

A small sample of each fraction was hydrolysed at 70° for two hours with hydrochloric acid ($\frac{n}{10}$), neutralised with silver carbonate and the hydrolysate examined on the paper chromatogram, using ammoniacal silver nitrate for the detection of the sugars on the paper. It was found that 1:3:4:6–tetramethyl fructofuranose had exactly the same R_G value as 2:3:4:6–tetramethyl glucopyranose. The tetramethyl fructofuranose is only slightly reducing to ammoniacal silver nitrate. Both Fractions I and II appear to be pure tetramethyl sugar.

The Methylation of Sucrose.

The method is based on that used by Haworth (7). Sucrose (25 g.) was dissolved in the minimum of water and sodium hydroxide solution (350 ml.; 30%) added. The mixture was vigourously stirred and dimethyl sulphate (100 ml.) added dropwise during seven hours. The temperature of the surrounding water bath was maintained at 45° throughout. After stirring overnight a second treatment was given using the same quantities of reagents. The partially methylated sucrose was obtained by the extraction of the alkaline solution after the second methylation with chloroform. The extracts were combined and dried over anhydrous sodium sulphate. On distillation of the solvent at 40°/15 mm. a very viscous syrup remained (9.0 g. η_D^{18} 1.4722).

This syrup was taken into methyl iodide (40 ml.) and methylated by the addition of silver oxide (30 g.) over five days. The product was recovered by extraction with chloroform, which on distillation gave a syrup

(8.8 g. η_D^{16} 1.4619). This was treated as before with Purdie's reagents, and an amber coloured syrup (8.26 g. η_D^{17} 1.4590) obtained from chloroform. The syrup was subjected to distillation in a high vacuum (0.05 mm.) when the following fractions were obtained.

Fraction I	200° (bath temperature)	0.48 g.	η_D^{18} 1.4562
Fraction II	200–205° "	6.89 g.	η_D^{18} 1.4584
Residue		0.7 g.	η_D^{18} 1.4610

The Hydrolysis of Octamethyl Sucrose.

A sample of fraction II was treated with aqueous hydrochloric acid ($\frac{n}{10}$) at 60° for 8 hours (8). The neutralised hydrolysate was examined on the paper chromatogram when only one spot due to the presence of reducing sugars was observed. This spot had exactly the same R_G value as 2:3:4:6-tetramethyl glucose, thus no separation of these two fully methylated sugars is possible by this method.

The Use of Urea Oxalate and Aniline Oxalate to Distinguish between Methylated Derivatives of Glucose and Fructose on the Developed Paper Chromatogram.

a) Preparation and Use of Urea Oxalate.

Urea (crystals) were added to a saturated solution of oxalic acid in ethanol at 40° until the approximate neutral point was reached. The white insoluble salt was filtered and washed with a little ethanol then dried in a vacuum desiccator over phosphorus pentoxide. When a paper chromatogram containing samples of octamethyl sucrose hydrolysate, synthesised tetramethyl fructofuranose and pure tetramethyl glucose was sprayed with a saturated aqueous solution of urea oxalate and heated at 105° for 30 minutes, the positions

of the octamethyl sucrose hydrolysate and tetramethyl fructofuranose were revealed as grey-green spots against a white background of the paper. The tetramethyl glucose control gave no colour reaction with this reagent.

b)Preparation and Use of Aniline Oxalate.

The preparation of aniline oxalate is exactly analogous to that of urea oxalate. When a duplicate paper chromatogram to the above was sprayed with a saturated aqueous solution of this reagent, and heated at 105° for 30 minutes, the spots of octamethyl sucrose hydrolysate and tetramethyl glucose gave a red colour reaction but no colour reaction was obtained with the tetramethyl fructofuranose.

The Re-examination of the Fractions of Methylfructosides Obtained from Hydrolysed Trimethyl Inulin.

Samples of each of the five fractions were hydrolysed as before and separated on duplicate paper chromatograms, using both tetramethyl fructofuranose and tetramethyl glucose as standards for the calculation of the R_G values. That paper containing the tetramethyl fructose control was sprayed with urea oxalate and the other, with aniline oxalate. The following results were obtained.

Fraction	Constituent Sugars:	R_G values.
A	Tetramethyl fructose:	1.00
	Trimethyl fructose	0.88
	Trimethyl glucose	0.88
B	As for "A"	
C	As for "A"	
D	Tetramethyl fructose	1.00 (trace)
	Trimethyl fructose	0.88
	Tetramethyl glucose	1.00
	Trimethyl glucose	0.88
E	As for "D"	
	and Dimethyl fructose	0.635 (trace)

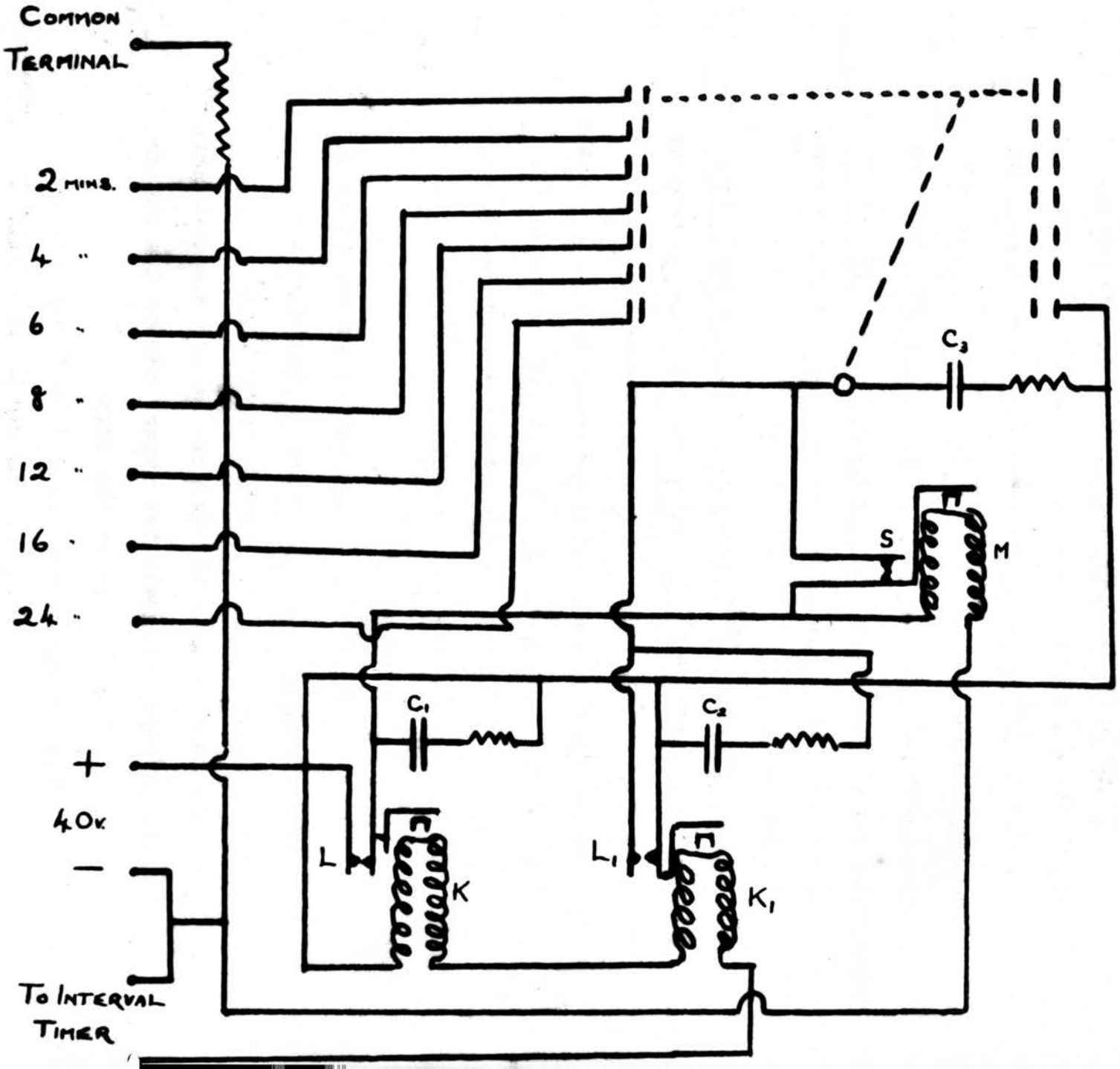
Fractions A, B and C appearing identical they were combined to give Fraction I, as were Fractions D and E, to give Fraction II.

The Separation of the Constituents of Fractions I and II on a Column of Powdered Cellulose.

The experimental procedure is briefly described by Hough, Jones and Wadman (10). The column consists of a glass tube 50 cm. × 3.5 cm. the bottom of which is drawn out into a dropping tube. A porcelain filter disc is lodged where the main tube narrows and on this is placed a thin layer of cotton wool. Upon this is packed powdered cellulose, about an inch at a time, so that the even packing which is essential for the efficient working of the column, is obtained. In this way about 40 cm. of the glass tube is packed with the cellulose powder. Finally a thin layer of cotton wool is placed on the top of the column.

Soluble impurities are removed from the cellulose by washing the column with n-butanol saturated with water, the solvent being contained in a constant head apparatus lodged in the top of the column. After thoroughly washing with this solvent, the washing is continued with the solvent to be used in the separation. In the present work a mixture of light petroleum (100–120°) 70%, n-butanol 30%, saturated with water, was the solvent used to develop the column. The petroleum was purified before use by shaking overnight with concentrated sulphuric acid, followed by washing with alkali and finally distilling.

The column was clamped vertically over a large aluminium plate around the circumference of which were


COMMON TERMINAL
2 MINS.
4 "
6 "
8 "
12 "
16 "
24 "
+
40V.
—
TO INTERVAL TIMER
C1
C2
C3
L
K
L1
K1
S
M

holes to take small test-tubes (3×½"). The eluate was collected in a test-tube for a pre-determined length of time, then the plate was automatically moved and the next receiver in the row came under the dropping tube of the column. This was in position for the same length of time as the previous one before it, in it's turn, was moved on.

The interval timer consists of a synchronous electric clock motor, so geared that the ultimate spindle speed is one revolution per four minutes. On this spindle two cams are mounted, one of which can make contact with the leaf switch every four minutes and the other, every two minutes. The leaf switch is mounted in such a way that it can be made to operate on either cam.

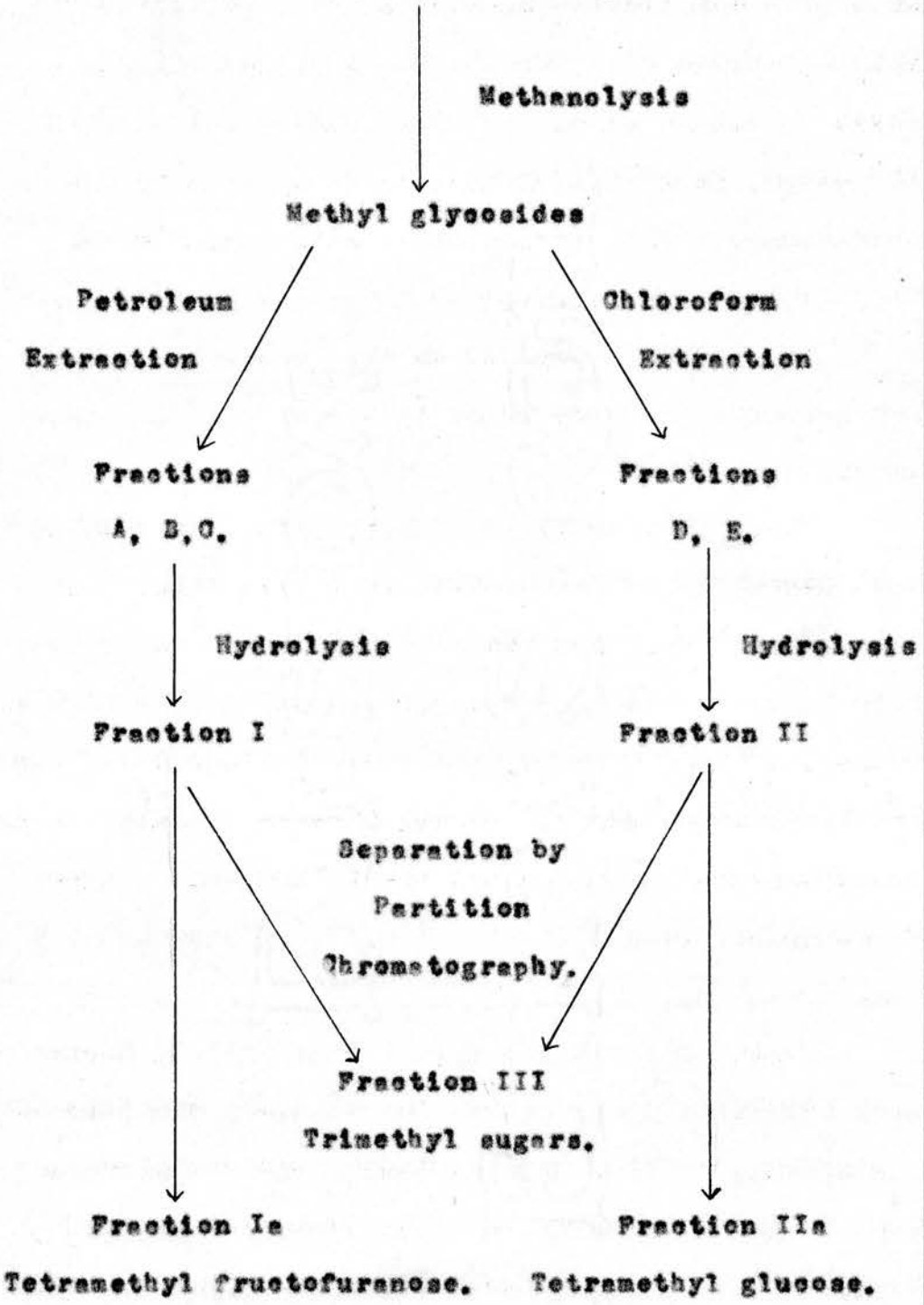
K is a relay of 600 ohms resistance operating a leaf switch L. K_1 is a relay of 200 ohms resistance operating a leaf switch L_1. M is an electromagnet which operates the selector switch by pawl and ratchet mechanism. A spring switch S is incorporated in the electromagnet. The function of the condensers C_1, C_2 and C_3 in conjuction with suitable resistances, is to obviate sparking.

The closing of the leaf switch on the interval timer results in the completion of the circuits through L and L_1 respectively. The closing of the circuit at L carries the current to the electromagnet thus operating the selector switch moving the wipers from one set of knives to the next.

The closing of the circuit at L_1 carries the current through the wipers to the sets of knives of

Scheme for the Examination of:

Trimethyl Inulin (Sample 1)



the selector switch, thence to the table mechanism which consists on an electromagnet operating by means of a pawl and ratchet mechanism. This ratchet is fitted with a spindle carrying the 28" diameter aluminium plate, which is bored with four concentric sets of 104 holes. Each time an impulse is applied to the electromagnet the ratchet wheel moves through one cog, thus changing the position of the plate by one hole.

Impulses are applied to this electromagnet at any selected times as shown by the various tappings on the diagram.

Since continuity of operation depends upon an even number of sets of knives it is essential that the wipers must move from the 24th set to the 1st automatically. This is done by taking a positive lead to the 25th set of knives and continuing the circuit through the wipers to the spring switch S, thence through the coil of the electromagnet. The movement of the armature of the electromagnet automatically breaks the circuit at S thus moving the wipers.

Fraction I was hydrolysed with aqueous oxalic acid (120 ml.; 4.0%) at 80° for 3 hours. The acid was neutralised with calcium carbonate and the mixture heated at 90° for 30 minutes to decompose any calcium bicarbonate which may have been formed. The filtered solution was evaporated to a syrup and dried with ethanol and benzene (1.192 g.)

This syrup was dissolved in the minimum of solvent (petroleum ether 100–120°, 70%; n-butanol, 30%; saturated with water.) and the solution introduced on to the top of the column with a pipette, allowing

each drop to soak in before adding the next. After replacing the cotton wool, the column was developed using 200 ml. of solvent which was collected in a receiver at the bottom. The column was then clamped over the aluminium plate and 400 ml. solvent allowed to run through, samples being collected at 6 minute intervals.

When the plate had moved through a circumference, the contents of each tenth tube were concentrated on a watch glass placed on a boiling water bath, and the concentrate analysed on the paper chromatogram. The tetramethyl fructofuranose was found in tubes 50–55, and 65 onwards contained trimethyl fructofuranose. There being now no sugar other than trimethyl fructose present on the column, the latter was washed with water to obtain the remainder of the trimethyl fraction.

The tetramethyl fructofuranose was obtained as a brown syrup (Fraction Ia; 196.3 mg.) on evaporation of the solutions present in tubes 50–55. The trimethyl fructofuranose solutions were also concentrated (40°/15 mm.) and the concentrate added to that obtained in the next separation.

Fraction II was hydrolysed with aqueous oxalic acid (300 ml.; 4.0%) at 80° for 3 hours, and the syrup of free sugars obtained as before (3.358 g.). This syrup was separated on the cellulose column, the procedure being exactly as described previously.

The tetramethyl hexose portion of this syrup was obtained on evaporation, under reduced pressure, of the contents of tubes 45–55. A brown syrup remained (Fraction IIa; 161.2 mg.) which did not crystallise on standing. The trimethyl fructose extended from tube 56 onwards, the

column being finally washed out with water as before. Evaporation of the solvent and combination of the trimethyl fractions from the two separation experiments gave a brown syrup (Fraction III; 8.789 g.).

The recovery from these two experiments is 91%.

The Examination of these three Fractions.

a) Fraction Ia

By examination of this syrup on the paper chromatogram it was shown to consist of a chromatographically pure sample of tetramethyl fructofuranose.

$[\alpha]_D^{15} = +39.9^\circ$ (c = 1.25 in water). OMe = 40.8%.

Oxidation by Alkaline Hypoiodite.

Ia (62.8 mg.) was dissolved in water (5ml.) to give an opalescent solution. The greasy impurity was removed by the addition of a little "Filter Cel", when on filtration a clear solution resulted.

Approximately 500 mg. of this solution were weighed into boiling tubes fitted with B 24 joints, pH 11.4 buffer (5.0ml.) was added followed by iodine solution (0.965 $\frac{n}{10}$; 2.5ml.). The stoppers were sealed in with a drop of 10% aqueous potassium iodide solution and the tubes allowed to stand at room temperature for 4 hours. Sulphuric acid (2.5 ml.; 4 n) was added and the iodine titrated with sodium thiosulphate (0.0196 n) Tubes containing water (500 mg.) were treated in an identical manner.

The following volumes of thiosulphate were required for the iodine present.

Water blank 1	12.402 ml.	Sugar solution 1	12.390 ml.
Water blank 2	12.390 ml.	Sugar solution 2	12.385 ml.

Thus, with respect to sugars, Fraction Ia is a

pure sample of tetramethyl fructofuranose.

With an methoxyl content of 40.5% the true weight of sugar is:

$$193.6 \times \frac{40.3}{52.5}$$

$$= 147.4 \text{ mg.}$$

The Preparation of tetramethyl fructofuronamide.

This transformation was carried out by the method of Avery, Haworth and Hirst (11). The aqueous solution of tetramethyl fructofuranose used for the hypoiodite oxidation was evaporated to a syrup at 40°/15 mm. and dried with ethanol and benzene. This was combined with the bulk of Fraction Ia and the total (170 mg.) was oxidised with concentrated nitric acid (2.5 ml.; d=1.42) on a water bath, the temperature of which was slowly raised. The reaction commenced at a bath temperature of 60°, and the heating was continued upto the boiling point where it was maintained for 2½ hours. The cooled solution was diluted with water, and distilled at 40°/15 mm. to remove the acid. This process was repeated in all 10 times before substituting methanol for the water, and finally anhydrous methanol was used.

The resulting syrup was dissolved in methanol containing 4% hydrochloric acid, and gently refluxed overnight; neutralised with silver carbonate and the filtered solution evaporated to a syrup which was methylated by treatment with Purdie's reagents (methyl iodide 15 ml.; silver oxide 5 g.) for 8 hours. The material was recovered by chloroform extraction and after evaporation of the solvent the syrup was treated

with methanol saturated with ammonia (10 ml.) and the solution allowed to stand at 0° for 3 days. Removal of the methanol left a crystalline solid (140 mg.)

After two recrystallisations from light petroleum (30–40°) m.p. 98–99°.

Mixed melting point with an authentic specimen of tetramethyl fructofuranamide: 99°.

Found: C, 45.9; H, 7.6%

Calc. for $C_{10}H_{19}O_6N$, C, 45.3; H, 7.16%

b) Fraction IIa

This remained as a syrup, and on examination on the paper chromatogram was shown to be a mixture of tetramethyl glucose and tetramethyl fructofuranose.

$[\alpha]_D^{15}$ = +52.0° (c=1.11 in water) OMe = 40.0%.

Oxidation by Alkaline Hypoiodite.

The experimental procedure was exactly as described for Fraction Ia.

Volumes of thiosulphate (0.0196 n) required for the iodine present:

Water blank 1) 12.402 ml. Sugar solution 1) 11.355 ml.

" " 2) 12.390 ml. " " 2) 11.360 ml.

The volume of iodine consumed by the sugar is equivalent to (12.39 − 11.36) ml.

= 0.93 ml. 0.0196 n thiosulphate.

Now 2000 ml. n thiosulphate ≡ 236 g. tetramethyl sugar and 0.93 ml. 0.0196 n thio. ≡ 0.0163 ml. n thio.

∴ 0.0163 ml. $= \frac{236}{2000} \times 0.0163$ g. sugar

= 1.92 mg.

5 ml. solution contain 0.0358 g. of Fraction IIa and 0.3236 g. of this solution was used for this analysis.

0.3236 g. contain $\frac{0.0358}{5} \times 0.3236$ g.

= 5.84 mg. material

This contains $5.84 \times \frac{40}{52.5}$ mg. sugars

= 4.45 mg.

Whence the percentage of tetramethyl glucose is:

$$\frac{1.92}{4.45} \times 100$$

= 43.1%

The total weight of Fraction IIa is 161.2 mg.

This contains $161.2 \times \frac{40}{52.5}$ mg. sugars

= 123 mg. tetramethyl sugars

43.1% of the this is tetramethyl glucose, whence the composition of the Fraction is:

Tetramethyl glucose	53.0 mg.
Tetramethyl fructofuranose	70.0 mg.

Therefore the total weight of tetramethyl fructofuranose isolated from this sample of Trimethyl inulin is:

(147 + 70) mg.

= 217 mg.

The Isolation of Pure Tetramethyl Glucose.

The syrup of Fraction IIa was extracted with successive small quantities of light petroleum (38–40°) and on removal of the solvent on a watch-glass fine white needle shaped crystals were obtained, contaminated with a small quantity of colourless syrup which did not crystallise. Thorough tiling removed the thin syrup and the crystals then had m.p. 90°.

Attempted Preparation of the Anilide.

Crystalline tetramethyl glucose (5.0 mg.) and freshly distilled aniline (1.8 mg.) were dissolved in absolute ethanol (1.5 ml.) and the solution gently refluxed for 2 hours. The solvent was removed in a

vacuum desiccator when a crystalline product was obtained. In an attempt to remove a syrupy impurity the crystals were carefully titurated with a very small quantity of dry ether, but the anilide went into solution. On evaporation of the solvent the process was repeated using light petroleum (30–40°), but again it was found impossible to purify the crystals.

c) Fraction III.

Using urea oxalate and aniline oxalate to spray duplicate paper chromatograms, this fraction was found to contain a trimethyl fructose and a trimethyl glucose.

$[\alpha]_D^{14}$ + 26.5° (c=1.43 in water) OMe = 38.0%

Oxidation by Alkaline Hypoiodite.

The method is as described previously.

Two experiments gave results of 3.6% and 3.2% for the aldose contents of this fraction.

The totalweight of this fraction was 5.759 g.

This contains $5.759 \times \frac{38}{41.9}$ g. sugars

$= 5.225$ g.

∴ Weight of trimethyl glucose:

$\frac{5.225}{100} \times 3.4$

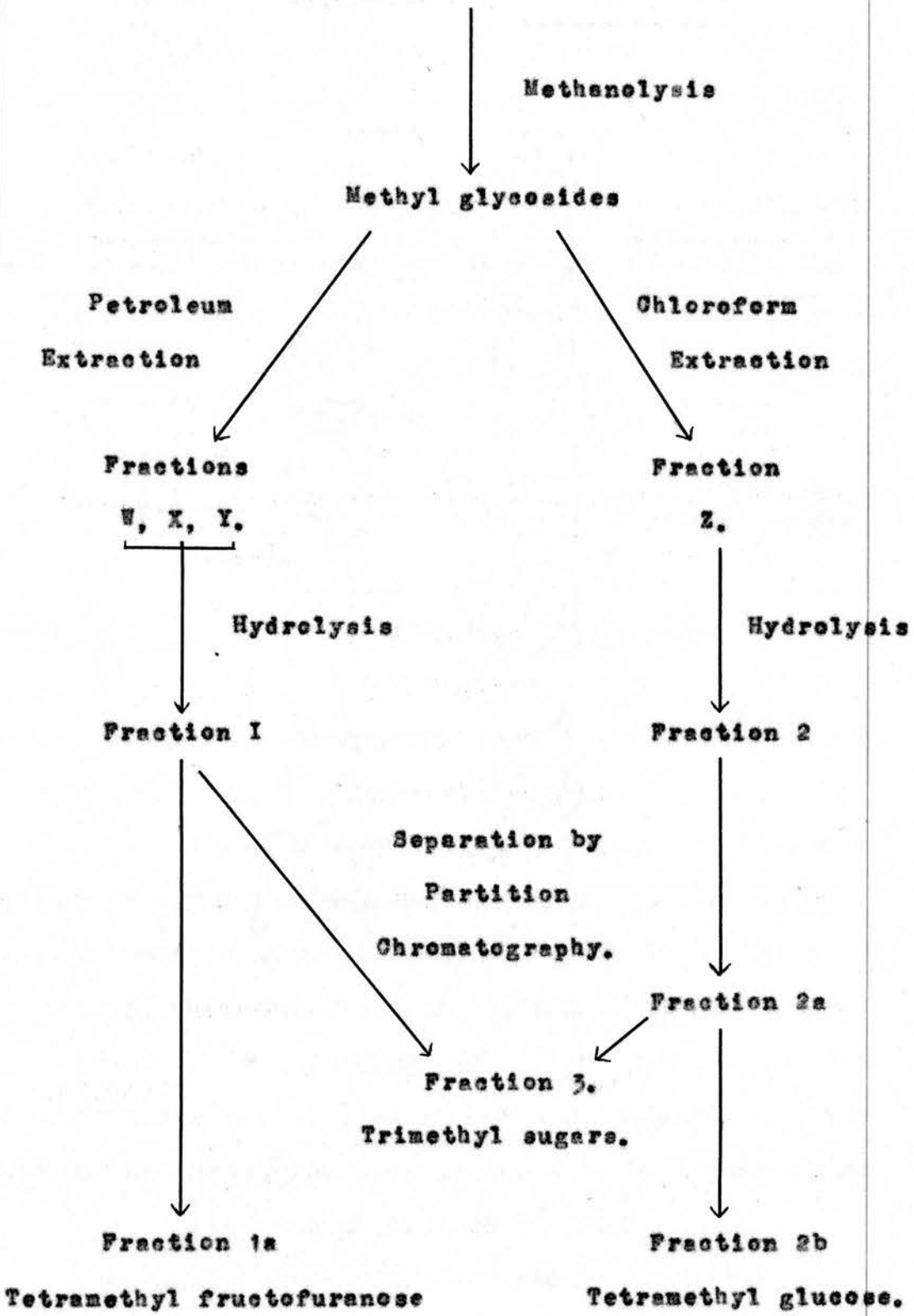
$= 177.7$ mg.

Total recovery of sugars form the cellulose column:

Tetramethyl fructofuranose	0.217 g.
Tetramethyl glucose	0.053 g.
Trimethyl fructose	5.048 g.
Trimethyl glucose	0.177 g.
Total	5.495 g.

Scheme for the Examination of:

Trimethyl Inulin (Sample 2)



The percentage composition of the first sample of hydrolysed trimethyl inulin is as follows:

Tetramethyl fructofuranose	3.98%
Tetramethyl glucose	0.97%
Trimethyl glucose	3.22%
Trimethyl fructose	91.86%

The Examination of the Second Sample of Trimethyl Inulin.

This sample , which had been prepared in an inert atmosphere, was analysed in exactly the same way as the first sample.

The Preparation of the Methylfructosides.

Trimethyl inulin (7.0 g.)(OMe = 45.6%) was treated with methanol (210 ml.), water (70 ml.) and crystalline oxalic acid (3.5 g.) on a water bath at 80° for 18 hours. The solution was neutralised with calcium carbonate and evaporated to a syrup at 40°/15mm. The syrup was extracted with warm chloroform, the extract dried over anhydrous sodium sulphate, and evaporated to leave a syrup which was taken up in methanol containing 0.25% hydrogen chloride and allowed to stand at 20° for 70 hours. The acid was neutralised with barium carbonate and the unfiltered solution evaporated. The residue was extracted with chloroform, which on distillation (40°/15 mm.) gave a syrup (7.57 g.) which was dried in a vacuum over phosphorus pentoxide.

Fractionation by Solvent Extraction.

The whole of the methylfructoside syrup was dissolved in water (30 ml.) and this solution was continuously extracted, in an all-glass apparatus, with light petroleum (40°) for 3 periods of 3 hours. Thus three fractions W, X and Y were obtained. Samples of

each of these were hydrolysed as before with aqueous oxalic acid (0.4 ml.; 2.25%) at 80° for 2 hours. The neutralised hydrolysates were examined on duplicate paper chromatograms, using urea oxalate and aniline oxalate to show the positions of the sugars after development. In this way the three fractions were shown to contain the following sugars:

Fraction W Tetra- and trimethyl fructose and trimethyl glucose
Fraction X As for "W"
Fraction Y As for "W" plus a trace of tetramethyl glucose.

The original aqueous solution was now exhaustively extracted with chloroform to give Fraction 2. (3.80 g.)

This was found, by the paper chromatogram method, to contain tetra- and trimethyl glucose and trimethyl fructose.

Fractions W, X and Y were combined to give Fraction 1 (1.76 g.)

The Hydrolysis to the Free Sugars.

Fraction 1 was hydrolysed by treatment with aqueous oxalic acid (120 ml.; 4.0%) at 80° for 3 hours. The acid was neutralised with calcium carbonate and the mixture heated to decompose any calcium bicarbonate. The filtered solution was evaporated to a syrup at 40°/15 mm., which was dried with ethanol and benzene. It was purified free from some remaining solid material by chloroform extraction. Removal of the solvent gave a syrup (1.43 g.) Fraction 1a.

Fraction 2 was hydrolysed similarly using 300ml. of the oxalic acid solution. After treatment as above a syrup (3.28 g.) was obtained, Fraction 2a.

The Separation of the Mixture of Sugars on a Column of Powdered Cellulose.

The dimensions of the column and the solvent were as used for the first sample of trimethyl inulin. (Page 39)

That fraction containing the tetramethyl fructose (1a) was separated first, and this sugar was isolated chromatographically pure (199 mg.) Fraction 1b. The trimethyl sugars were washed out of the column with water and this solution added to the solution of the trimethyl fraction obtained in the second separation, below.

The tetramethyl glucose fraction was obtained as a brown syrup (198.4 mg.)(Fraction 2b) from the separation of Fraction 2 on the cellulose column. On evaporation of the solution of the trimethyl sugars, a syrup (5.585 g.) was obtained, Fraction 3.

Examination of the tetramethyl glucose fraction on the paper chromatogram showed it to be contaminated with trimethyl fructose. In consequence of this, this syrup was taken up in the minimum of solvent and re-separated on the column. A perfect separation was achieved, the tetramethyl glucose crystallising completely on leaving in a vacuum desiccator over phosphorus pentoxide overnight, (135.4 mg.)(Fraction 2c). The trimethyl sugar from this separation was combined with that already isolated, and which had been found to be chromatographically pure, to give a total weight for Fraction 3 of 5.783 g.

The percentage recovery from this fractionation was 91.5%.

The Examination of these three Fractions.

a) **Fraction 1b.**

Examination on the paper chromatogram showed this syrup to be a mixture of tetramethyl glucose and tetramethyl fructofuranose.

$[\alpha]_D^{14}$ + 42.1° (c=1.04 in water) OMe = 41.8%

The Estimation of the Tetramethyl glucose Content by Hypoiodite Oxidation.

The method is as used for the analysis of the Fractions obtained from the first sample of trimethyl inulin (Page 43)

The total weight of this Fraction is 199 mg.

This contains $199 \times \frac{41.8}{52.5}$ mg. tetramethyl sugar.

= 158 mg.

Of this 13% is tetramethyl glucose.

= 20.6 mg. tetramethyl glucose.

And by difference 137.4 mg. tetramethyl fructose.

b) **Fraction 2c.**

Examination on the paper chromatogram showed this fraction to be tetramethyl glucose contaminated with a trace of tetramethyl fructofuranose.

$[\alpha]_D^{14}$ + 67.7° (c= 0.97 in water) OMe = 41.5%.

The Analysis for Aldose Content.

Hypoiodite oxidation showed Fraction 2c to contain 80.7% aldose.

The total weight of this Fraction was 135.4 mg.

This contains $135.4 \times \frac{41.5}{52.5}$ mg. tetramethyl sugar.

= 107 mg.

The composition of Fraction 2c by weight is therefore

85.6 mg. tetramethyl glucose

And by difference 21.4 mg. tetramethyl fructose.

Anilide Formation.

The whole of Fraction 2c was purified by solution in water, addition of a little "Filter Cel", followed by filtration and removal of the solvent. After drying with ethanol and benzene a crystalline compound (97 mg.) was obtained. To this was added redistilled aniline (55 mg.) and the whole dissolved in absolute ethanol (10 ml.) and refluxed for 3 hours. The solvent was removed in a vacuum desiccator when a crystalline material was obtained; m.p. 127–128°. After three recrystallisations from light petroleum it had m.p. 134–135° yield: 43 mg. A mixed melting point with an authentic specimen of tetramethyl glucose anilide was unchanged.

Found: C, 61.7; H, 7.9; N, 4.61; OMe, 38.2%

Calc. for $C_{16}H_{25}O_5N$: C, 61.5; H, 8.3; N, 4.3; OMe, 39.8%.

c) Fraction 3.

Examination on the paper chromatogram showed Fraction 3 to contain a trimethyl fructose and a trimethyl glucose of identical R_G value.

$[\alpha]_D^{14}$ + 38.1° (c=0.94 in water) OMe = 36.0%.

Estimation of the Aldose Content.

Fraction 3 (148.2 mg.) was dissolved in water and purified as before using "Filter Cel". 500 mg. portions of this solution were weighed out and their aldose contents estimated by hypoiodite oxidation.

Sodium thiosulphate is 0.0096 N.

A water blank required 24.78 ml. of this solution.

Solution taken in gms.	Thiosulphate required.	Aldose equivalent.
0.4996 g.	24.21 ml.	4.8%.
0.5392 g.	24.37 ml.	3.2%.

These experiments were repeated on the same solution, using three times the volume of buffer solution as iodine solution; instead of equal volumes as had been used previously.

A water blank required 24.70 ml. thiosulphate (0.0096 N)

Solution taken in gms.	Thiosulphate required	Aldose equivalent.
0.4986 g.	24.29 ml.	3.6%
0.5214 g.	24.19 ml.	4.1%

Averaging these four results we obtain a value of 3.9% for the concentration of the trimethyl glucose in this Fraction 3.

Total weight of Fraction 3 as isolated 5.783 g.

This contains $5.783 \times \frac{36.0}{41.9}$ g. trimethyl sugar. +2%

$= 4.970$ g.

But 3.9% of this is trimethyl glucose

= 194 mg. trimethyl glucose

And by difference 4.776 g. trimethyl fructose.

Percentage Composition of the Second Sample of hydrolysed Trimethyl Inulin.

Total Sugars Isolated:-

Tetramethyl fructofuranose	0.1588 g.
Tetramethyl glucose	0.1062 g.
Trimethyl glucose	0.1940 g.
Trimethyl fructofuranose	4.7760 g.
Total	5.2350 g.

Whence: Tetramethyl fructofuranose 3.0%

Tetramethyl glucose 2.0%

Trimethyl glucose 3.7%

Trimethyl fructofuranose 91.3%

The Identification of the Trimethyl Glucose.

Preparation of the Aldonic Acid.

The two "Trimethyl" fractions obtained from the two samples of trimethyl inulin were dissolved in water (55–60 ml.) and bromine (2 ml.) added to each solution. They were allowed to stand , with occasional shaking, for 4 days at room temperature. The bromine was removed by aeration and the acid neutralised by the addition of silver carbonate. After filtration, any silver remaining in solution was removed as the sulphide. The two solutions were combined and evaporated at 40°/15 mm. to a thin syrup which was examined on the paper chromatogram. Trimethyl glucose was still present as was shown by a red colouration when the paper was sprayed with a saturated solution of aniline oxalate.

The syrup was further oxidised by treating the solution, in 50 ml. water, with bromine (3 ml.) for 3½ days at room temperature. The bromine and hydrobromic acid were removed as before, and the syrup obtained on concentration of the solution examined on the paper chromatogram, when trimethyl glucose was again found to be present.

The aqueous solution of this syrup was further treated with bromine (5 ml.) in the presence of lead carbonate for 4 days at room temperature. The bromine was removed as above and excess acid neutralised with silver carbonate. Any silver or lead remaining in solution were removed as the sulphides. Concentration of the filtered solution gave a thin syrup which was found to be acid. The syrup was dissolved in a little water and the acid neutralised with silver carbonate, excess

silver being removed as before. Concentration of this solution gave a thin syrup which was examined on the paper chromatogram.

No trimethyl glucose was demonstrated on spraying the developed paper with aniline oxalate solution. Trimethyl fructose was still present as was shown with urea oxalate. A paper was also run in an acidic solvent (acetic acid, 10%, n-butanol, 40%, water, 50%) and the paper, after drying, sprayed with an ethanolic solution of bromo-phenol blue (0.05%). Two distinct yellow spots were observed against the blue background of the paper. These were centered 4.5 cm. and 5.8 cm. above the solvent boundary.

The acids were separated from the trimethyl fructose on a cellulose column; 11 of solvent, the same as used previously, being used to develop the column. The upper 15 cm. of packing were removed from the column and thoroughly extracted with hot water. On evaporation of the water at 40°/15 mm. a brown solid remained which was dried with ethanol and benzene, in which it was only partially soluble.

Preparation of the Methyl Ester.

This solid material was treated with methanolic hydrogen chloride (15 ml.; 2.0%) under reflux for 9 hours, the acid neutralised with silver carbonate and the silver in solution removed as the sulphide. On distillation of the methanol a brown syrup remained which was extracted with hot chloroform and the trimethyl gluconic acid methyl ester, which remained on distillation of the chloroform, was subjected to distillation in a high vacuum. The distillate was divided into two fractions.

Fraction I 90°–120° (bath tempre.) 40 mg. syrup OMe 53.0%

Fraction II 120°–open flame 170 mg. solid and syrup.

The solid portion of Fraction II was found to be sulphur which was removed by extraction of the sugar component with hot water, addition of a little "Filter Cel" and evaporation of the filtrate.

Fraction IIa syrup OMe 45.0% 50 mg.

Preparation of the Amide.

Fraction I was dissolved in methanolic ammonia (3 ml.) and allowed to stand at 0° for 4 days. At the end of this time a crystalline growth had appeared at the bottom of the tube. This was filtered off and washed with a little cold methanol. m.p. 273° decomp.

$[\alpha]_D^{16} = -90°$ (c=1.23 in water)

Found: C, 40.7, H, 6.7, OMe 34.0%.

Calc for $C_6H_{12}O_4N_2$: C, 40.9, H, 6.8, OMe 35.2%

There was insufficient material for an estimation of N.

On evaporation of the filtrate from the above a syrup remained which was combined with Fraction IIa and the solution in methanol saturated with ammonia (4 ml.) allowed to stand at 0° for 4 days. On removal of the solvent in a vacuum desiccator a small quantity of crystalline material and a larger amount of syrup remained. The syrup was extracted with warm petroleum ether (40–60°) containing a little absolute ethanol, removal of the solvent gave a light brown syrup (49.0 mg.) Fraction IIb. The crystalline material was proved by mixed melting point and rotation to be identical with the above.

Examination of the Amide, Fraction IIb.

OMe = 35.0%, calc. for a trimethyl gluconamide 36.7%.

Oxidation with Periodic Acid (15).

A portion of the amide (7.4 mg.) was dissolved in water (2 ml.) and sodium bicarbonate (2 ml. of N) added, followed by periodic acid (2ml. of 0.3 M). The solution was mixed and allowed to stand at room temperature for 1 hour, when hydrochloric acid (3 ml. of N) and sodium arsenite (2 ml. of N) were added with mixing. When the precipitate and yellow colour had completely disappeared sodium acetate (2 ml. of N) and dimedon reagent (1 ml. of a solution containing 85 mg./ml. of 95% ethanolic solution) were added with mixing. A control experiment using gluconamide (8.2 mg.) was treated in exactly the same way.

A precipitate of the formaldehyde-dimedone complex appeared immediately in the control experiment but both were allowed to stand overnight to complete the precipitation. The control gave 8.4 mg. of complex which is equivalent to (8.4 × 0.1027) mg. formaldehyde, representing a yield of 70% of the theoretical. No precipitation was observed in the experiment on Fraction IIb.

Weerman Test (16).

The trimethyl gluconamide under investigation (12.0 mg.) and gluconamide (11.2 mg.) as a control, were each dissolved in water (0.2 ml.) and sodium hypochlorite solution (0.4 ml.) added to each of the above solutions. After standing at 0° for 3 hours 6 drops of a saturated solution of sodium thiosulphate were added and the solutions were then saturated with sodium acetate, filtered, and the flasks and filter washed out with a saturated solution of semicarbazide hydrochloride

(2.0 ml.). A white precipitate appeared immediately in the control, and both mixtures were allowed to stand overnight at 0° to complete precipitation, but Fraction IIb gave a negative reaction.

Estimation of the Uptake of Periodate.

Fraction IIb (18.0 mg.) was dissolved in water (1.5 ml.) and sodium periodate (2 ml.; approx. $\frac{M}{4}$) added with mixing, and the reaction allowed to proceed at room temperature for 5 hours. A control experiment using authentic 2:3:6-trimethyl gluconamide (15.2 mg.) and a water blank were similarly treated. The excess periodate was estimated by titration of the iodine liberated from potassium iodide with standard sodium arsenite solution.

The control had taken up 0.95 moles. of periodate per $C_9H_{19}O_6N$.; while the unknown trimethyl gluconamide had consumed 0.274 moles. of periodate per $C_9H_{19}O_6N$.

DISCUSSION

The classical method of complete methylation followed by hydrolysis and identification of the methylated sugars obtained, was used to determine the part played by glucose in the structure of inulin. The polysaccharide was methylated by treatment with sodium hydroxide and dimethyl sulphate under mild conditions of temperature in order to reduce as far as possible the risk of degrading the molecule. Three such treatments gave a compound of OMe 43%. A further, similar, treatment in acetone solution raised the methoxyl content by 0.6%. In order to obtain trimethyl inulin, this material was methylated by Purdie's method, when, after purification by precipitation from chloroform solution by light petroleum, a compound of OMe 45.2% was obtainedd.

This trimethyl inulin was hydrolysed in two stages, firstly by methanolic-aqueous oxalic acid at 80° , and finally by methanol containing 0.25% hydrogen chloride, in the cold. The mixture of methyl fructosides was fractionated by extraction a) with light petroleum to give three fractions; and b) with chloroform to give two fractions.

A sample of each of these fractions was hydrolysed with aqueous oxalic acid (2.25%) at 80° for 2 hours, and the neutralised hydrolysates examined on the paper chromatogram. It was found that each fraction contained two methylated sugars, one of which had an R_G value of 0.88 and the other had an R_G value equal to that of tetramethyl glucose used as a control i.e. 1.00.

At first sight these chromatograms, which were sprayed with ammoniacal silver nitrate solution, would

seem to indicate that no preferential extraction of the fully methylated glycosides from the trimethyl glycosides had been effected; and that the only glucose derivative present is the fully methylated compound, as no trimethyl derivatives of glucose having an R_G value as high as 0.88 are listed (14). There remains the possibility that these two spots present on the developed paper chromatogram are due to mixtures of sugars having identical R_G values.

To explore the possibility that tetramethyl glucose and tetramethyl fructofuranose, whose R_G value had not then been estimated, have the same R_G value, a sample of octamethyl sucrose was prepared. On hydrolysis and examination of the hydrolysate on the paper chromatogram, only one spot was observed on spraying with ammoniacal silver nitrate solution and heating at 100° for 10–15 minutes. Its R_G value was identical with that of tetramethyl glucose. Thus it was demonstrated that the fully methylated derivatives of glucopyranose and fructofuranose have the same R_G value. This observation was confirmed using a synthetically prepared specimen of tetramethyl fructofuranose. In view of these results the following work was undertaken.

The Use of Amine Oxalates for the Detection of Sugars on the Developed paper Chromatogram.

Following a report by Dr. Jones (9) that sugars on a developed paper chromatogram showed up as coloured spots against the white background of the paper when the chromatogram was sprayed with saturated solutions of these reagents and heated in an oven at 105° for 15 minutes, a number of these compounds were tested in

order to find, if possible, a colour reaction which would distinguish between tetramethyl glucopyranose and tetramethyl fructofuranose. In addition to the fact that these sugars are indistinguishable when a silver nitrate spray is used, the methylated derivatives of fructose are only faintly reducing to ammonaical silver nitrate and a more delicate test is necessary.

It was found that when a saturated aqueous solution of urea oxalate was sprayed onto a developed paper chromatogram a sample of authentic tetramethyl fructofuranose gave a greyish-green colouration. A sample of tetramethyl glucopyranose on the same paper gave no colour reaction whatsoever. If a duplicate paper to the above was sprayed with a saturated aqueous solution of aniline oxalate only the tetramethyl glucose sample was detected as a pink spot, the tetramethyl fructofuranose giving no colour reaction with aniline oxalate. As was to be expected, a spot of octamethyl sucrose hydrolysate gave a characteristic colour with each reagent.

Thus it will be seen that by use of these two reagents on duplicate paper chromatograms of hydrolysed inulin fractions it will be possible to determine the nature of the sugar having an R_G value identical with that of tetramethyl glucose, information that could not be obtained by the use of the single reagent ammonaical silver nitrate. Furthermore, it was found that the urea oxalate solution was a far more delicate test for the presence of methylated derivatives of fructose than was ammonaical silver nitrate.

The synthetically prepared tetramethyl fructofuranose

was purified by distillation in a high vacuum and thus divided into two fractions. The higher boiling of the two had a refractive index 0.0007 higher than the lower boiling of the two fractions. This trace of trimethyl fructofuranose in the higher boiling fraction was not detected using ammonaical silver nitrate, but on substituting urea oxalate solution a definite, though faint, spot due to the trimethyl sugar was observed.

On re-examination of the five fractions of methyl fructosides obtained by solvent extraction of the hydrolysed trimethyl inulin, on the paper chromatogram, and using the two amine oxalates to detect the sugars, it was found that fractions A, B and C, extracted by light petroleum, all contained the same derivatives; tetramethyl frutose, trimethyl glucose and trimethyl fructose. Those two fractions D and E, which had been obtained by chloroform extraction, consisted of tetramethyl glucose, together with a trace of tetramethyl fructose, and the two trimethyl compounds as above.

Thus while there has been only slight preferential extraction of the tetramethyl over the trimethyl fructose from the aqueous solution by light petroleum, the tetramethyl fructose has been separated from the tetramethyl glucose by this extraction. The reason for the removal of the tetramethyl fructose in the light petroleum while the tetramethyl glucose remains in the aqueous solution is unknown.

The Separation of the Mixture of Sugars obtained on Hydrolysis of Trimethyl Inulin by Partition Chromatography on a Column of Powdered Cellulose.

Prior to the development of this technique

fractional distillation of the methyl glycosides was the only method available for the separation of a methylated polysaccharide hydrolysate into its individual components. This latter method suffers from two disadvantages in that it requires large quantities of material and that it is very difficult to obtain a complete separation of the methyl glycosides. Chromatography overcomes these two disadvantages and it is rapidly assuming a place of importance as a standard technique of carbohydrate chemistry.

Jones (18) reported that tetramethyl methylglucoside could be quantitatively separated from a mixture containing 3.0 g. trimethyl methylglucoside and 150 mg. of the fully methylated compound, by adsorption on activated alumina from solution in ether-light petroleum. MacDonald (19) had shown that the partition coefficients of 2:3:4:6-tetramethyl glucose and 2:3:6-trimethyl glucose differed by a factor of 100, thus allowing a separation of the two, though the manipulations involved are, however, unsuitable for small quantities. By partitioning between chloroform and water, the latter held in a rigid column of silica gel, Bell (12) achieved on a small scale, an absolute separation of these two methylated sugars. It was also found possible to separate the trimethyl and dimethyl glucoses by partition on a similar column between water and a chloroform-n-butanol mixture.

More recently, Bell and Palmer (13) have reported the quantitative separation of a mixture of 1:3:4:6-tetramethyl, 1:3:4-trimethyl and 3:4-dimethyl fructoses using a column of silica gel and eluting the sugars

with toluene containing 0.33% ethanol, chloroform containing 5.0% n-butanol and methanol respectively. A quantitiative recovery was not obtained with 3:4:6-trimethyl fructofuranose.

The method used in the present work was essentially as described by Hough, Jones and Wadman (10). The column was tightly packed with powdered cellulose and washed thoroughly before use. The light petroleum extracts were combined to give FractionI which thus contained tetramethyl fructose, 3:4:6-trimethyl fructose and a trimethyl glucose. This syrup was dissolved in the minimum of the solvent used (70% 100-120 petroleum ether, 30% n-butanol, saturated with water) and the solution transferred to the top of the column. The sugars were eluted by allowing more of the same solvent to flow down the column. The receivers were changed every five minutes by an automatic electrical mechanism and the contents of every tenth receiver were concentrated and analysed on the paper chromatogram. A picture of the distribution of the sugars throughout all the receivers was thus obtained. The contents of those tubes containing the same sugars were combined and the solvent distilled under reduced pressure. The quantity of each sugar was obtained directly by weight. In this way the tetramethyl fructofuranose was isolated chromatographically pure as Fraction Ia.

Fractions D and E, containing tetramethyl glucose and the two trimethyl compounds were combined to give Fraction II. This mixture was separated into its components on the cellulose column as before, the tetramethyl glucose being obtained free from trimethyl

compounds as Fraction IIa. This did not crystallise on standing.

It was found necessary to wash both the columns with water in order to elute all the trimethyl sugars. These solutions of trimethyl compounds were combined and the solvents distilled to give a syrup free from fully methylated sugars, Fraction III.

The recovery from these two chromatographic separations was 91% using a total of 6.114 g. sugars.

The samples isolated directly from the column by distillation of the solvents under reduced pressure were found to be contaminated with a waxy material. In order, therefore, to ascertain the true quantitative relationship of the monosaccharide derivatives isolated from the trimethyl inulin hydrolysate, it was essential to analyse the individual syrups for their true sugar contents. In addition, Fractions IIa and III consisted of mixtures of aldose and ketose derivatives and an accurate knowledge of the quantity of each sugar present is required in order to throw as much light as possible on the constitution of the inulin molecule.

Since each fraction contains only one class of sugar derivative i.e. either tetramethyl or trimethyl hexose, a knowledge of the methoxyl content of the fraction will enable us to calculate accurately the sugar content of each syrup.

The aldose derivatives can be estimated in the presence of ketoses by utilising the quantitative oxidation of the aldose to the aldonic acid by alkaline hypoiodite. The method used was modified from Hirst, Hough and Jones (14). 5 ml. of a solution

of accurately known concentration (1-2%) of each of the three Fractions was made up and purified free from grease by the addition of a little "Filter Cel" followed by filtration. Approximately 500 mg. samples of the resulting, clear, solutions were weighed out and analysed with alkaline hypoiodite.

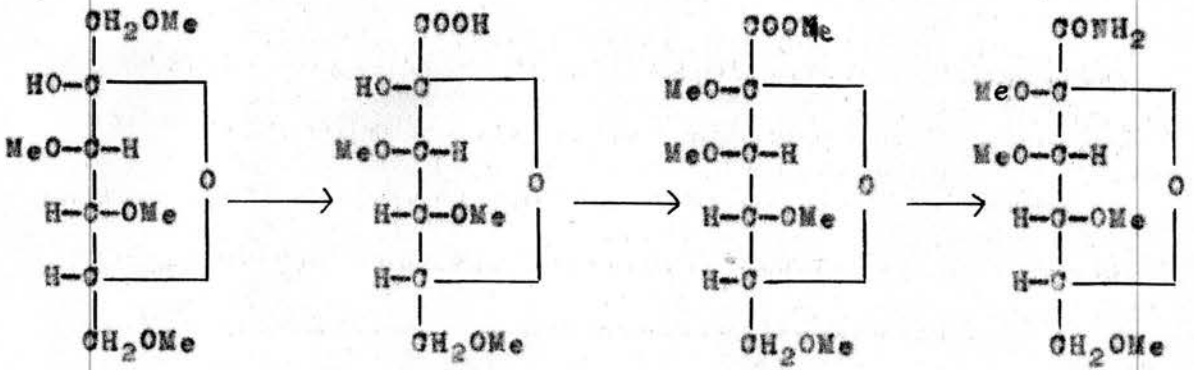
It was found that the chromatographically pure sample of tetramethyl fructofuranose, Fraction Ia, took up no iodine, and therefore the assumption upon which these analyses are based, uptake of iodine by the aldose derivatives only, is justified.

From the results of these estimations the absolute quantities of the various monosaccharide derivatives obtained on the hydrolysis of trimethyl inulin were calculated. The total tetramethyl fructofuranose isolated was equivalent to 3.95% of the total products of hydrolysis of trimethyl inulin. Assuming, for the moment that the glucose is derived from other than the inulin molecule, then these results would lead to the conclusion that this sample of trimethyl inulin, methylated in the normal atmosphere, consisted of a straight chain of 25 fructofuranose residues. Possible sources of the glucose will be discussed later on.

The end group had been assumed to be tetramethyl fructofuranose solely by comparison of its rate of travel on the paper chromatogram with that of an authentic, synthesised, specimen, and from a knowledge of the previous work on inulin,(3), in which the end group was characterised by conversion to tetramethyl fructofuronamide.

That sample of end group isolated from the

present sample of trimethyl inulin was also characterised as tetramethyl fructofuranose by its conversion to tetramethyl fructofuronamide, the transformation being carried out by the method of Avery, Haworth and Hirst (11).



The quantity of tetramethyl glucose in Fraction IIa was estimated as 43.1% using the hypoiodite oxidation method for the analysis. This is equivalent to 0.97% of the total hydrolysed trimethyl inulin.

An attempt was made to characterise the tetramethyl glucose isolated from this Fraction by the preparation and identification of the anilide. Only 5 mg. of relatively pure crystalline sugar could be obtained by extraction of Fraction IIa with light petroleum. After preparation of the anilide, the derivative could not be purified free from a small quantity of contaminating syrup.

The work of Haworth and Learner (1) established the constitution of the trimethyl fructose beyond doubt as the 3:4:6- isomer, and therefore the structure of the trimethyl compound isolated during the present work was not investigated.

Fraction III was analysed for its trimethyl aldose content by oxidation of the aldose component with alkaline hypoiodite. It was estimated as 3.4%

of this Fraction and therefore 3.22% of the total hydrolysed trimethyl inulin.

A total of 4.2% of methylated glucose has been isolated from the trimethyl inulin compared with 5.7% for the glucose content of the inulin before methylation.

A second sample of inulin ($[\alpha]_D$ - 40.0°) was methylated by a series of reactions similar to that used for the first sample except that the preliminary methylation with dimethyl sulphate in the presence of excess sodium hydroxide, was carried out in an atmosphere of nitrogen. A comparison of the quantities of end group obtained from the two samples will show if any oxidative breakdown had taken place during the methylation of the first sample in air, as has been found in the case of cellulose. A compound of the required methoxyl content for trimethyl inulin (45.6%) was obtained after purification of the crude product by precipitation from chloroform solution by light petroleum (40-60°).

This second sample was hydrolysed to the methyl fructosides by exactly the same methods as had been used for the first sample. An approximately 4% aqueous solution of the syrupy fructosides was extracted in an all-glass apparatus with three successive quantities of purified light petroleum (38-40°), each extraction being continued for 3 hours. On evaporation of the solvents, three syrups, Fractions W,X and Y were obtained. The fructosides remaining in solution were extracted exhaustively with chloroform to give Fraction Z.

Although the tetramethyl and trimethyl components

of the mixture obtained on hydrolysis could be separated completely and easily by partition chromatography on the cellulose column, this solvent extraction was performed in the hope that the two fully methylated derivatives would be separated from each other by this method as had been found in the investigation of the first sample of trimethyl inulin.

On examination of hydrolysed samples of Fractions W, X, Y and Z on duplicate paper chromatograms sprayed with aniline oxalate and urea oxalate, this separation was found to have been effected as before.

Fractions W and X contained tetramethyl and trimethyl fructose together with trimethyl glucose. Fraction Y contained the above sugars plus a trace of tetramethyl glucose. Fraction Z contained tetramethyl and trimethyl glucose and trimethyl fructose.

Fractions W, X and Y were combined, since they contained the same compounds, to give Fraction 1, which was hydrolysed to the free sugars and then separated into its components by partition chromatography on the column of powdered cellulose, using the same solvent as before. The tetramethyl fructofuranose was obtained free from any trimethyl sugars as fraction 1b. Similarly the tetramethyl glucose was obtained chromatographically pure as fraction 2b, from the separation of Fraction 2 (hydrolysed Fraction Z). The trimethyl fractions from both columns were combined to give fraction 3.

Fraction 2b was found, by examination on the paper chromatogram, to contain a little trimethyl fructose, and consequently this fraction was

re-separated on the column, when the tetramethyl glucose was obtained crystalline on evaporation of the solvent, fraction 2c. It would appear that a sample as large as Fraction 2 (5.28 g.) will not separate completely on a column of these dimensions when the R_G values of the components are as close as 0.88 and 1.00.

At this stage, therefore, the trimethyl inulin hydrolysate had been divided into three fractions, similar to those obtained from the first sample.

Fraction 1b was shown, by analysis on the paper chromatogram, to contain tetramethyl fructose together with a much smaller quantity of tetramethyl glucose.

Fraction 2c consisted largely of tetramethyl glucose, with a small quantity of tetramethyl fructose.

Fraction 3 contained 3:4:6-trimethyl fructofuranose and a trimethyl glucose.

Each syrup was found, as before, to be contaminated with a greasy impurity, and the methoxyl content was taken as giving an estimate of the actual sugar content of each fraction.

The tetramethyl glucose contents of fractions 1b and 2c were estimated by alkaline hypoiodite, and the fructose end group estimated by difference. The latter was found to be equal to 3.0% of the total hydrolysed trimethyl inulin, thus giving a value of between 30 and 35 residues for the chain length of inulin, assuming, as before, that the glucose end group does not originate in the inulin molecule.

Comparing this estimate of the end group (3.0%)

with that found in the first sample of trimethyl inulin (2.5%) there is little substantial evidence for the oxidative breakdown of inulin during methylation in sodium hydroxide solution in the air.

The whole of the tetramethyl glucose fraction was purified free from grease as before, and a white crystalline mass obtained. This was treated, under reflux, with the theoretical quantity of aniline in absolute ethanolic solution. After 3 hours the solvent was removed, when the crystalline anilide remained. It was recrystallised from light petroleum, and a mixed melting point with an authentic specimen of tetramethyl glucose anilide was unchanged.

The unknown trimethyl glucose was estimated, by alkaline hypoiodite, to constitute 3.9% of the trimethyl hexose fraction, and therefore 3.7% of the total hydrolysed trimethyl inulin. The recovery of methylated glucose represents 5.7% of the hydrolysed trimethyl inulin, the same concentration as was estimated in the unmethylated polysaccharide.

The Structural Investigation of the Trimethyl Glucose.

Since this compound could not be separated from the large amount of 3:4:6-trimethyl fructofuranose by physical methods, the whole of the trimethyl fraction was treated with bromine in an attempt to isolate the trimethyl aldonic acid and leave the trimethyl fructose unchanged. Three treatments with bromine were necessary before the trimethyl glucose was completely oxidised, as was shown by the disappearance of the pink spot on a paper chromatogram sprayed with aniline oxalate solution. The third treatment with

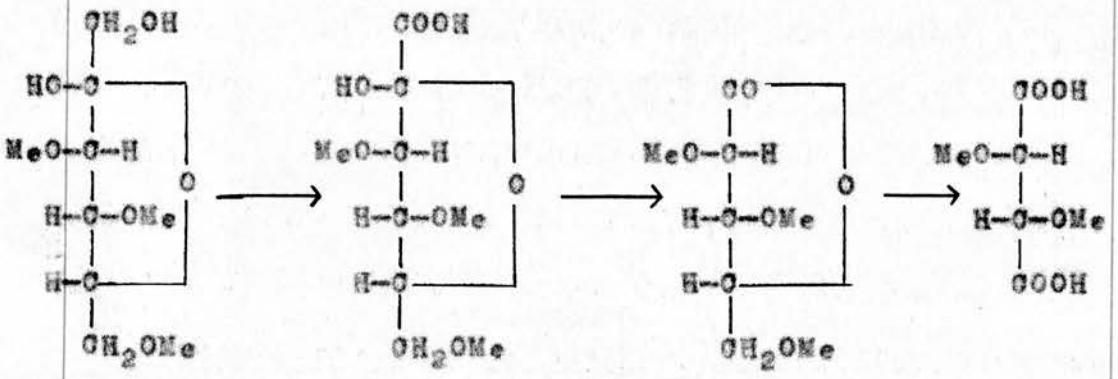
bromine was given in the presence of lead carbonate, and the overall period of oxidation was 12 days.

The trimethyl gluconic acid was separated from the 3:4:6-trimethyl fructofuranose by partition chromatography on the cellulose column, the sugar being washed down the column with the butanol-petroleum solvent, while the acid remained at the top and was extracted from the first 15 cm. of packing with hot water. On concentrating the extract and examining qualitatively on a paper chromatogram developed in an acidic solvent and sprayed with an indicator solution (bromo-phenol blue) two distinct yellow, acid, spots were observed.

These two acids were converted into their methyl esters by treatment with boiling methanolic hydrogen chloride and the syrups so obtained distilled in a high vacuum, to effect, if possible, a separation of the two components into the two fractions of the distillate.

Fraction I, a syrup of methoxyl content 4% high for a trimethyl gluconic acid methyl ester, was treated with methanol saturated with ammonia at 0° for 4days, when a crystalline growth had formed at the base of the tube. On examination of these crystals by melting point, rotation and analysis they were found to be D(-)-dimethoxy succinamide. It is considered so unlikely as to be unworthy of further consideration that bromine would oxidise an aldose right down to this compound, and the D(-)-dimethoxy succinic acid is thought, therefore, to be a result of the oxidative breakdown of some of the 3:4:6-trimethyl fructofuranose by the following

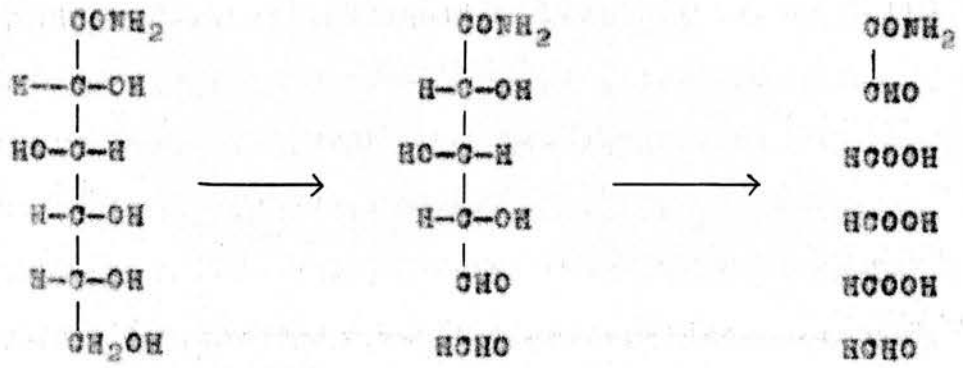
series of reactions, during the 12 days treatment with bromine.



Fraction II of the ester distillate was contaminated with sulphur, which was removed by extraction of the ester in water. Evaporation of the solvent left a syrup of OMe 45%. Concentration of the mother liquor from the D(−)-dimethoxy succinamide gave a syrup which was combined with Fraction II and re-treated with methanolic ammonia at 0° for 4 days. A syrupy amide remained on removal of the solvent, together with a further, smaller, crop of crystals of D(−)-dimethoxy succinamide.

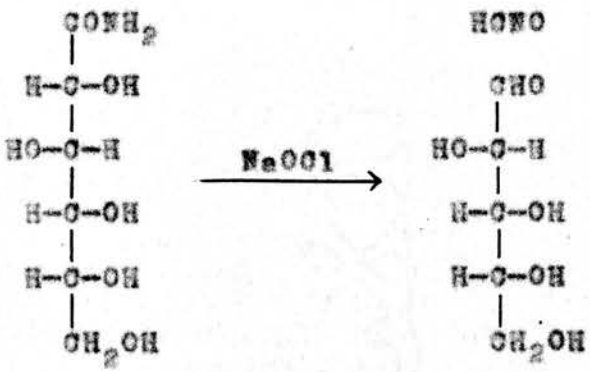
This syrupy amide had almost the correct methoxyl content for a trimethyl gluconamide and was examined by three series of reactions.

A sample of the syrupy amide, and a sample of gluconamide as a control, were oxidised with periodic acid, by Reeves method (15). If position C_6 is unmethylated, it will be split off the molecule as formaldehyde, which can be detected and quantitatively estimated as the dimedone complex.



The dimedone complex was obtained from the control in a 70% yield, but no precipitate was obtained from the amide under investigation. Therefore it must concluded that position C_6 is occupied, and that the trimethyl gluconamide is not the 2:3:4- isomer.

The unknown gluconamide was subjected to the Weerman reaction, given by all α-hydroxy amides (16). An α-hydroxy amide is oxidised by sodium hypochlorite to give hydrocyanic acid, which reacts quantitatively with semicarbazide to give a precipitate of the water insoluble hydrazodicarbonamide.



$$HCNO + NH_2{-}CO{-}NH{-}NH_2 \longrightarrow NH_2{-}CO{-}NH{-}NH{-}CO{-}NH_2$$

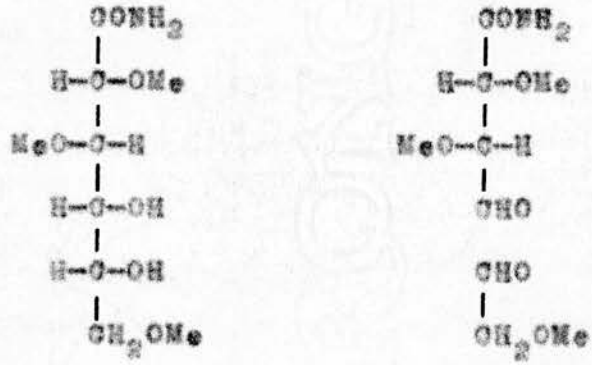
Thus by means of the above reaction a quantitative estimate of the concentration of α-hydroxy amide in a mixture is obtained.

A distinct precipitate of the white hydrazodicarbonamide was observed from a control test on gluconamide, but the unknown gave a negative reaction; therefore position C_2 must be occupied by a methoxyl

group and the compound is therefore not the 3:4:6- isomer.

The Estimation of the Uptake of Periodate Ion.

Two possibilities for the trimethyl gluconamide remain; either it is the 2:3:6- or the 2:4:6- isomer. 2:3:6-trimethyl gluconamide will take up 1 mole. of periodate per mole. according to the following reaction.



2:4:6-trimethyl gluconamide will not be attacked by periodate.

In the experiment, after 5 hours oxidation, a control sample of 2:3:6-trimethyl gluconamide had taken up 0.95 moles. of periodate per mole. of amide. the unknown compound, under identical conditions, consumed 0.274 moles. of periodate per mole. Thus it would appear that the reaction had gone almost to completion and that the gluconamide obtained from trimethyl inulin was a mixture of approximately

30% 2:3:6-trimethyl gluconamide

and 70% 2:4:6-trimethyl gluconamide.

The foregoing series of results do not point to any definite molecular structure for the inulin complex, but they do lead us to a limited number of possibilities which will now be considered in detail.

The first possibility which is open to consideration is that the inulin chain, proved straight by the non-detection of any dimethyl compounds, consists of a mixture of fructofuranose and glucopyranose residues.

In order, however, to account for the occurence of tetramethyl glucose, the reducing group of the fructose chain must be combined with a glucose residue through the reducing group of the latter; giving, at one end of the straight chain, a disaccharide having the non-reducing configuration of sucrose. A structure having the terminal grouping of sucrose has, in fact, been suggested for artichoke inulin by Dr. J.S.D.Bacon (17) as a result of enzymatic degradative studies on the polysaccharide. An alternative possible structure is one in which the fructofuranose chain is terminated by three or four glucose units, containing possibly two types of glycosidic linkage (Pages 58 & 75) and with the end two residues linked through their reducing groups to give a non-reducing polysaccharide. Again, alternatively, on the evidence available, the residues which give rise to the trimethyl glucose on methylation and hydrolysis might be distributed along the fructose chain.

It must be pointed out, however, that no sample of inulin yet prepared has had a negative reducing action on Fehling's solution, and the sample used in the present investigation was found to have a slight but definite reducing action. The amount of reduction reached a maximum after two minutes boiling and did not increase on continuing the heating for a further 13 minutes. It seems likely, therefore, that the reduction of Fehling's solution by inulin is due to a free reducing group at the end of each molecular chain, and not, as assumed by Pringsheim (Introduction; Page 4) to be due to the hydrolysis of the polysaccharide to free fructose. These structures suggested here would be non-reducing,

and they must therefore be regarded with a certain amount of doubt.

In addition, these structures, on methylation, will give rise to equal proportions of tetramethyl glucose and tetramethyl fructose, and this has not been found in practice (Pages 48 & 53). A molecular weight determination would probably provide valuable evidence here, as if these suggestions represent a true picture the molecular size as determined by the classical "end group" method will be half of the true molecular weight.

These results may also be interpreted as arising from a structure consisting of a short, straight chain glucosan associated in a physical manner with a longer, straight chain polyfructosan molecule. From a quantitative estimation of the tetramethyl fructofuranose end group arising from the non-reducing end of the chain, we may conclude that the polyfructosan molecule consists of approximately 33 anhydrofructose residues linked, as was shown by Haworth and Learner (1), through the 1:2-positions. This is the structure that had previously assigned to inulin following upon the work of Haworth, Hirst and Percival (3).

From the results of this present work there is now the possibility that this represents too simple a picture; the classical inulin complex of $[\alpha]_D$ -40° being built up of the short chain glucosan held on to the larger molecule either by hydrogen bonding or simply by adsorption, being carried down out of the aqueous solution when the polyfructosan separates on chilling.

From the analysis of the sugars produced on hydrolysis of trimethyl inulin, this glucosan, if in fact it exists,

is probably a tetrasaccharide (Pages 48 & 53) containing possibly two types of glycosidic linkage, 1:3- and 1:4-, (Pages 58 & 75). Valuable evidence for or against this type of structure for the inulin complex would probably be obtained by examination of a solution of the polysaccharide by electrophoresis in the Tiselius apparatus. It must be borne in mind, however, that if evidence was obtained here for the existance of two molecules in the solution, there is no reason to suppose, unless an accurate quantitative estimation of each was made, that one was the glucosan. The chain length given to the inulin molecule as a result of end group determinations might be an average value obtained from a mixture containing molecules of varying molecular dimensions.

In conclusion it might be pointed out that evidence has been obtained (Pages 58 & 74) to show that the trimethyl glucose isolated from an hydrolysate of trimethyl inulin is not the 3:4:6- isomer, as was claimed by Irvine and Montgomery (20).

SUMMARY

1) Two samples of pure inulin ($[\alpha]$ -40.0°) have been methylated by treatment with dimethyl sulphate in the presence of strong sodium hydroxide solution; one sample in the air and the other in an inert atmosphere. Further methylation of these by Purdie's method gave two samples of trimethyl inulin.

2) Methanolysis and fractionation by extraction with light petroleum lead to the separation of the tetramethyl methylfructofuranoside and the tetramethyl methylglucoside present in the hydrolysate. Each of these samples was contaminated with trimethyl compounds.

3) The two fully methylated sugars were isolated and quantitatively estimated after separation from trimethyl compounds by partition chromatography on a column of powdered cellulose.

4) No evidence has been obtained which might indicate oxidative breakdown of the inulin molecule during methylation in the presence of sodium hydroxide in the air.

5) The isolation of a small quantity of trimethyl glucose from the trimethyl fraction of the methylated inulin hydrolysate is reported. Investigation showed this to be a mixture of 70% 2:4:6- and 30% 2:3:6-trimethyl glucose.

6) On the basis of the evidence available possible structures for the inulin complex of $[\alpha]$ -40.0° are discussed.

7) An instance is given of the usefulness of amine oxalates in distinguishing between methylated derivatives of fructose and glucose on the paper chromatogram.

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INTRODUCTION

Part II of this thesis is concerned with the attempted elucidation of the structure of a polysaccharide extracted from the fungus *Lycoperdon bovista*.

The unripe fruit body of the fungus has been extracted in order to obtain the polysaccharide. During growth the fruit body is yellowish or olive in colour, and globose or depressed oval in shape. It is sessile, and attached at the base to a cord-like mycelium. The outer layer of the fruit is downy at first, then becoming smooth, glossy and fragile. Finally it cracks and falls away from the inner layer, which is brittle, and disappearing above it exposes the compact spore mass, which is at first white, then yellowish, and finally olivaceous. These fruit bodies appear between May and November, in woods and pastures, and measure approximately 12 x 11 inches. They are said to be edible.

This is one of the Higher Fungi, and exceedingly little work has been done on the polysaccharides of these plants, with the exception of the researches of K. Takeda (1), who studied a polysaccharide, which he called β-pachyman, isolated from the fruits of the fungus "Bukuryo."

In the Introduction to his booklet, Takeda gives a brief account of the work on fungal polysaccharides up to 1933. The small amount of work that had been done was only of a very preliminary nature, and the various authors held conflicting views. This research, by Takeda, was the first serious attempt to determine the structure of a fungal polysaccharide.

Bukuryo, whose technical name is given as

Pachyma hoelen, occurs in China and Japan, where it grows on the roots of pine, bamboo, mulberry and other trees. It is lump shaped, large or small, brown-black in colour externally, and either red or white internally.

The polysaccharide was extracted from the powdered "Bukuryo" with 5% sodium hydroxide solution, and precipitated in ethanol. It was purified by continued re-precipitation. It was a pure white amorphous powder, insoluble in water, did not reduce Fehling's solution, and contained no sugar acids. Glucose was the only hydrolysis product, either when acid or the enzyme Takadiastase was used.

Methylation was effected by the direct treatment of the polysaccharide with sodium hydroxide and dimethyl sulphate, followed by reaction with silver oxide and methyl iodide. The fully methylated compound, whose methoxyl content was 4% low for a trimethyl hexosan, was hydrolysed with methanolic hydrogen chloride and the glucosides distilled in a high vacuum. No tetramethyl methylglucoside could be detected. The trimethyl fraction was proved, by oxidation of the free sugar to the lactone with bromine water, to consist solely of 2:3:6-trimethyl methylglucoside. This was obtained in a 95% yield from the methylated β-pachyman. A still residue of low methoxyl content was not examined. β-pachyman would therefore appear to consist of a chain of glucose units linked through the 1:4- positions.

In his discussion of these results, Takeda points out that 2:3:6-trimethyl glucopyranose could arise as a result of a shift of the oxygen ring of glucofuranose

from the position C_4 to the free C_5 position, when 2:3:6-trimethyl glucofuranose is acted upon by acid during the hydrolysis of methylated β-pachyman. There is thus the posibility of the chain consisting of 1:5-linked glucofuranose residues. He finds support for this in anomalous data for products isolated after acetolysis of β-pachyman, and also in the isolation of a peculiar glucosazone from these acetylated products, which, after treatment with hydrochloric acid, give a glucosazone whose constants agree with those of a known sample. He postulates that the octa-acetyl hexobiose, isolated after acetolysis, contains furanose rings.

On the data given there is a real possibility that β-pachyman consists of a chain of 1:5- linked glucofuranose residues, but such a polysaccharide has not, as yet, been found elsewhere. Indeed, if one were isolated, it is to be expected that it would undergo hydrolysis with extreme ease, as does galactosarolose (2). Takeda states that β-pachyman is difficult to hydrolyse, so that in all probability it is not a chain of glucofuranose residues; but knowledge of the non-reducing end group is required before this question can be definitely decided either way.

Other work on fungal polysaccharides has been confined to compounds isolated from Lower Fungi, the moulds. These polysaccharides appear to be true extracellular products, and those so far studied present interesting types of complex carbohydrate structure. When produced in a liquid medium, mould polysaccharides usually have small molecular weights, due possibly, to the comparatively

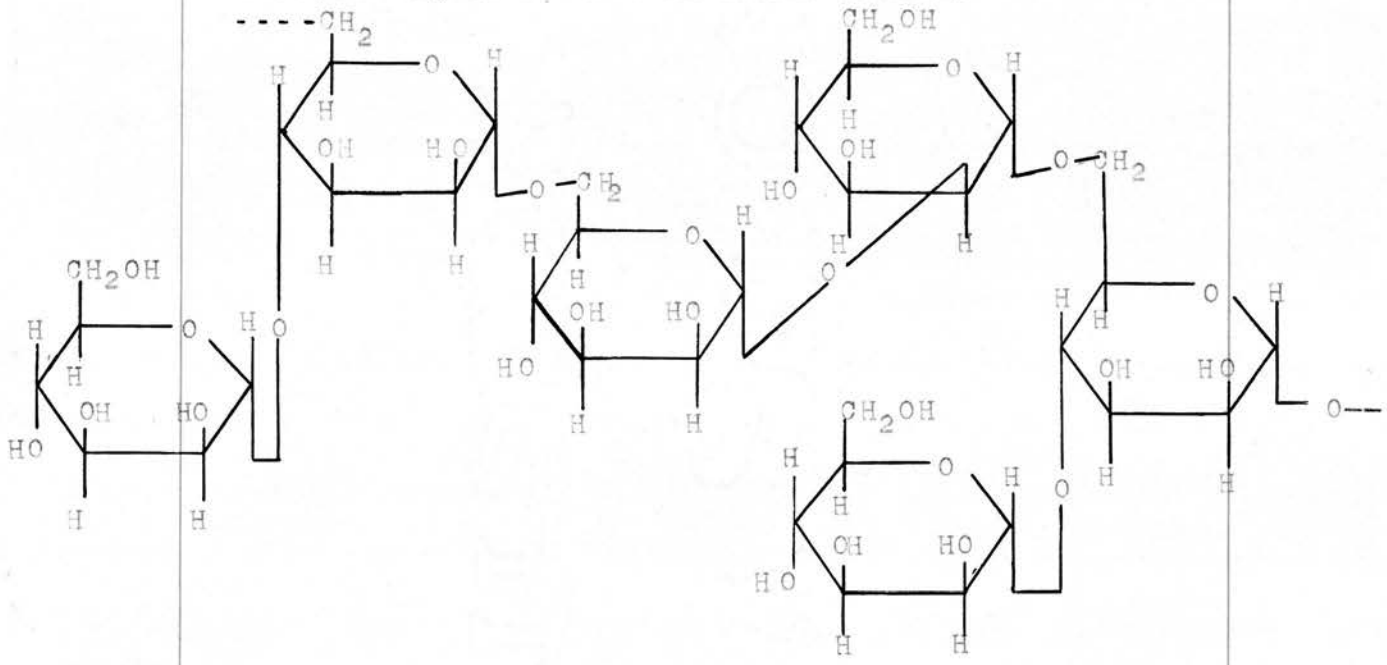
long period required for metabolism, during which the lytic enzymes degrade the initial macromolecules. Most moulds produce a complex mixture of polysaccharides, the proportion of any one component depending on the period required for growth. The biological function of the extracellular mould polysaccharides is unknown, but they are generally assumed to act as reserve carbohydrate. When the mould is grown on a solid medium these compounds appear to behave as mucilaginous "defensive colloids".

The well-tried methods known in polysaccharide chemistry have been used to elucidate the structures of the repeating units of mould polysaccharides, and it will be of interest, as a background to the present work, to review some of the types of structure met with in these compounds.

Mannocarolose is the name given to a polysaccharide isolated by Clutterbuck (3) from the mould Penicillium charlesii G.Smith, when grown on a liquid Czapek-Dox medium, containing glucose as the only source of carbon. Its structure was investigated by Haworth, Raistrick and Stacey (4) who reported that it consisted of a straight chain of nine D-mannopyranose units, linked through the 1:6- positions. 10% of 2:3-dimethyl mannose was isolated, which they assumed was due to incomplete methylation.

The trimethyl mannose constituent of the methylated mannocarolose was noted by Haworth, Hirst and Isherwood and Jones (5) to differ from an authentic specimen of 2:3:4-trimethyl mannose. A recent re-investigation of the hydrolysed methylated polysaccharide, by Stacey (6),

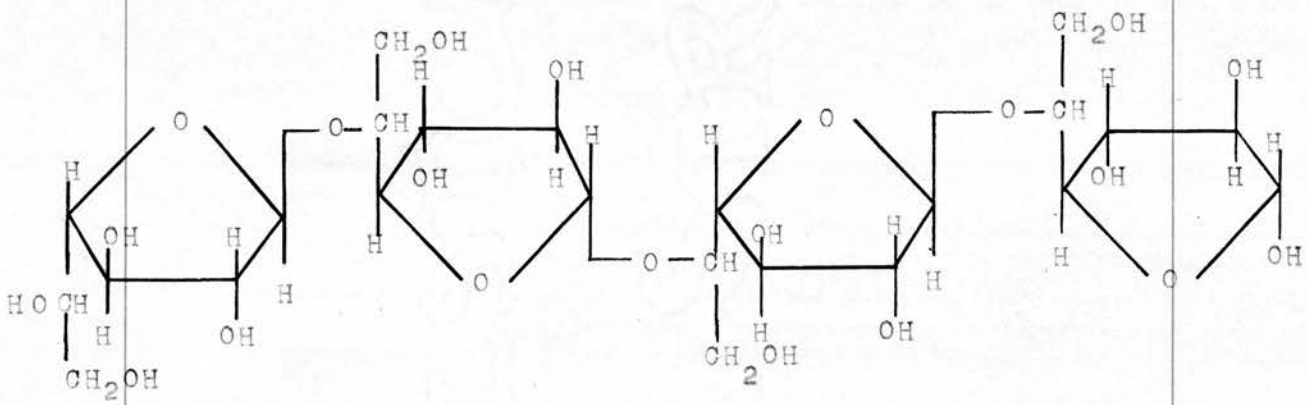
has revealed that the trimethyl mannose fraction consists of equimolecular amounts of the 2:3:4- and the 3:4:6-trimethyl derivatives, and it was shown also that the origin of the 2:3-dimethyl mannose component was not due to incomplete methylation. Accordingly, a branched chain structure is now thought to represent more closely the repeating unit of this mould mannan.



Mannocarolose

A galactan, Galactocarolose, is also produced from D-glucose by P. charlesii G.Smith (3). The structure of the polysaccharide was investigated by Haworth, Raistrick and Stacey (2), who showed that it was hydrolysed by $\frac{n}{100}$ hydrochloric acid to give D-galactose only. Hydrolysis of the methylated compound followed by the direct distillation of the galactosides led to the isolation of 12.4% tetramethyl, and 80% trimethyl derivatives. The trimethyl fraction was identified as 2:3:6-trimethyl galactose, therefore the original polysaccharide could consist of either galactofuranose or galactopyranose residues, but since it is hydrolysed by $\frac{n}{100}$ acid, and the end group was identified as

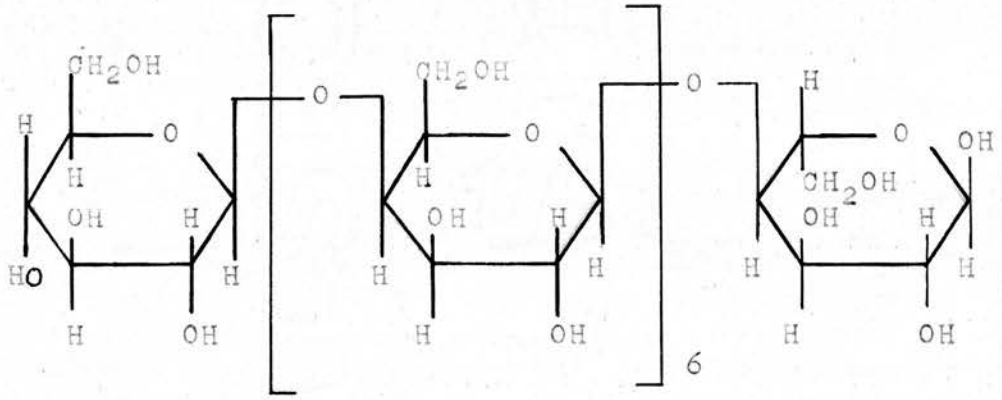
2:3:5:6-tetramethyl galactose, the linkage in the polysaccharide would appear to be 1:5-, with the galactose present in the furanose form. As the rotation is low, $[\alpha]_D = -84^\circ$, these authors consider it probable that the linkage is of the β-configuration. From the iodine number, and the isolation of 12.4% end group, the chain is probably 9 to 10 units in length. This appears to be one of the rare cases where D-galactose is found to occur naturally in the furanose form.



Galactocarolose

Varianose is a polysaccharide produced from D-glucose by Penicillium variane G.Smith. After incubation, the mould is filtered from the medium, the filtrate concentrated in a partial vacuum and the polysaccharide precipitated in ethanol. It was shown by Haworth, Raistrick and Stacey (7) to be hydrolysed by $\frac{n}{10}$ hydrochloric acid to give 70% D-galactose, 14% D-glucose and 14% of an unidentified hexose. On methylation and hydrolysis the glucose was isolated as the end group in an amount which indicated a chain of eight members. The remainder was mainly 2:3:6-trimethyl galactose, and the 14% unidentified hexose was isolated as trimethyl methylhexoside, representing the reducing end of the molecule. This

gave a liquid trimethyl hexose, and a liquid trimethyl hexonolactone; this latter gave a crystalline phenylhydrazide which was not identical with any known. Oxidation of the lactone gave no mucic acid, and no product showing any relationship to galactose. Treatment of the ester of this oxidation product with ammonia gave D-dimethoxy succinamide, thus showing the positions of the methyl groups on C_2 and C_3 of the trimethyl hexose, which would appear to be either L-altrose or D-idose. It would now seem to be identified as L-altrose (6).



Varianose

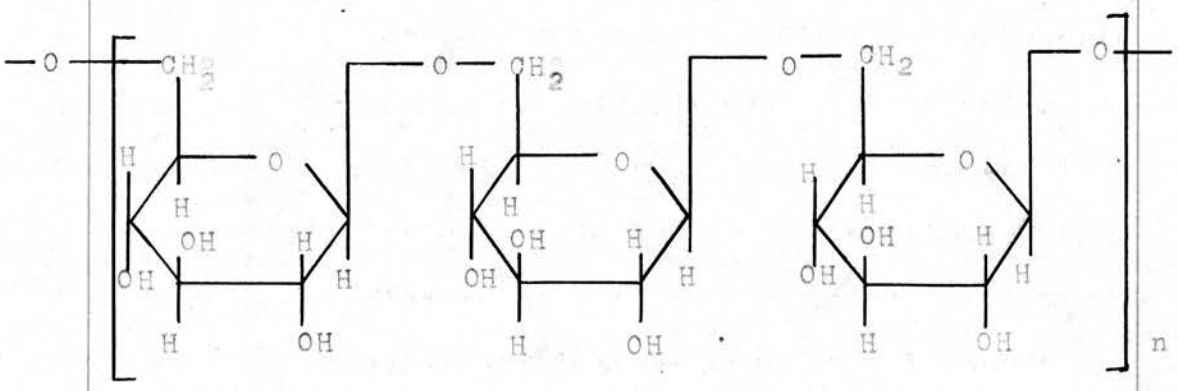
Luteic acid is a highly mucilaginous polysaccharide isolated by Raistrick and Rintoul (8). It is a metabolic product of Penicillium luteum Zukal cultured on any variety of common sugars. Mild acid or alkaline hydrolysis readily destroyed the remarkable viscosity of aqueous solutions of luteic acid, and split malonic acid off the molecule, leaving a neutral polysaccharide luteose. Further hydrolysis yielded glucose only. Quantitative estimations have shown that luteose is combined with malonic acid in the proportions of two molecules of glucose to one of malonic acid.

The structure of luteose was investigated by Anderson, Haworth, Raistrick and Stacey (9) who isolated

80% 2:3:4—trimethyl glucose and 10% dimethyl glucose after methylation and hydrolysis of the neutral polysaccharide. No end group could be detected. As the rotation is low, $[\alpha]_D = -38^\circ$ in aqueous sodium hydroxide they assigned the β-configuration to the 1:6- linkages.

Since no end group was isolated, either the molecular chain is too long to give a detectable quantity, or the molecule is in the form of a loop. As a result of osmosis experiments the molecule would appear to consist of 80 units, which would give tetramethyl glucose in sufficient quantities to be isolated considering the sensitivity of the method; therefore a closed chain structure must be envisaged. The authors consider that the isolation of a relatively large dimethyl fraction is in accord with this, the dimethyl glucose, whose structure was not proved, arising from cross linkages between short chains of glucose residues. On the other hand this yield of dimethyl glucose may be accounted for on the basis of incomplete methylation.

Although much work remains to be done concerning the fine structure of luteic acid, it is already apparent, by virtue of its being a combination between a glucan and an organic acid, that it is a polysaccharide unique in this field.

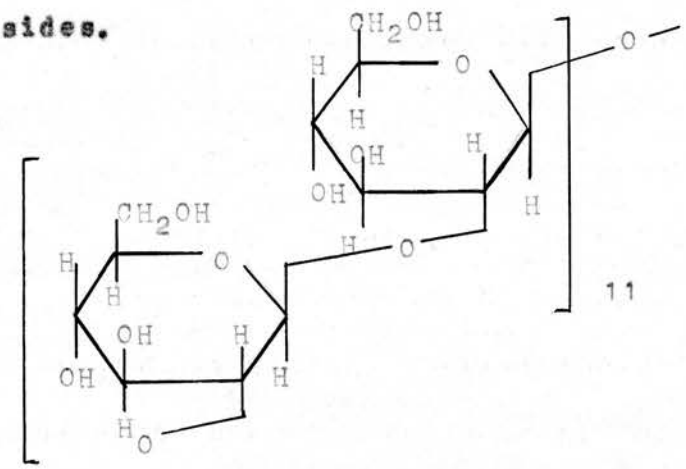


Luteose

Another interesting polysaccharide is that isolated from Phytomonas tumifaciens, the crown gall organism which produces an abnormal growth proliferation, a type of tumor, on plant cells of numerous types. The polysaccharide was investigated by McIntire, Peterson and Riker (10) who showed that glucose was the only product of hydrolysis. From diffusion and sedimentation-velocity studies, these authors conclude that the compound consists of a chain of 22 anhydroglucose residues.

Reeves (11) studied its optical behaviour in water and cuprammonium solution, and compared these results with those obtained from the four possible monomethyl -β-methylglucopyranosides. The optical activity of 2-methyl--β-methylglucopyranoside in water and cuprammonium so closely resembled that of the polysaccharide from Phytomonas tumifaciens that it is suggested that this polysaccharide is composed of glucopyranose units linked through the 1:2- positions.

This would appear to be a valid assumption, considering that the behaviour of a 1:3- linked compound, Laminarin, and 1:4- linked compounds, starch and cellulose, is similar to that of the corresponding substituted methylglucosides.



The Phytomonas tumifaciens polysaccharide.

The only fungal polysaccharide isolated up to the present which has been found to contain a uronic acid, is the specific polysaccharide obtained from Coccidioides immitis, and investigated by Hassid, Baker and McCready (12). It was prepared by precipitation in ethanol, and was estimated to contain 10.5% uronic acid. On hydrolysis with N sulphuric acid it gave D-glucose, D-galacturonic acid and an unidentified amino sugar, not glucosamine, in the approximate ratio 6:3:1. Both the original polysaccharide, and that regenerated from the acetyl compound, gave positive precipitin reactions, but only the former gave a positive skin reaction.

This review covers briefly the whole field of our present knowledge of the Fungal Polysaccharides. The great diversity of these compounds will be immediately obvious, both with regard to monosaccharide constituents, and to the variety in positions of linkage by which the units are bound together to form either simple straight chains, or complex branched structures. It is evident that a considerable gap in our knowledge exists here, which must be filled before we can have anything approaching a complete account of the naturally occuring polysaccharides. It would appear too, from past experience, that many new and interesting compounds await discovery, and it was with a view to exploring further this virtually untouched field in natural products chemistry, that the research described in Part II of this thesis was persued.

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EXPERIMENTAL

The fungus was obtained as a dry white powder by dehydrating the puff-ball in ethanol, and finally removing the ethanol in a vacuum oven. This sample of Lycoperdon bovista was collected in Chorlton-cum-Hardy, Manchester.

The Extraction of a Polysaccharide from the Dried Fungus.

Firstly, hot aqueous extraction was attempted. Dried powder (1 g.) was refluxed with water (25 ml.) for 12 hours. It was found necessary to add a few drops of capryl alcohol to act as wetting agent. The extract was filtered, and protein removed from the filtrate by the method of Sevag, Lackman and Smollens (1). Eight treatments were sufficient, using chloroform (10 ml.) and n-butanol (4 ml.) for each extraction. Nucleotides were removed from the solution by the method of Kerr and Blish (2), a method involving their precipitation by uranyl acetate. The polysaccharide was precipitated in ethanol and dried by tituration with ether. A cream coloured powder (14 mg.) was obtained, representing 1.4% of the dried fungus.

The polysaccharide gave a pale brown colour with iodine, and on hydrolysis glucose was the only sugar produced, as was shown by analysis on the paper chromatogram.

Secondly, a solution of sodium hydroxide (5%) was used for the extraction, and the above processes employed for the purification of the extract. A similar low yield of polysaccharide was obtained. This also gave only glucose on acid hydrolysis.

The most efficient extraction was obtained using the method developed by Bell(3) for the extraction of

glycogen from animal livers. The dried fungus (20 g.) was heated on a steam-bath for 3 hours with potassium hydroxide (300 ml.; 30%). The extract was filtered through cloth and the residue washed with water on the filter. The polysaccharide was precipitated by pouring the filtrate directly into 2.5 volumes of ethanol, and purified by re-precipitation, firstly in ethanol then in glacial acetic acid. After washing with absolute ethanol and drying with ether, polysaccharide (3.55 g.) was obtained. It was a white powder, soluble in water to give an opalescent solution. A further fraction of 0.5 g. of polysaccharide was obtained on addition of ethanol to the acetic acid mother liquor.

This total yield of 4.0 g. represents 20% of the fungus powder.

A further 25 g. of the powder was extracted with potassium hydroxide solution and 2.2 g. of pure polysaccharide obtained by precipitation in glacial acetic acid.

Aqueous extract found: N, 1.6%; ash, 2.1%.

Strong alkaline extract, found: N, 0.1%; ash, 3.8%.

The Determination of the Specific Rotation.

An aqueous solution being opaque, solutions in sodium hydroxide (0.5 n) were employed.

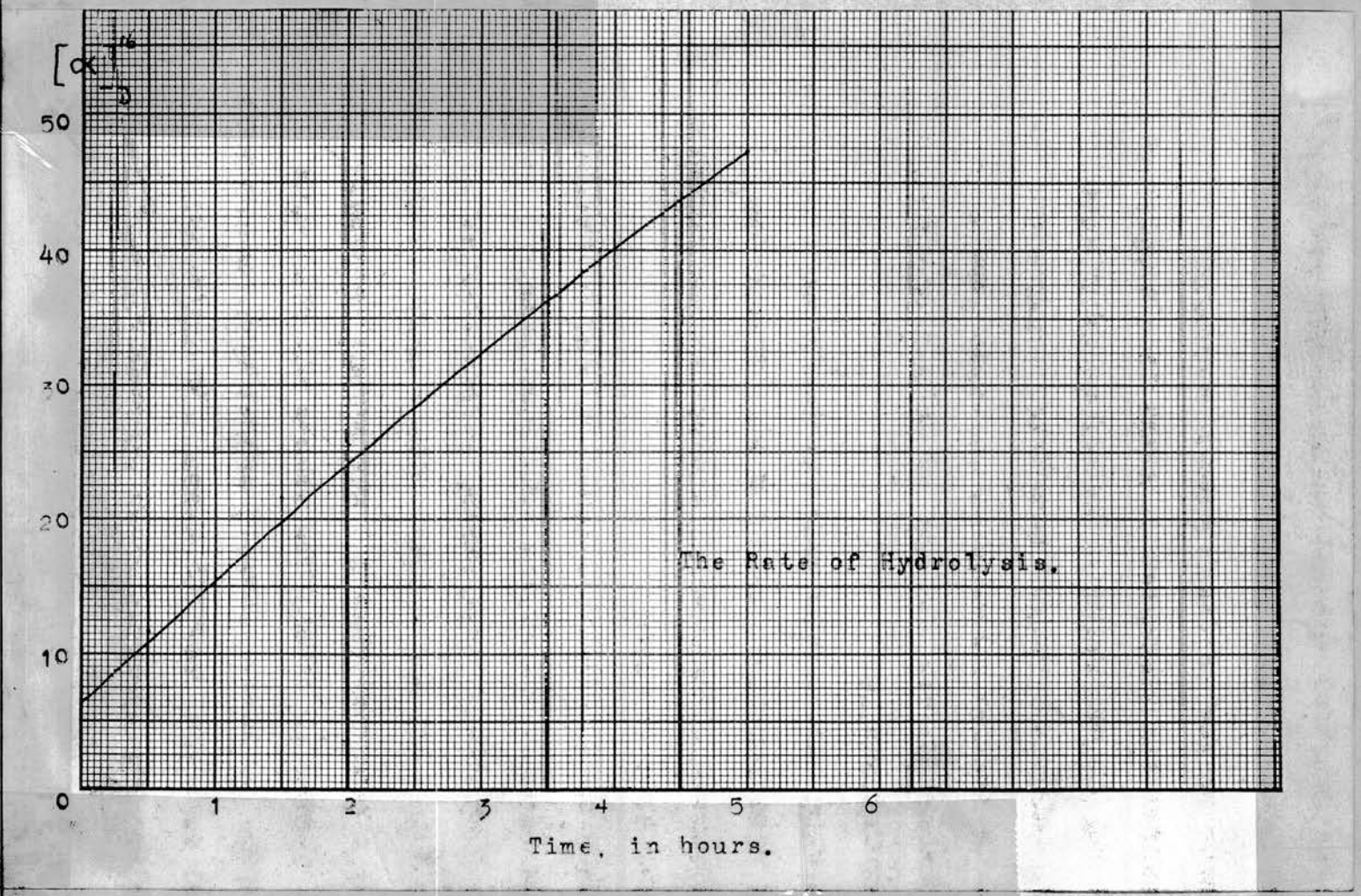
Aqueous extract, $[\alpha]_D = + 5.0^\circ$ (c=1.01)

1st. Strond alkaline extract, $[\alpha]_D = + 4.7^\circ$ (c=0.84)

2nd. Strong alkaline extract, $[\alpha]_D = + 3.4^\circ$ (c=1.04)

A sample of each of these fractions was hydrolysed with sulphuric acid (N) at 100° for 3 hours, and the neutralised hydrolysates analysed on the paper chromatogram.

Aqueous extract hydrolysate contained glucose with


$[\alpha]_D^{16}$
50
40
30
20
10
0
1
2
3
4
5
6
The Rate of Hydrolysis.
Time, in hours.

traces of arabinose and xylose.

1st Strong alkaline extract contained glucose only.

2nd Strong alkaline extract contained glucose only.

Colouration with Iodine.

A small sample of each of these extracted polysaccharides was dissolved in water (2–3 drops) on a glazed tile. On the addition of iodine solution a strong red-brown colouration developed, which faded in 10 minutes. A similar solution of glycogen gave an identical colour on the addition of iodine solution.

The Rate of Hydrolysis.

Polysaccharide (105.6 mg.; ash 3.8%) was hydrolysed with sulphuric acid (10 ml.; N) and the rotation observed at intervals. A graph of the increase in $[\alpha]_D$ with time is shown.

The Oxidation of this Polysaccharide by the Periodate Ion.

a) Estimation of the Formic acid Liberated.

As a preliminary experiment, polysaccharide (40.6 mg.; ash 3.8%) was weighed into a small bottle and potassium chloride (0.5 g.) and sodium periodate (3.0 ml.; approx. $\frac{M}{4}$) added. The volume was made upto 10 ml. and the oxidation carried out at room temperature, in diffused daylight, for 140 hours. A faint brown, iodine, colour was apparent after approximately 90 hours. Glycol (2 drops) was added and the acid titrated with standard sodium hydroxide ($\frac{n}{100}$).

1 mole. of formic acid was liberated from every 2.13 anhydroglucose residues.

In a repeat experiment, polysaccharide (57.2 mg.) was taken, together with potassium chloride (0.5 g.) and sodium periodate (3 ml.; $\frac{M}{4}$) and the volume made

upto 20 ml.. 2 ml. samples were extracted at intervals and after the addition of glycol, the acid present was titrated with sodium hydroxide ($\frac{n}{100}$). This oxidation was carried out in the dark. A blank experiment, omitting the polysaccharide, was run concurrently.

Sodium hydroxide = 0.010 N.

Duration of oxidation.	Alkali Titre.	No. of residues / mole. of formic.
42 hours	11.2 ml.	3.03
70 "	14.88 ml.	2.28
96 "	18.30 ml.	1.85
130 "	19.55 ml.	1.74
153 "	20.90 ml.	1.62
176 "	21.10 ml.	1.61

b) Estimation of the Uptake of Periodate.

Polysaccharide (approx. 45 mg.) was oxidised with sodium periodate (10 ml.; $\frac{M}{5}$) for varying lengths of time in the dark and at room temperature. The excess periodate was estimated by the addition of solid potassium iodide and titration of the liberated iodine with standard sodium arsenite ($\frac{n}{10}$).

Weight of Polysaccharide	Duration of Oxidation	Uptake in moles/$C_6H_{10}O_5$
48.8 mg.	17 hours	1.51
44.0 mg.	27 "	1.57
44.0 mg.	41 "	1.54

The Estimation of the Reducing Power.

The reducing power of this polysaccharide has been estimated by oxidation with sodium hypoiodite, using the method of Linderstrøm-Lang and Holter (4).

Polysaccharide (30–50 mg.) was dissolved in sodium carbonate solution (10 ml.; 0.4 n) and hydrochloric acid (2 ml.;0.4 n) added, giving a carbonate-bicarbonate buffer of pH 10.6. Iodine solution (3 ml.;0.1 n) was added from a microburette and the flasks set aside in the dark for a predetermined length of time. A blank experiment was made up exactly the same except for the polysaccharide. After acidifying with sulphuric acid (2 n) the iodine was titrated with sodium thiosulphate ($\frac{n}{50}$).

Weight of Polysaccharide	Duration of Oxidation	No. of residues /reducing group.
24.2 mg.	30 minutes	40
24.0 mg.	1 hour	33
32.8 mg.	2 hours	48
40.6 mg.	1 hour	33
51.6 mg.	1 hour	48

Average value: 40 anhydroglucose residues per reducing group.

The Extraction of Sample II of Fungal Polysaccharide.

An unripe fruit-body of Lycoperdon bovista, collected late in August 1948 in Linton Parish, Roxburghshire, (900 g.) was peeled and minced and extracted with potassium hydroxide (1200 ml.; 30%) on a steam bath for 3 hours. The solid residue was filtered off on cloth and washed with a little hot water, and the filtrate poured directly into 3 volumes of ethanol. The precipitate was allowed to settle and the mother liquor decanted. The solid was taken up into the minimum of water, and re-precipitated in ethanol. This process was then repeated three times, using glacial acetic acid for the precipitation. The white, flocculent solid was separated on the centrifuge and titurated with

water and some solid impurity removed on the centrifuge. The pure polysaccharide was obtained by precipitation out of this solution by ethanol, dried by washing on the centrifuge with absolute ethanol, and finally removal of the solvent in a vacuum desiccator. 2.65 g. of a fine white powder were obtained. Ash = 2.0%.

$[\alpha]_D$ = + 7.5° (c=1.65 in 0.5 n NaOH)

The Rate of Hydrolysis.

The rate of hydrolysis of this second sample of fungal polysaccharide with sulphuric acid (N) was estimated by polarimetric observation, and found to be complete in 3–5½ hours at 100°. This result is the same as that found for the first sample of fungal polysaccharide.

The Oxidation by the Periodate Ion.

a) The Estimation of the Formic acid Liberated.

Polysaccharide (50.8 mg.) was weighed into a small bottle, potassium chloride (0.5 g.) added followed by sodium periodate solution (3 ml.; $\frac{M}{4}$) and the volume was made upto 20 ml. The bottle was left in the dark at room temperature, and occasionally shaken. Samples (2 ml.) were removed at intervals, glycol (2 drops) added, and the acid titrated with sodium hydroxide ($\frac{n}{100}$) using methyl red indicator. A blank experiment, omitting the polysaccharide, was run concurrently.

The formic acid liberated was equivalent to 25.8 ml. 0.9172 $\frac{n}{100}$ sodium hydroxide, i.e. 1 mole. of formic is released per 1.43 anhydroglucose residues.

b) The Estimation of the Uptake of Periodate.

Polysaccharide (52 mg.) was oxidised with sodium periodate (10 ml.; $\frac{M}{8}$). Samples were withdrawn at intervals and the iodine liberated on the addition of solid

potassium iodide titrated with standard sodium arsenite ($\frac{n}{10}$).

Duration of Oxidation	Uptake in Moles/$C_6H_{10}O_5$
26 hours	1.27
45 "	1.35
67 "	1.42
99 "	1.43

DISCUSSION

At the commencement of research in this new field of the naturally occuring plant polysaccharides, an investigation was undertaken into those compounds occuring in the fungus Lycoperdon bovista. A polysaccharide has been extracted from the unripe fruit bodies of this fungus, which are, at this time, white in colour and downy to the touch. The interior of the puff-ball consists of a white spongy mass of tissue, the spores not having as yet been formed.

The fruit bodies were dried and powdered, and three methods were tried for the extraction of the polysaccharide. Both aqueous and dilute alkaline extraction gave poor yields of carbohydrate material, but on treatment of the dried fungus with potassium hydroxide in 30% aqueous solution, a good yield of polysaccharide was obtained. Bell (3) found that glycogen was unaffected by the strong alkali; and as the rotations of the materials extracted by water and potassium hydroxide are identical it would appear that this polysaccharide, too, is not affected by the alkali.

The polysaccharide is a cream coloured powder, which gives an opalescent solution in water, but a clear one in dilute sodium hydroxide. The rotations of the three samples in this solvent vary between +3.4° and 5.0°. The discrepancy may be ascribed to the difficulty in obtaining an accurate reading of the polarimeter when the observed rotation is so low. In aqueous solution the polysaccharide gives a strong red-brown colouration with iodine, which is identical with that given by glycogen.

The rate of hydrolysis has been studied, and the uninflected line obtained for increase in $[\alpha]_D$ with time suggests that the polysaccharide is homogeneous.

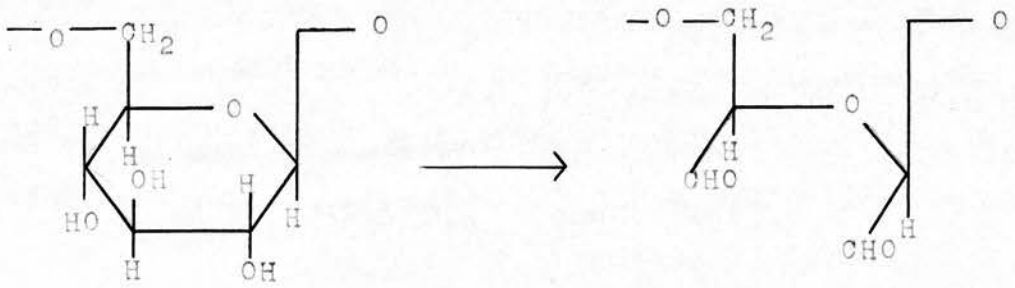
The acid hydrolysates of the three samples have been analysed on the paper chromatogram. The aqueous extract shows traces of arabinose and xylose, which would not be detectable by any other method, and which are removed by further precipitation; otherwise glucose is the sole product of hydrolysis.

The polysaccharide has been oxidised on a semi-micro scale with potassium periodate, and the course of the liberation of formic acid observed. In the first experiment free iodine was released, which according to Halsall, Hirst and Jones (5) will give a low value for the yield of formic acid. The second experiment was carried out in the dark, and the reaction proceeded normally. The results show that when oxidation is complete the amount of formic acid that has been released is equivalent to 1 mole. per 1.61 anhydroglucose residues.

On estimating the uptake of periodate by the polysaccharide, it was found that 1.54 moles. were consumed per residue.

These results would be obtained with two different types of polysaccharide structure. The molecule can be either highly branched, containing a large proportion of end group, or composed of anhydroglucose residues linked in a variety of ways.

The polysaccharide must contain some 1:6- linkages, as this type of linked residue is the only one that can give rise to formic acid on periodate oxidation.



If the polysaccharide consisted solely of units linked in this manner, 1 mole. of formic acid would be liberated per residue on oxidation with potassium periodate, but as this is not the case 1:2-, 1:3-, or 1:4- linkages must be present also. These linkages do not allow of the system of three contiguous hydroxyl groups necessary for the liberation of formic acid.

The reducing power of this polysaccharide has been estimated by treatment with sodium hypoiodite in a buffered solution at pH 10.6. The reactions were carried out on a semi-micro scale and a series of moderately consistent results were obtained. Allowing for a maximum experimental error of 6%(1 drop,0.04 ml. in a titration difference of 0.65 ml.) a value of 38-42 anhydroglucose residues per reducing group is obtained.

The uptake of iodine appears to be independent of the time of oxidation, and we may therefore reasonably conclude that the reaction is proceeding normally, and that the value obtained is a true one. The wide variation in the results may be attributed to the small quantities of polysaccharide used. The method is known to be very accurate for the estimation of monosaccharides (6) but it is doubtful whether a high degree of accuracy can be expected in this instance, as the polysaccharide itself reacts with iodine, giving a red-brown colouration.

A second sample of this polysaccharide has been extracted from a fresh unripe fruit body of the fungus by strong aqueous alkali, and on purification by re-precipitation the rotation was found to be identical with that of the first sample. On estimating the rate of hydrolysis with the same strength of acid and at the same temperature as used for the first sample, a similar result was obtained. Hydrolysis was complete in 5 hours, and glucose was the only sugar detected in the neutralised hydrolysate, using the paper chromatogram. From the two facts of low positive rotation and difficulty of hydrolysis, we may conclude that the anhydroglucose residues are linked in the β-configuration.

Periodate oxidation, both for the estimation of the formic acid released and for the consumption of periodate ion, have shown similar results to those obtained from the first sample of polysaccharide. We may therefore conclude that these two samples, extracted from different batches of fungus which may have grown in conditions vary far from identical, have a similar, if not the same, molecular structure.

SUMMARY

1) A polysaccharide has been extracted from the unripe fruit-bodies of the fungus Lycoperdon bovista. It is an amorphous powder, having $[\alpha]_D$ +5.0° in dilute alkali. It is completely hydrolysed by sulphuric acid (N) in 5 hours at 100°. In aqueous solution, a colouration is given with iodine which is identical with that given by glycogen.

2) Estimation of the reducing power by hypoiodite oxidation gave a value of 40 anhydroglucose residues per reducing group.

3) Oxidation by the periodate ion indicated either a very highly branched structure for the polysaccharide, or one containing a proportion of 1:6- linkages.

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EXPERIMENTAL

The Methylation Of The Fungal Polysaccharide.

This polysaccharide was methylated directly by the thallium method. Polysaccharide (1.736 g.) was dissolved in dilute sodium hydroxide (50 ml.; 0.5 n) and thallous hydroxide (35 ml.; 1.5 n) added. The heavy white precipitate of the polysaccharide-thallium complex was separated on the centrifuge, and, when tested, it was found to be appreciably soluble in water. The clear liquor from the centrifuge was made 50% with respect to ethanol, and the resulting precipitate centrifuged. It gave a positive Molish reaction, and therefore the two precipitates were combined, washed with absolute ethanol on the centrifuge and dried in a vacuum desiccator in the dark.

The complex (6.37 g.) was gently refluxed with methyl iodide (30 ml.) for 64 hours when the methyl iodide was distilled off under atmospheric pressure, leaving a bright yellow solid. This was extracted with boiling chloroform (4 times; 75 ml.) and a clear pale yellow solution obtained after filtration. Evaporation of the solvent at 35°/15 mm. gave a pale brown solid (0.422 g.).

The yellow material from the methylation was further extracted with hot water (4 times; 75 ml.) and the aqueous extracts, after filtration, taken down to dryness, leaving a pale brown friable solid (1.60 g.).

The extracted, partly methylated polysaccharide (2.022 g.) in aqueous solution (60 ml.) was again treated with thallous hydroxide (35 ml.; 1.5 n). As no precipitate was obtained the solution was taken down to

dryness at 40°/15 mm. and the residual solid dried with absolute ethanol and benzene. This solid, after powdering, was treated with methyl iodide (40 ml.) under reflux for 62 hours. The methyl iodide was distilled and the yellow solid extracted with chloroform (4 times; 75 ml.). On distillation of the solvent, partially methylated polysaccharide (1.88 g.) remained. The yellow solid was further extracted with hot methanol, when another 0.2 g. of partially methylated compound was obtained. No partially methylated polysaccharide could be extracted from the yellow solid by hot water.

These extracts of partially methylated polysaccharide (2.08 g.) were dissolved in an equiproportional mixture of absolute ethanol and benzene (100 ml.) and thallium ethoxide (5 g.) added slowly with shaking. The solution was evaporated to dryness, and the resulting dark brown solid powdered and refluxed with methyl iodide (50 ml.) for 80 hours. The methyl iodide was distilled and the partially methylated polysaccharide extracted as before with hot chloroform, when 1.97 g. were recovered.

This extract was treated under reflux with methyl iodide (40 ml.) containing a little acetone. An appreciable quantity of the solid did not go into solution, and this was filtered off and dried in a vacuum desiccator (1.42 g.). That portion of the compound which was in solution in the methyl iodide-acetone mixture was methylated over 18 hours by the addition of silver oxide (15 g.) and isolated in the usual manner (0.51 g.)

The remainder of the partially methylated

polysaccharide was given a further treatment with thallium ethoxide in ethanol-benzene solution as above. After refluxing with methyl iodide (40 ml.) for 60 hours the product was recovered by extraction in chloroform, (1.35 g.). It was found to be insoluble in hot methanol, and in pure methyl iodide. It was therefore taken up into the minimum of chloroform (7 ml.) and methyl iodide added until a precipitate just appeared, (30 ml.) Silver oxide (20 g.) was added at intervals while the mixture was refluxed for 24 hours. The partially methylated compound was recovered by extraction of the residue with hot chloroform, (1.34 g.).

This material, together with that which had previously been treated with Purdie's reagents, were combined in chloroform solution, centrifuged to remove some solid matter, and the solvent evaporated at 35°/15mm. leaving a brown solid of waxy appearence (1.79 g.). This was purified by extraction of the impurities in boiling light petroleum (40°), when a readily friable, pale buff coloured solid remained (1.38 g.; OMe = 39.0%).

This incompletely methylated polysaccharide, which was still insoluble in pure methyl iodide, was extracted with boiling acetone, in which it was only partially soluble. The insoluble portion, when dried, had OMe = 42.2% Fraction I (0.691 g.).

The solid extracted in the acetone was given two consecutive treatments with Purdie's reagents, then purified by extraction with light petroleum as before, and had OMe = 41.0%, Fraction II (0.388 g.).

Since the ash contents of these two fractions were so low as to have no effect on the methoxyl values,

they were re-methylated by Purdie's method. Fraction I was first treated with chloroform (2 ml.) giving a thick syrup to which methyl iodide was added (30 ml.), followed by silver oxide (5 g.) added at intervals during the 24 hours refluxing. Fraction II was treated in pure methyl iodide (20 ml.) in which it was completely soluble. Each sample was given a total of five methylations by this method, after the fractionation by acetone. Fraction I remained almost insoluble in pure methyl iodide. They were finally purified as before by extraction with boiling petroleum (40°), and had the following methoxyl constants:

Fraction I	0.62 g.	OMe = 42.2%
Fraction II	0.35 g.	OMe = 41.0%

The Methylation of the Second Sample of Fungal Polysaccharide

In this instance the method employed was direct treatment of the polysaccharide with sodium hydroxide and dimethyl sulphate in an atmosphere of nitrogen.

Polysaccharide (2.2 g.) was dissolved in aqueous sodium hydroxide (100 ml.; 30%) and nitrogen passed into the vigorously stirred solution while dimethyl sulphate (25 ml.) was added dropwise during 3 hours. The temperature was maintained at 40° by means of an external water bath. The stirring was continued for 6 hours, when further aqueous sodium hydroxide (45 ml.) was added, followed by dimethyl sulphate (25 ml.). In all, seven such treatments were given with these quantities of reagents and in the same flask.

After the seventh methylation the partially methylated polysaccharide separated from the solution as an amorphous precipitate. The alkali was neutralised

with sulphuric acid (30%) and the precipitated sodium sulphate was filtered and thoroughly extracted with hot chloroform. The neutral filtrate was evaporated to dryness at 40°/15 mm. and the residual solid dried with absolute ethanol and benzene, and then extracted with hot chloroform. The chloroform extracts were cooled to deposit sodium methyl sulphate, filtered, and evaporated to dryness, leaving a friable solid (2.40 g.)

This material which was insoluble in methyl iodide was extracted with boiling acetone, in which it was only partially soluble. A fractionation was thus achieved on the same basis as for the first sample of polysaccharide.

Fraction Y Acetone insoluble 1.751 g. OMe = 39.1%

Fraction Z Acetone soluble 0.630 g. OMe = 36.0%

Each fraction was given three consecutive treatments with Purdie's reagents. Fraction Y was first transformed into a thick syrup by the addition of chloroform (2–3 ml.) followed by methyl iodide (30–35 ml.) until precipitation just occured. Fraction Z was soluble in pure methyl iodide. The compounds were recovered from the methylation mixture in the usual manner.

Fraction Y 1.772 g. OMe = 40.3%

Fraction Z 0.454 g. OMe = 41.1%

A further three treatments with methyl iodide and silver oxide were given to each Fraction and under the same conditions as the above.

Fraction Y 1.734 g. OMe = 41.0%

Fraction Z 0.407 g. OMe = 41.5%.

<u>The Determination of the Rotations of these Samples of Methylated Polysaccharide.</u>

a) Sample I Fraction I (Acetone insoluble)

$[\alpha]_D = -9.3°$ (c = 1.29 in chloroform)

Fraction II (Acetone soluble)

$[\alpha]_D$ = +33.8° (c = 1.6 in chloroform)

b) Sample II Fraction Y (Acetone insoluble)

$[\alpha]_D$ = -3.4° (c = 1.8 in chloroform)

Fraction Z (Acetone soluble)

$[\alpha]_D$ = +17.7° (c = 2.6 in chloroform)

Sample II was re-fractionated with boiling acetone and the extracts taken to dryness to give Fraction Z_1, the residue being dried in a vacuum desiccator, Fraction Y_1.

Repeated Determination of the Rotation.

Sample II Fraction Y_1

$[\alpha]_D$ = -3.2° (c = 1.88 in chloroform)

Fraction Z_1

$[\alpha]_D$ = +14.1° (c = 1.36 in chloroform)

The Determination of the Molecular Weights by Viscosity.

A closed Ostwald's viscometer was used, and the determinations were carried out in a thermostat bath at 20°. A 2% solution in chloroform was employed.

$$\eta_{sp} = \frac{t - t_1}{t_1}$$

where t = time of flow of solution, in seconds.

and t_1 = time of flow of solvent, in seconds.

η_{sp} is known as the Specific Viscosity.

Sample I	Fraction I	η_{sp} = 0.238
	Fraction II	η_{sp} = 0.269
Sample II	Fraction Y_1	η_{sp} = 0.538
	Fraction Z_1	η_{sp} = 0.597

The Determination of the Constant K_m for a Chloroform Solution.

The molecular weight of Laminarin has been determined by viscosity measurements in m-cresol solution (1) using the constant $K_m = 10^{-5}$ for molecules of weights between 1,000 and 20,000, in the Staudinger equation:

$$\frac{\eta_{sp}}{C} = K_m M$$

where C = gram moles. per litre

and M = molecular weight of the methylated compound.

For a 2% chloroform solution of trimethyl laminarin η_{sp} = 0.261, and the molecular weight of trimethyl laminarin is known (1) to be 3120. Substituting in the equation above, we obtain the constant K_m for a molecule of this magnitude:

$$8.54 \times 10^{-4}$$

Using this constant we obtain the following values for the molecular weights of the fractions of fungal polysaccharide.

Sample I	Fraction I	2,270 or 14	anhydroglucose residues
	Fraction II	2,560 or 16	" "
Sample II	Fraction Y_1	5,100 or 31	" "
	Fraction Z_1	5,700 or 35	" "

The Hydrolysis of Sample I of Methylated Polysaccharide.

1) Fraction I (610 mg.) was treated under reflux with methanolic hydrogen chloride (60 ml.; 5.0%) in which it was insoluble, for 28 hours. The concentration of hydrogen chloride was maintained at approximately 5% by the addition, at intervals, of a 20% solution. The hydrolysate was centrifuged to remove the large quantity of insoluble material (Fraction Ia) and the supernatant liquor neutralised and evaporated to leave a syrup (344 mg., Fraction Ib). Examination of Fraction Ib on a paper chromatogram showed it to contain a large proportion of unhydrolysed material, and three sugars of R_G values 0.83, 0.654, and 0.517.

Both Fractions Ia and Ib were combined and further hydrolysed with formic acid (30 ml.; 50%) in which Ia was

immediately soluble, at 100°, until the rotation became constant. Three hours heating was found to be sufficient. The bulk of the formic acid was distilled off at 40°/15 mm. and the remaining acid removed by the continual addition and distillation of water. The syrup was finally dried by the addition of absolute ethanol and benzene, followed by the distillation of the solvents.

Examination on the paper chromatogram showed the syrup to be contaminated with inorganic ions, which were removed by treating an aqueous solution of the syrup with "Amberlite" ion exchange resins IR 100 and IR 4B. The pure syrup of reducing sugars (Fraction A, 510 mg.) was obtained on evaporation of the water and drying with absolute ethanol and benzene. Fraction A was found, by examination on the paper chromatogram, to contain five different methylated derivatives of glucose having the following R_G values: a) 1.00, b) 0.855, c) 0.775, d) 0.658, e) 0.54.

2) Fraction II (340 mg.) was treated in exactly the same way as Fraction I. It was found to be incompletely hydrolysed by methanolic hydrogen chloride and was therefore hydrolysed directly to the free sugars by formic acid (90%). Six hours heating at 100° was necessary before the rotation became constant. The formic acid was removed as before, and after final purification with ion exchange resins, a syrup (Fraction B, 300 mg.) was obtained. This was shown to contain five methylated derivatives of glucose having the same R_G values as those in Fraction A.

The Hydrolysis of Sample II of Methylated Polysaccharide.

Fraction Y_1 (1.734 g.) was hydrolysed directly

with formic acid (120 ml.; 90%) at 100° until the rotation became constant (3 hours). The acid was removed as previously and after purification with ion exchange resins, a colourless syrup was obtained (Fraction C, 1.27 g.).

Fraction Z_1 was treated exactly similarly to give Fraction D (351 mg.).

Fractions C and D were found, by qualitative analysis on the paper chromatogram, to contain the same five methylated derivatives of glucose as had been found in Fractions A and B.

The Separation of these Glucose Derivatives by Partition Chromatography on a Column of Powdered Cellulose.

The technique and apparatus used are exactly as described for the separation of the trimethyl and tetramethyl components of the mixture of methylated fructoses obtained on hydrolysis of trimethyl inulin, and described in Part I of this thesis (Page 39ff).

Fraction C (1.27 g.) was separated into its components by partition chromatography on the cellulose column, using, as solvent, a mixture of light petroleum (100-120°, 70%), n-butanol (30%) saturated with water.

The contents of those tubes containing the same sugars were combined and evaporated, and the syrup so obtained was purified free from grease by the extraction of the sugar in hot water followed by the addition of a little "Filter Cel." On evaporation of the filtered solution a chromatographically pure sample of each sugar was obtained, which was estimated gravimetrically.

Tetramethyl (crystalline)	86.0 mg.	OMe = 46.0%	R_G = 1.00
Trimethyl I (syrup)	328.8 mg.	OMe = 38.9%	R_G = 0.855
Trimethyl II(crystalline)	162.6 mg.	OMe = 33.5%	R_G = 0.775
Dimethyl I (syrup)	156.2 mg.	OMe = 25.9%	R_G = 0.658
Dimethyl II (syrup)	224.0 mg.	OMe = 29.6%	R_G = 0.540
Recovery	1157.6 mg.		
	= 91.0%.		

Weight of sugar in each Fraction (corrected by methoxyl values).

Tetramethyl	75.4 mg. or	7.1%
Trimethyl I	490.0 mg. or	46.3%
Trimethyl II	138.0 mg. or	13.0%
Dimethyl I	135.4 mg. or	12.8%
Dimethyl II	220.0 mg. or	20.8%

The Quantitative Estimation of these Components by the Paper Chromatogram Method.

The method used was a modification of that due to Hirst, Hough and Jones (2). The whole of each fraction A, B and D was dissolved in a little water, and this solution spotted on to the quantitative chromatogram using the butanol-ethanol-water solvent. The absolute weights of the various derivatives were not calculated, but the uptake of iodine by each component was used to give an estimate of the relative proportions of the five derivatives present, taking the tetramethyl component as 1.00.

The sodium thiosulphate used was approx. $\frac{n}{100}$

$\triangle$ = The titration difference between a sugar strip from the chromatogram and a paper blank of identical size.

Fraction A.

Tetramethyl	Δ = 0.23 ml.	=	1.00	= 7.7%
Trimethyl I	Δ = 1.46 ml.	=	6.35	= 48.8%
Trimethyl II	Δ = 0.54 ml.	=	2.34	= 17.9%
Dimethyl I	Δ = 0.45 ml.	=	1.95	= 14.2%
Dimethyl II	Δ = 0.48 ml.	=	2.08	= 15.1%

An average of three estimations on Fraction (A) gave the following values:

Tetramethyl	7.8%
Trimethyl I	48.0%
Trimethyl II	18.3%
Dimethyl I	9.8%
Dimethyl II	16.1%

Fraction B

An average of three estimations gave:

Tetramethyl	7.5%
Trimethyl I	40.6%
Trimethyl II	18.5%
Dimethyl I	11.8%
Dimethyl II	21.7%

Fraction D

An average of three estimations gave:

Tetramethyl	10.0%
Trimethyl I	40.0%
Trimethyl II	23.0%
Dimethyl I	8.5%
Dimethyl II	18.8%

The identification of the Methylated Sugars.

a) The tetramethyl fraction isolated form the paper column was recrystallised from light petroleum (40°). The long colourless needles had m.p. 92°, and a mixed

melting point with an authentic sample of 2:3:4:6-tetramethyl glucose was unchanged.

Anilide Formation.

The tetramethyl sugar (66.0 mg.) was treated under reflux at 80° with freshly distilled aniline (1 mole.; 23 mg.) in ethanolic solution (5 ml.) for 3 hours. The solvent was removed in a vacuum desiccator and the anilide recrystallised from light petroleum. After two recrystallisations it had m.p. 134-5,° a mixed melting point with an authentic sample of 2:3:4:6-tetramethyl glucose anilide was unchanged.

b) Trimethyl I.

Oxidation with Periodic acid,(3).

The trimethyl sugar (30.0 mg.) was dissolved in water (2 ml.) and sodium bicarbonate (2ml. of N) added, followed by periodic acid (2 ml. of 0.3M). The solutions were mixed and allowed to stand at room temperature for 1½ to 2 hours. Hydrochloric acid (3 ml. of N) and sodium arsenite (2 ml. of N) were added, and when the precipitate and iodine colour had completely disappeared sodium acetate (2 ml. of N) and dimedone reagent (1 ml. of a solution containing 85 mg./ml. of 95% ethanolic solution) were added. A fine precipitate appeared immediately and the mixture was allowed to stand overnight at room temperature to complete the precipitation. A control experiment, using pure glucose, was run concurrently. The precipitated dimedone-formaldehyde complex was filtered on a tared gooch crucible, dried for 30 minutes and weighed.

The control gave a 47% yield of formaldehyde, while the test experiment gave a yield of 6% of the theoretical.

Optical Behaviour in Methanolic Hydrogen Chloride.

Trimethyl I (8.2 mg.) was dissolved in methanolic hydrogen chloride (0.5 ml.; 2.0%) and the rotation observed at hourly intervals.

Over 24 hours $[\alpha]_D^{15}$ fell from +73.2° to +71.0°.

Anilide Formation.

Trimethyl I (106 mg.) in ethanolic solution (6 ml.) was refluxed for 3 hours at 80° with freshly distilled aniline (49.4 mg.). Some insoluble impurities were removed by filtration and the solvent evaporated in a vacuum desiccator. The crystals obtained were recrystallised from ether-petroleum ether, m.p. 142-3°.

Found: C, 60.5; H, 7.6; N, 4.6; OMe 31.9%.

Calc. for $C_{15}H_{24}O_5N$: C, 60.6; H, 7.8; N, 4.7; OMe 31.3%.

c) Trimethyl II.

This fraction was recrystallised from a mixture of ether-petroleum ether when fine white needles were obtained, m.p. 123-5°. Mixed melting point with authentic 2:4:6-trimethyl glucose: 124°.

The recrystallised sugar (6.6 mg.) dissolved in water (514.6 mg.) was examined at intervals on the polarimeter. $[\alpha]_D^{16}$ +100° (initial), +73.5° (constant in 19 hours)

Oxidation with Periodic Acid.

Using the method as described above, 20.6 mg. of this sugar gave no precipitate of a dimedone-formaldehyde complex.

Anilide Formation.

The recrystallised sugar (19 mg.) in absolute ethanolic solution (3 ml.) was heated under reflux for 3 hours at 80° with redistilled aniline (2 moles.; 18 mg.). The solvent was removed in a vacuum desiccator and the

needles recrystallised three times from ether -light petroleum, m.p. 154-6°.

2:4:6-trimethyl glucose anilide was prepared on a somewhat larger scale (100 mg.), from the authentic sugar, using 2 moles. of aniline as above. After six recrystallisations, m.p. 165-8°; mixed melting point with the above sample prepared from the polysaccharide hydrolysate: 156-7°.

$[\alpha]_D^{15}$ -136.8° (initial), +40.4° (constant 26 hours)

c = 1.04 in methanol.

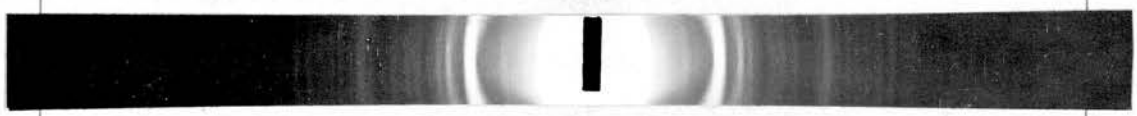
Found: C, 59.9; H, 7.75; N, 4.75; OMe 30.9%.

Calc. for $C_{15}H_{24}O_5N$: C, 60.6; H, 7.8; N, 4.7; OMe 31.3%.

X-ray Powder Photograph.

The prints of X-ray powder photograph negatives given by Trimethyl II and an authentic sample of 2:4:6-trimethyl glucose are shown. It is evident that the lines on both the photographs correspond, and therefore the two samples are assumed to be identical.

2:4:6-trimethyl glucose



"Trimethyl II"

d) Dimethyl I.

Oxidation with Periodic Acid.

Using the same method as was described for the oxidation of Trimethyl I, 24.4 mg. of this dimethyl sugar gave a 41% yield of formaldehyde as estimated by

the dimedon complex.

Optical Behaviour in Methanolic Hydrogen Chloride.

Dimethyl I (10.8 mg.) was dissolved in methanolic hydrogen chloride (0.6 ml.; 2.0%) and the rotation observed at hourly intervals.

$[\alpha]_D^{17}$ +48.9° (initial), +66.7° (constant 12 hours).

The Preparation of the Amide.

Dimethyl I (80 mg.) was dissolved in water (8 ml.) excess bromine (5 ml.) added, and the mixture allowed to stand at room temperature, with occasional shaking, for 3½ days. The bromine was removed by aeration and the solution neutralised with silver carbonate, silver ions in the solution being removed as the sulphide. On evaporation of the water at 40°/15 mm. a syrup remained which was dried with absolute ethanol and benzene, and taken up in methanol saturated with ammonia (15 ml.) and allowed to stand at 0° for 5 days. The solvent was distilled under diminished pressure and the residual syrup extracted with ether-light petroleum. On removal of this solvent a viscous syrup remained (66 mg.)

Weerman Reaction (4).

The amide (20.6 mg.) was dissolved in water (0.2 ml.) and sodium hypochlorite solution (0.4 ml.) added. The mixture was allowed to stand at 0° for 3 hours. Six drops of a saturated solution of sodium thiosulphate were added and the solution saturated with sodium acetate, filtered, and the flask and filter washed out with a saturated solution of semicarbazide hydrochloride (2 ml.) The mixture was allowed to stand overnight at 0° to complete the precipitation. A control experiment on gluconamide was run concurrently, using 26.8 mg.

The control gave 7.4 mg. of hydrazodicarbonamide. The amide under investigation gave a negative reaction.

The Estimation of the Uptake of Periodate Ion.

Dimethyl I (20.2 mg.) was dissolved in water (1.5 ml.) and sodium metaperiodate solution (2 ml.; $\frac{M}{4}$) added and the mixture allowed to stand at room temperature for 11 hours. Solid sodium bicarbonate and potassium iodide were added and the liberated iodine titrated with standard sodium arsenite.

Sodium arsenite = 0.926 $\frac{n}{10}$

The periodate uptake was 0.41 moles. per $C_8H_{16}O_6$

The estimation was repeated using 13.1 mg. sugar and allowing 48 hours for the reaction to go to completion. The periodate uptake was then 0.75 moles. per $C_8H_{16}O_6$.

e) Dimethyl II.

Oxidation with Periodic Acid.

The reaction was carried out exactly as for the other partially methylated monosaccharides. 27.9 mg. of this sugar gave a 49% yield of formaldehyde estimated by the dimedone complex.

Optical Behaviour in Methanolic Hydrogen Chloride.

Dimethyl II (8.0 mg.) was dissolved in methanolic hydrogen chloride (0.5 ml.; 2.0%) and the rotation observed at hourly intervals.

$[\alpha]_D^{17}$ + 57.5° (initial), +37.5° (constant 12 hours).

The Preparation of the Amide.

The amide was prepared exactly as for "Dimethyl I". Dimethyl II (196 mg.) was oxidised with excess bromine to the dimethyl gluconic acid, which was taken up into methanol saturated with ammonia and allowed to react at 0° for 5 days. The amide was obtained as a viscous

syrup (90.0 mg.) on distillation of the solvent.

Weerman Reaction.

The syrupy amide (25.0 mg.) gave a 7.5% yield of hydrazodicarbonamide. A control test on gluconamide (26.0 mg.) gave a 50% yield of this compound.

The Estimation of the Uptake of Periodate Ion.

This was run simultaneously with the estimation on "Dimethyl I" Dimethyl II (24.4 mg.) was dissolved in water (1.5 ml.) and sodium periodate (2 ml.; $\frac{M}{4}$) added and the reaction allowed to proceed for 11 hours, after which time solid sodium bicarbonate and potassium iodide were added and the liberated iodine titrated with standard sodium arsenite (0.926 $\frac{n}{10}$).

The periodate uptake was 0.99 moles. per $C_8H_{16}O_6$

This estimation was repeated using 13.9 mg. sugar and allowing 48 hours for the reaction.

The uptake was then 1.70 moles. per $C_8H_{16}O_6$.

Estimation of the Amount of Demethylation Caused by Subjecting 2:3:4-trimethyl glucose to the Hydrolytic Procedure as used for the Methylated Polysaccharide.

2:3:4-trimethyl glucose (Trimethyl I, 77.5 mg.) was heated with formic acid (7 ml.; 90%) on a boiling water bath under reflux for 4 hours. The acid was removed in a vacuum desiccator and the residual syrup taken up in a little water and the solution examined on the paper chromatogram. No dimethyl compounds were detected, but another derivative had been formed giving a spot of R_g value 0.96. On heating the slightly acidic aqueous solution on a boiling water bath for 2 hours and again running a qualitative paper chromatogram, this spot was no longer apparent. Again no dimethyl compounds could be detected.

DISCUSSION

The polysaccharide extracted from the first sample of *Lycoperdon bovista* was methylated by four treatments of the thallium complex with methyl iodide. This is a method which has been found to be particularly effective for the primary methylation of complex polysaccharides (5) giving a partially methylated compound soluble in methyl iodide so that the methylation can be completed by Purdie's method. In the present work the partially methylated compound was found to be insoluble in pure methyl iodide, but was fractionated into two portions depending on solubility in hot acetone. That fraction which was soluble in acetone was also found to be soluble in pure methyl iodide and its methylation then proceeded normally. That fraction which was insoluble in acetone was insoluble in pure methyl iodide and its further methylation was carried out by the addition of silver oxide to the solution in methyl iodide-chloroform.

Five consecutive treatments with Purdie's reagents failed to raise the methoxyl contents of either of these fractions from 42.2% for the insoluble portion (Fraction I); and 41.0% for the soluble portion (Fraction II). It is not clear why Fraction I should remain insoluble while Fraction II is soluble, as it has the higher methoxyl content and viscosity measurements show it to be apparently the smaller molecule. It is clear that a separation of at least two components has been achieved by this extraction with hot acetone, Fraction I having a negative rotation (-9.3°) while that of Fraction II is positive (+33.8°).

That polysaccharide which was obtained by the

extraction of the second sample of Lycoperdon bovista was methylated firstly by treatment with dimethyl sulphate in strong sodium hydroxide solution, and in an atmosphere of nitrogen. Seven consecutive treatments with double quantities of reagents were given in the same flask, in order to cut down losses to a minimum; only one extraction of partially methylated polysaccharide from sodium sulphate was then necessary. After the seventh treatment the partially methylated compound separated from the solution as an amorphous precipitate.

This partially methylated polysaccharide was separated into two portions as previously, by extraction with boiling acetone, and the methylation continued by Purdie's method. Fraction I, being insoluble in pure methyl iodide, was methylated in a solution containing a little chloroform. Two sets of three treatments were given, the second series failing to raise the methoxyl content above that attained after the first series.

It was clear in this instance also that a separation had been effected by the acetone extraction, though the rotations of the two Fractions were numerically smaller than the corresponding Fractions of the first sample of methylated polysaccharide. Both Fractions were re-extracted with acetone to test the possibility that the fractionation was incomplete, but the rotations remained the same. It is possible that the difference in rotations between the two samples is due to a smaller degree of degradation in the second sample, methylated in an inert atmosphere.

By comparison of the specific viscosities of trimethyl laminarin in m-cresol and chloroform solutions,

the value of the constant K_m in the Staudinger equation $\frac{\eta_{sp}}{C} = K_m M$ for a chloroform solution has been obtained. Using this value ($K_m = 8.54 \times 10^{-4}$) it has been possible to make an estimate of the molecular weights of the four Fractions of polysaccharide from a knowledge of the specific viscosities of the methylated Fractions in a chloroform solution.

The possibility mentioned above, that the two Fractions of polysaccharide which were methylated in an atmosphere of nitrogen are less degraded than those methylated by the thallium method, is borne out by a comparison of the molecular weights. Those of the Fractions methylated by the latter method being approximately half of those methylated by Haworth's method.

It was found that the two Fractions of sample I of methylated polysaccharide were only partially hydrolysed by treatment with boiling 5% methanolic hydrogen chloride for 28 hours. The incompletely hydrolysed material was recovered and was subjected to further hydrolysis by 90% formic acid at 100° until a constant rotation was obtained, three hours being necessary in each case. The syrups of free sugars from each Fraction were purified from contaminating inorganic ions by treatment of the aqueous solutions with "Amberlite" ion exchange resins. On analysing each syrup on the paper chromatogram five different methylated derivatives of glucose were detected.

By comparison of the R_G values determined for these sugars with those given by Hirst, Hough and Jones(6) we can tentatively identify tetramethyl glucose

and 2:4:6-trimethyl glucose, but the identity of the remaining three compounds is uncertain.

The two Fractions of sample II of methylated polysaccharide were hydrolysed directly to the free sugars by treatment with 90% formic acid at 100° until the rotation reached an equilibrium. Three hours heating was required for each Fraction. The five different methylated sugars which had been detected in the hydrolysates of sample I were found to be present in these two hydrolysates of sample II.

These partially methylated derivatives of glucose were separated by partition chromatography on a column of powdered cellulose, using as solvent a mixture of light petroleum (100-120°, 70%), n-butanol (30%) saturated with water. The apparatus and technique used are exactly as described in Part I of this thesis (Page 39). The components of Fraction C (hydrolysed sample II, Fraction I) were separated by this method and the individual sugars estimated gravimetrically. After allowing for the impurity of each component as estimated by the methoxyl content, the percentage composition of the hydrolysate was calculated.

The percentage compositions of the remaining three Fractions were estimated by the paper chromatogram method of Hirst, Hough and Jones (6).

In a consideration of these quantitative data two point arise which are worthy of comment. In a comparison of the proportions of tetramethyl to dimethyl sugar it is apparent that far more of the latter is present than can be accounted for on theoretical considerations. In a branched polysaccharide the

proportion of dimethyl to tetramethyl sugar isolated after methylation and hydrolysis can never exceed a 1:1 relationship. One non-reducing end group i.e. a fully methylated sugar, would be isolated from every branch of a complex polysaccharide; and every point of branching will give rise to a dimethyl compound. Thus, in a molecule consisting of a single branch from a main chain, the ratio of dimethyl to tetramethyl sugar will be 1:2, and this ratio will tend towards the maximum value of 1:1 as the complicity of the polysaccharide increases.

That the large quantity of dimethyl sugar isolated in the present work is due to the under methylation of the polysaccharide, can be shown if the methoxyl content of a partially methylated polysaccharide having the given constitutions is calculated. Summing the proportions estimated of the methoxyl values for the tetramethyl, trimethyl and dimethyl anhydroglucoses we obtain calculated values which compare favorably with those values found by experiment.

Thus Fraction I contains: 7.8% tetramethyl glucose
66.3% trimethyl glucose
25.9% dimethyl glucose

and the methoxyl content of such a mixture, calculated on the anhydro sugars, is 42.9%. The methoxyl content determined experimentally is 42.2%.

Similarly: Fraction II Calc. 43.0% Found 41.0%
Fraction Y Calc. 41.8% Found 41.0%
Fraction Z Calc. 42.9% Found 41.5%

The second point which is worthy of mention is that in those two Fractions of negative rotation the

quantity of "Trimethyl I" is 7–8% higher than in those Fractions of positive rotation. This might only be the result of chance, considering the large amount of under methylation, but on the other hand it may be taken, together with the differing sign of rotation, to indicate the presence of two polysaccharides having very similar structures.

That component of R_G value 1.00 was confirmed to be 2:3:4:6–tetramethyl glucopyranose by the preparation of the anilide. After two recrystallisations it had m.p. 134–5°, and a mixed melting point with an authentic specimen was unchanged.

From the table of R_G values published by Hirst, Hough and Jones (6) it was thought possible that "Trimethyl I" might be the 2:3:6– isomer, however as no inversion of rotation took place in 2% methanolic hydrogen chloride this could not be the case, and position C_4 must be blocked. This Fraction readily gave an anilide of melting point 142–3°, corresponding to 2:3:4–trimethyl glucose anilide. On estimating, by the dimedone complex, the yield of formaldehyde obtained on periodate oxidation a control sample of glucose gave a 47% yield while that obtained from the sugar under investigation was 6% of the theoretical. It has recently been pointed out (7) however, that the yield of formaldehyde from 2:3:4–trimethyl glucose under these conditions of oxidation falls very far short of the theoretical.

This sugar has been proved not to be the 2:3:6–isomer, and from the fact that 3:4:6–trimethyl glucose is said not to give an anilide which might be obtained crystalline(8), and the melting point of the anilide

prepared corresponds to that given for 2:3:4-trimethyl glucose anilide, it may be concluded that "Trimethyl I" is 2:3:4-trimethyl glucose.

"Trimethyl II" was recrystallised from a mixture of dry ether and light petroleum when it showed $[\alpha]_D$ +73.5° in water (constant in 19 hours). The crystals had m.p. 123-5° and a mixed melting point with a sample of authentic 2:4:6-trimethyl glucose was unchanged.

No trace of a precipitate of the formaldehyde-dimedone complex was observed after periodic acid oxidation of a sample of this sugar. Position C_6 must, therefore, be occupied by a methoxyl group.

The anilide was prepared and after three recrystallisations from an ether-petroleum ether mixture it had m.p. 154-6.° A mixed melting point with an authentic specimen of 2:4:6-trimethyl glucose anilide (m.p. 163-5°) which had been prepared under identical conditions was 156-7.° The authentic compound had $[\alpha]_D$ -126.8° (initial), +40.4° constant in 26 hours, in methanol.

Two moles of aniline had inadvertantly been used for the preparation of the anilide of the sugar obtained from the polysaccharide, and the melting point of the recrystallised product was 10° below that given for 2:4:6-trimethyl glucose anilide (8). The mixed melting point with an authentic specimen prepared under identical conditions indicated that the two compounds were the same. The rotation of the authentic specimen is, however, anomalous, the equilibrium rotation in methanol being given as -115° (8) and +20° (1). This evidence is, therefore, inconclusive.

The sugar isolated from the polysaccharide was

finally proved to be 2:4:6-trimethyl glucose by comparison of its X-ray powder photograph with that of an authentic sample of 2:4:6-trimethyl glucose; the spatial distribution of the lines of scattered X-rays corresponding, one with the other.

The first of the dimethyl fractions was shown, by periodic acid oxidation, to have a free C_6 position. Position C_4 was shown to be occupied; the rotation in 2% methanolic hydrogen chloride rising during 12 hours from +48.9° to 66.7°.

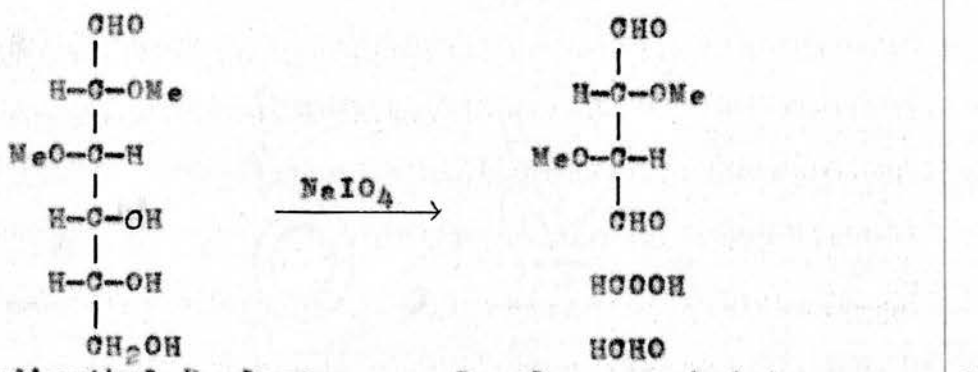
The amide was prepared, by bromine oxidation of the sugar to the dimethyl gluconic acid and reaction of this with methanolic ammonia at 0° for 5 days. Removal of the solvent left a syrupy amide which was subjected to the Weerman reaction. A negative result was obtained and position C_2 must, therefore, be occupied by methoxyl. From the results of these analyses we must conclude that "Dimethyl I" is 2:4-dimethyl glucose.

The second dimethyl fraction was subjected to periodic acid oxidation and position C_6 demonstrated to be free. The optical behaviour in 2% methanolic hydrogen chloride, a fall in $[\alpha]_D$ from +57.5° to 37.5° in 12 hours, would suggest that this fraction is a mixture of 2:3-dimethyl glucose and 3:4-dimethyl glucose, the other remaining isomeric dimethyl glucose having a free C_6 position.

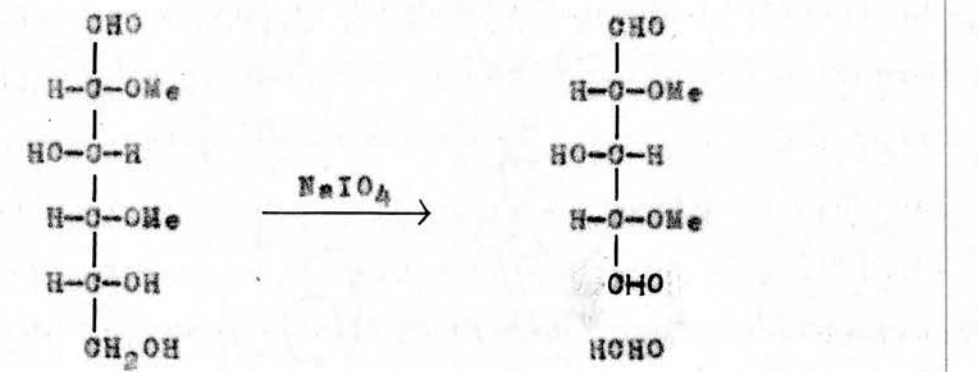
Preparation of the dimethyl gluconamide, a syrup, and quantitative estimation of the hydrazodicarbonamide given on hypochlorite oxidation in the Weerman reaction suggest, for the composition of this fraction, a mixture of 15% 3:4-dimethyl glucose and 85% 2:3-dimethyl glucose.

"Dimethyl I" was confirmed as the 2:4- isomer

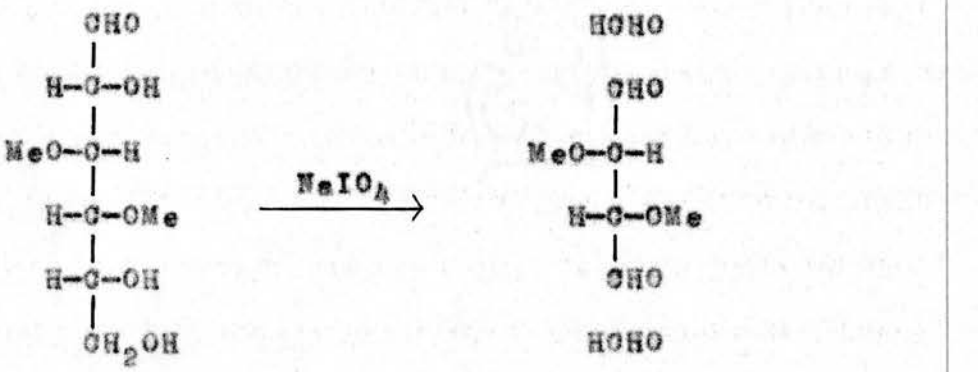
by estimation of the uptake of periodate after oxidation with sodium periodate. The three isomeric dimethyl glucoses having a free C_6 position are the 2:3-, 2:4- and 3:4-dimethyl compounds. They will react with periodate in the following ways:-



2:3-dimethyl-D-glucose — 2 moles. periodate consumed



2:4-dimethyl-D-glucose — 1 mole. periodate consumed



3:4-dimethyl-D-glucose — 2 moles. periodate consumed

Thus it is evident that the 2:3- and 3:4-dimethyl compounds each take up 2 moles. of periodate, while the 2:4-dimethyl compound will only consume 1 mole.

"Dimethyl I" was oxidised with sodium periodate (Page 129) using "Dimethyl II" as a control. It was found that when "II" had taken up 1.70 moles per $C_8H_{16}O_6$,

"I" had consumed 0.75 moles. per $C_8H_{16}O_6$, i.e. "I" must be the 2:4-dimethyl compound.

In order to estimate the extent, if any, of the demethylation of the products of hydrolysis of the methylated polysaccharide, a sample of chromatographically pure 2:3:4-trimethyl glucose ("Trimethyl I") was treated with 90% formic acid at 100° for 4 hours. On removal of the acid and examination on the paper chromatogram, no dimethyl compounds were detected; but another compound had been formed giving a spot of R_g value 0.96. This compound was decomposed by heating the syrup with dilute (1-2%) formic acid at 100° for 2 hours; it must, therefore, have been an ester, the strong formic acid having esterified the free hydroxyl group on position C_6. The non-detection of any dimethyl compounds during this experiment indicated that while 90% formic acid is a very strong hydrolytic agent for the glycosidic linkage, it has a negligible attack on the ether, methoxyl linkage.

Sample I of this fungal polysaccharide was methylated and divided into two fractions whose molecular sizes were estimated by viscosity measurements on a chloroform solution and comparison of the figures obtained with those obtained under identical conditions from trimethyl laminarin, whose molecular size had been estimated by viscosity measurements on a m-cresol solution. Fraction I of this sample was estimated to consist of 14 anhydroglucose units and Fraction II of 16 such units. These values compare very favourably with the number of residues per non-reducing end group as estimated by the paper chromatogram method on the hydrolysed Fractions; Fraction A giving a value of 13 and Fraction B 15 residues

per non-reducing end group.

Although the evidence from viscosity determinations might not be very reliable, the shape of the molecule being unkown, this evidence and that from the quantitative analysis would seem to support oneanother.

In the case of sample II of the fungal polysaccharide the two Fractions were estimated by viscosity determinations to have molecular sizes of 31 and 35 anhydroglucose units respectively. The number of residues per non-reducing end group, as found by analysis from the paper chromatogram, were 14 and 11 respectively. Fraction I would appear, therefore, to contain two non-reducing end groups per molecule, and Fraction II, three such groups per molecule; and a branched chain structure appears to be indicated.

Estimation of the reducing power of sample I of the fungal polysaccharide, by hypoiodite oxidation, gave a value of one reducing group per approximately 40 residues. This corresponds more closely to the molecular size of sample II, where we may assume the presence of one reducing end group per molecule, than to that of sample I; and we may reasonably conclude that sample I was degraded down to its "unit" size during the methylation process using the thallium method.

Due to the large amount of under-methylation present in the samples of methylated fungal polysaccharide, no definite molecular structure can be assigned either to the polysaccharide as a whole or to a possible repeating unit. We may conclude, however, that there are not more than two main types of glycosidic linkage present in these molecules; in the 1:6- and 1:3- positions.

As has been pointed out (Page 100) a high proportion of 1:6- linkages was expected from a consideration of the results of the periodate oxidations on the original polysaccharides. From the low positive rotation of the original polysaccharide and from its rate of hydrolysis, it is considered very probable that the constituent residues are linked in the β-configuration. The two Fractions of methylated polysaccharide are considered to be different molecules; their rotations differ in sign, and on quantitative analysis of the hydrolysis products those Fractions having the negative rotation have a higher content of 2:3:4-trimethyl glucose than those Fractions of positive rotation.

It is evident that these molecules must have a very complex steriochemical structure, and this steric hindrance is considered to be the reason why the methoxyl contents could not be raised above the 41-42% region. It may well be that we have here a limit to the classical method for the determination of polysaccharide structure. It is obvious that in order to obtain a "fully methylated" polysaccharide a much more drastic methylation procedure is necessary, but after such treatment we have no guarantee that the "fully methylated" compound is identical with, or bears any close relationship to, the original polysaccharide.

SUMMARY

1) The primary methylation of these two samples of *Lycoperdon bovista* polysaccharide was accomplished in the case of the first sample, by the thallium method; and in the second instance by the use of dimethyl sulphate and sodium hydroxide in an inert atmosphere. A complete methylation could not be achieved by the subsequent use of Purdie's reagents.

2) An estimation of the probable molecular weights of these methylated polysaccharides was obtained by measurement of the viscosities of 2% solutions in chloroform.

3) Efficient hydrolysis of these methylated polysaccharides directly to the free sugars was attained by the use of 99% formic acid at 100°. Five different methylated derivatives of glucose were demonstrated on a paper chromatogram of the hydrolysate.

4) The relative proportions of these five derivatives were estimated by use of the paper chromatogram method, and they were separated in bulk by partition chromatography on the cellulose column. The five fractions obtained were identified as a) tetramethyl glucose, b) 2:3:4-trimethyl glucose, c) 2:4:6-trimethyl glucose, d) 2:4-dimethyl glucose, e) 15% 3:4- and 85% 2:3-dimethyl glucoses.

5) The structural applications of these results, which are limited owing to the under methylation of the original polysaccharide, are briefly discussed.

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