UNIVERSITY OF EDINBURGH

INVESTIGATIONS ON THE CARBOHYDRATE CONSTITUENTS OF COUCH GRASS ROOTS (*AGROPYRON REPENS*).

A

THESIS

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by

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INTRODUCTION

Many plants store carbohydrates in their tubers in the form of polyfructosans which generally serve as reserve polysaccharide material in place of, or in addition to, starch. It is only within the past 25 years, however, that the widespread occurrence of fructosans in nature, both in the plant and microbiological kingdoms, has been recognised. Due mainly to Schlubach and his school in Hamburg, a number of fructosans have been isolated in a state of purity from widely different sources, and their structures investigated. But in this country carbohydrate workers have, as yet, failed to give fructosan polysaccharides the attention merited by their diverse sources and varying structures.

The fructosan polysaccharides differ from one another in the mode of linkage between the D-fructose residues, and in molecular size. As a result of their series of investigations, however, Schlubach and Sinh(1) have been able to show that fructosans may be divided into two classes.

(a) The inulin group, in which the second, or reducing, C atom of 1 fructose residue is linked to the first C atom of the adjacent residue, and

(b) the phlein group, in which the second, or reducing, C atom of one fructose residue is linked to the sixth

C atom of the adjacent residue.

For members of group (b), the acetyl derivatives have positive rotations and, according to Schlubach and Sinh, the difference between the rotations of the acetate and the corresponding fructosan appears to be related to the proportion of 1:3:4-trimethyl fructose produced from the methylated fructosan. The smaller the difference, the smaller is the yield of 1:3:4-trimethyl fructose and, therefore, the more branched is the chain.

e.g. Fructosan	Diff. in rotations	% 1:3:4-trimethyl fructose
phlein	71°	100
levan	66°	80
secalin	41°	50

For members of the phlein group it was also observed that the number of fructose units in the polysaccharide, and therefore the molecular weight, decreases with increasing branching of the chain, whereas the ease of hydrolysis increases.

In group (a) the same relationships were found between molecular weight, ease of hydrolysis, and the amount of branching. In this group, however, the difference in rotations of the acetyl compound and the corresponding fructosan increases with decreasing yield of 3:4:6-trimethyl fructose. In other words, the greater the difference, the more branched is the molecule.

e.g. Fructosan	Diff. in rotations	% 3:4:6-trimethyl fructose.
Inulin	3°	100
Asparagosin	12°	80
Sinistrin	21°	60
Graminin	33°	50

It is indeed astounding that nature is capable of building such a wide variety of polymers of D-fructose units. Yet it is even more astounding that all these polymers can, with one or two exceptions, be placed in one of 2 groups, and that in each of these groups the properties of the various members bear a definite relationship to one another. It emphasises that nature builds on the simplest and most regular of patterns.

It is believed by the Schlubach school that the fructosans have loop structures, rather than open-chain structures. In other words, the D-fructose units are linked together in the form of a ring. By hydrolysis of the fully-methylated fructosan polysaccharides, considerable quantities of tetramethyl- and dimethyl-D-fructose are invariably obtained, a feature which necessitates the presence of highly-branched structures.

It is a matter of great difficulty to extract a fructosan in a high state of purity from its natural source, and great credit must be given to the Hamburg school for the painstaking manner in which they tackled the purification of their materials. It was a common feature to reprecipitate the polysaccharide 300 times until a constant rotation was obtained. To ensure that the material was in fact homogeneous, the acetate was prepared and was itself reprecipitated many hundreds of times, and the polysaccharide regenerated from the acetate with sodium methoxide and compared with the original material.

In their investigations on the structures of the various polyfructosans, the same general technique was employed by Schlubach and his colleagues. Once the material had been isolated in a homogeneous state, it was methylated by the action of dimethyl sulphate and sodium hydroxide on the acetate, and the fully-methylated derivative hydrolysed. The components of the hydrolysate were separated by direct distillation in high vacuum, or by conversion to the benzoyl derivatives of the partially-methylated sugars and separation of these from the fully-methylated sugar and from each other by high vacuum distillation. The components were then identified and the relative proportions of tetramethyl-, trimethyl- and dimethyl-D-fructoses produced were estimated. With this knowledge at hand, together with investigations into the molecular size of the polysaccharide by various physical techniques, they were able to suggest a structure for the polysaccharide.

Although it cannot be denied that Schlubach and his school have done admirable work in this field, it must be admitted that their results are not always convincing and much work has still to be done.

Firstly, several of the partially-methylated sugars obtained by hydrolysis of the fully-methylated material could not be identified, with the result that the whole mode of linkage was left open to doubt. Secondly, the nearest whole-number ratio of the various methylated fructoses obtained from the hydrolysates of the methylated fructosans frequently gave rise to more than one possible interpretation. The chief importance of Schlubach's work is that it provided the initial impetus required for systematic investigations in fructosan chemistry. He has shown that a basic pattern underlies the structures of all the fructosans and it is the duty of the modern carbohydrate chemist to continue these investigations, and to assist in filling the many gaps which at present exist.

It is now proposed to give a short account of the results obtained in the investigations of the various fructosans, and we shall consider firstly the members of the inulin group, secondly the members of the phlein group, and finally the anomalous fructosans.

(a) The Inulin Group.

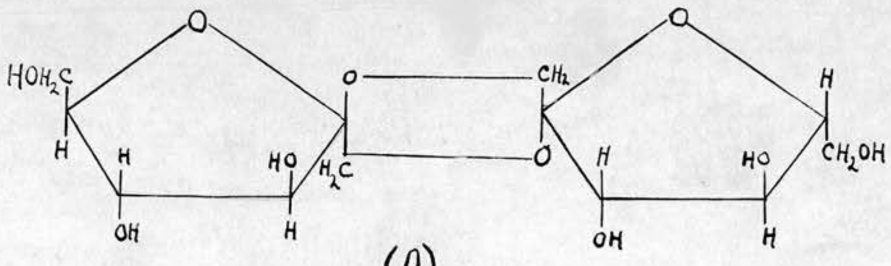
Inulin.

Inulin was the first of the fructosans to be isolated and has been investigated much more fully than any of the others. First isolated from artichoke tubers by Rose in 1804, it is widely distributed in the plant kingdom, being stored as reserve polysaccharide in the roots of the *Compositae*, the largest group of flowering plants. In addition to artichoke tubers, good yields have also been obtained from dahlia tubers, dandelion roots and chicory. Quite recently, inulin was isolated from a rubber-producing plant guayule called *Parthenium argentatum Gray*,(2) whilst another rubber-producing plant called *Taraxacum Kok-saghyz* has also been shown to contain inulin.(3)

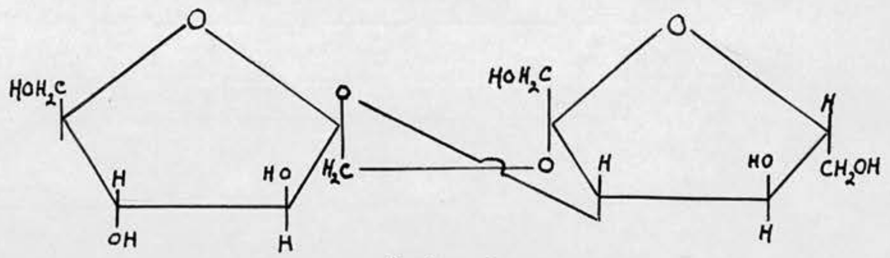
By the hydrolysis of trimethyl inulin, Haworth and Learner(4) identified the trimethyl sugar produced as 3:4:6-trimethyl-*D*-fructose, thereby showing that the *D*-fructose units in inulin are linked through C_1 and C_2.

Using Haworth's end group assay method, Haworth, Hirst and Percival(5) obtained 3·7% of tetramethyl-*D*-fructofuranose from fully-methylated inulin. They therefore concluded that inulin is composed of a chain of about 30 D-fructofuranose units and has a molecular weight of 5000. Molecular weight determinations by osmotic pressure measurements on acetylated and methylated inulin gave values of 8880 and 6210.(6) Using the ebullioscopic method in aqueous solution, Drew and Haworth(7) determined the molecular weight to be not less than 3200 or 3600, which corresponds to a molecule containing 20 or 24 D-fructose units.

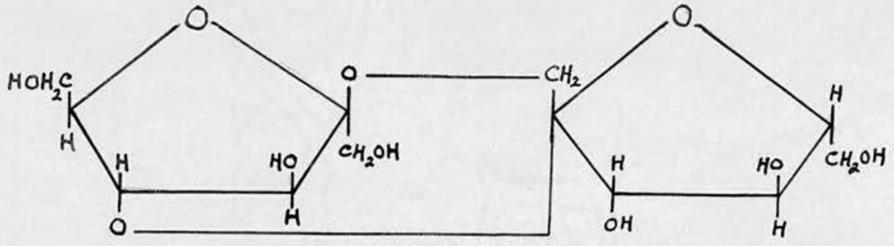
Inulin is very readily hydrolysed, even in the presence of mere traces of acid, to yield fructose, glucose, and difructose anhydrides. The difructose anhydrides occur to the extent of about 5% in an inulin hydrolysate and are much more resistant to acid hydrolysis than inulin itself. The difructose anhydrides have been isolated as 3 distinct compounds by fractional precipitation.(8) Difructose anhydride I has been shown by Haworth and Streight(9) to be 1,2':2,1'-di-D-fructofuranose anhydride (A), whilst Jackson and McDonald(10) have identified difructose anhydride III as the 1,2':2,3'-isomer (B). More recently the remaining one was identified as 2,1':4,2'-di-D-fructofuranose anhydride(11) (C).



(A)



(B)

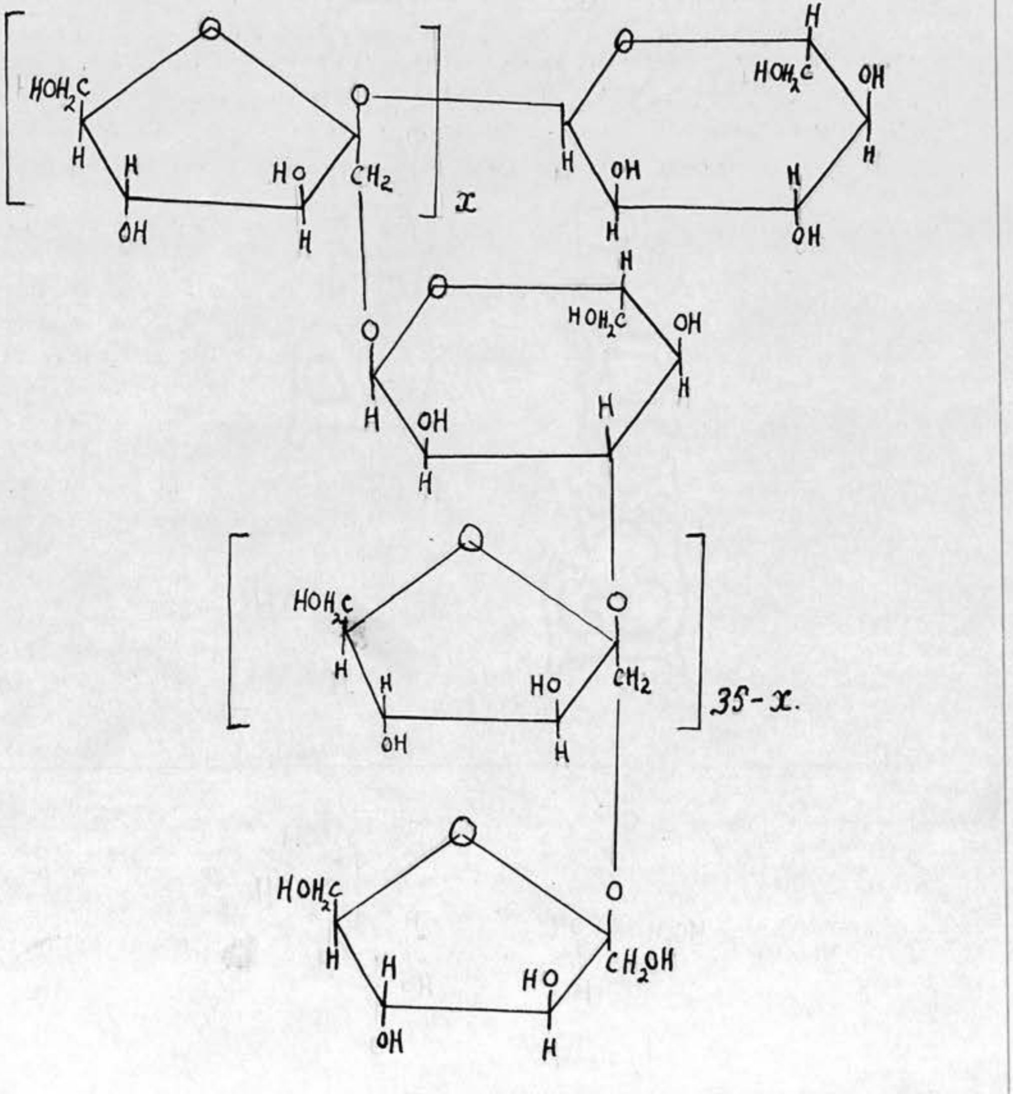


(C)

Linkages, represented as β- above, may be α- or β-.

Much controversy has taken place as to the relationship of the difructose anhydrides to inulin. It is now considered almost certain that they arise as by-products in the hydrolysis of inulin. A large amount of evidence in support of this view allows one to discard the original belief that they occur preformed in the inulin molecule.

Many workers have noted the presence of glucose in inulin hydrolysates. The percentage of glucose produced seems to vary between the limits of 1.9% and 4.4%, the average value being 3·3%. Hirst, McGilvray and Percival (12) estimated the glucose produced by hydrolysis of dahlia inulin as 5.7%, using the method of quantitative paper chromatography. By determining the total quantities of tetramethyl- and trimethylglucose produced from methylated inulin, using alkaline hypoiodite oxidation, a value of 5·7%, calculated as glucose, was again obtained. As a result of their investigations, the authors suggest that the glucose does not arise from an associated glucosan, since the proportion of tetramethyl- to trimethyl-glucose is too great to permit this. They suggest, as a possible structure, a chain of about 35 fructofuranose residues with the potentially reducing fructose residue linked to a glucopyranose unit by a sucrose-type linkage and with a second glucopyranose residue linked through C_1 and C_3 at some undetermined position in the chain, as indicated below.



Although most workers favour the idea that the fructofuranose units are linked in a chain in inulin, Schlubach claims that a non-reducing sample of inulin isolated by him necessitates a closed-loop structure.(13) Owing to the extreme ease of hydrolysis of inulin, it is quite possible that completely undegraded inulin exists in the plant in a cyclic structure which is very readily ruptured to give the open-chain structure normally observed.

It will be seen, however, that the open-chain model of Hirst, McGilvray and Percival, terminated by a sucrose residue, could also account for the non-reducing properties of inulin.

Asparagosin.

This fructosan was first isolated from asparagus roots by Tanret,(14) and later by Schlubach and Böe.(15) The latter clarified the aqueous extracts with lead acetate and the polysaccharide was then fractionally precipitated with alcohol. By reprecipitating 180 times to constant rotation, a product with $[\alpha]_D = -32{\cdot}6°$ in water was obtained. This product had a molecular weight of 1635 when determined by the cryoscopic method, corresponding to 10 fructose residues.

Methylation of the acetate and hydrolysis of the fully-methylated polysaccharide yielded dimethyl-, trimethyl- and tetramethyl-D-fructose in the ratio 1:8:1

(calculated as D-fructose). The trimethyl fraction was identified as 3:4:6-trimethyl-D-fructose by preparation of its crystalline osazone, showing that in asparagosin the fructofuranose units are linked through C_2 and C_1 as in inulin. Since the sample of Schlubach and Böe was non-reducing, they claimed that the molecule consists of a closed ring of D-fructofuranose units. Furthermore, because asparagosin is almost identical with inulin, except that its molecular weight is one third of the latter, the authors claimed that this work on asparagosin provides good evidence in support of Schlubach's closed-ring theory for inulin.

Sinistrin.

The polyfructosan obtained from the sea onion (Scilla maritima, Urginea maritima) has been studied by several workers.(16,17,18)

Schlubach and Loop precipitated the polysaccharide 200 times from water with alcohol when the resulting product had $[\alpha]_D^{20} = -44\cdot5^\circ$ in water. The fully-methylated polysaccharide prepared from the acetate was hydrolysed and the components of the hydrolysate separated by high-vacuum distillation of the benzoyl derivatives. Dimethyl-, trimethyl-, and tetramethyl-D-fructose were obtained in the ratio 1:3:1 or 2:5:2. The trimethyl fraction was identified as 3:4:6-

trimethyl-D-fructose by means of its osazone. However the greater part of the osazone could not be crystallised, and, since the trimethyl fraction itself did not have a specific rotation in good agreement with that of 3:4:6-trimethyl fructose, it is highly probable that this trimethyl fraction was a mixture. Moreover the dimethyl fraction could not be identified.

Molecular weight determinations gave a value of 2526 for sinistrin, corresponding to 15-16 fructose units. This indicates that the ratio 1:3:1 is correct, since the number of fructose residues is a multiple of 5, rather than a multiple of 9.

Since Sinistrin was found to be non-reducing, the authors stated that the molecule is in the form of a closed ring, or that the reducing group may be concerned in inner anhydride formation in the terminal fructose unit. However, from the fact that there is uncertainty as to the identity of the methylated fructoses and their ratio, the structure of sinistrin must still be considered doubtful and open to further investigation.

Graminin.

In 1870 Popp(19) isolated a carbohydrate, which he called Synanthrose, from dahlia tubers and tubers of other members of the *Compositae*, this carbohydrate being present in addition to inulin. In 1878 Müntz(20) isolated a non-reducing carbohydrate from unripe rye which he said was identical with Popp's "Synanthrose." At the same time Ekstrand and Johanson(21) studied a carbohydrate which they isolated from roots of members of the <u>Gramineae</u> and which they called Graminin. It was obtained from the roots of many common grasses, e.g. <u>Trisetum alpestre</u>, <u>Agrostis</u> and <u>Festuca</u>.

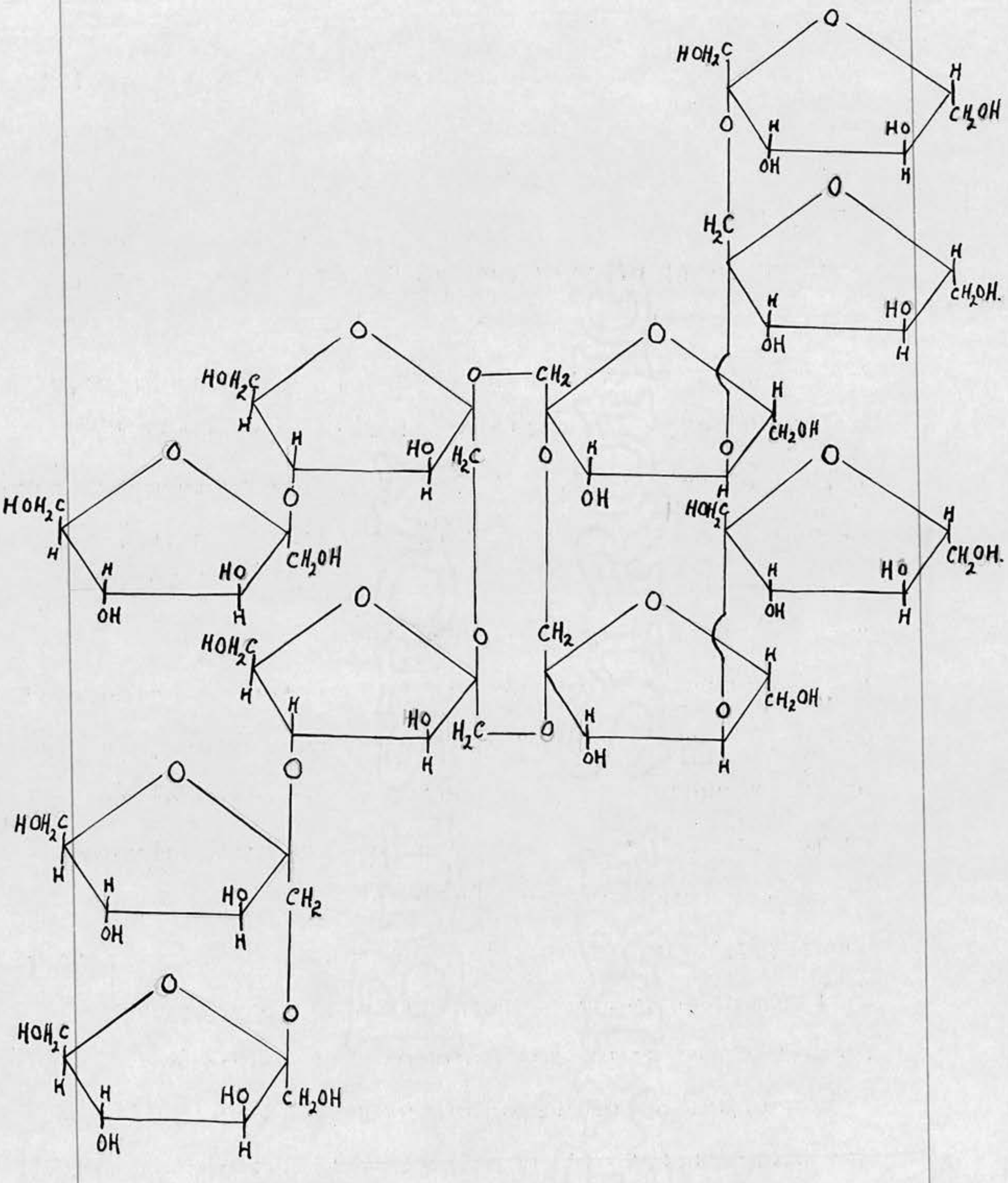
Much conflicting work ensued, when a large number of carbohydrates were isolated from rye, wheat and barley under various names. Schlubach and Koenig(22) applied the name Graminin to the material from rye and endeavoured to prepare a truly homogeneous sample for their constitutional studies. The polysaccharide was reprecipitated 100 times from water with alcohol and final purification achieved by acetylation, followed by deacetylation, when a non-reducing material with $[\alpha]_D = -40°$ in water was obtained.

Molecular weight determinations indicated that graminin was built up from 9-10 <u>D</u>-fructose units.

The polysaccharide was methylated in the normal way and the methylated fructoses produced by hydrolysis of the fully-methylated graminin were separated by fractional distillation of the benzoyl derivatives in high vacuum. The trimethyl fraction appeared to be 3:4:6-trimethyl fructose, although definite proof of this was not obtained. The dimethyl fructose underwent decomposition on distillation, and because of this, it was not possible to determine the exact ratio of the tetramethyl-, trimethyl-, and dimethyl-fructoses. They appeared to be present in the ratio 1:1:1, or, more probably, 2:1:2.

Assuming this latter ratio to be correct, and the number of fructose residues in the molecule to be ten, a large ring structure seems the most likely possibility for graminin. The fact that the material is non-reducing excludes the possibility of a long chain structure. Explanation of the disappearance of the reducing end groups by the formation of inner anhydrides was also rejected by Schlubach as this would demand the presence of 10% monomethyl fructose, whereas no monomethyl material was detected.

The authors were able to construct a large structure, involving 10 fructose units, such that tetramethyl-, trimethyl-, and dimethyl-fructoses would be produced in the ratio of 2:1:2. The whole formulation however must be accepted at present with great reserve, until more conclusive evidence is brought to light from the methylation studies of graminin.



GRAMININ (according to Schlubach and Koenig).

The linkages are represented as β-linkages, although it is not known whether the linkages are α- or β- in graminin.

Kritesin.

The latest of the inulin-type polyfructosans to be investigated is Kritesin which was isolated from ears of barley (Ackermanns isaria) by Schlubach and Rathje.(23)

The methylated fructoses produced by hydrolysis of the fully-methylated kritesin were separated by fractional distillation of the benzoyl derivatives in high vacuum, when tetramethyl-, trimethyl- and dimethyl-D-fructose were obtained in the ratio 1·12:2·02:1. The amount of tetramethyl fructose was assumed to be high owing to cleavage of the molecule during methylation.

The trimethyl fraction was undoubtedly a mixture. It gave a mixture of 2 osazones, one containing 3 methoxyl groups and apparently identical with that of 3:4:6-trimethyl-D-fructose, and the other containing 1 methoxyl group. The dimethyl fraction appeared to be 3:6-dimethyl fructose and is probably identical with that obtained from graminin.

It would appear that kritesin is an inulin-type fructosan, although the obvious lack of homogeneity of the trimethyl fraction must cast a shadow of doubt on this statement.

b) The Phlein Group.

Phlein.

Phlein is considered the parent of the group of polyfructosans in which the D-fructose residues are linked through C atoms 2 and 6.

In 1887 Ekstrand and Johanson(24) isolated a carbohydrate from the haulm of *Phleum pratense*. The haulm is thickened at the lower end to a bulb which in autumn expands and becomes filled with a concentrated solution of a carbohydrate. The authors called this carbohydrate graminin. In a later paper, however,(21) they stated that this carbohydrate is different to the one occurring in the *Gramineae* and to the new polysaccharide they gave the name phlein.

A constitutional study of phlein was undertaken by Schlubach and Sinh.(25) They isolated the product from the tubers of *Phleum pratense*, nodosum L variety (timothy) by aqueous extraction of the ground roots. After 6 reprecipitations from an aqueous solution with alcohol, and final purification by acetylation followed by deacetylation, the material had $[\alpha]_D = -50°$ ($c = 1$ in H_2O). Cryoscopic determinations of molecular weight indicated 15-16 fructose units.

Hydrolysis of trimethyl phlein yielded 1:3:4-trimethyl fructose with a small amount of dimethyl fructose (1·9%) which was attributed due to undermethylation. Since 1:3:4-trimethyl fructose alone was produced, the authors stated that phlein consists of a closed ring structure of 15-16 fructose units, the second, or reducing, C atom of each residue being linked to the 6th C atom of the adjacent residue.

Just as phlein, the parent of group (b) fructosans, is considered to be a closed ring with no branching, so inulin, the parent of group (a) fructosans, is considered by the Hamburg school to have such a structure. Since phlein is hydrolysed more readily than inulin, it follows that the $C_2 - C_6$ linkage in the phlein ring is more readily disrupted than the $C_2 - C_1$ linkage in the proposed inulin ring. Moreover, in each group, all the members except the parent have branches radiating from the ring and, as has been shown by Schlubach and Sinh (loc.cit.), become increasingly easily hydrolysed as branching increases. It follows, therefore, that the side-chain $C_2 - C_6$ or $C_2 - C_1$ linkages are more readily disrupted than the corresponding radial linkages.

Levan.

Many polysaccharides have been synthesised by the action of bacteria on simple carbohydrate substances, such as hexoses and disaccharides. The bulk of these bacterial polysaccharides were found to be glucosans, yielding D-glucose only on hydrolysis (e.g. bacterial cellulose, dextran). One bacterial fructosan, however, called Levan has been obtained, yielding D-fructose only on hydrolysis.

Levan has been synthesised by the action of a wide variety of organisms on sucrose, and, to a lesser extent, raffinose, and chemical investigations have shown that the resulting polysaccharides are on the whole very similar.

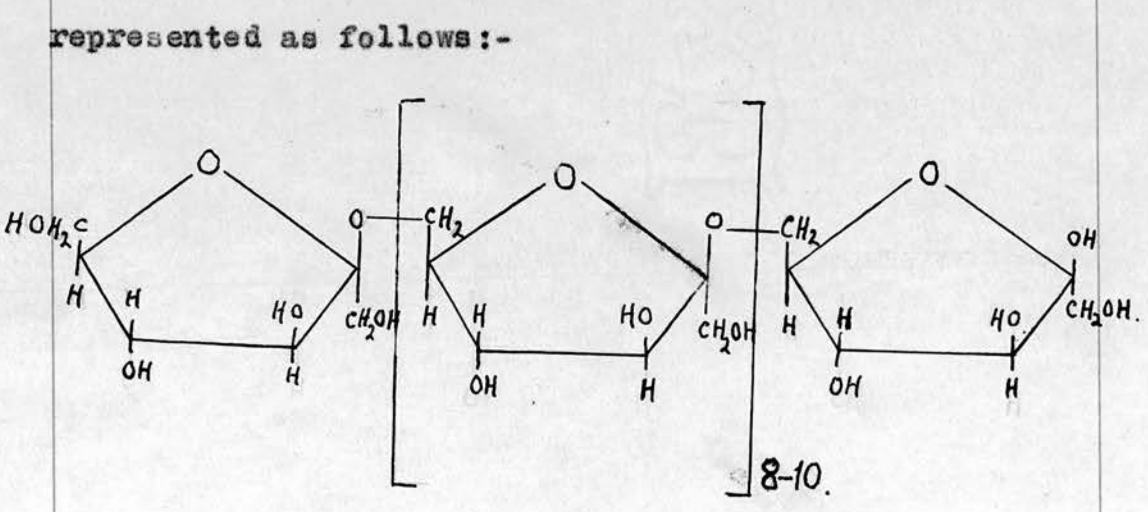
Much of the early work on levan is highly confusing, owing to the isolation of impure products. In 1901 Greig Smith(26) stated that levan could be produced from sucrose in the presence of Bacillus levaniformans, but not from D-glucose, D-fructose, lactose or maltose. He noted that hydrolysis of levan yielded D-fructose only and that levan was closely related to inulin but not identical with it.

In 1930, Harrison, Tarr and Hibbert(27) investigated the production of levan from sucrose by the action of B.subtilis and B.mesentericus. Levan formation was found to occur in suitable nutrient solutions with sucrose and raffinose, but not with melezitose, lactose, maltose, D-glucose or D-fructose. The authors, therefore, suggested that only carbohydrates with a terminal D-fructofuranose unit were satisfactory substrates for levan formation.

Recently, in 1945, Hehre(28) synthesised levan from sucrose and raffinose by means of *Streptococcus salivarius* enzyme preparations.

A chemical investigation of the structure of the levan produced by the action of *B.subtilis* on sucrose was made by Hibbert and Brauns.(29) The levan was obtained by precipitation of the concentrated culture in methanol and purified by reprecipitation and electrodialysis. Hydrolysis of trimethyl levan gave a 98·5% yield of crystalline 1:3:4-trimethyl-D-fructose, showing that levan is a D-fructose polymer. Since the properties of the polysaccharide support the idea that it is of the furanose type, then the D-fructofuranose residues must be linked through C_2 and C_6.

Challinor, Haworth and Hirst(30) investigated the structure of the levan produced by the action of *B.mesentericus* on sucrose. Fractional distillation of the products of hydrolysis of their methylated levan yielded tetramethyl-D-fructofuranose in an amount corresponding to a chain length of 10-12 D-fructose units. From this work, the structure of levan may be represented as follows:-



It will be seen that this formula contains a reducing end group but, as there is uncertainty as to the reducing or non-reducing properties of levan, this formula must be accepted with a certain amount of reserve.

Certain differences in properties of levans produced by different organisms are no doubt due to variations in particle size, which has been shown to occur by sedimentation, ultra-microscope and electron microscope studies. The presence or absence of "end groups" in the two investigations outlined above may be due to different sources yielding different levans or due to the employment of different techniques.

Poain.

A fructosan very similar in properties to levan has been isolated from Poa trivialis (roughstalk bluegrass) by Challinor, Haworth and Hirst.(31) This fructosan, which has been named Poain, is also present in barley leaves and seems to be an important component of the diet of farm animals.

The methylated poain on hydrolysis yielded 1:3:4-trimethyl fructose as the main product. Unfortunately the analysis was not performed quantitatively and consequently it was impossible to state whether poain and levan are distinct substances or whether they are one and the same material in different states of purity.

It seems appropriate to mention at this point that the chemistry of the fructosans presents three main difficulties.

(1) The process of purification of a fructosan is, as has already been mentioned, a long and tedious one. The tendency of chemically-similar compounds to cling persistently to the fructosan under investigation makes the homogeneity of the product a constant source of dubiety. Much of the conflicting work in the literature is undoubtedly due to the employment of materials which were not homogeneous. As stated by Schlubach and Koenig, for example, in the introduction to their paper on graminin (loc.cit.) a large number of workers isolated from rye a variety of carbohydrates differing slightly from one another, and each worker gave his own material a name he himself chose. They were almost certainly not studying homogeneous materials. Such a state of affairs leads naturally to confusion rather than to progress.

(2) The generally non-crystalline nature of the partially-methylated fructoses produced by the hydrolysis of methylated fructosans calls for careful manipulation in any quantitative study.

(3) The methods of analysis of mixtures of methylated fructoses have only recently been developed to the degree of accuracy of the analytical methods applicable in the glucose series.

Bell and Palmer(32) have developed a small-scale analytical separation of mixtures of 1:3:4:6-tetramethyl-, 1:3:4-trimethyl-, and 3:4-dimethyl-fructose by partition chromatography, using a column of silica gel. The method gives a recovery of 98% and gives quantitative recoveries from as little as 100 mg. of material.

Hirst, McGilvray and Percival(12) obtained an excellent separation of the components of a hydrolysate of methylated inulin on a column of powdered cellulose, using the technique developed by Hough, Jones and Wadman.(33)

In the glucose series, the ratio of tetramethyl-, trimethyl- and dimethyl-aldose produced by hydrolysis of the methylated polysaccharide can be determined directly by oxidation with alkaline hypoiodite,(34) thus providing a check on the ratio obtained by quantitative separation and gravimetric estimation of each component. Unfortunately, however, there is as yet no reagent corresponding to alkaline hypoiodite in its ease of application in the ketose series. Results must be based entirely on the gravimetric estimation of each component after separation. Furthermore, in the aldose series, oxidation by alkaline hypoiodite provides a convenient method for the estimation of the methylated sugar content of each syrup produced after separation of the components of the methylated polysaccharide hydrolysate. For methylated fructose syrups, however, there is no method available for the determination of the methylated sugar content unless one utilises the methoxyl content of the syrup for this purpose.

It is when one meets two very similar compounds like levan and poain, which may or may not be identical, that these difficulties make themselves truly apparent.

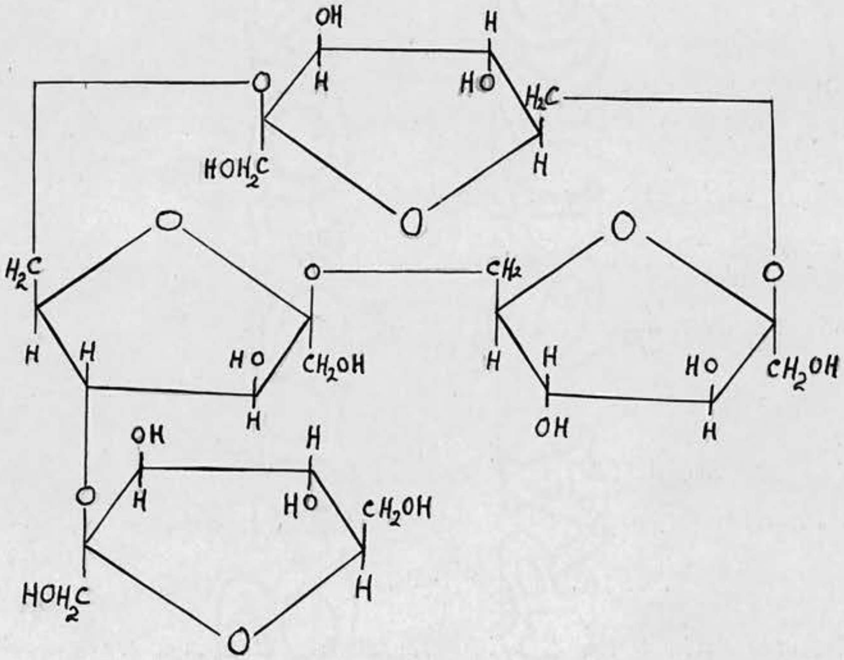
Secalin.

This was isolated from the stems of unripe rye by Belval(35) and its constitution investigated by Schlubach and Bandmann.(36) They encountered great difficulty in obtaining homogeneous specimens of the polysaccharide and its acetate. After reprecipitation of the acetate to constant rotation and deacetylation with sodium methoxide, a non-reducing polysaccharide was obtained with $[\alpha]_D = -37{\cdot}5°$ in water.

Molecular weight determinations on this material suggested that the secalin molecule is composed of four fructose residues.

The acetate was methylated and the products of hydrolysis of the methylated secalin were separated by fractional distillation in high vacuum of the β-naphthoyl derivatives. 1:3:4:6-Tetramethyl fructofuranose, 1:3:4-trimethyl fructose and an unidentified (probably 1:3-) dimethyl fructose were obtained in the ratio 1:2:1.

The non-reducing property of secalin together with the above results led the authors to suggest the formula indicated below for the polyfructosan, the molecule being represented as a tetrafructose anhydride. Although the linkages are represented as β-linkages, no claim can be made as to whether the linkages in secalin are α- or β-.



SECALIN (according to Schlubach and Bandmann).

Schlubach and Koenig (*loc.cit.*) isolated graminin from rye flour in considerable yield. Secalin, isolated from the stems of unripe rye, was shown by Schlubach and Bandmann to be quite different from graminin. It has been observed that, on the ripening of rye grains, starch is produced at the expense of the graminin content, thereby suggesting that the graminin is transformed into starch on ripening. In oats it seems that this transformation goes a stage further since no graminin at all is found in the ripe oat granule. It has been postulated that secalin and graminin are intermediates in the biosynthesis of starch from cane-sugar. The only regular pattern observed however in the series cane sugar - secalin - graminin - starch appears to be in the regular increase in the number of $C_6H_{10}O_5$ units; namely 2-4-10-starch.

Pyrosin.

The structure of the fructosan pyrosin, isolated from wheat stalks, was investigated by Schlubach and Huchting,(37) who showed it to be a phlein-type fructosan.

The carbohydrate was shown to be non-reducing and composed only of fructose units. Molecular weight determinations gave very irregular results. Determinations by the cryoscopic method indicated 5 fructose units while osmotic pressure determinations indicated a value of 205-225 fructose units. Solubility properties, however, indicated that the number of fructoses units was not greater than 200.

Hydrolysis of methylated pyrosin and separation of the components by benzoylation, followed by fractional distillation in high vacuum, yielded 1:3:4:6-tetramethyl-D-fructofuranose, 1:3:4-trimethyl-D-fructose, and a dimethyl fructose, the rotation of which indicated that it was the 1:3(?) dimethyl fructose which was believed to be produced by hydrolysis of methylated secalin. Tetramethyl-, trimethyl- and dimethyl-fructose were obtained in the ratio 1·4:3:1, the value for the tetramethyl fraction being considered high owing to partial cleavage of the molecule on methylation.

The above results led these workers to suggest that the structure of pyrosin was either a four-membered ring of fructose units linked through C_2 and C_6, with a single fructose unit attached to one member through C_4, or, less probably, a ring of fructose units with a chain of 4 units attached to each ring unit.

It was observed that pyrosin does not fit into Schlubach's rule connecting the rotations of the polysaccharide and its acetate with the yield of 1:3:4-trimethyl fructose produced on hydrolysis of the methylated fructosan.

Anomalous Fructosans.

There were 3 fructosans which appeared to fall outside of the 2 general groups, as described by

Schlubach and Sinh (loc.cit.). These were Asphodelin, Irisin and Triticin.

Asphodelin.

Isolated from asphodelus tubers, asphodelin was first intensively investigated by Colin and Neyron.(38) They described it as a non-reducing polysaccharide yielding fructose (6 parts) and glucose (1 part) on hydrolysis. This indicated that asphodelin was either a glucofructosan or an intimate mixture of a glucosan and fructosan with the latter predominating.

Schlubach and Lendzian(39) hydrolysed methylated asphodelin and obtained tetramethyl-, trimethyl- and dimethyl-fructose in the ratio 1·1:8·32:1·65. The tetramethyl derivative was shown to be tetramethyl-D-fructofuranose and the dimethyl derivative appeared to be identical with that obtained from irisin (see below) and graminin. The trimethyl fructose fraction, however, was found to contain an appreciable methylated glucose content. The authors stated that, in their belief, the glucose probably does not form an integral part of the molecule but is due to a glucosan which was not completely removed during the purification process. Owing to the highly probable lack of homogeneity of the asphodelin sample, little can be deduced from the work as to its structure.

Irisin.

In 1886, Wallach(40) isolated a white hygroscopic compound from the roots of Iris pseudoacorus which yielded D-fructose on hydrolysis. Noticing differences in certain properties from those of inulin, he called the new carbohydrate irisin.

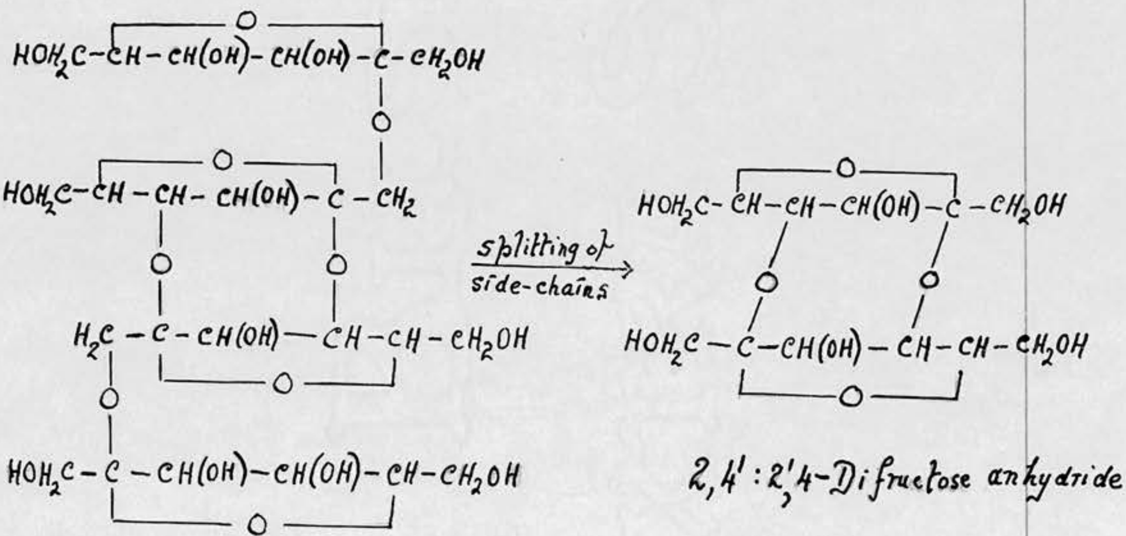
An investigation of the structure of irisin was made by Schlubach, Knoop and Liu.(41) By hydrolysis of methylated irisin, tetramethyl-D-fructofuranose and a dimethyl fructose, which could not be conclusively identified, were obtained in equal amounts, with no trimethyl fructose. To explain these results, the structure postulated for irisin was a chain of D-fructose units, each unit having another D-fructose unit attached to it. The tetramethyl-D-fructose is therefore derived from the "dangling" D-fructose units. However, since the authors were unable to identify the dimethyl fraction, the linkages within the molecule could not be fixed with any certainty.

The authors observed discrepancies in the rate of hydrolysis of irisin when measured by reducing value and by change in rotation.(42) This may be accounted for by the readier hydrolysis of the fructose units suspended from the chain with the formation of intermediate products of hydrolysis. By studying the controlled acid hydrolysis of the original irisin and the material regenerated from the acetate of the intermediate hydrolysis product, the authors concluded that the hydrolysis of irisin may be represented by the following scheme:-

Irisin → Tetrafructose anhydride → Difructose anhydride (B)
(A)
(Di-irisan)

→ D-Fructose.

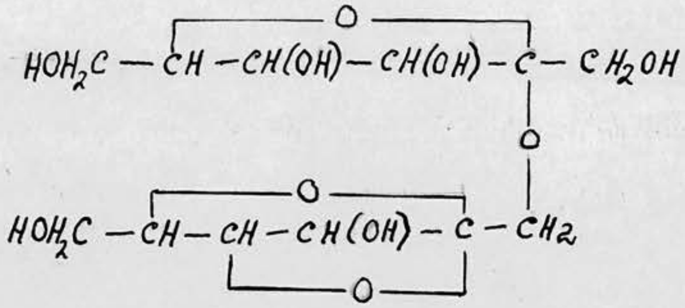
The intermediate di-irisan was isolated in pure form and the structure investigated by studying the hydrolysis products of the methylated di-irisan, Tetramethyl-D-fructofuranose, 3:6-dimethyl-D-fructose and a trimethyl-D-fructose containing a methoxyl group on C_1 were obtained, the latter arising from methylated (B)



2,4':2'4-Difructose anhydride

(A) (B)

It would appear, therefore, that the fundamental building unit of Irisin, which is called Irisan, is:



Recently, however, Bell and Palmer(43) have made an investigation of methylated irisin, using partition and paper chromatography. The dimethyl fructose fraction was identified as the 3:4-isomer, but, in addition, they found an appreciable trimethyl fraction, which consisted of 2 parts of 1:3:4-trimethyl-*D*-fructose and 1 part of (probably) 3:4:6-trimethyl-*D*-fructose.

Triticin.

The other fructosan which appears to be anomalous is known as triticin.

Occurrence in Nature.

Triticin occurs in the rhizomes of *Agropyron repens*, commonly known as couch grass, quack grass or twitch. Couch grass is notoriously known to all gardeners as the weed whose rapidly-spreading roots or rhizomes can quickly become uncontrollable.

In spite of this, feeding experiments have shown couch grass rhizomes to be of high nutritional value. According to Weibull,(44) the nutritive value of couch grass as cattle food equals that of hay, as well as forming useful feeding stuff for horses and sheep. Experiments have also shown that couch grass rhizomes fed to rabbits form a satisfactory substitute for hay.(45) A Norwegian publication in 1941 describes the feeding of couch grass roots to hens. It was found that the roots gathered in spring contained a considerable quantity of free sugars which by autumn were completely replaced by starch.

Herbalists frequently recommend couch grass roots to alleviate pains arising from bladder disorders, a cure which the ancient Greeks and Romans are known to have used.

Previous Investigations of Triticin.

The first reference to the extraction of carbohydrate material from couch grass is found in a paper published by Stenhouse(46) in 1843. In an article entitled "The occurrence of mannite in *Laminaria saccharina* and other seaweeds," Stenhouse draws attention to an article in the 8th volume of Berzelius' "Lehrbuch" in which Pfaff states that he obtained mannite from the roots of *Triticum repens*, or couch grass. Stenhouse was unable to find mannite in the extracts of couch grass, although he states that "the grass roots certainly contain a great deal of an uncrystallisable sugar which readily ferments."

An article by Ludwig and Müller(47) in 1872 entitled "The Constituents of Couch Grass Roots (Triticum repens L)" states that the roots contain

1) A strongly laevo-rotatory fructose derivative.
2) A dextro-rotatory sugar (not cane sugar).
3) A gum produced by decomposition of the laevorotatory sugar, together with a nitrogenous organic substance.
4) A sweet-tasting product intermediate between this gum and fructose, together with a nitrogenous organic substance.

Continuing this work, Müller(48) in 1873 isolated the fructose derivative from couch grass roots as a gummy material. He called the new carbohydrate triticin, from the botanical name of its source. Müller obtained his product by extracting the well-powdered roots with hot 25-30% alcohol, washing the extract repeatedly with water and then removing protein material from the aqueous solution with lead acetate. He precipitated the carbohydrate from a concentrated aqueous solution with alcohol and washed the product with cold, and finally hot, alcohol. From 1000 gm. dry grass roots, Müller obtained 15-20 gm. of triticin with $[\alpha]_D = -41°$ in water. As a result of his analytical studies, Müller concluded that the new carbohydrate was isomeric with cane sugar with a molecular formula $C_{12}H_{22}O_{11}$.

In 1880 Reidemeister(49) made a comparative study of "lavulin," sinistrin and triticin, comparing, or rather contrasting their action on polarised light and their sensitivity to heat. Triticin, he said, was readily decomposed by boiling its aqueous solution, to yield fructose, whereas sinistrin and "lavulin" were not decomposed by heating their aqueous solutions in a sealed tube at 100°. This agrees with Schlubach's observations that triticin is the most readily hydrolysed of all the naturally occurring fructosans.

In 1887 Ekstrand and Johanson(24) isolated a polysaccharide from Dracaena australis which they claimed to be very similar to triticin from Triticum repens L. The only significant difference in their properties lay in their optical rotatory powers. The authors quoted $[\alpha]_D$ as - 36·6° for the polysaccharide from Dracaena australis whilst for triticin $[\alpha]_D$ is quoted as - 41°. Considering the possibility that the two materials may differ only in their homogeneity, the authors stated that if they are not identical polysaccharides, they are at least very closely related.

Continuing this work, Ekstrand and Mauzelius,(50) using Raoult's method of molecular weight determination, concluded that the molecular formula of triticin (?) from Dracaena rubra ($[\alpha]_D = - 36{\cdot}6°$) is $C_{36}H_{60}O_{30}$. As far as is known, no further work has been done on this carbohydrate material from Dracaena.

Another material which was considered to be very similar to triticin from couch grass is levosin. Levosin was isolated from rye (0·8%), wheat and barley (0·2%) by Tanret⁽⁵¹⁾ in 1891, but was not found to be present in oats or maize. This material had $[\alpha]_D$ = -36° and was readily hydrolysed by heating its aqueous solution in a sealed tube, to yield fructose (3 parts) and glucose (1 part). Tanret pointed out the similarity of levosin to triticin and sinistrin. He compared the values of $[\alpha]_D$ in water and found $[\alpha]_D$ = - 47° for triticin, $[\alpha]_D$ = - 40·7° for sinistrin and $[\alpha]_D$ = - 36° for levosin. He believed that, although they differed in their rotatory powers, they yielded the same sugars on hydrolysis in different proportions.

The variation of the levosin content of cereals with maturation was also studied by Tanret. In rye, the content was found to remain unchanged on maturation (0·8%). In green wheat, the levosin content was found to be 0·8%, but dropped to 0·2% on ripening, and, in barley, an even greater fall was detected; viz. from 2% (green) to 0·1% (ripe).

Colin and Belval⁽⁵²⁾ isolated a product from wheat and rye flours which appeared identical with Tanret's levosin. This product, a white amorphous powder, with $[\alpha]_D$ = - 36° in water, yielded fructose (9 parts) and glucose (1 part) on hydrolysis.

The above work on levosin indicates the importance of the time of harvesting of the material from which a carbohydrate is to be extracted. From spring to autumn there is a gradual decrease in free sugar content with a corresponding increase in starch content. Also, as shown by Schlubach and Bandmann,(36) decreases in fructosan contents, and, in some cases, complete disappearances, have been observed during the ripening of cereals, with a corresponding increase in starch content.

It was not until 1937 that any chemical investigation into the structure of the polyfructosan from Triticum repens L. was carried out, although, as has been seen, the isolation of the material and a comparative study of certain properties received a good deal of attention from chemists of the 19th Century. Schlubach and Peitzner(53) studied the chemical nature of the acetate and methyl ether of triticin. By the high vacuum fractional distillation of the methyl-fructosides produced by hydrolysis of the fully-methylated triticin, these workers were able to propose a somewhat tentative structure for the molecule. Schlubach and Peitzner isolated a very pure sample of triticin from couch grass roots by 300 reprecipitations from water with alcohol. Their product was described as a white, nearly tasteless, almost non-hygroscopic solid with $[\alpha]_D^{20} = -51{\cdot}4°$ in water. This is in direct contrast to previous workers, all of whom described triticin as a very hygroscopic material.

The acetate was prepared by the action of acetic anhydride on this material in pyridine solution and after 490 reprecipitations from chloroform solution with petroleum ether, the product had $[\alpha]_D = -15{\cdot}5^\circ$ in chloroform. The action of potassium methoxide on the acetate yielded triticin in the same state of purity as the original sample.

Trimethyl triticin was prepared from the acetate by the action of dimethyl sulphate and caustic soda in acetone solution, according to Haworth, followed by silver oxide-methyl iodide methylations, according to Purdie. The resulting product had a methoxyl content of 45% and a specific rotation of - 61·2° in chloroform. This was hydrolysed with a solution of oxalic acid in aqueous alcohol, and the hydrolysate fractionated by distillation in a high vacuum to yield tetramethyl-, trimethyl-, and dimethyl-D-fructose in the ratio 3:1:3. A well-defined separation was finally achieved by benzoylation, high vacuum fractional distillation of the benzoyl derivatives and unchanged material, followed by debenzoylation and hydrolysis of each fraction.

The tetramethyl fraction was shown to be 1:3:4:6-tetramethyl-D-fructofuranose and the dimethyl fructose was thought to be identical with that obtained by hydrolysis of trimethyl sinistrin. The trimethyl fructose ($[\alpha]_D$ = - 10·5° ⟶ - 13·8° in water) however could not be identified, since 3:4:6-trimethyl-D-fructose, (from the inulin-type fructosans), has $[\alpha]_D$ = + 31°, and 1:3:4-trimethyl-D-fructose (from the phlein type) has $[\alpha]_D$ = - 47·6°, according to Schlubach and Sinh,(25) and - 52°, according to Challinor, Haworth and Hirst.(30) The authors therefore state that the trimethyl fructose obtained from methylated triticin is neither of the two which arise from other naturally-occurring fructosans, and triticin itself cannot be placed in either of the two groups of fructosans.

Cryoscopic determinations of the molecular weight of the polysaccharide gave values of 2600-2830, indicating that the molecule is built up from 16-17·5 fructose units. Since methylation studies indicated that the polysaccharide is composed of groups of 7 fructose residues, and since the pure material was found to be non-reducing, Schlubach and Peitzner concluded that the fructose units are arranged in closed rings, which are highly ramified, such that each unit ring structure contains 14 or 21 fructose units. Such a formulation must, however, be accepted with considerable reserve. Furthermore, since the trimethyl and dimethyl fractions obtained by the hydrolysis of the methylated polysaccharide could not be identified, the linkages between the fructose units are unknown.

It is the main object of the work for this thesis to attempt to clarify the structure of triticin, and to attempt to elucidate the type of linkage in the molecule, in order to confirm or refute Schlubach's statement that triticin apparently bears no relationship to either of the two general groups of fructosans.

Within the past decade there have been great advances in the field of carbohydrate chemistry with the development of partition chromatography. The application of paper partition chromatography to carbohydrates has made possible the detection and identification of minute quantities of sugar materials, in addition to providing an elegant method for the quantitative assay of sugars on the micro-scale. A method for the estimation of reducing sugars, using the Somogyi reagent, was developed by Hirst, Flood and Jones,(54) and this was rapidly followed by the development of the use of alkaline hypoiodite for the estimation of methylated aldoses by Hirst, Hough and Jones.(34) An alternative method for the determination of hexoses, pentoses, and methyl-pentoses by determination of the formic acid liberated on oxidation with sodium meta-periodate was developed, almost at the same time, by Hirst and Jones.(55) Paper partition chromatography has also proved to be a powerful tool in the separation of the methylated sugars produced on hydrolysis of a methylated polysaccharide, as has already been outlined (p. 23)

As great controversy has arisen over the presence or absence of glucose in the inulin molecule, it is desired to investigate whether inulin is an isolated example of a fructosan producing glucose on hydrolysis, or whether glucose is invariably produced and escaped detection previously, owing to the limitations of the classical chemical methods. Paper chromatography will be of immense importance in this respect.

Furthermore, the classical methods of high vacuum fractional distillation of the components of the hydrolysate of a methylated polysaccharide cannot be expected to give as clear-cut and as quantitative a separation as does elution through a column of powdered cellulose. As already mentioned, the whole number ratio of tetramethyl-, trimethyl- and dimethyl-fructoses produced frequently gave rise to more than one possibility, thus demanding a more accurate technique for their separation. It is believed that partition chromatographic separation will provide this improved accuracy.

It may be said, therefore, that a second primary object of this work is to discover how the results obtained using the various modern techniques and refinements in carbohydrate chemistry compare with those obtained using the older classical methods. The results obtained in this work will therefore be closely compared with the results obtained by Schlubach and Peitzner (loc.cit.) in their investigations into the structure of triticin.

In this survey of studies of extracts of the roots of couch grass, mention must finally be made of an aromatic enyne hydrocarbon extracted from the roots as a steam-volatile oil by Treibs.(56) A distillation of the roots yielded 0·052% of a volatile oil which, by steam distillation fractionation, yielded a homogeneous fraction in 95% yield. Treibs showed this substance to be 1-phenyl-n-hex-2ene-4-yne ($C_{12}H_{12}$) and called it agropyrene, from its botanical origin.

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EXPERIMENTAL.

Raw Material.

The couch grass (ca.7 Kg.) was gathered from a field under cultivation at New Graden, Roxburghshire, between April 9th and 12th, 1948. The rhizomes were cleaned and milled in a "Christy-Norris" hammer mill, using a No.18 mesh screen (0·0336 ins.).

The milled roots strongly reduced Fehling's solution and, in addition, a cold aqueous extract of the roots gave a positive test for fructose on application of the Seliwanoff test.

The Extraction and Identification of the Free Sugars.

Milled couch grass roots (5 g.) were subjected to continuous extraction with 80% aqueous alcohol in a Soxhlet extractor for 14 hours. The yellow alcoholic extract was filtered and concentrated under reduced pressure to small bulk, water being added at regular intervals. The aqueous solution was clarified by filtration through 'filter cel' and evaporated under reduced pressure to yield a dark brown viscous syrup (0·8 g.).

A paper chromatogram of the syrup run in butanol/ethanol/water and sprayed with aniline oxalate indicated the presence of fructose (R_G = 0·12), glucose (R_G = 0·093) and a probable disaccharide (R_G = 0·05), the relative amounts decreasing in the order given.

Estimation of Glucose and Fructose.

The glucose and fructose in the syrup obtained by alcoholic extraction of the couch grass roots were estimated on the chromatogram, using Somogyi's reagent, according to Flood, Hirst and Jones.(1)

Estimation I. Fructose = 16·3%, Glucose = 3·0%,
Total = 19·3%.

Estimation II. Fructose = 18·4%, Glucose = 3·8%,
Total = 22·2%.

Further Investigations of the Free-Sugar Extract.

The syrup gave a positive test for nitrogen, but the biuret reaction for proteins was inconclusive.

The Ninhydrin Test.

The syrup was refluxed with 35% sulphuric acid for 18 hours, in order to hydrolyse any protein. The deep red solution was neutralised by the addition of baryta, followed by excess barium carbonate, any bicarbonate being decomposed by heating on the water bath at 80° for 1 hour. The barium sulphate was removed by filtration, washed well with water and the aqueous solution evaporated to a syrup, which gave a positive nitrogen test, and a positive ninhydrin reaction. This indicates the presence of protein in the original alcoholic extract.

Study of the Disaccharide present.

Paper chromatograms of the alcohol extract indicated, in addition to glucose and fructose, a spot with $R_{\underline{G}}$ = ca. 0·05, which is probably due to a disaccharide.

Using the technique employed by Flood, Hirst and Jones(1) in quantitative paper chromatography, the suspected disaccharide was extracted from the paper with water (5 ml.) and hydrolysed by heating at 80° for 1 hour with 2N sulphuric acid (5 ml.). After neutralisation with barium carbonate and concentration to a syrup, paper chromatograms indicated the presence of glucose and a spot with $R_{\underline{G}}$ = 0·053, presumably unchanged material. After hydrolysis for a further 3 hours, paper chromatograms indicated the presence of glucose (mainly), fructose and a trace of xylose, with no disaccharide spot.

Uronic Acid Investigations.

A series of paper chromatograms run in butanol/acetic acid/water, and sprayed with a saturated solution of o-phenetidine and trichloracetic acid in alcohol, showed no evidence for the presence of uronic acid.

The naphthoresorcinol test for uronic acid was carried out on the syrup against a galacturonic acid control. This gave a negative reaction for the syrup under investigation.

Large-Scale Extraction of Free Sugars.

Couch grass roots (800 g.) were extracted with 80% alcohol (3 litres) for 20 hours in a copper Soxhlet extractor, in exactly the same way as described above. This yielded a syrup (103 g.), corresponding to a yield of 13% from the original grass roots. This agreed well with yields of 13-14% obtained from a series of small-scale extractions.

The residue from the alcoholic extraction (660 g.) was available for the extraction of the polysaccharide.

Extraction of the Polysaccharide.

a) Removal of protein with basic lead acetate.

Milled couch grass roots (10 g.), from which free sugars had been removed as described above, were shaken with cold water (100 ml.) for 4 hours, in the presence of barium carbonate and a little toluene.

The aqueous extract was removed by filtration through a wad of cotton wool, and to this solution was added a saturated solution of basic lead acetate in water at 60°, until precipitation appeared complete. The precipitate was removed by filtration through a pad of 'filter cel,' and Pb^{++} removed from the filtrate by passage of hydrogen sulphide for 5 minutes. The lead sulphide was removed by filtration through 'filter cel,' hydrogen sulphide again passed through the solution to ensure that Pb^{++} removal was complete, and hydrogen sulphide removed from the solution by aeration for 1 hour.

The aqueous solution was then concentrated under reduced pressure (40°/15 mm.) and the polysaccharide precipitated by pouring the concentrated solution (20 ml.) dropwise into alcohol (600 ml.), with constant stirring. The polysaccharide precipitated as a white fibrous material, which readily filtered, and was reprecipitated a further twice. After washing with ether, the polysaccharide was obtained as a fine white hygroscopic powder (1·48 g.; $[\alpha]_D^{16} = -43°$ in water), which was dried in a vacuum desiccator on porous tile over phosphoric oxide, and finally in a high vacuum. [Sample A.].

Ash Determination.

The ash content of the polysaccharide was determined by ignition to constant weight in a platinum crucible.

Ash value = 3·53% (direct ignition)
= 3·47% (as sulphate).

Nitrogen Estimation.

Nitrogen estimations were performed on the polysaccharide sample (0.15 g.), using the micro-Kjeldahl apparatus.

$$\text{Analysis: } \left.\begin{array}{l} \text{a) Nitrogen} = 0{\cdot}320\% \\ \text{b) Nitrogen} = 0{\cdot}327\% \end{array}\right\} \text{Protein} = 2{\cdot}02\%$$

Estimation of Adsorbed Alcohol.

The alcohol adsorbed by the polysaccharide sample was estimated by the method of Newman,(2) as applied to fucoidin by Percival and Ross.(3)

Results.

Wt.of polysac.	Blank Titre	Test Titre	% Alcohol
53·8 mg.	20·35 ml.	16·9 ml.	1·84
45·1 mg.	20·35 ml.	17·33 ml.	1·93

mean value = 1·89% adsorbed alcohol.

On correction for the ash content (3·5%), protein content (2·0%) and alcohol content (1·9%), the value of $[\alpha]_D$ for the polysaccharide sample becomes:

$$\frac{100}{92\cdot6} \text{ of } -\frac{43^\circ}{1} = -\underline{46\cdot4^\circ}$$

b) Removal of protein by the Sevag method.

Milled couch grass roots (5·3 g.), which had been subjected to a preliminary alcoholic extraction, were shaken with water (75 ml.) for 5 hours, and the aqueous solution filtered from the residue (4·3 g.) through a pad of cotton wool.

Protein was removed from the aqueous extract by a technique described by Sevag, Lackmann and Smollens.(4) This consists of shaking the aqueous solution with chloroform (0·25 volumes) and butanol (0·1 volumes) for 2 hours, whereby protein is removed as a white stable gel which may be removed from the aqueous solution in a separating funnel. The process was repeated 10 times until no gel was produced on shaking. The aqueous solution was then concentrated under reduced pressure, and the polysaccharide precipitated from alcohol, as described earlier.

The product was obtained as a fine white hygroscopic powder (0·75 g.; $[\alpha]_D^{16} = -44°$ in water) after 2 reprecipitations. [Sample B].

Ash Determination.

Ignition to constant weight in a platinum crucible gave an ash value of 4·95% (direct ignition)
4·97% (as sulphate).

Nitrogen Content.

This was determined using the micro-Kjeldahl apparatus.

$$\text{Analysis: } \left.\begin{array}{l} \text{a) Nitrogen} = 0{\cdot}261\% \\ \text{b) Nitrogen} = 0{\cdot}266\% \end{array}\right\} \text{Protein} = 1{\cdot}65\%.$$

Alcohol Content.

Using the method of Newman,(2) the alcohol content was estimated as 1·5%.

On correction for the above ash, protein and alcohol contents the value of $[\alpha]_D$ becomes:

$$\frac{100}{92{\cdot}0} \text{ of } -\frac{44°}{1} = \underline{-48°}$$

Application of the Sevag Method on a larger scale.

The method was repeated using 30 g. of dry grass roots as starting material. On this occasion, a total of 23 treatments in all were required by the Sevag process, before protein removal appeared complete. The polysaccharide was then precipitated from the concentrated aqueous solution, as before, and reprecipitated 3 times. This yielded a heavy, fibrous product in high yield (8·4 g.; $[\alpha]_D^{17} = -29°$ in water). [Sample C].

c) Removal of protein with neutral lead acetate, followed by the Sevag method.

Milled couch grass roots (29 g.) were extracted with water (200 ml.), as before, and protein removed by the addition of neutral lead acetate solution dropwise at 40° until no further precipitation occurred. The precipitate was removed by filtration, Pb^{++} removed as lead sulphide and hydrogen sulphide removed from the solution by aeration.

To remove last traces of protein, the aqueous solution was concentrated to 100 ml. and shaken with chloroform (25 ml.) and butanol (10 ml.). After repeating once, no gel was produced. The polysaccharide was then precipitated in the normal manner and reprecipitated twice, to yield a fine white powder (6·1 g.; $[\alpha]_D^{16} = -33°$ in water). [Sample D].

Ash Content = 3·5% (by direct ignition)

= 3·5% (as sulphate).

d) Removal of protein with cadmium hydroxide.

The effect of cadmium hydroxide as a deproteinizing agent was studied, using the method outlined by Fujita and Iwatake$^{(5)}$ for the removal of proteins from the blood.

Four extractions of milled alcohol-extracted couch grass roots (10 g. each) were made and, in the clarification of the aqueous extracts with cadmium hydroxide, 4 different techniques were employed.

(i) To a solution of cadmium sulphate ($CdSO_4.8H_2O$; 1 g.) in water was added N.NaOH (7·8 ml.) and the resulting suspension of cadmium hydroxide was added dropwise to the boiling aqueous extract in the presence of barium carbonate. After filtration, Cd^{++} were removed from the solution as cadmium sulphide and hydrogen sulphide removed by the passage of compressed air. The last traces of protein material were removed by shaking with chloroform-butanol, according to Sevag, and the process repeated three times. The polysaccharide was precipitated in aqueous alcohol in the normal manner to yield a fine white hygroscopic powder (3·7 g.; $[\alpha]_D^{15}$ = - 28° in water). [Sample E].

(ii) A cadmium hydroxide suspension was added to the aqueous extract as in (i), but Cd^{++} were removed by passage of the solution through zeocarb-deacidite ion-exchange columns, when the extract was colourless. Precipitation of the polysaccharide from alcohol yielded a fine white powder (0·94 g.; $[\alpha]_D = -48°$ in water). [Sample F].

(iii) Here the cadmium hydroxide was formed '<u>in situ</u>' in the aqueous extract. To the boiling aqueous extract, containing barium carbonate, was added cadmium sulphate (1 g.), and, after 1 minute, N.NaOH (5 ml.) was added dropwise. Cd^{++} were then removed as sulphide, as in (i), and 1 Sevag treatment showed that complete removal of protein had occurred, using this technique. This yielded a fine white powder (1·7 g.; $[\alpha]_D^{16} = -36°$ in water). [Sample G].

(iv) The solution was deproteinized with cadmium hydroxide, using the technique outlined in (iii), and Cd^{++} were then removed by passage of the solution through zeocarb-deacidite columns. After shaking with chloroform-butanol, the polysaccharide was precipitated in alcohol as a fine white powder (1·4 g.; $[\alpha]_D^{18} = -44°$ in water). [Sample H].

<u>The Purification of the Polysaccharide by Acetylation followed by Deacetylation</u>.

<u>The Acetylation of Triticin</u>.

The impure polysaccharide (3·5 g.; Sample C) was dissolved in the minimum amount of water (7 ml.) and redistilled pyridine (35 ml.) added. The pyridine-water azeotrope was removed at 40°/15 mm., more pyridine added, and the process repeated several times until finally a solution of the polysaccharide in dry pyridine was obtained. Acetic anhydride (40 ml.) was added to the solution, with stirring, over a period of 1 hour, the stirring continued for a further 3 hours and the mixture then shaken for 20 hours. The deep red solution was filtered from a small amount of insoluble material (0·3 g.; $[\alpha]_D = -27°$ in water) and the acetate precipitated by dropping the solution into water (1 l.) with constant stirring. After standing overnight, the acetate was removed by filtration and washed well with water for 7 hours until free from pyridine and acetic acid. The white powder was dried on porous plate in a vacuum desiccator over calcium chloride, dissolved in chloroform (25 ml.) and precipitated by pouring into light petroleum (b.p. 60° - 80°; 250 ml.). The product was then dried in a vacuum desiccator over phosphoric oxide and paraffin wax and, finally, in a high vacuum, to yield a fine white powder (2·2 g.).

$[\alpha]_D^{17} = -11°$ (c = 2 in chloroform).

% CH_3CO = 42·0.

The Deacetylation of Acetylated Triticin.

The acetate was deacetylated with sodium methoxide, according to Zemplén and Pacsu.(6)

The acetate (2·1 g.) was dried in a high vacuum, dissolved in dry chloroform (15 ml.) and cooled in a freezing mixture. A solution of sodium (0·1 g.) in absolute methanol (5 ml.) was added and the semi-solid mixture shaken for 5 hours. Ice water (5 ml.), 10% acetic acid (2 ml.) and water (13 ml.) were added.

Removal of the chloroform layer, concentration under reduced pressure and precipitation with light petroleum gave $<$ 10 mg. unchanged material.

The deacetylated product was precipitated from the concentrated aqueous layer with alcohol to yield a fine, white, hygroscopic powder (0·9 g.; $[\alpha]_D^{17} = -44°$ in water). [Sample J].

Ash content = 2·8% (by direct ignition)
= 4·3% (as sulphate).

If allowance is made for this ash content, the effect is to increase the value of the specific rotation by <u>ca</u>. 2°.

i.e. $[\alpha]_D^{17} = -46°$ (c = 1 in water).

<u>Fractional Precipitation of the Polysaccharide</u>.

A sample of the polysaccharide ($[\alpha]_D = -44°$), isolated in the same way as specimen H, was submitted to fractional precipitation from its aqueous solution with alcohol.

The polysaccharide (3·8 g.) was dissolved in water (30 ml.) and absolute alcohol (85 ml.) added to the solution with stirring, to yield a white turbidity. The solution could not be clarified by centrifuging but was clarified by filtration through a pad of 'filter cel.' Extraction of the pad with water yielded < 10 mg. material.

Addition of alcohol (300 ml.) to the above solution after clarification gave a gum-like precipitate, which hardened on trituration with alcohol, to yield a fine, white, slightly hygroscopic powder (2·9 g.; $[\alpha]_D^{18} = -43°$ (c = 1·6 in water)). [Fraction I].

Addition of a further 200 ml. alcohol to the filtrate from Fraction I gave a white turbidity, which could not be centrifuged. The solution was evaporated under reduced pressure, to yield a glass which, on trituration with alcohol, yielded a fine, white powder (0·6 g.; $[\alpha]_D^{18} = -42°$ (c = 3 in water)). [Fraction II].

Total recovery = 3·5 g. (92%).

Reducing Action of Triticin.

Both fractions of the fractionated polysaccharide gave a slight reduction of Fehling's solution on boiling for 15 minutes, a similar result being obtained with the purest sample of triticin available (Sample F; $[\alpha]_D = -48°$). Analar sucrose, however, also gave a slight reduction of Fehling's solution under these conditions, and it would appear that some degradation occurs on prolonged heating.

The Hydrolysis of the Polysaccharide.

A pure sample of polysaccharide (0·1 g.) was hydrolysed by heating on a water bath at 80° with 1% oxalic acid solution (10 ml.) for 4 hours. $[\alpha]_D^{17} = -48°$ (c = 1 in water) (zero) $\longrightarrow$ - 84° (10 minutes) constant.

The hydrolysate was neutralised with calcium carbonate, filtered, and inorganic ions removed from the solution by shaking with "amberlite" ion-exchange resins (IR-100(H) and IR-4B(OH)). The solution was then evaporated to a syrup under reduced pressure and spotted on the chromatogram. Paper chromatograms run in butanol/ethanol/water, and developed with aniline oxalate, indicated the presence of fructose (mainly) and glucose or galactose ($\underline{R}_G = 0{\cdot}085$). Paper chromatograms run in butanol/pyridine/benzene/water, however, indicated that glucose was present but that galactose was absent.

A series of hydrolyses of various samples of the polysaccharide invariably produced glucose in addition to fructose on the chromatogram. The extreme ease of hydrolysis of the polysaccharide was demonstrated by the fact that, with 0·1% oxalic acid solution as the hydrolysing agent, hydrolysis was complete in 10 to 15 minutes.

Attempted Preparation of Galactose 1-methyl 1-phenylhydrazone.

To decide whether any galactose was present in the polysaccharide hydrolysate, it was decided to treat the hydrolysate with asymmetrical methyl-phenylhydrazine, according to the method outlined by Hirst, Jones and Woods.(7)

For this purpose, a sample of polysaccharide (0·5028 g.) was hydrolysed with 1% oxalic acid (50 ml.), using the procedure described above. A synthetic mixture (0·50 g.) of fructose (90%) and galactose (10%) was prepared and used as a control experiment.

Water (10 ml.) was added to the hydrolysate and to the synthetic mixture, and to each solution the following reagents were added:-

2·5 g. redistilled 1-methyl 1-phenylhydrazine
10 ml. absolute ethanol
0·3 ml. glacial acetic acid.

The mixture was kept at 35° for 18 ~~hourx~~ hours and then cooled at 0° for 24 hours. The crystals were filtered into a weighed sintered glass crucible, washed with ice-cold ethanol and dried at 90° for 30 minutes.

Control experiment.

This yielded a yellow solid (41 mg.).

m.p. = 176° - 178° (d).

There was no depression of the melting point on admixture with an authentic specimen of galactose methylphenylhydrazone.

Hydrolysis Product from Polysaccharide.

This yielded only a minute amount of solid (ca.6 mg.).

Autohydrolysis of the Polysaccharide.

Fraction I of fractionated polysaccharide (0·1 g.) was dissolved in water (10 ml.) and heated on a boiling water bath to constant rotation.

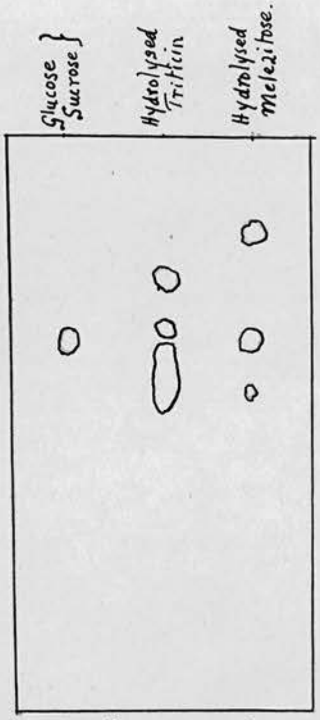
$[\alpha]_D^{16}$ = - 44° (zero) ⟶ - 46° (3 hours) ⟶ - 51° (12 hours) ⟶ - 54° (16 hours) ⟶ - 75° (19 hours) ⟶ - 81° (24 hours) constant.

The aqueous solution was concentrated under reduced pressure and spotted on the chromatogram. Duplicate papers were run in ethyl acetate/acetic acid/ water. One paper was sprayed with naphthoresorcinol reagent, prepared by mixing an 0·2% solution of naphthoresorcinol in ethanol (3 parts) with concentrated hydrochloric acid (1 part).(8) This showed the presence of fructose, 2 spots of approximately the same R_G value as sucrose (0·039) and a fructose derivative with R_G = 0·056. A solution of the fructosan itself gave only a pink spot on the starting line with naphthoresorcinol.

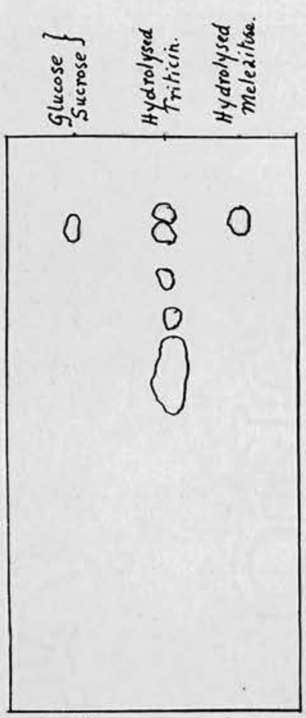
The other paper, sprayed with ammoniacal silver nitrate, showed the presence of fructose, glucose and a reducing spot with $\underline{R_G}$ = 0·056.

Fraction II of the fractionated polysaccharide yielded precisely the same results on hydrolysis with boiling water, viz. fructose, glucose, a reducing spot ($\underline{R_G}$ = 0·056) which gave a pink coloration with naphthoresorcinol, and 2 non-reducing spots which travelled at the same rate as sucrose.

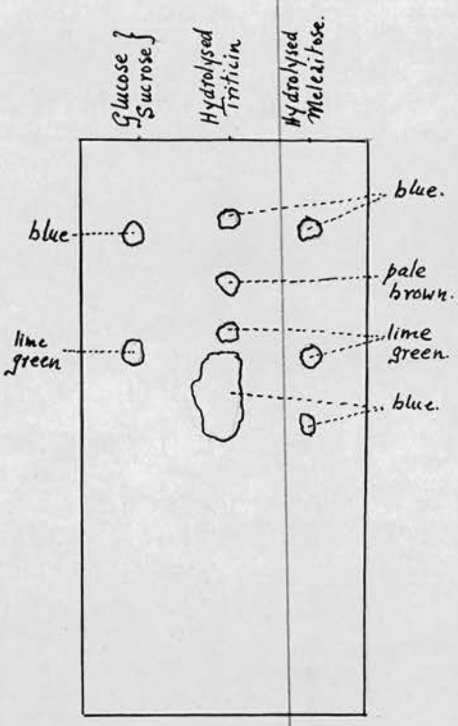
In order to determine the rate at which turanose travelled, as compared with sucrose, in ethyl acetate/acetic acid/water, melezitose was submitted to controlled hydrolysis.(9) A pure sample of melezitose was heated on a gently boiling water bath with concentrated sulphuric acid (0·7% $^{w}/_{v}$) for 20 minutes. The solution was immediately cooled, neutralised with barium carbonate, filtered and the filtrate freed from inorganic ions by shaking with "amberlite" resins. The solution was evaporated to a syrup under reduced pressure and spotted on the chromatogram. A series of chromatograms were run in ethyl acetate/acetic acid/water and developed with various spray reagents, the melezitose hydrolysate being compared with the aqueous hydrolysate of the polysaccharide. The results are summarised in the diagrams below.



Ammoniacal Silver Nitrate.



Naphthoresorcinol-Hydrochloric Acid.



Urea Oxalate.

Butanol/ethanol/ammonia/water gave no effective separation of the component sugars.

The syrup obtained by the autohydrolysis of triticin was dissolved in water (10 ml.), oxalic acid (0·1 g.) added and the aqueous solution heated on the water bath at 80° for 5 hours. The solution was neutralised with calcium carbonate, filtered, shaken with "amberlite" ion-exchange resins and the solution evaporated to a syrup under reduced pressure. This syrup was spotted on the chromatogram and the papers were run in ethyl acetate/acetic acid/water, when fructose and glucose only were detected. There was no trace of any material between glucose and the starting line when spraying with ammoniacal silver nitrate or urea oxalate.

The Colorimetric Estimation of the Fructose produced on hydrolysis of the polysaccharide.

Reagents.

Acid Reagent: 130 g. glycerol (B.P.), 100 ml. conc. hydrochloric acid (A.R.) containing 45 mg./l. $CuSO_4.5H_2O$, and 50 ml. water.

Resorcinol: 0·45% in water.

Stock Fructose Solution: 5 mg./ml. in saturated benzoic acid solution.

Standard fructose solutions, the concentrations of which ranged from 0·02 mg./ml. to 0·2 mg./ml., were prepared by dilution of the stock solution.

A standard curve for fructose was constructed over the range 0·02 mg./ml. - 0·20 mg./ml. The fructose used for the construction of the standard curve had $[\alpha]_D^{16} = -103° \longrightarrow -92°$ (constant).

The procedure, which was rigidly followed for each solution, was as follows:-

The acid reagent (5 ml.), followed by the resorcinol solution (1 ml.) and the standard fructose solution (2 ml.) were transferred by pipette to a pyrex test tube (6" x 3/4"), fitted with a close-fitting glass bulb at the mouth. After thorough mixing, the contents of the tube were heated in a boiling water bath for exactly 12 minutes. The solution was cooled at once in running water and then read against a blank carried through the procedure at the same time, using the Spekker absorptiometer (1 cm. cells and I.S. violet 601). It was observed that, provided the reagents were well-mixed before heating, there was no change in the colour intensity over a period of 2 hours.

Concentration of fructose. (mg./ml.)	$\log_{10} \frac{I}{I_o}$
0·02	0·143
0·05	0·367
0·075	0·481
0·10	0·636
0·15	0·900
0·20	1·195

A standard solution of pure polysaccharide ($[\alpha]_D$ = - 48°) containing 0·1 mg./ml. was made. The fructosan solution was treated according to the method outlined above, and the concentration of the fructose was determined from the standard graph.

Results of the estimation.

Concentration of fructosan solution = 0·111 mg./ml.

Determination I Spekker Reading = 0·715

Concentration of Fructose = 0·115 mg./ml

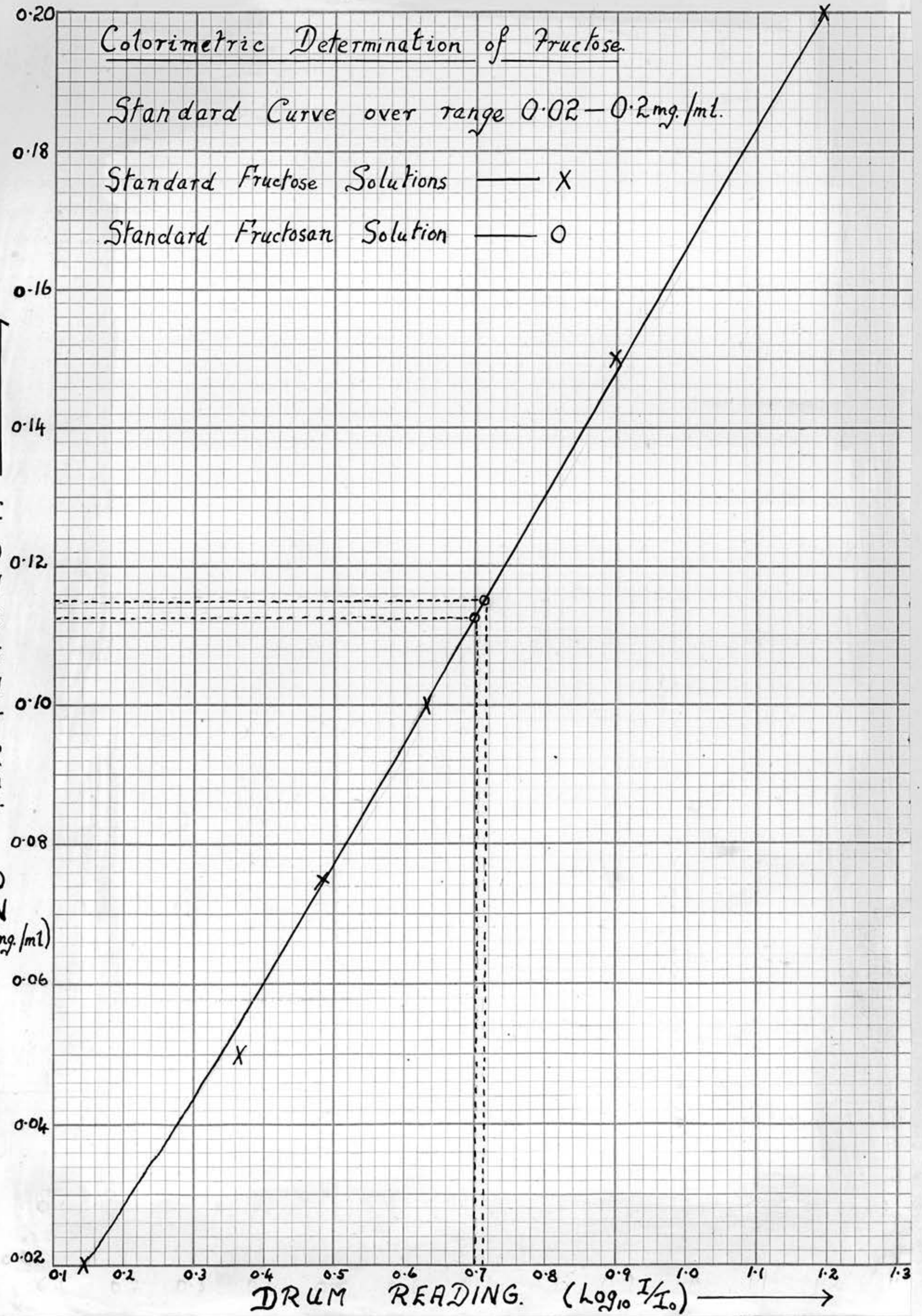
∴ % Fructose in hydrolysate = 92·6.

Determination II Spekker Reading = 0·702

Concentration of Fructose = 0·112 mg./ml

∴ % Fructose in hydrolysate = 91·0.

Mean value of fructose produced on hydrolysis = 91·8%



Colorimetric Determination of Fructose.

Standard Curve over range 0·02 – 0·2 mg./ml.

Quantitative Estimation of the glucose produced on hydrolysis of the polysaccharide.

The fractionated polysaccharide (0·1027 g.) was dissolved in water (10 ml.) and heated on a boiling water bath for 36 hours. The aqueous solution was then concentrated under reduced pressure to yield a thin, straw-coloured syrup which was dried in a high vacuum over phosphoric oxide. Yield 0·1034 g.

The syrup was dissolved in water (5 ml.) and spotted on the chromatogram. In order to be able to estimate accurately a small amount of glucose in the presence of a large amount of fructose, the hydrolysate solution was spotted on a number of paper chromatograms, using a micro-pipette. This ensured that each paper contained exactly the same weight of sugar material. 5 Papers were spotted thus, and the sugars separated by running in ethyl acetate/acetic acid/water for 40 hours. The paper strips were freed from acetic acid prior to aqueous extraction by evacuating in a desiccator over water overnight. A paper blank, on pulping in water, then showed no acid reaction to methyl red. Glucose, from 5 paper strips, and fructose, from 2 paper strips, were extracted by allowing cold water to run down the length of the strips, using the procedure described by Laidlaw and Reid.(10) Cold water extraction was continued for 30 minutes, paper blank strips being extracted alongside.

Sodium meta-periodate solution (1 ml.; 0·25M) was added to each solution, and the solutions oxidised by heating on a boiling water bath for 30 minutes. After cooling under the tap, ethylene glycol (0·2 ml.) was added and the liberated formic acid titrated against 0·01N sodium hydroxide using methyl red as indicator.

Result.

Paper Blank I titre = 0·290 ml. NaOH.

Paper Blank II titre = 0·240 ml. NaOH.

Glucose titre (from 5 paper strips) = 0·540 ml. NaOH.

∴ Formic acid liberated from glucose ≡ (0·540-0·270)ml.
= 0·270 ml. NaOH.

Fructose titre (from 2 paper strips) = 1·350 ml. NaOH.

∴ Fructose from 5 strips $= \frac{1\cdot350 \times 5}{2} = 3\cdot375$ ml. NaOH.

∴ Formic Acid liberated from fructose ≡ (3·375-0·270)ml.
= 3·105 ml. NaOH.

∴ % Glucose is $\frac{\cdot270 \times 3 \times 100}{5 \times 3\cdot105} = \underline{5\cdot2\%}$.

Now % Fructose in hydrolysate = 91·8% (by colorimetric assay).

∴ % Glucose in hydrolysate $= \frac{5\cdot2 \times 91\cdot8}{100}$

$= \underline{4\cdot8\%}$.

Studies on the Methylated Polysaccharide.

The Acetylation of Triticin.

A solution of the polysaccharide (34 g.) in pyridine (400 ml.) was made, using the method of Pacsu and Mullen(11) already described (p. 55), and the product was acetylated by the addition of acetic anhydride (400 ml.) with stirring, the procedure being the same as that described previously. The acetate was precipitated by pouring into water and washed free of pyridine and acetic acid. The product was then dried in a vacuum desiccator on porous plate over calcium chloride and paraffin wax, and finally in a vacuum oven at 40°/30 mm. over concentrated sulphuric acid. This removed last traces of pyridine and yielded a buff-coloured solid (42 g.). On reprecipitation twice from chloroform with light petroleum, the product (37·5 g.) still had a faint buff colour.

$[\alpha]_D^{15} = - 11°$ (c = 3 in chloroform).

Acetyl Content. a) 44·6%, b) 45·0%, (theory, 44·8%).

Fractionation of the Acetate.

The acetylated polysaccharide (32·7 g.) was dissolved in chloroform (350 ml.) and fractionally precipitated by the addition of light petroleum (b.p. 60° - 80°) in 100 ml. portions, the solution being stirred vigorously.

On addition of 650 ml. light petroleum, a red, fibrous material separated, the chloroform solution of which was not optically active.

Fractionation then occurred as follows:-

Fraction I	750 ml. light petroleum	8·3 g.
Fraction II	850 ml. " "	11·5 g.
Fraction III	950 ml. " "	5·0 g.
Fraction IV	1350 ml. " "	6.6 g.
Fraction V.	Concentration of solution to 50 ml. and addition of light petroleum.	0·6 g.
Fraction VI	Residue after distillation of solvent.	0·2 g.

Total recovery = 32·2 g. (98%).

The first four fractions were obtained as white powders, Fraction V was coloured yellow and Fraction VI was obtained as a yellow syrup.

The fractions had the following constants:-

Fraction I $[\alpha]_D^{17} = -$ 9° (c = 3 in chloroform)
% CH_3CO = 41·3.

Fraction II $[\alpha]_D^{17} = -$ 9° (c = 3 in chloroform)
% CH_3CO = 41·9.

Fraction III $[\alpha]_D^{17} = -$ 9° (c = 3 in chloroform)
% CH_3CO = 42·2.

Fraction IV $[\alpha]_D^{17} = -$ 7° (c = 3 in chloroform
% CH_3CO = 42·9.

Fraction V $[\alpha]_D^{17} = +$ 2·5° (c = 1·5 in chloroform)
% CH_3CO = 45·6.

Fraction VI $[\alpha]_D^{17} = +$ 25° (c = 1·5 in chloroform).

The specific viscosities of 1% solutions in chloroform of the various fractions were determined using a closed Ostwald viscometer immersed in a thermostat at 20°C.

Fraction	Time of flow.	η_{sp}/20°	c(g./l.)	η_{sp}/c	η_{sp}/C'
Chloroform	65·4 secs.				
I	69·2 "	·058	10·68	·0055	1·567
II	69·7 "	·065	11·50	·0057	1·647
III	68·5 "	·047	11·00	·0043	1·242
IV	67·5 "	·032	11·53	·0029	0·800
V	68·7 "	·050	16·56	·0030	0·877

where c' is the concentration in g.mols/litre of acetylated anhydrofructose residues, assuming the repeating unit to be a trihydroxy-anhydrofructose $(C_6H_{10}O_5)$.

It was decided to combine fractions I and II and to use fraction III for a trial methylation.

The Methylation of Acetylated Triticin.

Trial Methylation.

Fraction III of the fractionated acetate (4·6 g.) was dissolved in acetone (200 ml.) and methylated by a procedure similar to that described by Haworth and Streight(12) for the methylation of inulin.

Dimethyl sulphate (100 ml.) and 30% caustic soda (250 ml.) were added simultaneously to the solution with vigorous stirring, the rate of addition being

1 part of dimethyl sulphate to 2·5 parts of caustic soda. The flask was immersed in a water bath, the temperature of which was maintained at 40°, and the reaction was carried out in an atmosphere of nitrogen. The acetone was then removed in partial vacuum, and the temperature slowly raised to 75° and maintained thus for 1 hour. The white, slightly sticky solid was filtered hot, washed with a little boiling water (7 ml.) and returned to the flask.

After 3 such methylations, the cream-coloured solid was washed thoroughly with boiling water till free of sulphate, and dried at the pump. The solid was then dissolved in chloroform (100 ml.), the solution allowed to stand overnight over anhydrous sodium sulphate, and the methylated polysaccharide precipitated from solution with light petroleum (b.p. 60° - 80°). This yielded a fine, white powder which was dried in high vacuum (1·8 g.; OMe, 42·7% (theory, 45·6%)).

The methylated polysaccharide was then refluxed with methyl iodide (40 ml.) for 7 hours, silver oxide (18 g.) being added in 2 g. batches every 30 minutes. The methylated product was recovered from the silver oxide by repeated extraction with boiling chloroform. The combined extracts were dried over anhydrous sodium sulphate and the methylated polysaccharide precipitated from the concentrated chloroform solution with light petroleum. This yielded a fine, white solid (1·44 g.) OMe = 42·7% $[\alpha]_D^{22} = -57°$ (c = 1 in chloroform). The material was therefore unchanged after 1 methyl iodide-silver oxide methylation, and a further methylation by this method gave no further increase in methoxyl content.

Comparison of viscosities of methylated and acetylated triticin solutions.

The viscosity of the methylated polysaccharide (OMe, 42·7%) in m-cresol was, at this stage, compared with that of the acetylated polysaccharide (CH_3CO, 42·2%) from which it was derived.

The measurements were made in an Ostwald viscometer immersed in a thermostat at 20°.

Substance.	Wt. in 1000 ml. m-cresol (c).	Time of flow	$\eta_{sp/20°}$	η/c	η/c'
m-Cresol		433 secs.			
methylated triticin	3·711 g.	445 secs.	·028	·0075	1·54
acetylated triticin	3·711 g.	441 secs.	·018	·0050	1·44

where c' = concentration in g.mol./litre of methylated or acetylated anhydrofructose residues.

Methylation with Thallous Ethoxide - Methyl Iodide.

The partially-methylated polysaccharide (1·1 g.; OMe, 42·7%) was dissolved in chloroform (60 ml.) and 'filter cel' added to the solution. A solution of thallous ethoxide (1·3 g.) in chloroform (15 ml.) was then added and, after shaking well, the chloroform was removed from the mixture by distillation under reduced pressure, the flask being protected from light during the process. The grey solid residue was finely powdered in the dark and refluxed with methyl iodide (60 ml.) until the yellow thallous iodide was no longer alkaline to phenolphthalein. The methyl iodide was then removed under reduced pressure and the residue exhaustively extracted with boiling chloroform. The combined extracts were dried over anhydrous sodium sulphate, concentrated under reduced pressure and the methylated polysaccharide precipitated with light petroleum (b.p. 60° - 80°), to yield a fine, white powder (0·92 g.; OMe, 43%).

The product had:

$[\alpha]_D = -59°$ (c = 1 in chloroform).

Ash content = 0·42%.

Correction for the above ash content gives a methoxyl content of 43·2%.

Hydrolysis of Methylated Triticin.

The method adopted was similar to that described by Haworth, Hirst and Percival (13) for the hydrolysis of methylated inulin.

The methylated polysaccharide (16 mg.) was heated with methanol (0·45 ml.) and water (0·15 ml.) containing oxalic acid (6 mg.) in a sealed tube immersed in a water bath at 80° for 14 hours. The solution was neutralised with calcium carbonate and centrifuged, the solution removed by pipette and concentrated under reduced pressure.

Paper chromatograms sprayed with a saturated solution of urea oxalate in water indicated the presence of tetramethyl fructofuranose ($\underline{R_G}$ = 1·0), a trimethyl fructose ($\underline{R_G}$ = 0·84), a dimethyl fructose ($\underline{R_G}$ = 0·62) and a spot travelling faster than tetramethyl fructofuranose ($\underline{R_G}$ = 1·08).

There was no evidence as to the presence of monomethyl fructose or free sugar.

Large-Scale Methylation of Fractionated Acetate.

Fractions I and II of the fractionated acetate were combined and the total material (19 g.) methylated, the procedure being similar to that already described.

The acetate was dissolved in acetone (500 ml.) and dimethyl sulphate (210 ml.) and 30% sodium hydroxide (560 ml.) added simultaneously with efficient stirring. The reaction was performed in a water bath at 40° and in an atmosphere of nitrogen. The partially-methylated material was isolated in the manner described previously to yield a brown, hard, solid mass. This material was methylated thus a second time, the volumes of reagents being reduced by some 25%. The product was isolated in a more finely-divided form on this occasion.

The liquor from the 1st methylation was dialysed for 7 days when, on concentration, no turbidity was obtained with barium chloride. The concentrated deep yellow solution was filtered and poured into alcohol, but no precipitation occurred, and the alcoholic liquor, on evaporation, yielded no significant residue. It may, therefore, be assumed that practically no loss occurs in methylation due to material remaining in solution.

After a third methylation with dimethyl sulphate-sodium hydroxide, the temperature of the water bath was raised to 100° and maintained thus for 1 hour, in order to destroy any sodium methyl sulphate. The pale yellow solid was filtered rapidly while hot and washed thoroughly with boiling water (2 l.) till free of sulphate. The product was dried at the pump and then on porous plate in a vacuum desiccator over calcium chloride.

The crude material (11·9 g.) was then methylated directly with methyl iodide (85 ml.) and silver oxide (60 g.), according to Purdie, the procedure being identical to that already described (p.71.). The methylated polysaccharide was then precipitated from chloroform with light petroleum (b.p. 60° - 80°) to yield a white solid (9·7 g.; OMe, 43·3%).

$[\alpha]_D^{18} = -61°$ (c = 1 in chloroform).

Viscosity measurements.

A comparison was made of the viscosities of solutions of the methylated polysaccharide (OMe, 43·3%) and the acetylated polysaccharide (CH_3CO, 41·6%) in m-cresol.

Substance	Wt. in 1000 mls. m-cresol (c)	Time of flow	$\eta_{sp}/20°$	η/c	η/c'
m-cresol		429·6 secs.			
methylated polysaccharide	3·680 g.	440·4 secs.	0·025	0·0068	1·39
acetylated polysaccharide	3·770 g.	436·5 secs.	0·016	0·0043	1·23

Fractionation of the Methylated Polysaccharide.

The methylated polysaccharide (9·7 g.) was dissolved in chloroform (70 ml.) and light petroleum (b.p. 60° - 80°) added slowly and with constant stirring in batches of 100 mls.

Fractionation occurred as follows:-

Fraction I	700 ml. light petroleum	5·2 g.
Fraction II	1100 ml. light petroleum	2·44 g.
Fraction III	Residue after removal of solvent	1·06 g.

Total recovery = 8·7 g. (90%).

Fractions I and II both separated as a fine, white powder after trituration with light petroleum. Fraction III was obtained as a syrup which, after repeated trituration with light petroleum, yielded a pale yellow solid.

The fractions had the following constants:-

Fraction I $[\alpha]_D^{19}$ = - 60° in chloroform, OMe = 41·4%

Fraction II $[\alpha]_D^{19}$ = - 60° in chloroform, OMe = 41·0%

Fraction III $[\alpha]_D^{19}$ = - 55° in chloroform, OMe = 40·5%

The viscosities of the 3 fractions in m-cresol solution were determined.

Fraction	Wt.of solid in 1000 ml. m-cresol (c).	Time of flow	$\eta_{sp}/20°$	η/c	η/c'
m-cresol	3·36 g.	442·4 secs.			
I	3·36 g.	449·1 secs.	·01515	·0045	0·920.
II	3·52 g.	445·3 secs.	·0066	·0019	0·380.
III	3·42 g.	446·2 secs.	·0086	·0025	0·512.

where c' = concentration in g.mols./litre of methylated anhydrofructose residues.

From the above results it was decided to hydrolyse a portion of fraction I and to subject the remainder of that fraction to further methylation.

A) Remethylation of fractionated trimethyl triticin.

A portion of fraction I (2·05 g.) was methylated with thallous ethoxide-methyl iodide, using the procedure described previously (p.73). This yielded a fine, white powder (1·92 g.; OMe, 43·4%).

$[\alpha]_D^{17}$ = - 60·4° (c = 1 in chloroform) .

Ash content = 1·16%.

Correcting for this ash content, the methoxyl content becomes 43·9%.

This material was then combined with the sample from the preliminary trial methylation ($[\alpha]_D$ = - 59°; OMe = 43·2%) and the total material (2·70 g.) again methylated with thallous ethoxide-methyl iodide. The product precipitated from chloroform solution as a pale yellow fine powder (2·56 g.).

It was thought that the pale yellow colour might be due to some contamination with thallous iodide. When, however, an aqueous solution of sodium sulphide was added to a little of the material, no black precipitate was obtained. This, coupled with the fact that the material did not give a Tl^+ flame test, is good evidence that the methylated polysaccharide is free from thallous iodide.

The methylated material had OMe = (a) 44·5%
(b) 44·6%.

Ash content = 0·05%, unchanged on sulphation.

It is assumed that this sample of methylated polysaccharide is fully-methylated.

B) Hydrolysis of the Fractionated Methylated Polysaccharide.

A portion of Fraction I of the methylated polysaccharide (3·07 g.) was hydrolysed by heating under reflux on a water bath at 80° with methanol (90 ml.) and water (30 ml.) containing oxalic acid (1·2 g.), until the rotation was constant ($[\alpha]_D^{19} = + 16°$ after 24 hours).

The acidic solution was neutralised with calcium carbonate, the solution filtered and evaporated to a syrup under reduced pressure. Last traces of water were removed by the addition of alcohol and benzene and removal of the azeotrope by distillation under reduced pressure. The syrup was then extracted with boiling chloroform in the presence of anhydrous sodium sulphate, and the solution filtered and concentrated under reduced pressure. The residue was dried in a vacuum desiccator over phosphoric oxide to yield a mobile syrup (3·39 g.).

The Separation of the Components of the Hydrolysate.

The methylated sugars obtained by hydrolysis of the methylated polysaccharide were separated by elution through a column of powdered cellulose, using the technique developed by Hough, Jones and Wadman.(14)

A column of powdered cellulose was prepared (27" x 1·4") and the column eluted with water, butanol saturated with water and, finally, a mixture of butanol (50%)-light petroleum (50%) saturated with water. The light petroleum (b.p. 100° - 120°) had been purified by shaking overnight with concentrated sulphuric acid, followed by washing with water and sodium bicarbonate solution and distillation.

The hydrolysate of the methylated polysaccharide (3·39 g.) was then added to the column and, after 2 hours, elution was commenced with a 50% - 50% mixture of butanol-light petroleum saturated with water. The receiver was changed by an automatic timing devic every 24 minutes. Every tenth tube was evaporated on the water bath, 2 drops of water added and this solution spotted on the chromatogram. Each chromatogram was duplicated. One, using tetramethyl fructofuranose as the standard, was sprayed with an aqueous solution of urea oxalate in order to detect methylated fructose material. The other, using tetramethyl glucopyranose as the standard, was sprayed with an aqueous solution of aniline oxalate in order to detect any methylated aldose material which might be present. Fractionation occurred as follows:-

Tubes.	R_G value of syrup	Fraction.
11 - 31	1·09	I
31 - 41	mixture of F.I and F.II	
41 - 85	1·01	II
95 - 115	0·95	III
120 - 155	0·88	IV
165 - 201	0·85	V
218 - 245	0·80	VI
335 - 425	0·64	VII

The column was then eluted with water (500 ml.). The appropriate tubes were combined quantitatively, each tube being thoroughly washed with water and acetone.

Investigations of the Fractions from the Cellulose Column.

In the investigation of each fraction, the following procedure was employed.

The solution was evaporated to a syrup under reduced pressure (45° - 50°/15 mm.). The residue was extracted with water (ca.150 ml.) and the extract freed from a waxy material by filtration through a pad of 'filter cel.' The clarified aqueous solution was then evaporated to a syrup under reduced pressure, last traces of water being removed as an alcohol-benzene-water azeotrope. Final dewaxing was achieved by extraction of the syrup with acetone, followed by gravitational filtration and evaporation to a syrup under reduced pressure. The syrupy residue was then dried in a vacuum desiccator over phosphoric oxide and soda lime.

Fraction I.

This was obtained as a highly mobile non-reducing syrup (0·380 g.) and had:

$[\alpha]_D^{17}$ = + 56° (c = 1 in water).

n_D^{20} = 1·4415

OMe = 58·3%

The syrup was chromatographically pure (R_G = 1·09), giving a black colour with urea oxalate and no colour with aniline oxalate.

Although the syrup itself did not reduce Fehling's solution, acid hydrolysis followed by neutralisation gave a strong reducing action. The syrup is, in all probability, tetramethyl-methyl-D-fructofuranoside (OMe, = 62·0%).

Hydrolysis.

A portion of the syrup (0·12 g.) was dissolved in water (20 ml.) containing oxalic acid (0·2 g.) and heated on the water bath at 80° till the rotation was constant.

$[\alpha]_D^{19}$ = + 56° (c = 0·6 in 1% oxalic acid solution)(zero) $\longrightarrow$ + 34° (1 hour) $\longrightarrow$ + 31° (2 hours) constant.

The solution was neutralised with calcium carbonate, heated on the water bath to decompose bicarbonate, and filtered. The solution was evaporated to a syrup under reduced pressure and extracted with acetone to remove wax and inorganic material. The acetone extract was then evaporated under reduced pressure to yield a mobile syrup. Yield = 0·09 g. n_D^{20} = 1·4508.

The syrup was run on the chromatogram against standard tetramethyl fructofuranose. Development with urea oxalate showed the syrup to be a chromatographically pure sample of tetramethyl-D-fructofuranose.

Purification of Fraction I.

The remainder of the original glycosidic syrup (229 mg.) was submitted to distillation in a high vacuum, using a micro-distillation apparatus. A colourless, mobile syrup distilled (b.p. 82° - 85°/ 0·001 mm. bath temperature). Yield = 213 mg. The distillate had n_D^{22} = 1·4396

OMe = 61·5% (theory, for tetramethyl methylfructofuranoside, 62·0%).

A dark brown non-volatile gum-like residue remained in the distillation apparatus.

Fraction II.

This was obtained as a mobile reducing syrup (0·268 g.) with

η_D^{20} = 1·4493.

OMe = 49·1% (theory, for tetramethylfructofuranose, 52·5%).

$[\alpha]_D^{18}$ = + 45° (c = 2 in water) ($[\alpha]_D$ lit. = + 31·7° in water.)

The syrup was chromatographically pure, giving a black colour with urea oxalate and a faint pink colour with aniline oxalate.

Hydrolysis.

The high value obtained for $[\alpha]_D$ suggested the possible contamination of the tetramethyl fructofuranose with trimethyl methylfructoside.

To hydrolyse glycoside material, the syrup (0·182 g.) was dissolved in water (10 ml.) containing oxalic acid (0·1 g.) and the solution heated to constant rotation on a water bath at 80°.

$[\alpha]_D^{19}$ = + 45° (c = 2 in 1% oxalic acid) (zero) $\longrightarrow$ + 6·6° (30 minutes) $\longrightarrow$ + 5·5° (90 minutes) constant.

The solution was neutralised with calcium carbonate, heated on the water bath for 30 minutes and the filtrate evaporated to a syrup under reduced pressure. The syrup was extracted with acetone, evaporated to a syrup and dried in a vacuum desiccator over phosphoric oxide. This yielded a fairly mobile syrup (0·146 g.)

n_D^{14} = 1·4548

OMe = 44·5%.

The syrup was spotted on the chromatogram and was shown to be a mixture of tetramethyl fructofuranose and a trimethyl fructose (R_G = 0·84).

Fraction II is therefore a mixture of tetramethyl fructofuranose and a trimethyl methylfructoside which do not separate on the chromatogram.

Mixture of Fraction I and Fraction II.

This yielded a mobile reducing syrup (0·200 g.) n_D^{13} = 1·4499.

Paper chromatography indicated that the syrup was mainly tetramethyl fructofuranose contaminated with a little of the methylfructoside.

Hydrolysis.

This syrup (0·200 g.) was combined with the remainder of Fraction II (0·07 g.) and dissolved in water (20 ml.) containing oxalic acid (0·2 g.). The solution was heated on the water bath at 80° for 4 hours, and the same procedure followed as outlined previously. After acetone extraction, a fairly mobile syrup was obtained which was dried in a vacuum desiccator over phosphoric oxide.

Yield = 0·231 g.

n_D^{15} = 1·4540 OMe = 46·2%

Paper chromatography showed the presence of tetramethyl fructofuranose (mainly) and a trimethyl sugar ($\underline{R_G}$ = 0·84).

Fraction III.

This yielded a syrupy residue (18 mg.; n_D^{20} = 1·4604) The syrup gave a slight reduction of Fehling's solution and further reduction after acid hydrolysis.

A series of chromatograms showed that, with urea oxalate development, one diffuse spot ($\underline{R_G}$ = 0·94) was obtained, and with aniline oxalate development a single pink spot ($\underline{R_G}$ = 0·85) was obtained.

The syrup was thought to be a trimethyl methylfructoside which had undergone partial hydrolysis to a trimethyl fructose.

Hydrolysis.

The remainder of this fraction (ca. 10 mg.) was hydrolysed with 1% oxalic acid for 4 hours, using the technique described above.

The hydrolysate was spotted on the chromatogram and was shown to consist mainly of a trimethyl sugar ($\underline{R_G}$ = 0·84) which gave a pink colour with aniline oxalate and a blue-grey colour with urea oxalate. In addition, small amounts of a dimethyl sugar ($\underline{R_G}$ = 0·60) and what is presumably free fructose were found to be present.

Fraction IV.

This yielded a viscous pale yellow syrup (0·073 g.).

n_D^{18} = 1·4650.

OMe = 38% (theory, for a trimethyl fructose, 41·9%).

$[\alpha]_D^{14}$ = + 42° (c = 1 in water).

The syrup strongly reduced Fehling's solution. It was chromatographically pure (R_G = 0·86), and gave a blue colour with urea oxalate and a faint pink colour with aniline oxalate, and travelled at the same rate as authentic 3:4:6-trimethyl fructose.

The syrup was heated at 80° with 1% oxalic acid for 4 hours, neutralised with calcium carbonate, shaken with "amberlite" ion-exchange resins and concentrated under reduced pressure to yield a fairly mobile syrup ($[\alpha]_D^{16}$=+ 32° (c = 1·5 in water)). The syrup travelled at exactly the same rate as authentic 3:4:6-trimethyl fructose on the chromatogram, and was chromatographically pure with no trace of dimethyl sugar.

Fraction V.

This was obtained as a viscous reducing syrup (0·505 g.).

n_D^{15} = 1·4682

OMe = 38·0% (theory, for a trimethyl fructose, 41·9%).

$[\alpha]_D^{16}$ = + 31° (c = 1 in water).

The syrup was chromatographically pure, with an R_G value of 0·83. It gave a black colour with urea oxalate and a pink colour with aniline oxalate. The trimethyl sugars obtained by hydrolysis of fractions II and III travelled at the same rate on the chromatogram as this sugar but more slowly than the trimethyl sugar of fraction IV.

Hydrolysis.

The syrup (0·490 g.) was hydrolysed by heating with a 1% solution of oxalic acid in water at 80° for 4 hours.

$[\alpha]_D^{15} = +31°$ (zero) $\longrightarrow -55°$ (1 hour) constant.

The solution was neutralised with calcium carbonate, filtered, ionic material removed by shaking the solution with "amberlite" resins and the solution then concentrated to a syrup under reduced pressure. The residue was dried in a vacuum desiccator over phosphoric oxide, to yield a straw-coloured viscous syrup (0·408 g.).

Paper chromatography showed the hydrolysate to be a mixture of a dimethyl- and a trimethyl-fructose. The original syrup from the column was therefore a mixture of a trimethyl fructose and a dimethyl methyl-fructoside.

Fraction VI.

This was obtained as a pale yellow viscous syrup (0·088 g.).

$n_D^{16} = 1{\cdot}4768$ OMe = 37·5%

The syrup was chromatographically pure ($\underline{R_G} = 0{\cdot}78$), giving a pale yellow colour with aniline oxalate and a blue colour with urea oxalate.

The syrup gave a slight reduction of Fehling's solution, but was strongly reducing after acid hydrolysis.

Hydrolysis.

The syrup was hydrolysed with oxalic acid solution (1% in water), the procedure followed being the same as that already described. This yielded a pale yellow viscous syrup (0·045 g.).

$[\alpha]_D^{16} = -41^\circ \longrightarrow -43^\circ$ (c = 1 in water).

Paper chromatograms of this syrup, developed with urea oxalate, indicated 1 spot only and showed the dimethyl fructose of fraction VII to be present ($\underline{R_G} = 0{\cdot}63$). But paper chromatograms developed with aniline oxalate showed, in addition to the brown spot of the dimethyl fructose, a pink spot ($\underline{R_G} = 0{\cdot}78$). This material travelled at the same rate as an authentic specimen of crystalline 2:4:6-trimethyl glucose, and more slowly than 2:3:4-trimethyl glucose.

The syrup was seeded with authentic 2:4:6-trimethyl glucose, but no crystallisation occurred, even on prolonged standing in the refrigerator.

Oxidation by Alkaline Hypoiodite.

Hydrolysed F.VI (33·45 mg.) was dissolved in water (4·92 g.) and filtered through a little 'filter cel' in order to remove a greasy impurity. To 3 ml. of the filtrate were added phosphate buffer (pH 11·4; 5 ml.), and iodine solution (0·1N; 2·5 ml.), and the ground glass stopper was then sealed into the boiling tube with a little potassium iodide solution (10%). Two water blanks, omitting only the sugar, were treated identically and the tubes allowed to stand in the dark at room temperature for 5 hours. Sulphuric acid (4N; 2 ml.) was added to each tube and the iodine titrated with sodium thiosulphate (1·028 $\frac{N}{100}$), using starch indicator.

Ingles + Israel J. 810 (1948)

Water Blank I Titre = 25·275 ml.
Water Blank II Titre = 25·260 ml.
Sugar solution titre = 22·680 ml.

∴ volume of iodine consumed by sugar is equivalent to

(25·268 - 22·680) ml.

= 2·588 ml. 0·01028N sodium thiosulphate

= 0·02660 ml. N sodium thiosulphate.

Now 2000 ml. N sodium thiosulphate ≡ 222 g. trimethyl aldose.

∴ 0·02660 ml. N " " ≡ ·002953 g. trimethyl aldose.

Now, weight of syrup = $\frac{3 \times \cdot 03345}{4 \cdot 92}$ = 0·02083 g.

∴ aldose content of syrup = $\frac{0 \cdot 2953}{0 \cdot 02083}$ = <u>14·5%</u>.

Fraction VII.

This was obtained as a highly viscous syrup (0·66 g.), which, after prolonged drying in a high vacuum, had:

n_D^{18} = 1·4802

OMe = 27·0% (theory, for a dimethyl fructose, 29·8%).

$[\alpha]_D^{18} = -58° \longrightarrow -61°$ (constant) (c = 1 in water), after correction for low methoxyl content.

The syrup was chromatographically pure (R_G = 0·62), giving a grey-blue coloration with urea oxalate and a brown coloration with aniline oxalate.

The syrup was unchanged after hydrolysis with 1% oxalic acid solution.

Column Eluate. (Fraction VIII).

This contained a large amount of waxy material which was removed by repeated extraction of the syrup with acetone, filtration, and evaporation. A pale green viscous syrup remained (0·051 g.) with a strong odour

n_D^{21} = 1·4780 OMe = 7·2%.

The syrup reduced Fehling's solution and gave no further reduction after acid hydrolysis.

It gave no coloration on the chromatogram with urea oxalate but with aniline oxalate it gave a distinct pink coloration (R_G = 0·57) which was totally different from the brown colour given by the dimethyl fructose fraction from the column.

A series of chromatograms were run using dimethyl glucose syrups as standards. The material under investigation travelled more slowly than 2:3-dimethyl glucose, but at the same rate as 3:6-dimethyl glucose.

Column Recovery.

A summary of the weights and components of the fractions obtained from the cellulose column is given below.

Fraction.	Weight.	Components.
I	0·380 g.	tetramethyl methylfructofuranoside
II	0·268 g.	(tetramethyl fructofuranose (trimethyl methylfructofuranoside.
I - II	0·200 g.	(tetramethyl methylfructofuranoside (tetramethyl fructofuranose (trimethyl methylfructofuranoside.
III	0·020 g.	trimethyl methylfructofuranoside.
IV	0·073 g.	trimethyl fructose (A).
V	0·505 g.	(trimethyl fructose (B) (dimethyl methylfructofuranoside.
VI	0·088 g.	(trimethyl glucose (?) (dimethyl methylfructofuranoside.
VII	0·660 g.	dimethyl fructose.
VIII	0·051 g.	dimethyl glucose (?).

Total recovery = 2·245 g. (66%).

The Separation of the Components of the hydrolysed Fractions from Column I.

The methylated sugars obtained by hydrolysis of the major fractions from column I were separated by a further elution through a cellulose column (column II).

Hydrolysed F.II, hydrolysed F.I - F.II mixture and hydrolysed F.V. were combined to yield a fairly mobile syrup (0·774 g.).

This was separated into its components by elution through a column of powdered cellulose (22" x 1"), using as solvent a mixture of 70% light petroleum (b.p. 100° - 120°) and 30% butanol saturated with water, the technique otherwise being identical to that described previously (p.80).

Fractionation occurred as follows:-

Tubes.	R_G value of syrup.	Fraction.
110-140	1·01	I (Tetramethyl)
280-325	0·88	II (Trimethyl A)
335-445	0·85	III (Trimethyl B)
Solvent changed to 50% butanol-50% light petroleum, saturated with water.		
705-880	0·62	IV (Dimethyl).

Investigations of the Fractions from Column II.

Each fraction was submitted to the dewaxing process described previously (p.81) and the syrupy residue dried overnight in a vacuum desiccator over phosphoric oxide and finally in a high vacuum for 3 hours.

Fraction I (Tetramethyl).

This was obtained as a pale yellow mobile syrup

(0·209 g.) and was estimated directly by weight. The syrup, after drying in a high vacuum, had $n_D^{19} = 1{\cdot}4504$. $[\alpha]_D^{18} = + 35°$ (c = 2 in water).

The syrup was chromatographically pure. With aniline oxalate it gave a faint pink coloration, whereas standard tetramethyl fructofuranose gave no coloration.

Fraction II (Trimethyl A).

This was obtained as a fairly mobile, dark-coloured syrup (0·033 g.) which was submitted to distillation in a high vacuum, using a microdistillation apparatus. A pale yellow, fairly mobile syrup distilled (b.p. 70° - 75°/0·005 mm. bath temperature), leaving a non-volatile, dark-brown, gum-like residue.

The distillate had

$n_D^{23} = 1{\cdot}4560$

$[\alpha]_D^{18} = + 33° \longrightarrow + 29°$ (c = 0·5 in water).

The syrup was chromatographically pure and travelled at the same rate as an authentic sample of 3:4:6-trimethyl fructose.

Fraction III (Trimethyl B).

This was obtained as a fairly viscous syrup (0·187 g.). After drying at 40°/0·1 mm. for 3 hours, the syrup had

$n_D^{20} = 1{\cdot}4638$

$[\alpha]_D^{16} = - 51{\cdot}5°$ (c = 1 in water).

The syrup was hygroscopic and absorbed moisture noticeably during weighing on the semi-micro balance. It was chromatographically pure and gave a blue-grey coloration with urea oxalate and a faint pink coloration with aniline oxalate. It travelled more slowly on the chromatogram than authentic 3:4:6-trimethyl fructose and trimethyl A.

On seeding with a crystal of authentic 1:3:4-trimethyl fructose, the syrup crystallised on standing at room temperature overnight. A crystal scraped from the top of the flask had m.p. = 60° - 65°.

The partially-crystallised syrup was drained on porous tile, the crystalline material removed from the tile and triturated with cold light petroleum (b.p. 40° - 60°), and then crystallised from a mixture of light petroleum and carbon tetrachloride. The solid crystallised as long needles (m.p. 72° - 73°) which showed no depression of the melting point on admixture with an authentic crystal of 1:3:4-trimethyl fructose.

<u>Fraction IV</u> (Dimethyl).

This was obtained as a highly viscous, hygroscopic syrup (0·282 g.). After drying at 40°/0·1 mm. for 3 hours, the syrup had

n_D^{23} = 1·4780

OMe = 27·1% (theory, 29·8%)

$[\alpha]_D^{18} = -58° \longrightarrow -60°$ (c = 1·6 in water), after correction for a low methoxyl content.

The syrup absorbed water noticeably during weighing on the semi-micro balance.

All the syrupy fractions from this, and other, cellulose columns were found to have methoxyl contents of ca.2% below theory. This is attributed as being due mainly to the presence of some dissolved waxy material, for, on distillation in high vacuum, the syrups invariably gave theoretical values for methoxyl contents and left a non-volatile, gum-like residue in the distilling flask. It would appear, therefore, that the dewaxing process employed is not completely efficient. In quoting values for $[\alpha]_D$ of syrups obtained from cellulose columns throughout this work, corrections have been made for this dissolved waxy material. The methoxyl content has been used as a measure of the methylated sugar content of the methylated ketose syrup, for, whilst it is realised that this is not altogether satisfactory, there is no other method available for the estimation of a methylated ketose.

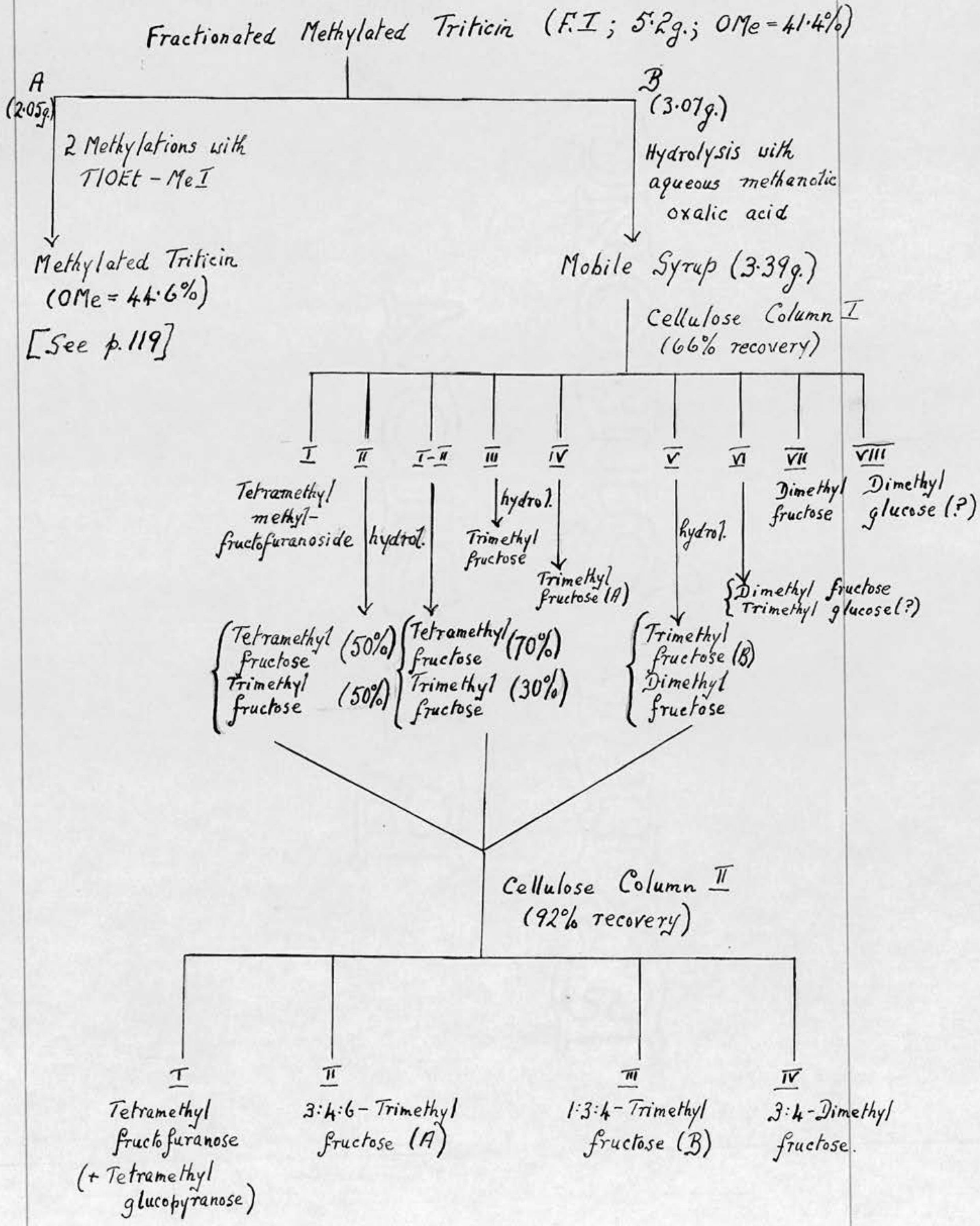
It has recently been suggested by Chanda (unpublished work) that this wax is due to polymerisation of aldehydes present in the n-butanol. These aldehydes are not removed by preliminary distillation of the butanol, but removal is effected by boiling under reflux over sodium hydroxide pellets for several hours, followed by distillation over sodium hydroxide prior to use.

Column II Recovery.

Fraction.	Component.	Weight.
I	Tetramethyl fructofuranose	0·209 g.
II	Trimethyl fructose (A)	0·033 g.
III	Trimethyl fructose (B)	0·187 g.
IV	Dimethyl fructose	0·282 g.

Total Recovery = 0·711 g. (92%).

Summary of Scheme of Separation.



Examination of the Fully-Methylated Polysaccharide (OMe = 44·6%).

As described earlier, a portion of Fraction I of fractionated methylated polysaccharide was subjected to 2 further methylations with thallous ethoxide-methyl iodide. This yielded a fine pale yellow powder (2·56 g. ; OMe = 44·6%).

Hydrolysis of Trimethyl Triticin.

(a) Hydrolysis with Aqueous Methanolic Oxalic Acid.

The fully-methylated polysaccharide (2·46 g.) was dissolved in methanol (75 ml.) and water (25 ml.) containing oxalic acid (1 g.). The solution was heated on the water bath at 80° for 24 hours.

A small amount of insoluble white gelatinous material (23 mg.) was removed by filtration through a No.4 sintered glass crucible. Thus the actual weight of methylated polysaccharide is 2·437 g. and correction for this impurity raises the methoxyl content to 45·1%. The insoluble impurity was not thallous salt material for it did not give a Tl^+ flame test or a black precipitate with sodium sulphide. It melted on a spatula to give a dark-brown syrupy product.

(b) Continuation of the Hydrolysis with Aqueous Oxalic Acid.

The solution from the above hydrolysis contained a mixture of methylated fructoses and their corresponding methylfructosides.

The solution was diluted with water and the methanol-water azeotrope removed by distillation at 35°/15 mm., water being added from a dropping funnel at the same rate as the azeotrope distilled. When all the methanol had been removed, the concentration of the aqueous solution was adjusted to 1% with respect to oxalic acid. The solution was then heated on the water bath at 80° for 5 hours when the rotation was constant.

The solution was neutralised with calcium carbonate, filtered, and the filtrate evaporated to a syrup under reduced pressure, last traces of water being removed as a water-alcohol-benzene azeotrope. The syrup was then extracted with boiling chloroform, the extract dried over anhydrous sodium sulphate and evaporated under reduced pressure to yield a mobile syrup (2·72 g.) which was dried in a vacuum desiccator over phosphoric oxide.

Recovery from hydrolysis = 97%.

The Separation of the Methylated Sugars on a Cellulose Column.

The syrup (2·7 g.) obtained by hydrolysis of the fully-methylated polysaccharide was separated into its components by elution through a column of powdered cellulose (15" x 1·25") with butanol (50%)-

petrol ether (50%) saturated with water. The technique employed was the same as that already described (p.80), the automatic timing device being adjusted such that the receiver was changed every 8 minutes. Every 10th tube was evaporated to small bulk, and spotted on the chromatogram. Paper chromatograms were duplicated, one being sprayed with urea oxalate and the other with aniline oxalate. Fractionation occurred as follows:-

Tubes.	R_G value of sugar.	Fraction.	Component.
51-110	1·01	I	Tetramethyl fructose.
184-451	0·87 - 0·83	II	Trimethyl fructose.
581-1020	0·62	III	Dimethyl fructose.

The trimethyl fructose fraction extended over a wide range of tubes and it was considered possible that the fraction was a mixture of 2 sugars, since there was a gradual decrease in R_G value from 0·87 to 0·83 in the series of chromatograms.

The tetramethyl fructose fraction, in addition to giving a blue coloration with urea oxalate, gave a pink coloration with aniline oxalate whereas pure tetramethyl fructofuranose gave no coloration with aniline oxalate. It is considered that the tetramethyl fructofuranose (R_G = 1·01) was, in all probability, contaminated with tetramethyl glucopyranose (R_G = 1·00).

There was however no evidence as to the presence of trimethyl- and dimethyl-glucoses in the aniline oxalate series of chromatograms. Trimethyl glucose should have appeared between Fraction II and Fraction III (Intermediate III - Tubes 452 - 580) and dimethyl glucose after Fraction III (Intermediate IV - Tubes 1021 - 1280).

Investigations of the Fractions from Column III.

Fraction I (Tetramethyl fructose).

It was proposed to estimate the end group colorimetrically using the modification of Roe's procedure outlined earlier in this thesis (p. 63).

Construction of Standard Curve for Tetramethyl-D-fructofuranose.

The standard tetramethyl-D-fructofuranose used had $[\alpha]_D^{16} = +28°$ in a saturated benzoic acid solution in water and was shown to contain a trace of trimethyl fructose on the chromatogram.

Reagents.

Acid Reagent.	130 g. glycerol B.P.
	100 ml. conc. HCl (A.R.) containing 45 mg. $CuSO_4 . 5H_2O$/litre.
	50 ml. H_2O.
Resorcinol	0·45% in H_2O.

Stock Tetramethyl fructose solution 5 mg./ml. in saturated benzoic acid solution.

Standard Tetramethyl fructose solutions.

0.25 mg./ml.
0.20 mg./ml.
0.15 mg./ml.
0.10 mg./ml.
0.075 mg./ml.
0.050 mg./ml.

prepared by dilution of stock solution.

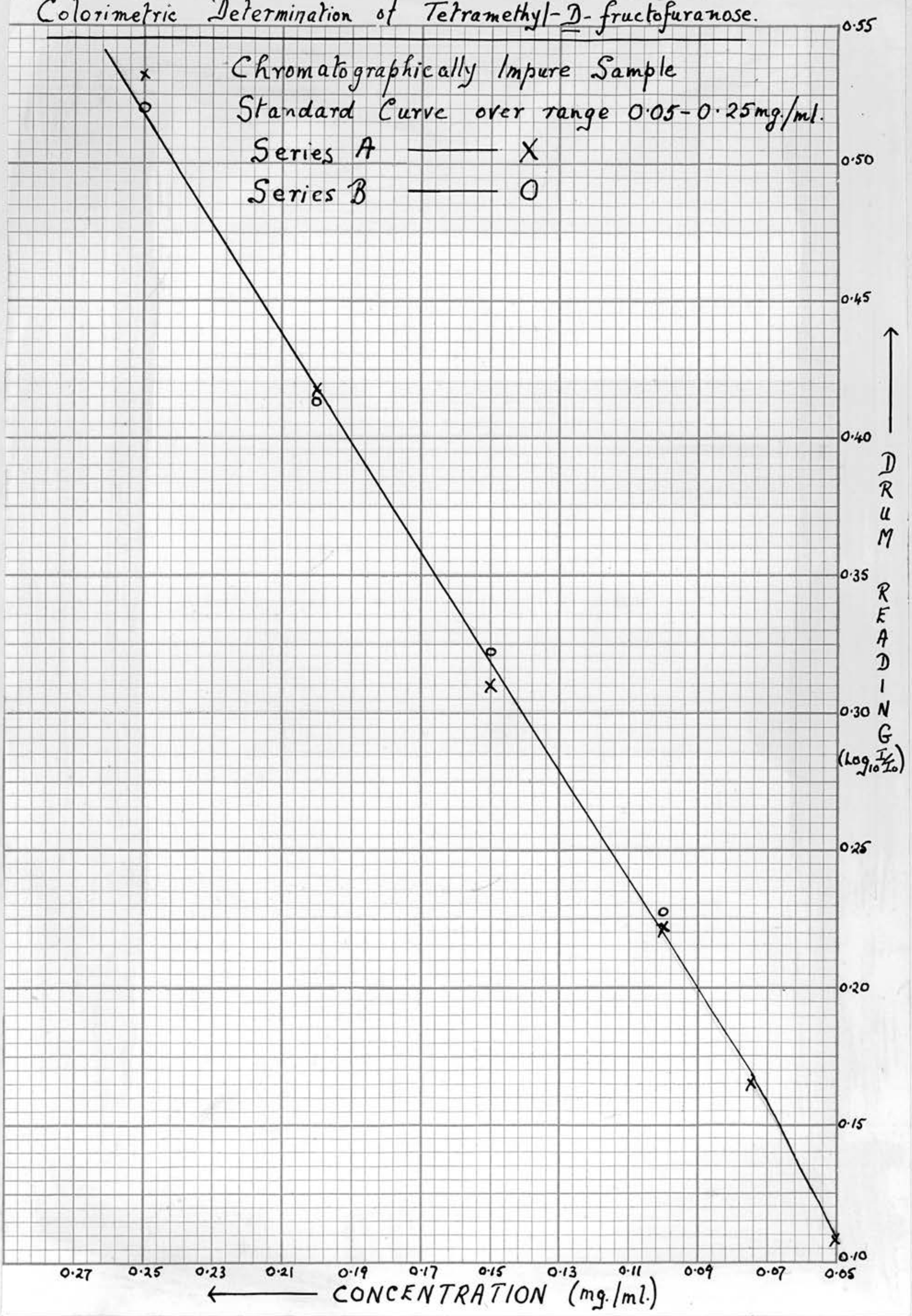
The following experimental procedure was rigidly followed:-

5 ml. of Acid Reagent, followed by 1 ml. of the resorcinol solution and 2 ml. of the standard tetramethyl fructose solution were transferred by pipette into a Pyrex test tube (6" x 3/4"), fitted with a close-fitting glass bulb and, after thorough mixing, heated in a boiling water bath for exactly 12 minutes. The solution was cooled at once in running water and read against a blank carried through the procedure at the same time, using a Spekker absorptiometer (1 cm. cells and I.S. violet 601).

The colour intensity was found to be stable over 2 hours and the change of colour intensity with concentration was found to be linear over the range 0·25 - 0·05 mg./ml., the latter being the lowest limit of concentration capable of being measured by the absorptiometer. (Series A).

The results were repeated using standard solutions prepared from a different stock solution of the same sample of tetramethyl-D-fructofuranose, when excellent agreement was found. (Series B).

Concentration (mg./ml.)	Spekker Reading ($\log_{10} I/I_o$) A.	B.
0·25	0·532	0·520
0·20	0·418	0·414
0·15	0·310	0·323
0·10	0·222	0·227
0·075	0·165	
0·05	0·108	



Colorimetric Determination of Tetramethyl-D-fructofuranose.

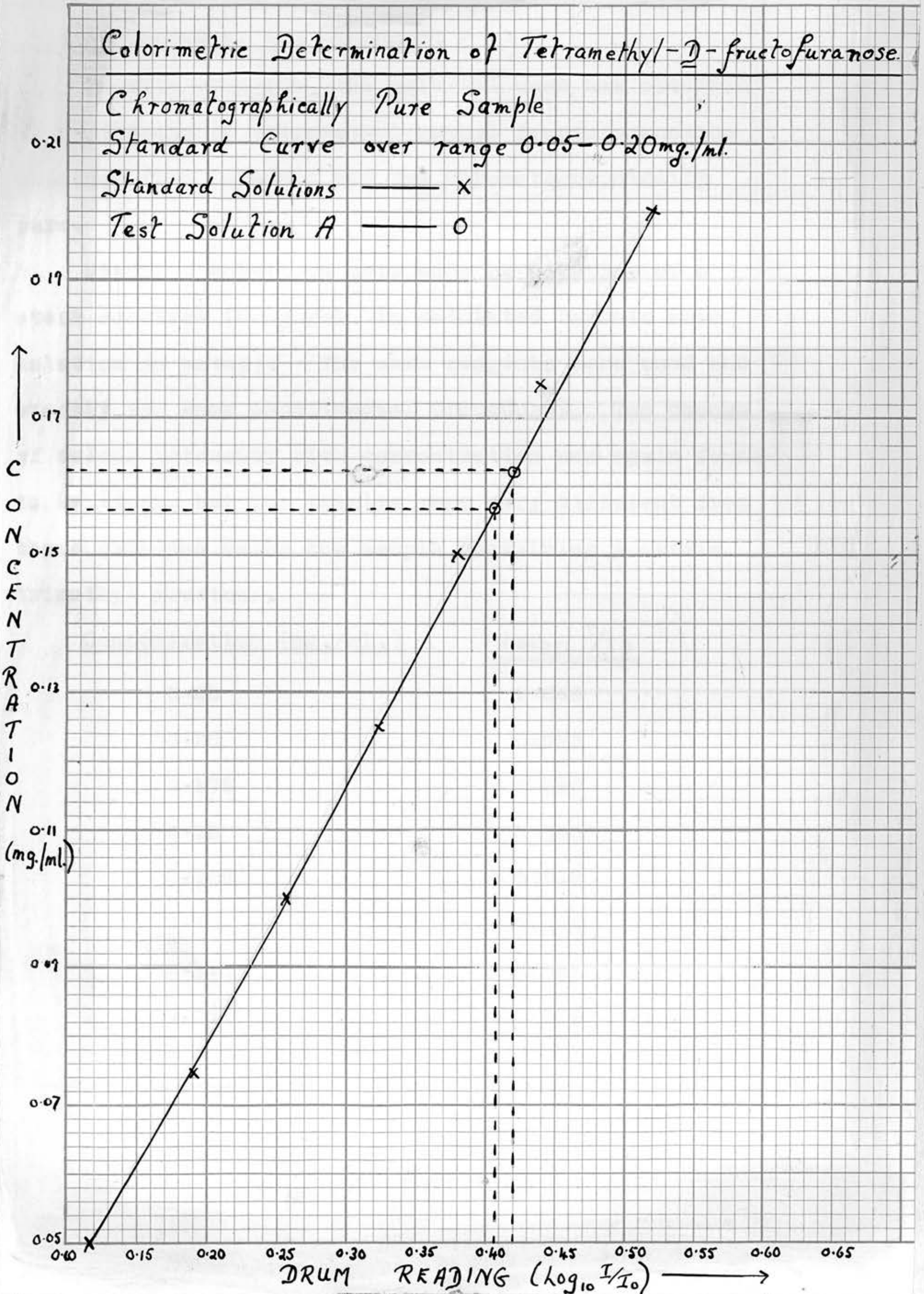
GRAPH X

The use of chromatographically pure tetramethyl-D-fructofuranose as the standard.

On this occasion, the material used was obtained by hydrolysis of tetramethyl methylfructofuranoside obtained from Column I and was chromatographically pure.

Standard solutions were made by dilution of a stock solution (5 mg./ml. in saturated benzoic acid solution in water). The same reagents were used and exactly the same experimental procedure. The change of colour intensity with concentration was again found to be linear but the results were very different from those obtained using the sample containing a little trimethyl fructose.

Concentration (mg./ml.)	$\text{Log}_{10}\ {}^{I}/_{I_o}$
0·25	0·642
0·20	0·523
0·175	0·440
0·15	0·380
0·125	0·323
0·10	0·256
0·075	0·191
0·05	0·116


Colorimetric Determination of Tetramethyl-D-fructofuranose.
Chromatographically Pure Sample
Standard Curve over range 0·05–0·20 mg./ml.
Standard Solutions —— x
Test Solution A —— O
0·21
0·19
0·17
0·15
0·13
0·11
0·09
0·07
0·05
CONCENTRATION (mg./ml.)
0·10
0·15
0·20
0·25
0·30
0·35
0·40
0·45
0·50
0·55
0·60
0·65
DRUM READING ($Log_{10} I/I_0$)

GRAPH Y

Estimation of the "Tetramethyl" fraction from the Cellulose Column.

Control Experiment.

A solution of chromatographically pure tetramethyl fructofuranose was made in n-butanol (50%)-light petroleum (50%) saturated with water. The solvent was removed by distillation under reduced pressure as an azeotrope with water, a saturated benzoic acid solution in water being added from a tap funnel. This was continued until a purely aqueous solution of the material was obtained. The solution was filtered into a 1-litre graduated flask and made up to the mark with washings of the flask and funnel. The tetramethyl fructofuranose was then estimated colorimetrically in aliquot portions of 2 ml. of solution.

Weight of tetramethyl fructofuranose taken = 0·1645 g.
Weight of tetramethyl fructofuranose estimated = 0·1682 g.
Error = 3·7 mg. excess in 164·5 mg.
= + 2·2%.

Column Solution.

The solution of tetramethyl fructose from the cellulose column was diluted to 1010 ml. with a saturated solution of benzoic acid in water. Two 15 ml. samples were transferred by pipette into 100 ml. R.B. flasks, and the solvent replaced by water, using exactly the same procedure as that described for the control experiment. The aqueous solution was then diluted to 100 ml. and the tetramethyl fructose estimated colorimetrically in 2 ml. portions, using the standard graph constructed for chromatographically pure tetramethyl fructofuranose.

Test Solution A.

Vol. of original standard solution used = 15 ml.

Spekker Readings (a) 0·419.

(b) 0·405.

(a) From standard graph, concentration = 0·1625 mg./ml.

∴ In 100 ml. solution (or 15 ml. original solution) are 16·25 mg.

∴ Total weight of tetramethyl fructofuranose = 1·094 g.

(b) From standard graph, concentration = 0·157 mg./ml.

∴ Total weight of tetramethyl fructofuranose = 1·057 g.

Mean value = 1·075 g.

Test Solution B.

Vol. of original solution used = 15 ml.

Spekker Readings (a) 0·408.

(b) 0·421.

(a) From standard graph, concentration is 0·1585 mg./ml.

∴ Total weight of tetramethyl fructofuranose = 1·067 g.

(b) From standard graph, concentration is 0·163 mg./ml.

∴ Total weight of tetramethyl fructofuranose = 1·097 g.

Mean value = 1·082 g.

Allowing for the error of + 2% which was estimated from the control solution, it may be stated that the total weight of tetramethyl fructofuranose in fraction I from the column is

$$\frac{98}{100} \text{ of } 1{\cdot}08 = 1{\cdot}06 \text{ g.}$$

Gravimetric Examination of Fraction I.

All the solutions from the colorimetric analysis were combined and evaporated to a syrup under reduced pressure. The syrup, which was contaminated with a large amount of benzoic acid, was extracted with ice-cold water and concentrated under reduced pressure. This process was repeated a further twice and the last traces of water were removed as an alcohol-benzene-water azeotrope. The syrupy residue was extracted with acetone, filtered and the acetone extract concentrated to a syrup under reduced pressure and dried in a vacuum desiccator over phosphoric oxide, to yield a very mobile, straw-coloured syrup (1·122 g.).
The syrup had
n_D^{16} = 1·4649. OMe, = 44% (theory, 52·5%).
$[\alpha]_D^{15}$ = + 34° (c = 1 in water) after correction for low methoxyl content.

Examination of the syrup on the paper chromatogram showed it to be a chromatographically pure sample of tetramethyl fructofuranose.

The syrup was dried in a high vacuum for 3 hours, in order to remove the last traces of benzene.

Loss in weight = 2·2%.

n_D^{19} = 1·4625. OMe = 44%.

Assuming the above loss in weight to be due entirely to loss of solvent, and none due to distillation of tetramethyl fructofuranose, then weight of Fraction I = $\frac{97 \cdot 8}{100}$ of 1·122 g. = 1·098 g.

The very low methoxyl content and very high refractive index found for this syrup are puzzling features. A similar observation was made when the standard tetramethyl fructose was recovered. The mobility of the syrup was found to have increased considerably as well as the refractive index (n_D^{20} = 1·4490 (before analysis); n_D^{20} = 1·4570 (after analysis)). This may be due to some benzoic acid remaining in solution in the mobile syrup.

Estimation of the Aldose Content of the Tetramethyl Fructofuranose Syrup by Alkaline Hypoiodite Oxidation.

The method used was a modification of that described by Hirst, Hough and Jones,(15) the reaction being carried out in phosphate buffer solution (pH 11·4).

The syrup (0·1225 g.) was dissolved in water (10 ml.) and greasy impurities removed by filtration through a pad of 'filter cel.'

5 ml. of this solution were transferred by pipette into a boiling tube fitted with a B.24 glass joint, phosphate buffer (6 ml.) added, followed by iodine

(0·1N; 3 ml.) and the stopper sealed in with a little potassium iodide solution (10%). Into a second tube were transferred by pipette 3 ml. of the solution, pH 11·4 buffer (4 ml.) and 0·1N iodine (2 ml.). Two water blanks containing 5 ml. and 3 ml. water respectively were treated identically. The tubes were allowed to stand in the dark at room temperature for 3 hours, sulphuric acid (3 ml. or 2 ml.; 4N) added and the iodine titrated with sodium thiosulphate ($0{\cdot}999\frac{N}{100}$), using starch indicator.

A) Volume of solution and water blank = 3 ml.
Water blank titre = 19·740 ml.
Sugar solution titre = 18·065 ml.
whence tetramethyl glucose content is 5·37%.

B) Volume of solution and water blank = 5 ml.
Water blank titre = 29·610 ml.
Sugar solution titre = 27·100 ml.
whence tetramethyl glucose content is 4·84%.

∴ Tetramethyl glucose content is 5·1% (mean value), so total weight of tetramethyl glucose is

$$\frac{5{\cdot}1}{100} \text{ of } \frac{1{\cdot}098}{1} = \underline{0{\cdot}056 \text{ g}}.$$

and, by difference, weight of tetramethyl fructose

$$= \underline{1{\cdot}042 \text{ g}}.$$

Fraction II (Trimethyl fructose).

The "trimethyl fraction" was estimated gravimetrically, the de-waxing process being identical to that described previously.

The last traces of wax were removed by extracting the syrup with acetone, filtering and evaporating to a syrup under reduced pressure. The residue was dried in a vacuum desiccator over phosphoric oxide to yield a viscous syrup (0·381 g.).

After drying in a high vacuum, the syrup had

$\eta_D^{17} = 1{\cdot}4688$

OMe = 38% (theory, for a trimethyl hexose, 41·9%).

$[\alpha]_D^{16} = -\ 28° \longrightarrow -\ 33{\cdot}5°$ (c = 1 in water).

The syrup gave one large spot on the chromatogram (<u>R_G</u> = 0·84) with a slight tailing effect at the top. This suggested the possibility of the syrup being a mixture of 2 trimethyl sugars with similar <u>R_G</u> values.

A sample of the syrup was hydrolysed with 1% oxalic acid by heating at 80° for 4 hours. After neutralisation and removal of ionic material, the syrup showed only a mere trace of dimethyl sugar on the chromatogram.

<u>Estimation of Aldose Content of the Syrup.</u>

The aldose content of the trimethyl fructose syrup was estimated by alkaline hypoiodite oxidation, using the procedure described previously (p. 111.).

The aldose content was estimated as <u>4·0%</u>.

Total weight of syrup = 0·381 g.

so weight of trimethyl aldose (assumed to be trimethyl glucose) $= \frac{4}{100}$ of $\frac{0{\cdot}381}{1}$ = <u>·015 mg.</u>

and, by difference, weight of trimethyl fructose

$= 0{\cdot}366$ g.

The trimethyl syrup was seeded with an authentic specimen of 1:3:4-trimethyl fructose when the syrup partially crystallised as long colourless needles on standing at room temperature for several days.

<u>Fraction III</u> (Dimethyl fructose).

The "dimethyl fraction" was estimated gravimetrically, the syrup being submitted to the dewaxing process already described. After complete removal of wax, the highly viscous syrup was dried in a vacuum desiccator over phosphoric oxide and then in high vacuum.

Yield = 1·047 g.

The syrup was considerably hygroscopic and readily absorbed moisture on weighing on the semi-micro balance.

After prolonged drying in a high vacuum the syrup had

$\eta_D^{18} = 1{\cdot}4782$.

OMe $= 27{\cdot}4\%$ (theory, for a dimethyl hexose, $29{\cdot}8\%$).

$[\alpha]_D^{15} = -61°$ (c = 1 in water) after correction for low methoxyl content.

Estimation of the aldose content of the syrup.

The % aldose (assumed to be dimethyl glucose) present in the syrup was determined by oxidation with alkaline hypoiodite, using the procedure already described (p. 111.).

The aldose content was estimated as 3·9%.

Total weight of syrup = 1·047 g.

so weight of dimethyl aldose (assumed to be dimethyl glucose) $= \frac{3\cdot 9}{100}$ of $\frac{1\cdot 047}{1}$ = ·041 g.

and, by difference, weight of dimethyl fructose = 1·006 g.

Examination of Intermediate Fractions from the Column for methylated aldose material.

Intermediate III (i.e. intermediate between trimethyl- and dimethyl-fructose fractions).

Trimethyl glucose (R_G = ca.0·78), if present, would be expected here. On evaporation of the solution and de-waxing a syrupy residue remained (17 mg.). Paper chromatograms of the syrup sprayed with aniline oxalate showed a pink spot, travelling at exactly the same rate as 2:4:6-trimethyl glucose (R_G = 0·79). Paper chromatograms sprayed with urea oxalate indicated the presence also of some trimethyl- and dimethyl-fructose material.

Estimation of the Aldose Content by Alkaline Hypoiodite Oxidation.

The syrup (5 mg.) was dissolved in water (6 ml.).

and the procedure followed as described previously, using 5 ml. of the sugar solution, phosphate buffer (4 ml.) and 0·1N iodine (2 ml.).

Water Blank (1) 19·920 ml. (2) 19·880 ml.

Test solution 18·06 ml.

Vol. iodine consumed $\equiv$ 1·84 ml. 0·999 $\frac{N}{100}$ sod.thiosulphate.
= ·0184 ml. N.sod.thiosulphate.

Now 2000 ml. N.sod. thiosulphate $\equiv$ 222 g. trimethyl sugar.
so 0·0184 ml. N sod. " $\equiv$ 2·04 mg. " "

Now 5 ml. solution contain 4·20 mg. syrup, so aldose content = <u>49%</u>

and weight of trimethyl glucose = <u>8·3 mg.</u>

$\therefore$ Total weight of trimethyl glucose = 15 mg. (from Fraction II)
+ 8·3 mg.
= <u>23·3 mg.</u>

<u>Intermediate IV</u> (i.e. after dimethyl fructose fraction).

This, on given the usual treatment, yielded a syrupy residue (29 mg.). Paper chromatograms of the syrup were duplicated. One, sprayed with urea oxalate, showed the presence of dimethyl fructose. The other, sprayed with aniline oxalate, showed that dimethyl glucose was absent.

<u>Column Eluate</u>.

The column was eluted with water (500 ml.), and the eluate evaporated and treated in the usual manner, to yield a syrupy residue (11 mg.). This gave no colour reaction on the chromatogram with urea oxalate, but with aniline oxalate faint brown spots ($\underline{R_G}$ = 0·85 and 0·67) were obtained which could not be identified. There was, however, no evidence as to the presence of any dimethyl glucose.

Column Recovery.

Fraction.	Component.	Weight.
I	Tetramethyl sugars	1·098 g.
II	Trimethyl sugars	0·381 g.
III	Dimethyl sugars	1·047 g.
Intermediate III	Dimethyl- and Trimethyl-sugars	0·017 g.
Intermediate IV	Dimethyl sugar	0·029 g.
Column Eluate	-	0·011 g.

Column Recovery = 2·583 g. (96%).

From studies of these fractions, the weights of the various methylated sugars present were estimated.

Sugar	Method of Estimation.	Weight	% of Total
Tetramethyl fructofuranose	colorimetrically	1·06 g.	41·6
"Tetramethyl glucose"	alkaline hypoiodite oxidation	0·056 g.	2·2
Trimethyl fructose	gravimetrically	0·366 g.	14·4
"Trimethyl glucose"	alkaline hypoiodite oxidation.	0·023 g.	0·90
Dimethyl fructose	gravimetrically	1·006 g.	39·4
"Dimethyl glucose"	alkaline hypoiodite oxidation	0·041 g.	1·6

The above weights, expressed in terms of glucose and fructose, become:-

	Weight	% of total.
isolated as Tetramethyl fructose	0·809 g.	39·0
" " "Tetramethyl glucose"	0·043 g.	2·1
" " Trimethyl fructose	0·297 g.	14·4
" " "Trimethyl glucose"	0·019 g.	0·9
" " Dimethyl fructose	0·870 g.	41·9
" " "Dimethyl glucose"	0·036 g.	1·3

whence, for the <u>fructose sugars</u>,

Tetramethyl: Trimethyl: Dimethyl = ·809: ·297: ·870

= 2·73: 1: 2·93.

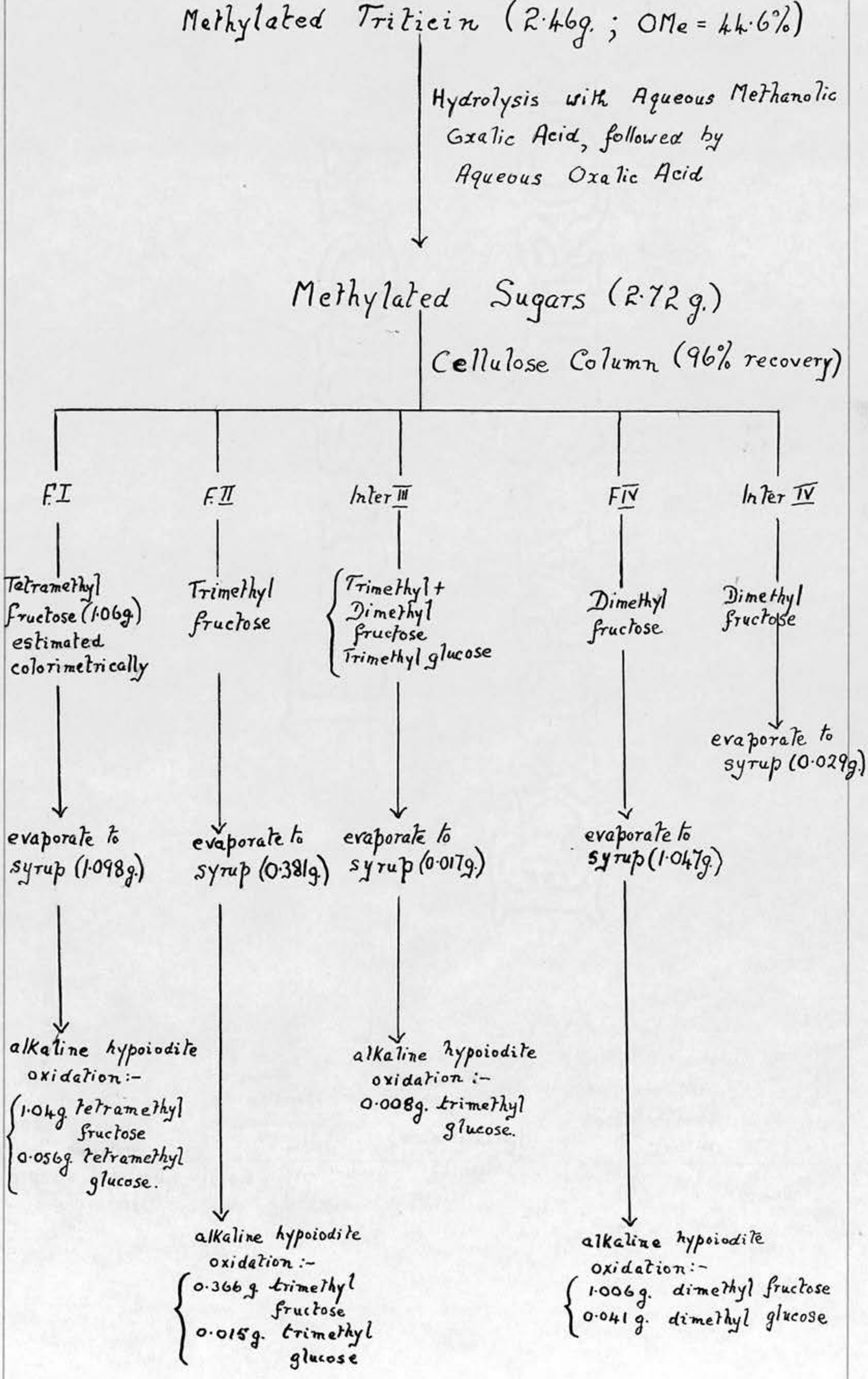
and for the <u>glucose sugars</u>,

Tetramethyl: Trimethyl: Dimethyl = ·043: ·019: ·036

= 2·3: 1: 1·9.

The Products of Hydrolysis of Fully-Methylated Triticin

(Summary)



Investigation into the Structure of the Tetramethyl Fructose.

The tetramethyl fraction was proved to be tetramethyl fructofuranose by conversion to the crystalline tetramethyl fructofuronamide, the method employed being similar to that described by Avery, Haworth and Hirst.(16)

The tetramethyl fructofuranose syrup from column III(1·0 g.) was oxidised with concentrated nitric acid (10 ml.; d = 1·42) on a water bath, the temperature of which was slowly raised. When the temperature reached 65° a vigorous reaction occurred with the evolution of nitrous fumes. The temperature was then gradually raised to the boiling point and maintained thus for 3 hours. The solution was cooled, diluted with water and the nitric acid removed by distillation at 40°/15 mm. Water was again added, the solvent removed by distillation under reduced pressure, and this process repeated 6 times in all, methanol then being added to remove water and, finally, anhydrous methanol. The residue consisted of a syrup intimately mixed with solid material.

The residue was then dissolved in methanolic hydrogen chloride (30 ml.; 4%) and boiled gently under reflux on a water bath for 12 hours. The solution was neutralised with silver carbonate, filtered and concentrated under reduced pressure to yield a syrup, mixed with a solid residue. The residue was methylated according to Purdie by boiling under reflux with methyl iodide (50 ml.) and silver oxide (20 g.). After removal of the methyl iodide by distillation, the residue was repeatedly extracted with boiling chloroform and the combined chloroform extracts dried over anhydrous sodium sulphate and evaporated to a syrup under reduced pressure.

The syrup was then submitted to distillation in a high vacuum.

Fraction I b.p. 110°/0.9 mm. (bath temp.) 0·61 g. n_D^{21} = 1·4454
Fraction II b.p. 85°/0·03 mm. (bath temp.) 0·08 g. n_D^{16} = 1·4530

Fraction I, a colourless, mobile, non-reducing syrup, was dissolved in anhydrous methanol saturated with ammonia (10 ml.) and the solution allowed to stand at 0° for 3 days. The solvent was then removed by distillation under reduced pressure to yield a syrupy non-reducing residue (0·58 g.) which partially crystallised on standing at room temperature. The partially-crystallised syrup was drained on porous tile for 2 weeks and the solid extracted with hot ether to yield a hard crystalline mass (m.p. 80° - 85°). The non-reducing residue was insoluble in hot ether and hot light petroleum and was obtained as plates (m.p. 175° - 176°).

The fraction extracted by hot ether was extracted several times with boiling light petroleum (b.p. 40° - 60°) and the insoluble residue crystallised from ether.

After 3 crystallisations, the material was obtained as long needles

m.p. = 98° - 100°

mixed m.p. with authentic tetramethyl fructofuronamide = 99° - 101°

$[\alpha]_D^{16} = -86°$ (c = 1 in water).

Found: C = 48·2%; H = 7·7%; N = 5·8%; OMe = 46·6%.

Calc. for $C_{10}H_{19}O_6N$: C = 48·2%; H = 7·6%; N = 5·6%; OMe = 49·8%.

Investigations into the Structure of the Trimethyl Fructose from Column III.

Oxidation of the syrup by sodium meta-periodate.

The formaldehyde liberated on periodate oxidation was estimated as the dimedon complex, using the methods of Reeves(17) and Bell.(18)

Oxidation by Reeves' Method.

The trimethyl fructose syrup (14·13 mg.) was dissolved in water (2 ml.), sodium bicarbonate (2 ml.; 1N) added, followed by sodium meta-periodate solution (2 ml.; 0·3M), and the solution allowed to stand overnight. Excess periodate and iodate were destroyed by the addition of hydrochloric acid (2 ml.; 2N) followed by sodium arsenite (2 ml.; 1M). After the yellow colour had completely disappeared, sodium acetate (2 ml.; 1M) was added, followed by dimedon reagent (2 ml.; 85 mg./ml. in 95% ethanol) and precipitation allowed to occur overnight.

Yield of Formaldehyde-Dimedone Complex = 37·35 mg.

m.p. = 143° - 169°.

This corresponds to a yield of 201% of theory, assuming 1 mole of formaldehyde per mole of trimethyl fructose.

A glucose control solution oxidised by this method gave "F-D" complex (m.p. = 186° - 187°) in 94% yield, assuming 1 mole of formaldehyde per mole of glucose.

Oxidation by Bell's Method.

The trimethyl fructose syrup (14·13 mg.) was dissolved in water (2 ml.), phosphate buffer (pH 7·5; 10 ml.) added, followed by sodium meta-periodate solution (2 ml.; 0·3M) and the solution allowed to stand overnight. Hydrochloric acid (2 ml.; 2N) and sodium arsenite (2 ml.; 1M) were added and, after reduction was complete, sodium acetate-acetic acid buffer (pH 4·5; 16 ml.), followed by dimedone reagent. Precipitation was allowed to occur overnight.

Yield of Formaldehyde-Dimedone Complex = 16·85 mg.

m.p. = 186° - 187°.

This corresponds to a yield of 91% of theory, assuming 1 mole of formaldehyde per mole of trimethyl fructose.

A series of glucose control solutions oxidised by this method gave yields of "F-D" complex (m.p. 186° - 187°)

corresponding to 35-57% of theory, assuming 1 mole of formaldehyde per mole of glucose. A formalin control experiment was also performed. Formalin (1 ml.; 40%) was diluted to 100 ml. and 1 ml. of this solution (4 mg. formaldehyde) was oxidised by sodium metaperiodate (2 ml.; 0·3M) in the presence of phosphate buffer (pH 7·5; 10 ml.), the procedure being the same as that outlined above. This yielded 37·39 mg. "F-D" complex (m.p. 187°), corresponding to 96% recovery of formaldehyde.

The Formation of 1:2-Isopropylidene 3:4:6-trimethyl fructose.

Authentic 3:4:6-trimethyl fructose ($[\alpha]_D$ = + 30° in acetone) was dissolved in a freshly-prepared solution of hydrogen chloride in anhydrous acetone (0·55%) and the solution allowed to stand at room temperature.

Time (mins.)	$[\alpha]_D$
0	+ 30°
5	+ 70·2°
10	+ 75·2°
25	+ 75·2°
60	+ 73·4°

After 2½ hours the product was poured into a saturated solution of sodium bicarbonate (150 ml.). The solution was extracted with chloroform, the chloroform extract dried over anhydrous sodium sulphate and concentrated to a syrup under reduced pressure.

The pale yellow mobile syrup was submitted to distillation in a high vacuum. The product distilled as a colourless, mobile syrup (bath temp. 65°/0·005 mm.).

$\eta_D^{19} = 1{\cdot}4446$

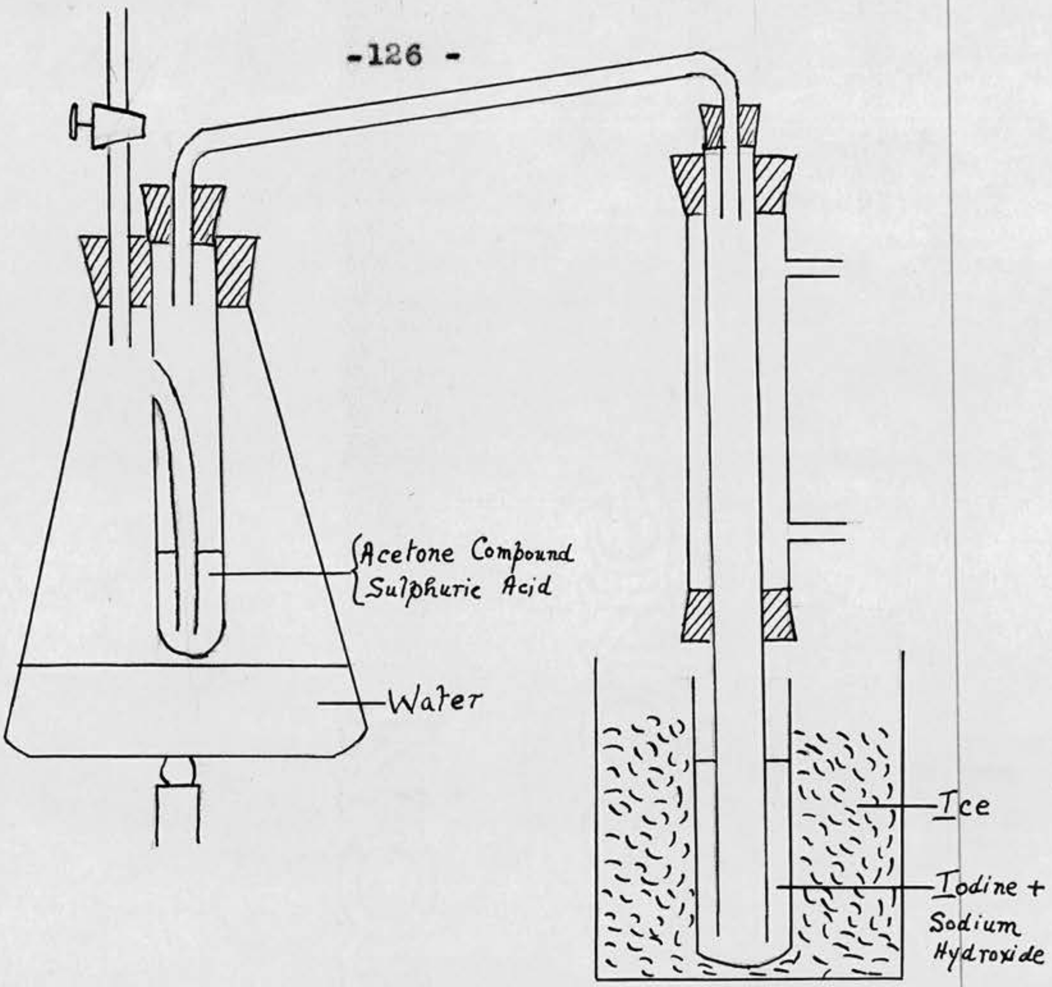
$[\alpha]_D^{18} = +\ 63{\cdot}6°$ (c = 0·4 in acetone).

$[\alpha]_D^{17} = +\ 71°$ (c = 1·2 in water).

A small amount of gummy non-volatile material ($\eta_D = 1{\cdot}4740$) remained in the distilling flask.

Micro-determination of Acetone.

The method used was a modification of that described by Bell and Harrison,(19) using an apparatus devised by Tordai and Percival.(20) The apparatus consists of a 500 ml. conical flask with a bubbler tube and an escape stop-cock fitted in the bung. The bubbler is connected to a condenser leading to a boiling tube immersed in an ice bath.



To the receiver, cooled in ice, were added N sodium hydroxide (10 ml.) and iodine solution (0·03N; 25 ml.). Into the bubbler were placed N sulphuric acid (3 ml.) and a solution containing 5-6 mg. of the acetone compound. The water in the boiler was heated gently and the temperature slowly raised to the boiling point. Steam was passed through the apparatus for 30 minutes, after which time the condenser was raised above the liquid and steam allowed to pass for a further 5 minutes. After washing the condenser inside and out, N sulphuric acid (15 ml.) was added and the excess iodine titrated with standard sodium thiosulphate (0·03N), using starch indicator. A tube containing the reagents only was titrated alongside.

Found: $(CH_3)_2CO$, 14·9%.

Calculated for 1:2-*iso*propylidene 3:4:6-trimethyl fructose, 22·1%.

The estimation was repeated, this time heating more strongly and for a longer period (50 mins).

Found: $(CH_3)_2CO$, 21·3%.

Treatment of Trimethyl Fructose Syrup with Acid Acetone.

A portion of the partially-crystallised trimethyl fructose syrup from Column III (75 mg.) was dissolved in anhydrous acetone, the rotation measured and a freshly-prepared solution of hydrogen chloride in anhydrous acetone added, such that the final solution contained 0·5% hydrogen chloride.

$[\alpha]_D^{15}$ = + 4° (c = 0·75 in acetone) (zero)

$\longrightarrow$ + 12·6° (c = 0·6 in acetone-hydrogen chloride) (5 mins.)

$\longrightarrow$ + 14·4° (25 mins.) constant.

The observation of a change in the value of $[\alpha]_D$ suggested acetone compound formation, and so the main bulk of the trimethyl fructose syrup was treated with hydrogen chloride in acetone.

The syrup (0·127 g.) was dissolved in acetone containing hydrogen chloride (0·5%) and the solution allowed to stand at 0° overnight ($[\alpha]_D$ = +4·7° $\longrightarrow$ + 17° constant). The combined solutions were then poured into a saturated solution of sodium bicarbonate and concentrated under reduced pressure to 50 ml.

In order to separate the monoacetone compound from unchanged trimethyl fructose, the aqueous solution was continuously extracted with redistilled light petroleum (b.p. 38° - 40°), using an all-glass apparatus of the type described by Brown and Jones.(21) The aqueous solution (50 ml.) was placed in the upper extractor whilst the lower extractor contained water (50 ml.) in order to wash the light petroleum extract. A little barium carbonate was added to each extractor, to prevent any local acidity arising.

The flask containing the solvent was changed at regular intervals and the solution evaporated to dryness under reduced pressure, and dried overnight in a vacuum desiccator over phosphoric oxide.

Duration of Extraction	Total Weight of Extract.
3 hours	16 mg.
6 hours	23 mg.
solvent changed to light petroleum (b.p. 40° - 60°)	
14 hours	41·2 mg.
20 hours	42 mg.

The aqueous solution was then removed from the top extractor and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield a pale yellow viscous syrup which was dried in a vacuum desiccator over phosphoric oxide (Residue A).

Yield = 23·5 mg.

η_D^{19} = 1·4660.

The syrup yielded no iodoform on hydrolysis with sulphuric acid and bubbling into sodium hypoiodite solution (see p. 126). The syrup partially crystallised on seeding with authentic 1:3:4-trimethyl fructose.

The aqueous layer from the chloroform extraction was concentrated to a syrup under reduced pressure and the residue extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield a fairly viscous syrup which was dried in a vacuum desiccator over phosphoric oxide (Residue B).

Yield = 75 mg.

η_D^{20} = 1·4640

OMe = 38% (theory, for trimethyl hexose, 41·9%)

$[\alpha]_D^{16}$ = - 53° (c = 1·4 in water) after correction for low methoxyl content.

The syrup was seeded with authentic 1:3:4-trimethyl fructose when crystallisation readily occurred at room temperature.

Residue A and residue B both gave a spot (<u>R_G</u> = 0·83) on a chromatogram developed with urea oxalate which travelled more slowly than authentic 3:4:6-trimethyl fructose (<u>R_G</u> = 0·86). Residue B also showed a trace of dimethyl fructose on the chromatogram due possibly to some demethylation during prolonged contact with hydrogen chloride in acetone.

Total recovery of fractionation = 141 mg. (70%). Fractionation yielded 42 mg. of monoacetone cpd. of Trimethyl X + 99 mg. of unchanged Trimethyl Y.

Investigation of the Monoacetone Compound.

The syrup obtained from the light petroleum extract (42 mg.) was distilled in a high vacuum, using a micro-distillation apparatus. The product distilled as a colourless mobile syrup (bath temp. 85°; pressure 0·04 mm.), leaving a gummy residue.

The distillate had

$n_D^{22} = 1{\cdot}4480$. $[\alpha]_D^{16} = +46°$ ($c = 1$ in H_2O).

OMe = 35·0% (theory, for 1:2-isopropylidene 3:4:6-trimethyl fructose, 35·5%).

The syrup, on hydrolysis with dilute sulphuric acid, yielded a positive iodoform test.

The acetone produced on hydrolysis was estimated using the apparatus of Tordai and Percival (p. 125). Found: $(CH_3)_2CO$ = 13·1% (theory, for 1:2-isopropylidene 3:4:6-trimethyl fructose, 22·1%).

Treatment of Pure Trimethyl Fructose (A) with Acid Acetone.

Fraction IV from Column I consisted of a chromatographically pure trimethyl fructose syrup, which appeared identical with 3:4:6-trimethyl fructose. To prove that it was 3:4:6-trimethyl fructose, and not 1:4:6-trimethyl fructose, the syrup was treated with acid acetone, and the change in rotation studied.

The syrup (22·5 mg.) was dissolved in anhydrous acetone (5 ml.), the rotation of the solution noted, and a freshly-prepared solution of hydrogen chloride in anhydrous acetone (1 ml.; 3·6%) added.

$[\alpha]_D^{16}$ = + 31·1° (c = 0·45 in acetone) (zero).
$\longrightarrow$ + 69·3° (c = 0·37 in acid acetone) (5 mins.).
$\longrightarrow$ + 74·6° (40 mins.) $\longrightarrow$ + 69·3° (12 hours).

The acetone solution was poured into a saturated sodium bicarbonate solution (100 ml.) and the mono-acetone compound isolated in the manner already described (p.124). The product was distilled in a high vacuum, using a micro-distillation apparatus, to yield a straw-coloured, mobile syrup (b.p. 80° - 85°/ 0·02 mm. (bath temp.)).

n_D^{13} = 1·4510 $[\alpha]_D^{13}$ = + 64° (c = 1 in water).

The liberation of acetone on hydrolysis of the syrup with dilute sulphuric acid was detected, using the apparatus of Tordai and Percival.(20) An uptake of iodine, due to the precipitation of iodoform, was obtained, but unfortunately there was not sufficient material to enable an accurate estimation of the acetone.

Investigation of the Structure of the Dimethyl Fructose.

Oxidation of the syrup by sodium meta-periodate and the estimation of the liberated formaldehyde.

The syrup was oxidised by sodium meta-periodate and the liberated formaldehyde estimated as dimedone complex, using the methods of Reeves (17) and Bell. (18)

Oxidation by Reeves' Method.

The dimethyl fructose syrup (10·09 mg.) was dissolved in water (2 ml.) and sodium bicarbonate (2 ml.; /N), followed by sodium meta-periodate solution (2 ml.; 0·3M) added. Oxidation was allowed to occur for 36 hours. Hydrochloric acid (2 ml.; 2N) and sodium arsenite (2 ml.; 1M) were added and, after the yellow colour had completely disappeared, sodium acetate (2 ml.; 1M) followed by dimedone reagent (2 ml.; 85 mg./ml. in 95% ethanol). Precipitation was allowed to occur for 24 hours.

Wt. of "F-D" complex = 28·38 mg. m.p. = 186° - 188°.

This corresponds to a yield of 100% of theory, assuming 2 moles CH_2O/mole of dimethyl fructose.

i.e. 1 mole of dimethyl fructose produces 2·0 moles of formaldehyde.

Oxidation by Bell's Method.

The dimethyl fructose syrup (10·62 mg.) was dissolved in water (2 ml.), phosphate buffer (pH 7·4;

10 ml.) and sodium meta-periodate solution (2 ml.; 0·3M) added, and oxidation allowed to occur for 24 hours. Hydrochloric acid (2 ml.; 2N) and sodium arsenite (2 ml.; 1M) were added and, after reduction was complete, sodium acetate-acetic acid buffer (pH 4·5; 16 ml.) and dimedone reagent (2 ml.). Precipitation was allowed to occur for 24 hours. Yield of "F-D" complex = 20·08 mg. m.p. 188° - 189°.
i.e. 1 mole of dimethyl fructose liberates 1·35 moles of formaldehyde.

Allowing 36 hours for periodate oxidation, and allowing crystallisation to occur over a period of 72 hours, the dimethyl fructose syrup (10·09 mg.) yielded 23·15 mg. "F-D" complex (m.p. 188° - 189°).
i.e. 1 mole of dimethyl fructose yields 1·64 moles of formaldehyde.

Osazone Formation.

To the dimethyl fructose syrup (0·18 g.) was added a solution of phenylhydrazine hydrochloride (0·7 g.) and sodium acetate (1·4 g.) in water (5 ml.). A little sodium bisulphite was added and the mixture heated on a water bath at 80° for 30 minutes. On cooling slowly, a red oil separated which, on trituration with water, rapidly hardened to yield a pale yellow solid (Fraction I). The aqueous solution was heated at 100° for 4 hours and cooled at 0° overnight, when a pale yellow solid separated (Fraction II).

The yellow solid was, in each case, removed by filtration, washed well with water and dried on porous tile in a vacuum desiccator over phosphoric oxide.

Fraction I 0·036 g. m.p. = 74° - 78° (amorphous)
Fraction II 0·045 g. m.p. = 78° - 79° (amorphous)

Found: C = 60·3%; H = 7·1%; N = 13·9%; OMe = 14·9%.
Calc. for $C_{20}H_{26}O_4N_4$: C = 62·2%; H = 6·7%; N = 14·5%; OMe = 16·0%.
Calc. for $C_{20}H_{26}O_4N_4.H_2O$:C = 59·4%; H = 6·9%; N = 13·9%. OMe = 15·3%.

Great difficulty was encountered in the purification of this compound. Attempts to crystallise the solid from aqueous alcohol yielded, in each case, a deep red oil which did not crystallise on standing for 3 weeks in the refrigerator.

A small sample of the product, however, crystallised as long, fine, yellow needles from a mixture of ether and light petroleum (b.p. 60° - 80°) after seeding with an authentic crystal of 3:4-dimethyl glucosazone. m.p. = 124° - 126°, mixed m.p. with authentic specimen = 124° - 125°.

When attempts were made to crystallise the main bulk of the product in this way, however, only 3 mg. of a yellow amorphous solid (m.p. 116° - 119°) could be obtained.

Found: C = 62·5%; H = 6·9%.

Calc. for $C_{20}H_{26}O_4N_4$: C = 62·2%; H = 6·7%.

The remainder of the product was obtained as a brown caramel-like substance.

Oxidation with Periodic Acid and isolation of D(-)-dimethoxysuccinic acid derivatives.

Formation of Dimethyl D(-)-dimethoxysuccinate.

To the dimethyl fructose syrup (0·82 g.) was added periodic acid (25 ml.; 1·15M) and the solution allowed to stand in the dark at room temperature for 36 hours. The solution was aerated for 24 hours to remove formaldehyde, and barium chloride (2·3 g.) added to the solution, followed by excess barium carbonate. After filtration, bromine (4 ml.) and barium carbonate (2 g.) were added to the solution, and the solution allowed to stand at room temperature for 36 hours. Bromine was then removed by aeration in the cold, the solution neutralised with silver carbonate and filtered. Silver ions were removed from the solution by the passage of hydrogen sulphide, the solution heated to coagulate the silver sulphide and filtered through a pad of 'filter cel.' The aqueous solution was evaporated to dryness under reduced pressure, last traces of water being removed by the addition of methanol, and finally anhydrous methanol. The residue consisted of a small amount of syrup embedded in a considerable amount of solid material.

The residue was boiled overnight with methanolic hydrogen chloride (60 ml.; 4%), the solution neutralised with silver carbonate, filtered and evaporated to dryness under reduced pressure. The syrupy residue, which was contaminated with a considerable amount of inorganic salt, was extracted with chloroform and evaporated to a syrup under reduced pressure. The syrup was distilled in a high vacuum to yield a colourless mobile material (0·55 g.).

b.p. = 100° - 110°/0·2 mm. (bath temp.).

n_D^{18} = 1·4325.

$[\alpha]_D^{16}$ = - 77·5° (c = 1·7 in methanol).

The Preparation of D(-)-dimethoxysuccinamide.

Redistilled dimethyl ester of D(-)-dimethoxysuccinic acid (0·167 g.) was dissolved in dry methanol saturated with ammonia (2 ml.) and the solution allowed to stand at 0° for 3 days. A crystalline precipitate was removed by filtration, washed with a little dry methanol and dried in a vacuum desiccator over phosphoric oxide. The product was obtained as long needles (0·14 g.) which became dark at 255° and melted at 277° - 278°, a mixed melting point with authentic D(-)-dimethoxy-succinamide showing exactly the same behaviour.

$[\alpha]_D^{16}$ = - 92° (c = 1 in water).

Found: C = 40·9%; H = 6·8%; N = 16·2%; OMe = 34·2%.

Calculated for $C_6H_{12}O_4N_2$. C = 40·9%; H = 6·8%; N = 15·9%; OMe = 35·2%.

The Preparation of D(-)-dimethoxysuccin-bis-methylamide.

Redistilled dimethyl D(-)-dimethoxy-succinate (0·343 g.) was dissolved in dry methanol saturated with methylamine (2 ml.) and the solution allowed to stand at 0° for 3 days. A crystalline precipitate was removed by filtration, washed with a little dry methanol and dried in a vacuum desiccator over phosphoric oxide. Yield = 0·123 g.

m.p. = 207° - 208°.

mixed m.p. with authentic specimen = 206° - 208°.

By evaporation of the filtrate and washings a further crop of crystalline material was obtained (0·218 g.) which was recrystallised from ethyl acetate to yield long needles (m.p. 208° - 209°).

Total recovery = 0·341 g.

For the recrystallised material:-

$[\alpha]_D^{16} = -134°$ (c = 1·2 in water).

Found: C = 47·3%; H = 7·85%; N = 13·7%; OMe = 29·5%.

Calculated for $C_8H_{16}O_4N_2$. C = 47·0%; H = 7·8%; N = 13·7%; OMe = 30·4%.

Molecular Weight Determinations.

a) Molecular Weight of methylated Triticin.

The molecular weight of the fractionated methylated polysaccharide (Fraction II) was determined using Barger's isopiestic method.(22)

A chloroform solution of known concentration of the methylated polysaccharide (1·126%) was compared with a series of chloroform solutions of sucrose octa-acetate of known molar concentration. For this purpose, a series of drops taken alternately from the methylated polysaccharide solution and one of the standard solutions were introduced into a capillary, and the ends sealed. A series of capillaries were made in this way to cover a molar concentration of 0·002M - 0·010M for the standard material. The capillaries were immersed in water in a constant temperature room, and the size of drops measured with a travelling microscope having a vernier accurate to 0·02 mm. Readings were taken after 1 hour and again after 20 hours, when the solutions had come to equilibrium. It was found essential to have the capillaries immersed in water, as highly inconsistent readings were obtained when they were allowed to rest on a grooved wooden block.

Concentration of methylated polysaccharide solution = 1.126%.

Molarity of sucrose octa-acetate drops	Change in size of drops (10^{-2} mm.)					Sum of changes with respect to methylated triticin
	T	S	T	S	T	
·002	+15	-14	+4	-8	+8	+49
·003	-5	+4	-5	+8	0	-22
·004	+4	+8	+1	+13	-7	-23
·005	-4	+4	-13	+3	0	-24
·006	-1	+4	-9	+12	-4	-30
·009	-5	+16	-8	+19	-16	-64

T = methylated triticin

S = sucrose octa-acetate.

From the above table, it appears that the isopiestic condition lies between ·002M and ·003M, whence

$$\text{Molecular Weight} = \frac{1{\cdot}126 \times 10}{{\cdot}002} \text{ to } \frac{1{\cdot}126 \times 10}{{\cdot}003}$$

$$= 5252 \text{ to } 3753.$$

Since the molecular weight of a methylated anhydrofructose unit is 204, the molecular size is 18 - 25 units.

When the concentration of the methylated polysaccharide solution was increased to 2·5%, no reproducible results could be obtained, all the drops of the methylated polysaccharide solution increasing in size between the range ·002M - ·010M.

b) Molecular Weight of Acetylated Triticin.

A solution of fraction I of the fractionated acetate in chloroform (2·082%) was compared with standard solutions of sucrose octa-acetate in chloroform, using the procedure described above.

Concentration of triticin tri-acetate solution = 2·082%

	Molarity of sucrose octa-acetate drops.	Change in size of drops (10^{-2} mm.)					Sum of changes with respect to acetylated triticin
		T	S	T	S	T	
42 hours.	0·002	+4	+5	+7	-4	+2	+12
	0·003	-8	+8	+2	+1	-8	-23
	0·004	-8	+7	-4	0	-9	-28
	0·005	-3	+21	-10	+8	-23	-65
	0·006	-38	+10	-32	+19	-17	-116

It appears that the isopiestic condition lies between ·002M and ·003M, whence

$$\text{Molecular Weight} = \frac{2{\cdot}082 \times 10}{{\cdot}002} \text{ to } \frac{2{\cdot}082 \times 10}{{\cdot}003}$$

$$= 10410 \text{ to } 6940$$

Since the molecular weight of an acetylated anhydrofructose unit is 288, the molecular size is 24 - 36 units.

In order to narrow the above range, the concentration of the acetylated triticin solution was increased to *ca*.5%, such that the isopiestic condition would lie somewhere in the range ·005M - ·008M.

Concentration of triticin tri-acetate solution = 5·028%.

Molarity of sucrose octa-acetate drops.	Change in size of drops (10^{-2} mm.)					Sum of changes with respect to acetylated triticin.
	T	S	T	S	T	
0·004	+15	-15	+33	-34	0	+97
0·005	+24	-34	+34	-7	+14	+113
0·006	-2	+8	-3	-3	-4	-14
0·007	-12	0	-2	0	-2	-16
0·008	-10	+8	-5	+7	-4	-34

It would appear that the isopiestic condition lies at an approximate concentration of ·006M, whence

$$\text{Molecular Weight} = \underline{\text{ca}}\,\frac{5\cdot028 \times 10}{\cdot006} = \underline{\text{ca}}.8380.$$

This corresponds to 29 acetylated anhydrofructose residues.

Oxidation of the Polysaccharide by the Periodate Ion.

For the periodate oxidation studies, the purest sample of polysaccharide available was used ($[\alpha]_D = -48°$).

Estimation of the Uptake of Periodate.

To a solution of the polysaccharide (ca.300 mg., accurately weighed) in water (35 ml.) was added sodium meta-periodate solution (15 ml.; 0·3M) and the solution allowed to stand in the dark at room temperature. A blank experiment, omitting only the polysaccharide, was run concurrently. At regular intervals, 5 ml. portions were removed by pipette and the periodate uptake determined by the addition of excess sodium arsenite solution ($\frac{N}{10}$) and potassium iodide, and titration of the excess arsenite with standard iodine ($\frac{N}{10}$).

Weight of polysaccharide = 0·2813 g. in 50 ml.

Duration	Titre of 0·1095NI_2		Periodate uptake in moles/fructose residue.
	Blank	Test.	
0·75 hours	2·02 ml.	4·80 ml.	0·88
1·5 "	2·19 ml.	5·08 ml.	0·92
3 "	1·95 ml.	5·01 ml.	0·96
6 "	2·06 ml.	5·155 ml.	0·976
20 "	1·97 ml.	5·14 ml.	1·00
50 "	2·00 ml.	5·16 ml.	1·00

Estimation of liberated formic acid.

Triticin ($[\alpha]_D$ = -48° in water) was subjected to oxidation by potassium periodate, according to Halsall, Hirst and Jones.(23)

The polysaccharide (ca.300 mg., accurately weighed) was dissolved in water (35 ml.), and potassium chloride (1 g.) and sodium meta-periodate solution (15 ml.; 0·25M) added, and the mixture shaken in the dark at room temperature. After 3-4 days, 5 ml. samples were withdrawn at intervals. After the potassium periodate had completely separated, ethylene glycol (0·3 ml.) was added to destroy excess periodate, and the formic acid titrated against standard sodium hydroxide (0·01358N), using methyl red as indicator.

A blank experiment, omitting only the polysaccharide, was run concurrently and treated in exactly the same way as the test solution. Furthermore, a solution of the fructosan in water, of the same concentration as the polysaccharide solution submitted to oxidation, was titrated directly against the standard sodium hydroxide.

Results.

Fructosan Blank. Titre <0·01 ml. of 0·01358 N NaOH for 33 mg. of fructosan.

Weight of polysaccharide = 0·2885 g. in 50 ml.

Duration	Alkali Titre after correction for Blank.	No. of residues/ mole of formic acid.
73 hours	0·346 ml.	37·9
120 hours	0·411 ml.	31·9
168 hours	0·436 ml.	30·1
240 hours	0·446 ml.	29·4
382 hours	0·451 ml.	29·1

Examination for the presence of Formaldehyde.

To the solution of periodate-oxidised polysaccharide (5 ml.) was added sodium arsenite (2 ml.; 1·2M) to destroy excess periodate, followed by phenylhydrazine hydrochloride solution (2 ml.; 1%) and potassium ferricyanide solution (1 ml.; 5%). On addition of a few drops of concentrated hydrochloric acid, no port wine coloration was obtained, whereas a strong wine colour was given by a standard formalin solution containing 0·001 mg. formaldehyde.

Treatment with Dimedon Reagent.

To 5 ml. of the solution of periodate-oxidised polysaccharide (containing 28·85 mg. polysaccharide) were added hydrochloric acid (2 ml.; 2N) and sodium arsenite (2 ml.; 1M). After the yellow colour had completely disappeared, sodium acetate solution (2 ml.; 1M) and dimedon reagent (2 ml.; 85 mg./ml. in 95% ethanol) were added and the solution allowed to stand at room temperature.

A blank experiment was performed simultaneously by the addition of the above reagents to 5 ml. of a solution of sodium meta-periodate and potassium chloride in water.

After standing for 48 hours, the test solution yielded a precipitate (11·44 mg.) which was filtered into a weighed sintered glass funnel.

m.p. = 215° - 225° (d)

[m.p. formaldehyde-dimedon complex = 188° - 189°].

Carbon, hydrogen and nitrogen micro-analyses of crystalline derivatives were performed by Drs. Weiler and Strauss. All other estimations were performed by the author. Methoxyl determinations were carried out by the gravimetric micro-method of F.Pregl, based on the Zeisel method.

DISCUSSION.

The couch grass roots under investigation were harvested in early spring and were, accordingly, found to contain a considerable free sugar content. Great difficulty, however, was found in isolating the water-soluble fructosan from the rhizomes in a high state of purity.

It was found essential to remove the free sugars prior to extraction of the water-soluble polysaccharide, and this was achieved by continuous extraction of the milled roots with 80% aqueous alcohol over a period of 2 days. This extraction produced a syrup in a yield corresponding to 13% of the dried raw material. This syrup was shown, by the technique of partition paper chromatography, to contain fructose (17%), glucose (3%) and a probable disaccharide. Attempts were made to investigate the nature of the latter material by extraction from the paper with water, followed by acid hydrolysis. No definite conclusions could be reached, however, although the material is certainly much more resistant to hydrolysis than sucrose.

The alcohol extract of the couch grass roots was shown to contain protein, since, by vigorous hydrolysis to amino acids, the hydrolysate yielded a positive ninhydrin reaction. Several proteins, such as Zein, which is present in maize, are alcohol-soluble and would be extracted under the above conditions. No material containing uronic acid was found to be present in the alcohol extract.

After alcohol extraction, the milled roots were extracted with cold water for several hours, the fructosan being extracted under these conditions, along with a large quantity of protein. Protein material was removed with basic lead acetate, after which the polysaccharide was isolated by precipitation from a concentrated aqueous solution with alcohol. This yielded a fine, white, hygroscopic solid in 14·8% yield, and the product had $[\alpha]_D$ = -46° in water (p. 50), after correction for ash, nitrogen and alcohol contents (Sample A). This method of extraction is essentially the same as that employed by Schlubach and Peitzner(24) (loc.cit.), who described their product, after 300 precipitations from alcohol, as a non-hygroscopic material with $[\alpha]_D$ = -51° in water.

Other methods of deproteinisation of the aqueous extracts were then studied in a series of small-scale extractions, and the purities of the polysaccharide samples closely compared. In all extractions, great care was taken that the medium never at any stage became acidic. Extractions were made in the presence of barium carbonate, a little toluene also being added to prevent bacterial action.

The method of Sevag, Lackmann and Smollens,[(4)] which consists of shaking the aqueous extract with chloroform (1/4 vol.) and butanol (1/10 vol.), thereby removing the proteins as a gelatinous layer, was employed. This gave very similar results to the basic lead acetate method when employed on a small scale, the product (yield 14%) having similar values for $[\alpha]_D$ and % N as sample A (Sample B. p. 51). On a large scale, however, the method of Sevag was found to be totally ineffective and time-consuming, a total of 23 treatments being necessary to ensure complete removal of the protein present in 30 g. of grass roots. The true advantage of this method lies in its application for the removal of the last traces of protein material. In all successive extractions, protein was removed by some other method and then the last remaining traces were removed by the Sevag method.

When neutral lead acetate, followed by chloroform-butanol, was used to remove protein, however, the resulting product was obtained in greater yield (21%) with a lower value for $[\alpha]_D$ (-33°) [Sample D]. This suggests the co-precipitation of some material along with the polysaccharide.

A series of extractions were then performed using cadmium hydroxide, as described by Fujita and Iwatake,[(5)] followed by chloroform-butanol, for deproteinisation. Various techniques were employed and it was found that methods which used hydrogen sulphide for the removal of inorganic ions gave larger yields and smaller values for $[\alpha]_D$ (Samples E and G) than methods which eliminated inorganic material by elution through ion-exchange columns (Samples F and H).

Attempts were finally made to purify the polysaccharide by acetylation, followed by deacetylation. Impure polysaccharide (Sample D) was acetylated with acetic anhydride in pyridine solution, the solution in pyridine being effected according to the method described by Pacsu and Mullen(11)(p.55). After reprecipitation of the acetate from chloroform with light petroleum, it was deacetylated with sodium methoxide in anhydrous methanol, according to Zemplén and Pacsu.(6) The deacetylated material had $[\alpha]_D = -46°$ after correction for ash content (Sample J). The acetylation-deacetylation reaction was therefore effective in raising the value of $[\alpha]_D$ from -33° to -46°.

From the above experiments, using different methods of deproteinisation, several samples of polysaccharide had values of $[\alpha]_D$ ranging from -44° to -48°. The effect of the normally-observed ash, nitrogen and adsorbed alcohol contents would be to raise the value of $[\alpha]_D$ by ca.3°, so that the calculated specific rotations of the fructosan samples compare favourably with the value quoted by Schlubach and

Peitzner(24) ($[\alpha]_D$ = -51° in water). Contrary to the observations of these workers, however, all the samples were found to be slightly hygroscopic. All the samples, also, reduced Fehling's solution slightly after prolonged boiling, but, as sucrose also gave a reducing action under these conditions, it is probable that some degradation of the molecule occurs.

It was also observed that, apart from the basic lead acetate method, techniques involving the use of hydrogen sulphide gave poor-quality products in high yield and should be avoided. It appears, therefore, that some material is co-precipitated which ion-exchange columns normally remove. As the ash contents of the poor-quality products were not abnormally high, this material cannot be inorganic salts.

Methods not involving hydrogen sulphide, however, gave pure products in 14% yield from the dry weight of powdered roots after free sugar removal. This corresponds to a 12% yield from the original dry weight.

A sample of one of these pure products was submitted to fractional precipitation from its aqueous solution with alcohol, and was found to be homogeneous (p. 56).

The Hydrolysis of Triticin.

The polysaccharide was very readily hydrolysed, hydrolysis being complete in 10 minutes with 0·1% oxalic acid solution. On hydrolysis of the purest sample of fructosan available, the final value of $[\alpha]_D$ was found to be -84°, which is only 91% of the equilibrium value of $[\alpha]_D$ for pure D-fructose.

A series of paper chromatograms of hydrolysates of various samples of triticin showed that a small amount of glucose was invariably produced in addition to fructose. No sample of polysaccharide could be prepared which, on hydrolysis, yielded fructose only on the chromatogram.

In an attempt to prove that the sugar which travelled more slowly than fructose on the chromatogram was, in fact, glucose, and not galactose, it was decided to treat the polysaccharide hydrolysate with asymmetrical methyl-phenylhydrazine. Galactose may be quantitatively determined thus, in the presence of other sugars, as a crystalline 1-methyl 1-phenylhydrazone.(7) A synthetic mixture of fructose (90%) and galactose (10%) yielded a crystalline solid, the melting point of which was not depressed on admixture with an authentic specimen of galactose 1-methyl 1-phenylhydrazone. The hydrolysate, however, yielded only a minute amount of crystalline material and it would appear that galactose, if present, occurs to the extent of less than 1% in the polysaccharide hydrolysate.

The hydrolysis of triticin was also found to occur by heating its aqueous solution under reflux on a boiling water bath, hydrolysis being complete after 24 hours, when the final rotation was $[\alpha]_D = -81°$. Paper chromatograms of the hydrolysate were duplicated, one being sprayed with ammoniacal silver nitrate and the other with the naphthoresorcinol spray reagent, as described by Partridge.(8) In addition to fructose and glucose, a spot travelling at the same rate as sucrose, and a spot with $\underline{R_G} = \cdot 056$ were observed.

It was considered that this latter spot might be turanose, and accordingly melezitose was submitted to controlled hydrolysis as described by Hudson and Pacsu.(9) It was found, however, that turanose travelled at almost the same rate as sucrose on the chromatogram, in ethyl acetate/acetic acid/water.

The spots intermediate between glucose and the starting line on the chromatogram of the aqueous hydrolysate of triticin could not be identified. The reducing spot ($\underline{R_G} = \cdot 056$) gave a pink colour with naphthoresorcinol and a pale brown colour with urea oxalate. The latter colour reaction is peculiar since all fructose derivatives give a blue coloration with urea oxalate.

As will be seen from the diagrams (p. 62), naphthoresorcinol indicated 2 spots travelling at the same rate as sucrose and turanose, both of which appeared to be non-reducing. No claim can be made as to the identity of these sugars however, although one is almost certainly sucrose. As a result of methylation studies (vide infra) it is reasonable to presume that they arise due to partial degradation of the triticin molecule to yield units with comparatively stable linkages. For, if there is, as would appear from the methylation studies, a non-terminal glucose unit linked through C^1 and C^3 to the 2 adjacent fructose units in the triticin molecule, one or both of these glucose-fructose linkages may be preserved on autohydrolysis.

The Quantitative Determination of Fructose and Glucose produced by hydrolysis of Triticin.

Colorimetric Determination of Fructose.

The method used was a modification of Roe's procedure(25) based on the original observation of Seliwanoff, which depends on the dehydration of fructose in hot, strong hydrochloric acid solution to give hydroxymethylfurfural. This then condenses with resorcinol to give an orange or red product. (Cole et al unpublished).

It was considered extremely probable that, under the experimental conditions of the colorimetric determination, the fructosan would be completely hydrolysed, with the result that the fructose produced could be directly estimated from a standard fructosan solution. An obvious advantage of such an estimation is that there is no danger of fructose undergoing a keto-enol tautomeric change with the production of glucose and mannose, since it never comes into contact with alkali.

The estimation was performed on a pure sample of triticin ($[\alpha]_D = -48°$) and the fructose produced on hydrolysis was estimated as (a) 92·6% and (b) 91·0%.

Quantitative Estimation of Glucose by Paper Chromatography.

The glucose produced on hydrolysis of the polysaccharide by boiling water was estimated by the technique of quantitative paper chromatography. The sugars were extracted from the paper strips by cold water, using the apparatus described by Laidlaw and Reid(10) and the sugars in solution estimated by titration of the formic acid liberated on oxidation by warm sodium meta-periodate.(26) In order to enable the accurate estimation of a small amount of glucose in the presence of a large amount of fructose, the solution of the mixture of sugars was spotted on a number of chromatograms, by means of a micro-pipette. This ensured that each paper contained exactly the same weight of sugar. The glucose strips from 5 papers were extracted and the glucose present in the combined extracts estimated. For the fructose, only 2 strips were extracted, but, by proportion, the fructose present in 5 papers could be calculated.

Using this technique, the glucose present in the aqueous hydrolysate of the polysaccharide was estimated as 4·8%.

i.e. Fructose = 91·8% (by colorimetric assay)
Glucose = 4·8% (by quantitative chromatographic estimation).

Studies of the Methylated Polysaccharide.

The Methylation of Triticin.

Triticin was methylated by simultaneous deacetylation and methylation of the acetate, using a procedure similar to that employed by Haworth, Hirst and Percival(13) for the methylation of inulin.

The acetylation of triticin was effected by the addition of acetic anhydride to a solution of the polysaccharide in pyridine, the solution in pyridine being obtained by the displacement process of Pacsu and Mullen.(11) The acetate, which was prĕcipitated by water and reprecipitated from chloroform solution by means of light petroleum, had $[\alpha]_D = -10°$ in chloroform, a value considerably lower than that quoted by Schlubach and Peitzner(24) (-15°) after 490 reprecipitations. The acetate was fractionally precipitated from chloroform with light petroleum, whereby 6 fractions were obtained. A study of the various constants led to the combination of the first two fractions, which were then submitted to methylation.

The acetate was methylated by the addition of 30% sodium hydroxide (3 parts) and dimethyl sulphate (1 part), the reaction being carried out in acetone solution at 40° and in an atmosphere of nitrogen (p. 71). After 3 such methylations, the product was methylated with silver oxide and methyl iodide, according to Purdie. The methylated polysaccharide was then precipitated from chloroform solution with light petroleum in a yield of 73% from the acetate, and had a methoxyl content of 43·3%. The product was, at this stage, fractionated from chloroform solution with light petroleum. 3 fractions were thus obtained, and it was found that the methoxyl content of each fraction had been reduced to <u>ca</u>.41%.

A portion of Fraction I of the fractionated methylated polysaccharide was submitted to methylation by the thallium method, using thallous ethoxide and methyl iodide. This technique was found to be very effective and to give quantitative recoveries. After 2 methylations according to this procedure, the methylated polysaccharide had a methoxyl content of 45% and was assumed to be fully-methylated.

The Hydrolysis of Trimethyl Triticin and the Examination of the products of hydrolysis.

Two separate studies were undertaken in this respect, the remainder of fraction I of the fractionated methylated polysaccharide (OMe = 41%) and the fully-methylated polysaccharide (OMe = 45%) being hydrolysed and studied independently.

In the former case, hydrolysis was achieved using a solution of oxalic acid in 80% aqueous methanol, as described by Haworth, Hirst and Percival(13) for the hydrolysis of trimethyl inulin. Unfortunately it was not possible to follow the hydrolysis polarimetrically as unhydrolysed material precipitated from the solution on cooling, but the reaction was allowed to proceed to constant rotation.

In order to separate the methylated sugars present in the hydrolysate, the classical method of high vacuum fractional distillation was dispensed with, in favour of one of the recently-developed methods derived from the principles of partition chromatography. Bell and Palmer(27) have obtained a quantitative separation of tetramethyl-D̲-fructofuranose, 1:3:4-trimethyl-D̲-fructose and 3:4-dimethyl-D̲-fructose on a column of silica gel.

A method which has received much application recently is the use of a column of powdered cellulose for the separation of a mixture of methylated sugars.(14)

The components of the hydrolysate of methylated inulin were separated in this way by Hirst, McGilvray and Percival,(28) and it was this latter technique that was employed for the separation of the components of the hydrolysate of the methylated triticin.

The mixture of methylated sugars was added to a column of powdered cellulose, of length 27", which had been subjected to preliminary washings. The solvent used was a mixture of butanol (50%) and light petroleum (50%) saturated with water, the light petroleum having been purified by treatment with concentrated sulphuric acid, followed by thorough washing and distillation. The course of the separation was followed by a series of paper chromatograms, every tenth tube being evaporated and spotted on the chromatogram. Owing to the fact that aniline oxalate was found to be a very poor reagent for the detection of methylated fructoses, paper chromatograms were duplicated. One was sprayed with aniline oxalate, in order to detect methylated aldoses and the other was sprayed with urea oxalate which was found to be very sensitive towards methylated fructoses.

Seven fractions were obtained from the cellulose column (p. 81) but examination of the individual fractions showed that some of the fractions were contaminated with methyl-glycosides.

A portion of <u>Fraction I</u> was distilled in a high vacuum to yield a mobile syrup, the constants of which agreed well with those quoted for tetramethyl methyl-D-fructofuranoside (p. 83).

Fraction II, on hydrolysis with aqueous oxalic acid, yielded a mixture of tetramethyl fructofuranose and a trimethyl fructose, showing that the original syrup from the column consisted of a mixture of tetramethyl-D-fructofuranose and a trimethyl methylfructoside having identical R_G values.

Fraction IV, appeared from its methoxyl content to be a trimethyl fructose. The syrup was unchanged on acid hydrolysis, indicating that it was not contaminated with a dimethyl methylfructoside. The value of $[\alpha]_D$ in water (+32°) was found to agree well with that quoted in the literature by various workers for 3:4:6-trimethyl-D-fructose and, in addition, the syrup was found to travel at the same rate as an authentic sample of 3:4:6-trimethyl-D-fructose on the chromatogram (p. 87).

Fraction V appeared, likewise, from its methoxyl content to be a trimethyl fructose, and the value of $[\alpha]_D$ in water (+31°) indicated, at first sight, that this fraction too was a pure sample of 3:4:6-trimethyl-D-fructose. On acid hydrolysis, however, the solution became strongly laevorotatory and was shown to consist of a mixture of a trimethyl fructose and a dimethyl fructose on the chromatogram, the former travelling more slowly than 3:4:6-trimethyl-D-fructose.

Fraction VI, on acid hydrolysis, yielded a dimethyl fructose, which was detected by spraying with urea oxalate and with aniline oxalate, and a trimethyl aldose, which was detected by aniline oxalate only. The trimethyl aldose travelled at exactly the same rate as crystalline 2:4:6-trimethyl glucose on the chromatogram.

The aldose content of this syrup was estimated as 14·5% by oxidation with alkaline hypoiodite,(15) this value being based on the assumption that a dimethyl fructose is completely unattacked by alkaline hypoiodite. It would appear then that this syrup is a mixture of a dimethyl fructose, which from later evidence is assumed to be 3:4-dimethyl fructose, (86%; $[\alpha]_D = -60°$) and 2:4:6-trimethyl glucose (14%; $[\alpha]_D = +89° \longrightarrow +71°$). On this assumption, the value of $[\alpha]_D$ for the syrup may be calculated as $-39° \longrightarrow -42°$, whereas the experimental value obtained was $-41° \longrightarrow -43°$.

The original syrup from the column would therefore appear to be a mixture of a dimethyl methylfructoside and a trimethyl aldose, which is, in all probability, 2:4:6-trimethyl glucose.

Fraction VII yielded a strongly laevorotatory dimethyl fructose which remained unchanged on aqueous acid hydrolysis, giving no trace of monomethyl fructose.

The value of $[\alpha]_D$ in water ($-58^\circ \longrightarrow -61^\circ$) agreed well with the value obtained by McDonald and Jackson(29) for pure synthesised 3:4-dimethyl fructose (-60·7°) and with the value obtained by Bell and Palmer(27) for the dimethyl syrup produced from methylated irisin (-59·4°).

The cellulose column was then thoroughly eluted with water and the eluate spotted on the chromatogram after the usual purification. The fact that no colour reaction was obtained with urea oxalate indicated that no methylated ketose was present. With aniline oxalate, however, a distinct pink coloration was obtained ($\underline{R_G}$ = 0·57), the material travelling more slowly than authentic 2:3-dimethyl glucose, but at the same rate as 3:6-dimethyl glucose. According to Connell,(30) 2:6-dimethyl- and 4:6-dimethyl-glucose travel at the same rate as 3:6-dimethyl glucose, the 4:6-isomer giving a brown coloration and the 2:6-isomer a pink coloration with aniline oxalate. It is possible therefore that the column eluate contains 2:6-dimethyl glucose or 3:6-dimethyl glucose or both.

The total column recovery (66%) was low, making quantitative interpretations of the results impossible. This may have been due to the fact that a large number of fractions (8) were studied independently, but, undoubtedly, the chief reason was due to the presence of considerable quantities of methylglycosides which escaped detection by the usual spray reagents. Nevertheless, one or two important results emerged from this work.

1) A complete separation of (probably) 3:4:6-trimethyl fructose and a second trimethyl fructose was obtained by elution through a cellulose column of length 27" with butanol (50%)-light petroleum (50%) saturated with water. This pointed to the fact that 2 types of linkage may be present in the triticin molecule and the reason why Schlubach and Peitzner (loc.cit.) were unable to identify their trimethyl fraction may have been due to the fact that they were in fact dealing with a mixture of 2 isomers.

2) By the use of aniline oxalate as a spray reagent in conjunction with urea oxalate, evidence arose as to the presence of small quantities of methylated glucoses in addition to the methylated fructoses.

Pure synthetic tetramethyl-D-fructose gave no coloration with aniline oxalate, but the tetramethyl fructose fraction from the cellulose column gave a pink coloration. This is doubtless due to the presence of some tetramethyl glucopyranose, which, by virtue of its R_G value (1·00), could not be separated chromatographically from tetramethyl fructofuranose (1·01). In addition, Fraction VI appears to contain a trimethyl glucose whereas the column eluate possibly contains dimethyl glucose(s).

The Separation of the Components of the hydrolysed Fractions from Column I.

Fractions II, I-II mixture and V were hydrolysed with aqueous oxalic acid and the methylated sugars produced separated by a further elution through a cellulose column (length 22"). The technique was the same as that employed previously except that the solvent was changed to butanol (30%)-light petroleum (70%). It was, however, found necessary to change the solvent to a 50:50 mixture in order to elute the dimethyl fraction from the column.

A tetramethyl, a dimethyl and 2 trimethyl fractions were collected, a complete separation of the 2 trimethyl fractions again being obtained (p. 93). The total column recovery was 92%.

Trimethyl fructose A, after distillation in a high vacuum, had $[\alpha]_D = +33° \longrightarrow +29°$, a value which agreed well with the value quoted in the literature for 3:4:6-trimethyl-D-fructose [+31°]. The fact that it travelled at the same rate on the chromatogram as authentic 3:4:6-trimethyl fructose provided further evidence as to the identity of trimethyl fructose A.

Trimethyl fructose B, which travelled more slowly on the chromatogram than 3:4:6-trimethyl fructose, was strongly laevo-rotatory, a feature which indicated the presence of a pyranose ring due to C_6 being unsubstituted. The value of $[\alpha]_D$ (-51°) did in fact agree well with the values quoted by various workers for 1:3:4-trimethyl-D-fructose obtained from levan-type polyfructosans.(31,32)

The syrup readily crystallised on seeding with a crystalline specimen of 1:3:4-trimethyl-D-fructose, kindly supplied by Dr. D.J. Bell. After tiling and crystallisation from a mixture of light petroleum and carbon tetrachloride, the material was obtained as long white needles (m.p. 72° - 73°), the melting point of which was not depressed on admixture with an authentic specimen of 1:3:4-trimethyl-D-fructose.

The tetramethyl fructose fraction again gave a slight pink coloration with aniline oxalate, suggesting the presence of a little tetramethyl glucopyranose. The value of $[\alpha]_D$ reported for pure tetramethyl-D-fructofuranose is +31°, whereas the value found experimentally for this fraction was +35°, a feature which again suggests the presence (ca.8%) of tetramethyl-D-glucopyranose ($[\alpha]_D$ = +83° in water).

The dimethyl fructose fraction was obtained as a viscous, hygroscopic syrup which could not be obtained in crystalline form. The syrup was strongly laevorotatory, suggesting the presence of a pyranose ring, due to non-substitution on C_6, and the value of $[\alpha]_D$ (-55°)

agreed reasonably well with that reported for 3:4-dimethyl fructose (-60°), better agreement being obtained on correction for the low methoxyl content of the syrup (p. 96).

Study of the Fully-methylated Triticin (OMe = 45%).

As a result of the investigations outlined above, it is obvious that hydrolysis of the methylated polysaccharide with aqueous methanolic oxalic acid yields mainly the methylated free sugars, along with some methylfructosides. On this occasion, in order to ensure the complete hydrolysis of glycosides, the aqueous methanolic hydrolysis of methylated triticin (2·46 g.) was followed by an aqueous oxalic acid hydrolysis.

The mixture of methylated sugars (2·7 g.) so obtained was separated into its components on a 15" column of powdered cellulose, using the same technique as described previously (p. 157). The solvent employed was a mixture of butanol (50%)-light petroleum (50%) saturated with water. Three fractions, namely a tetramethyl- (1·1 g.), a trimethyl- (0·38 g.) and a dimethyl- (1·05 g.) fraction, were collected. It appeared highly probable, however, that the trimethyl fraction, which extended over a long range, was a mixture of 2 components, since there was a gradual fall in the R_G value from 0·87 to 0·83. The tetramethyl fraction gave a pink coloration with aniline oxalate, again indicating the presence of some tetramethyl glucopyranose, but there was, on this occasion, no evidence for the separation of a trimethyl- and a dimethyl-glucose from the trimethyl- and dimethyl-fructose respectively. This is doubtless due to the fact that a shorter column was employed.

Each fraction was then investigated quantitatively, in order to find the ratio "tetra":"tri":"di" produced from the fully-methylated triticin.

The Colorimetric Determination of Tetramethyl Fructofuranose.

It has been pointed out by Bell and Palmer,(27) that 1:3:4:6-tetramethyl-D-fructofuranose is volatile, a pure sample undergoing a regular decrease in weight on drying in a vacuum desiccator.

The hygroscopic nature of the material demands thorough drying for an accurate gravimetric assay and it is obvious that the volatility of the material, even at the water pump, will not permit of this.

It was decided therefore to attempt to estimate the tetramethyl fructofuranose by a colorimetric determination in dilute solution. It has been seen that a modification of Roe's procedure based on the Seliwanoff colour reaction provided a satisfactory means for the determination of fructose in solution (p.152)

It seemed reasonable to presume that this method would also provide a satisfactory means of estimation of tetramethyl fructofuranose.

A standard curve was constructed for a concentration ranging from 0·05 to 0·25 mg./ml., when a linear relationship was found between Log_{10} I/Io and concentration. When the results were repeated using a different set of reagents and different standard solutions, excellent agreement was obtained (Graph X).

The material used for the construction of the above standard curve was shown, by paper chromatography, to contain a trace of trimethyl fructose. In order to discover whether this had any injurious effect in the colorimetric estimation, it was decided to check this curve using, as a standard, tetramethyl fructofuranose which had been obtained by the hydrolysis of the tetramethyl-methyl-D-fructofuranoside from Column I, and which was chromatographically pure. A linear relationship between $\log_{10}{}^{I}/_{Io}$ and concentration was again found (Graph Y), but a big discrepancy in the numerical values was obtained.

It will be seen, therefore, that, whereas the method gives excellent concordant results for the determination of tetramethyl fructofuranose in dilute solution, a chromatographically pure sample is essential for the success of the method.

The estimation of the Tetramethyl Fructofuranose from the Cellulose Column.

Since the "end group" from the cellulose column was in a solution of butanol-light petroleum-acetone, whereas the standard curve for the tetramethyl fructofuranose was constructed using a saturated benzoic acid solution in water, it was necessary to consider first a quantitative method of extraction of the material.

Rather than concentrate the solution from the column and extract repeatedly with water in a process similar to that described by Bell and Palmer (loc.cit.) it was considered advisable to remove the butanol-light petroleum-acetone as an azeotrope with water, a saturated solution of benzoic acid in water being added at the same rate as the azeotrope distilled off. In order to test the effectiveness of this method, a control solution of chromatographically pure tetramethyl fructofuranose was carried through the process. After the material had been partitioned into water in the method outlined, the solution was made up to a fixed volume and the substance estimated colorimetrically, using 2 ml. aliquot portions (Graph Y). Under these conditions, the method was found to give a satisfacoty recovery with an error of +2%.

The tetramethyl fructofuranose in Fraction I was then estimated colorimetrically, using exactly the same procedure, the final weight (1·06 g.) being the mean of 2 concordant results (p. 109).

Examination of Fraction I (Tetramethyl fraction).

After the tetramethyl fructofuranose had been estimated by this colorimetric method, the solution was evaporated to a syrup and purified in the usual manner. The value of $[\alpha]_D$ (+34°) in water indicated the presence of ca.7% tetramethyl glucopyranose in the syrup (vide p. 163).

In order to estimate the aldose content of the syrup, it was submitted to oxidation by alkaline hypoiodite, using a modification of the method described by Hirst, Hough and Jones.(15) Under the conditions described by these workers, aldose material is oxidised quantitatively to aldonic acid by alkaline hypoiodite, whereas, theoretically, ketose material is unattacked. The stability of tetramethyl fructofuranose to alkaline hypoiodite was demonstrated by Hirst, McGilvray and Percival.(28) Using a sample of tetramethyl fructofuranose, from methylated inulin, which had been separated from tetramethyl glucopyranose by light petroleum extraction of an aqueous solution of the methylglycosides, they found no iodine uptake. It is apparent, therefore, that oxidation by alkaline hypoiodite provides an excellent method for the estimation of tetramethyl glucopyranose in the presence of tetramethyl fructofuranose.

When a sample of Fraction I from the cellulose column was oxidised by alkaline hypoiodite, the aldose content was estimated as 5·1%. Since the total weight of the syrup, estimated gravimetrically, was 1·098 g., the weight of tetramethyl glucose may be calculated as 0·056 g., which corresponds to 2·2% of the total products of hydrolysis of methylated triticin. By difference, the weight of tetramethyl fructofuranose may be calculated as 1·042 g., which agrees well with the value obtained by colorimetric assay. This corresponds to 41·6% of the total products of hydrolysis.

Examination of Fraction II (Trimethyl fraction).

Since 1:3:4-trimethyl fructose and 3:4:6-trimethyl fructose are not volatile at the water pump, they may be readily estimated gravimetrically.

There was no definite evidence to suggest the presence of 2 trimethyl fructoses in this fraction, only one diffuse spot being obtained on the chromatogram. A tailing effect at the top of this spot, however, indicated that the syrup might, in fact, be a mixture of 2 trimethyl sugars. Furthermore, the value of $[\alpha]_D$ (-33·5°) did not correspond with the values already quoted for 1:3:4-trimethyl-D-fructose or 3:4:6-trimethyl-D-fructose (p.p. 162-163), but was intermediate between them. This would further indicate the possibility of the syrup being a mixture of the two isomers.

On seeding with an authentic crystal of 1:3:4-trimethyl-D-fructose, kindly supplied by Dr. D.J.Bell, the syrup partially crystallised. Pure 3:4:6-trimethyl-D-fructose has never been obtained in crystalline form.

The aldose content of the syrup was estimated as 4·0%, by oxidation with alkaline hypoiodite. If the aldose be assumed as trimethyl glucose, this would represent 0·6% of the total products of hydrolysis, and, by difference, the trimethyl fructose may be calculated as 14·4%. The validity of these figures, of course, depends on the assumption being true that a trimethyl fructose is completely unattacked by alkaline hypoiodite in the same way as is tetramethyl fructofuranose. Laidlaw and Reid,[33] using a pure crystalline sample of 1:3:4-trimethyl-D-fructose obtained from the levan from perennial rye grass (*Lolium perenne*) did, in fact, get a very small, but practically negligible, iodine uptake.

Examination of Fraction III (Dimethyl fraction).

This fraction was estimated gravimetrically and yielded a strongly laevorotatory, hygroscopic syrup which could not be crystallised. The value of $[\alpha]_D$ in water (-61°) agreed well with the value quoted for the dimethyl fraction obtained from Column I and with the value quoted for pure synthetic 3:4-dimethyl-D-fructose.

The aldose content of the syrup was estimated in the normal manner as 3·9%, and, assuming this to be dimethyl glucose, this corresponds to 1·6% of the total hydrolysis products. By difference, the recovery of dimethyl fructose may be calculated as 39·4%. Again it must be stressed that these figures are dependant upon the complete non-oxidation of a dimethyl fructose by alkaline hypoiodite. Unfortunately, no figures are available to prove that this is in fact the case for a dimethyl fructose.

In order to obtain as complete a recovery as possible from the cellulose column and to explore the possible occurrence of further quantities of methylated aldoses, all the eluates collected between the three main fractions were investigated.

The eluate between the trimethyl and dimethyl fractions yielded a syrupy residue which gave a pink spot on the chromatogram travelling at the same rate as 2:4:6-trimethyl glucose. The aldose content of the syrup was estimated as 49% by oxidation with alkaline hypoiodite, and, together with the aldose content of Fraction II, the "trimethyl glucose" may be calculated as 0·9% of the total hydrolysis products. Investigations of the other eluates showed no evidence on the chromatogram for further quantities of methylated aldoses, or for significant quantities of methylated ketoses.

The separation of the component sugars of the hydrolysate of the fully-methylated triticin by means of a cellulose column gave a total recovery of 96%. Doubtless due to the fact that a shorter column was employed than in Columns I and II, there was no separation of the trimethyl fraction into 2 components. Moreover, the suspected trimethyl- and dimethyl-glucoses were for the most part eluted along with the trimethyl- and dimethyl-fructoses respectively, and were estimated by oxidation with alkaline hypoiodite.

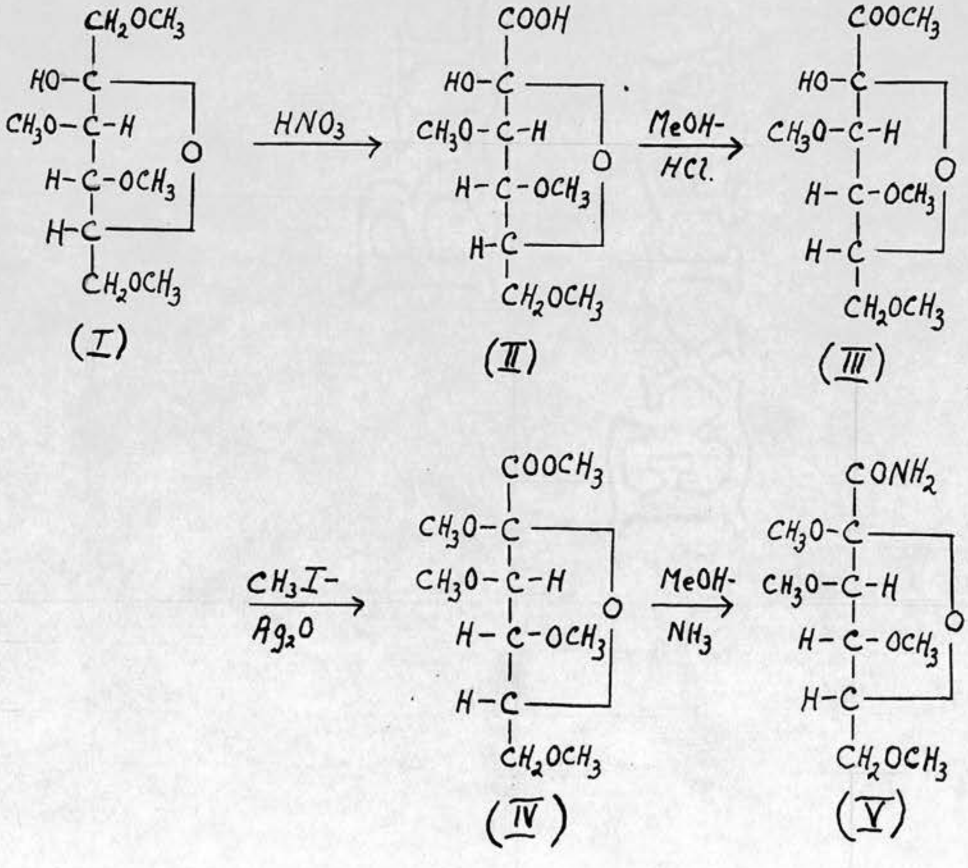
When the weights of the individual fractions were expressed in terms of hexose, rather than methylated hexose, it was found that tetramethyl fructose, trimethyl fructose and dimethyl fructose were obtained from methylated triticin in the ratio 2·73:1:2·93, or 3:1:3, expressed in the nearest whole number ratio (p.118) This figure is precisely the same as that obtained by Schlubach and Peitzner[(24)] by high vacuum fractional distillation of the methyl-fructosides and would indicate that the triticin molecule is composed of multiples of 7 fructose units.

An additional interesting feature is that the total recovery of methylated glucose from the hydrolysate of methylated triticin was 4·3% (expressed as glucose).

This is in excellent agreement with the value of 4·8% obtained when the glucose in a hydrolysate of the polysaccharide was estimated by quantitative paper chromatography.

Identification of the Tetramethyl Fructose.

Definite proof that the "end group" was tetramethyl-D-fructofuranose was obtained by conversion to the crystalline tetramethyl-D-fructofuronamide (p.120). This was achieved by oxidation of the syrup with nitric acid, followed by esterification and methylation, and the amide was produced by the action of methanolic ammonia on the methyl ester, as described by Avery, Haworth and Hirst.(16) The series of reactions may be represented by the following scheme:-



The partially-crystalline solid (V) was allowed to drain on porous tile, extracted with hot ether and hot light petroleum (b.p. 40° - 60°) and crystallised from ether. After 3 crystallisations the required product was obtained as long needles (m.p. 98° - 100°), the analysis figures and specific rotation agreeing well with those reported for tetramethyl-D-fructofuronamide. A series of methoxyl determinations gave a concordant value of *ca*.47%, (theory 49·8%). A pure authentic sample of tetramethyl fructofuronamide (m.p. 99° - 100°) gave a methoxyl content of 47·4%, whilst Haworth, Hirst and Nicolson(34) quote a value of OMe = 47·7% for their pure material. The low values may be due to a tendency for the glycosidic methoxyl group to come off as methanol, immediately on heating, and this may be partly lost by distillation.

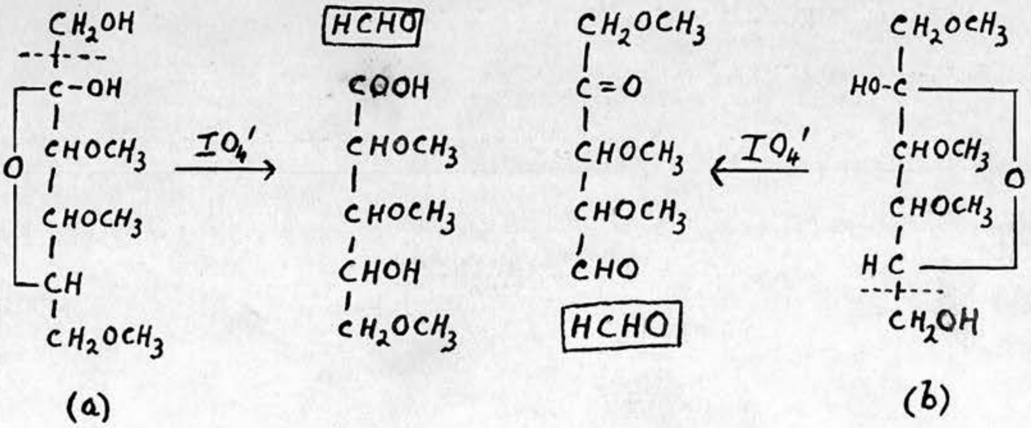
A byproduct (m.p. 175°-176°), insoluble in hot ether and hot light petroleum, was also obtained. A byproduct (m.p. 177° - 178°) which was insoluble in all the usual organic solvents was reported by Haworth, Hirst and Nicolson(34) and they believe it to be an amide derived from a byproduct of the original oxidation. An alternative possibility is that the tetramethyl glucose present in Fraction I was oxidised by nitric acid to yield a xylo-trimethoxyglutaric acid which formed the diamide (m.p. 194° - 195°).

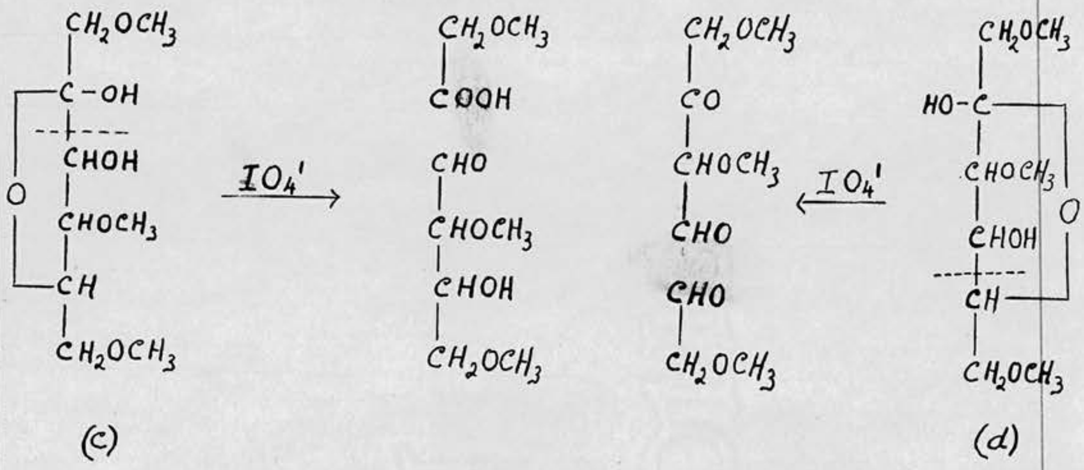
Investigations into the Structure of the Trimethyl fraction.

As already mentioned, by separation of the products of hydrolysis of methylated triticin on a long cellulose column (27"), 2 trimethyl fructose fractions, which, by virtue of their specific rotations, appeared to be 3:4:6- and 1:3:4-trimethyl-D-fructose, were obtained. On this occasion, using a shorter column, only one fraction was obtained and it was desired to prove that it was a composite mixture of these 2 isomers.

The Oxidation of the Syrup by Sodium meta-Periodate.

If, in a methylated ketose, C_6 or C_1 are unmethylated, then, provided C_5 or C_2 respectively are also free, oxidation by the periodate ion will liberate formaldehyde, whereas, if the terminal $-CH_2OH$ groups are both methylated, no formaldehyde yield will occur. Thus, of the 4 possible trimethyl fructoses, the 3:4:6- (a) and the 1:3:4-(b)isomers will liberate formaldehyde, whereas the 1:4:6-(c) and the 1:3:6-(d) isomers will not.





Formaldehyde produced on periodate oxidation was originally estimated by steam distillation but Reeves (17) introduced a very elegant process whereby formaldehyde was estimated gravimetrically as a crystalline dimedon complex, a small weight of formaldehyde giving approximately 10 times its weight of the crystalline complex.

When the trimethyl fructose syrup was oxidised and the formaldehyde estimated using the technique described by Reeves, the weight of complex represented a 201% yield, assuming 1 mole of formaldehyde to be produced per mole of trimethyl sugar. Since the product melted over a wide range, it would appear that, under the conditions employed, the aldehyde produced on periodate oxidation itself formed an insoluble dimedon complex.

It is generally recognised that, in order to obtain theoretical yields of formaldehyde, the periodate oxidation must be carried out in a neutral medium.

Reeves used the carbon dioxide-bicarbonate buffering system at pH ca. 7·5. Alternatively the oxidation may be carried out in the presence of phosphate buffer (pH 7·4 - 7·5), as described by Bell.(18) Bell made a study of the oxidation of partially-methylated sugars under these conditions, and found that, whereas some rapidly yielded the theoretical amount of formaldehyde, others reacted more slowly and gave a yield of formaldehyde considerably below the theoretical. Continuing these observations, Bell, Palmer and Johns(35) found that in some cases the "missing" formaldehyde in the anomalous oxidations could be accounted for as formic acid. They found that 1:3:4-trimethyl fructose, when oxidised in the presence of phosphate buffer (pH 7·4), gave 1 mole of formaldehyde per mole of sugar. On the other hand, 3:4:6-trimethyl fructose, when oxidised under the same conditions, could only be made to yield 0·82 moles of formaldehyde per mole of sugar.

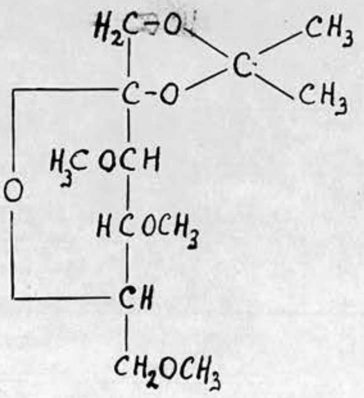
When the trimethyl syrup from hydrolysed trimethyl triticin was oxidised under these conditions, the weight of formaldehyde-dimedon complex corresponded to a 91% yield, assuming 1 mole of formaldehyde per mole of trimethyl syrup. The melting point of the product indicated that no interference from other aldehydes had occurred when oxidation was allowed to take place under these conditions.

A series of glucose control experiments showed that, when glucose was oxidised in the presence of phosphate buffer, theoretical yields of formaldehyde were never obtained, values varying between 35% and 57% of the theoretical being obtained. On the other hand, when oxidised in the presence of bicarbonate, glucose gave theoretical yields of formaldehyde.

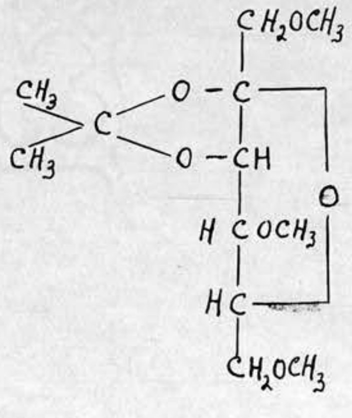
From an estimation of the formaldehyde liberated on periodate oxidation of the trimethyl fraction, it would appear that if the syrup is a mixture of 1:3:4-trimethyl fructose and another trimethyl fructose, then the other one must be 3:4:6-trimethyl fructose, which will itself liberate formaldehyde.

<u>The treatment of the syrup with acid acetone.</u>

Of the 4 theoretical trimethyl fructoses, 2 will form mono-acetone compounds. 3:4:6-Trimethyl fructose will form 1:2-*iso*propylidene 3:4:6-trimethyl fructose (i) whilst 1:4:6-trimethyl fructose will form 2:3-*iso*propylidene 1:4:6-trimethyl fructose (ii).



(i)



(ii)

The condensation of these 2 isomers with acetone has been studied by Montgomery(36) who found that, when 3:4:6-trimethyl fructose was treated with a solution of hydrogen chloride in dry acetone, the specific rotation increased from +31° to +74·8°. Since 1:3:4-trimethyl fructose does not form a mono-acetone compound, it was considered that a change in rotation on treatment of the trimethyl fraction with acid acetone would provide good evidence for the presence of 3:4:6-trimethyl fructose.

When authentic 3:4:6-trimethyl fructose was treated with a solution of hydrogen chloride in dry acetone (0·6% w/v), the specific rotation was found to increase from +30° to +73·4° (p.124), which is in good agreement with the results of Montgomery.

When the partially-crystalline trimethyl syrup from the cellulose column was treated with hydrogen chloride in acetone, the specific rotation increased from +4° to +14·4° in 25 minutes. This increase of 10° in the specific rotation, as against an increase of 44° for the authentic 3:4:6-trimethyl fructose would indicate _ca_.22% 3:4:6-trimethyl fructose in the total syrup. This figure agrees reasonably well with the figure obtained from rotational measurements of the trimethyl syrup itself. The specific rotation of the syrup in water was -33°. Taking -61·6° as the value for $[\alpha]_D$ in water for pure 1:3:4-trimethyl fructose, as quoted by Bell and Palmer,(27) and +30° as the value for $[\alpha]_D$ for pure 3:4:6-trimethyl fructose, this would indicate that the syrup contains approximately 30% 3:4:6-trimethyl fructose and 70% 1:3:4-trimethyl fructose.

In order to extract the 1:2-isopropylidene 3:4:6-trimethyl fructose from the 1:3:4-trimethyl fructose, the aqueous solution was continuously extracted with light petroleum in an all-glass apparatus of the type described by Brown and Jones.(21) This removed the mono-acetone compound, and the 1:3:4-trimethyl fructose was obtained by evaporation of the aqueous solution and extraction of the residue with chloroform. The syrup crystallised readily on seeding with authentic 1:3:4-trimethyl fructose and had $[\alpha]_D$ = -53° in water.

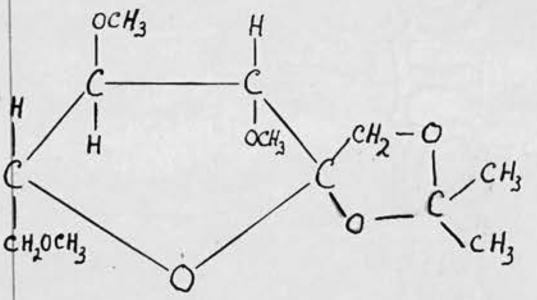
The mono-acetone compound obtained from the light petroleum extract was finally submitted to distillation in a high vacuum.

The acetone liberated on hydrolysis of the mono-acetone compound was estimated on the micro-scale using the apparatus of Tordai and Percival,(20) modified from that described by Bell and Harrison.(19) The acetone was estimated as 13·1%, whereas the theoretical value for 1:2-isopropylidene 3:4:6-trimethyl fructose is 22·1%. However, using exactly the same experimental conditions, a value of 14·9% was obtained for the material prepared from the authentic 3:4:6-trimethyl fructose. By hydrolysis for a longer period, however, a value of 21·3% was obtained for the authentic material but, unfortunately, no more material was available for a repeat estimation on the syrup under investigation. It is felt however to be highly probable that the compound was the mono-acetone derivative of a trimethyl hexose.

The constants observed for the authentic 1:2-*iso*propylidene 3:4:6-trimethyl fructose and the syrup obtained from the light petroleum extract are compared below with each other, and with the constants quoted by Montgomery (*loc.cit.*).

	Authentic 1:2-isopropylidene 3:4:6-trimethyl fructose.	Syrup from methylated triticin.	Montgomery's products.	
			from synthetic 3:4:6-trimethyl fructose.	from 3:4:6-trimethyl fructose from inulin.
b.p.	65°/0·005 mm.	85°/0·04 mm.	110°/0·2 mm.	-
n_D	1·4446 (19°)	1·4480 (22°).	1·4450	1·4483
% OMe	-	35·0	33·7	-
$[\alpha]_D$ in acetone	+64°	-	+70°	+74°
$[\alpha]_D$ in water	+71°	+46°	-	-

The methoxyl content and the refractive index certainly agree well with the values expected for a mono-acetone derivative of a trimethyl hexose. The main discrepancy lies in the value of the specific rotation in water. This constant is not recorded in the literature and was determined on the authentic sample prepared, when it was found to be of the same order as the value in acetone. The low value of $[\alpha]_D$ observed for the syrup under investigation could be explained by the preferential formation of one isomer. As stated by Montgomery (loc.cit.), there are, theoretically, 2 possible isomers of the *iso*-propylidene compound derived from the α and β forms of 3:4:6-trimethyl fructose. viz:-



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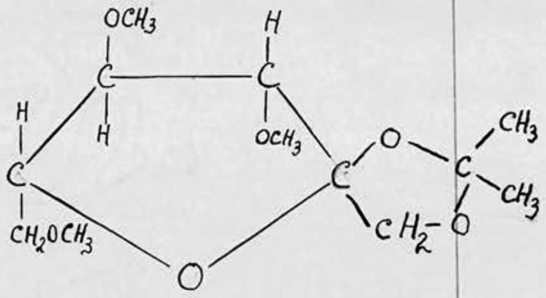


II

Hydrolysis experiments give evidence for the existence of these 2 isomeric forms, for, by arresting the hydrolysis of the mono-acetone compound, it is possible to isolate unhydrolysed material with $[\alpha]_D$ as low as +32° in acetone. This may be explained as being due to preferential hydrolysis of one of the isomers. If, in the original formation of the mono-acetone compound from the trimethyl sugar, one isomer was formed preferentially, there could well be a variation in the value of $[\alpha]_D$ although all the other constants would agree.

Another possibility, which cannot be ignored, is that the syrup is a 50% mixture of the mono-acetone derivatives of 3:4:6- and 1:4:6-trimethyl fructoses. The value of $[\alpha]_D$ in acetone for 2:3-<u>iso</u>propylidene 1:4:6-trimethyl fructose (ii) is +17·8° and, assuming the value in water to be of the same order, as was found for 1:2-<u>iso</u>propylidene 3:4:6-trimethyl fructose (i), then a 50% mixture of the 2 materials would give a specific rotation of the order found. The refractive index, boiling point and, of course, methoxyl content found for (i) are practically identical with those found for (ii), and it would be extremely difficult to identify one in the presence of the other.

The treatment of pure Trimethyl Fructose (A) with acid acetone.

Fraction IV from Column I (p. 87) consisted of a chromatographically pure trimethyl fructose ($[\alpha]_D$ = +32° in water) which appeared identical with 3:4:6-trimethyl-D-fructose.

In an attempt to obtain definite proof that the second trimethyl fructose produced on hydrolysis of methylated triticin was, in fact, the 3:4:6-isomer, the syrup was treated with acid acetone and the change in rotation studied (p. 131). The value of $[\alpha]_D$ increased from +31° to +70° on condensation with acetone, which is in good agreement with the change observed by Montgomery when 3:4:6-trimethyl-D-fructose underwent acetone condensation.

The syrup was distilled in a high vacuum when the specific rotation in water was found to be +64°, the authentic 1:2-*iso*propylidene 3:4:6-trimethyl fructose having a value of +71° in water. It would appear almost certain that the mono-acetone compound is derived from 3:4:6-trimethyl fructose, the low value of $[\alpha]_D$ quoted (+64°) being attributed to the presence of a little vaseline in the syrup derived from the glass joints during the high vacuum distillation.

Investigations into the Structure of the Dimethyl Fructose.

It has already been mentioned that the fact that this material was strongly laevorotatory indicated that C_6 was free, thereby making possible the formation of a pyranose ring. The value of the specific rotation did in fact agree well with the values quoted in the literature for pure 3:4-dimethyl-D-fructose.

The Oxidation of the Syrup with Sodium meta-Periodate.

3:4-Dimethyl fructose, having C^1 and C^6 both free, liberates 2 moles of formaldehyde on oxidation by the periodate ion, and, of all the possible dimethyl fructoses, only the 3:4-isomer is capable of doing this.

The syrup, on prolonged oxidation in bicarbonate buffer solution, yielded 2·0 moles of formaldehyde per mole of dimethyl fructose (p. 132), the method of estimation being that described by Reeves.(17) When oxidation was allowed to proceed in phosphate buffer solution, as described by Bell,(18) no value higher than 1·64 moles of formaldehyde per mole of dimethyl fructose could be obtained. This agrees with the observations of Bell, Palmer and Johns,(35) who found that, by using this method, pure 3:4-dimethyl fructose yielded 1·72 moles of formaldehyde per mole of dimethyl fructose.

The Attempted Formation of 3:4-Dimethyl Glucosazone.

3:4-Dimethyl fructose, having C_1 and C_2 unsubstituted, will yield 3:4-dimethyl glucosazone on treatment with excess phenylhydrazine. This compound was first prepared by McDonald and Jackson(29) from 3:4-dimethyl fructose. They found the material very difficult to purify, but, on crystallisation from ether-light petroleum, the product had a melting point of 126°. Similar results were recently obtained by Bell and Greville(37) on treatment of 3:4-dimethyl glucose with excess phenylhydrazine.

When the syrup under investigation was treated with excess phenylhydrazine hydrochloride and sodium acetate, in the presence of a little sodium bisulphite.

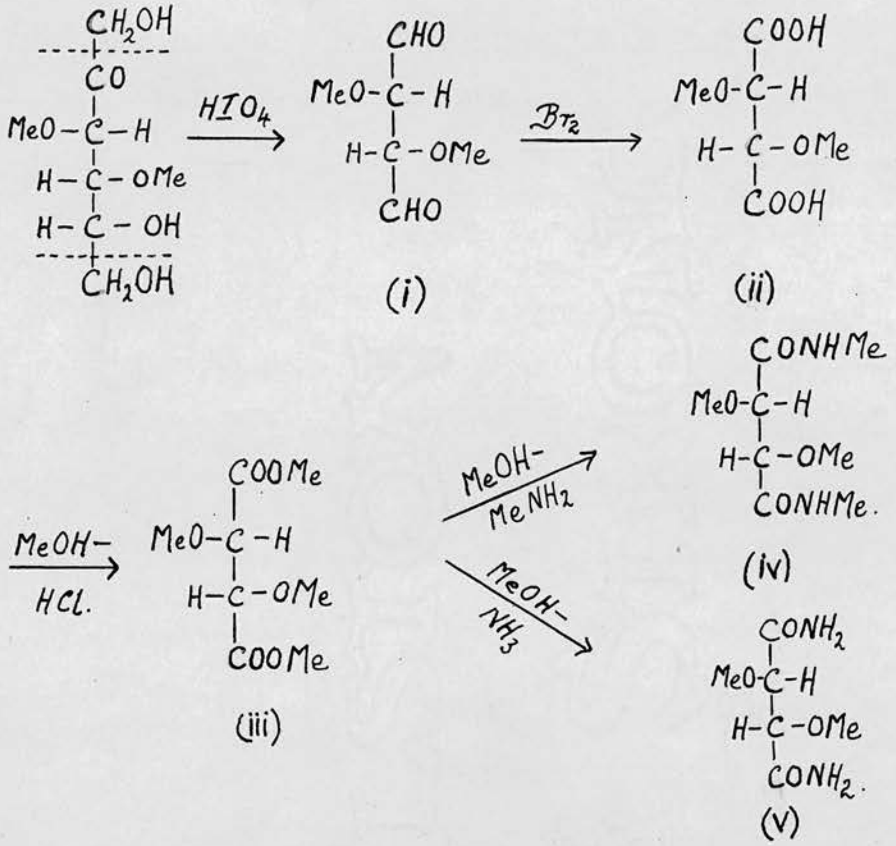
the product was obtained as a yellow, amorphous solid, which melted in the region of 80°.

Great difficulty was obtained in the purification of this material. Attempts to crystallise the solid from aqueous alcohol were unsuccessful, as the product invariably precipitated as a red oil. A trial sample of the product, however, crystallised from ether-light petroleum as long, fine, yellow needles, on seeding with an authentic crystal kindly supplied by Dr. D.J. Bell. This product had a melting point of 124° - 126°. However, attempts to purify the main bulk of the material in this way were unsuccessful, as the product was obtained as a brown caramel-like substance, even when dry solvents were employed.

The Oxidation of the Syrup with Periodic Acid and the Isolation of D(-)-Dimethoxysuccinic Acid Derivatives.

Final proof that the methoxyl substituents are located on C_3 and C_4 in the dimethyl fructose was obtained by the isolation of characteristic derivatives of D(-)-dimethoxysuccinic acid after periodic acid oxidation. Of all the possible dimethyl fructoses, only the 3:4-isomer can split between C_1 and C_2 and between C_5 and C_6, on periodate oxidation, to yield dimethoxysuccindialdehyde. The isolation of crystalline derivatives of dimethoxysuccinic acid, therefore, fixes the methoxyl substituents on C_3 and C_4.

The series of reactions involved is outlined below:-



The aldehyde (i) produced after oxidation with periodic acid was oxidised to the acid (ii) by bromine water and the acid esterified with methanolic hydrogen chloride. The ester (iii) was distilled in a high vacuum when the constants recorded were found to be in good agreement with those quoted by Haworth and Jones (38) for dimethyl D(-)-dimethoxysuccinate (p. 136).

A portion of the redistilled ester (iii) was converted to D(-)-dimethoxysuccinamide (v) by the action of methanolic ammonia, the remainder being converted to D(-)-dimethoxysuccin-bis-methylamide (iv) with methanolic methylamine. The constants for these 2 crystalline derivatives agreed well with those recorded by Haworth and Jones (loc.cit.) and the identity of each was established beyond doubt.

General Conclusions from the Experiments on the Hydrolysis of Methylated Triticin.

The yields of the various methylated sugars obtained by the hydrolysis of methylated triticin are computed to be, tetramethyl fructofuranose (41·6%), trimethyl fructoses (14·4%, comprising 3:4:6-trimethyl fructose (3·6%), 1:3:4-trimethyl fructose (10·8%)), 3:4-dimethyl fructose (39·4%), tetramethyl glucopyranose (2·2%), trimethyl glucose (0·9%) and dimethyl glucose (1·6%).

Molecular Weight Determinations on Acetylated and Methylated Triticin.

Barger's isopiestic method(22) for the determination of molecular weights was originally confined to compounds of low molecular weight, but recently Caesar, Gruenhut and Cushing(39) found it possible to examine polymers of molecular weight 1000-100,000 by this technique.

When fraction I of acetylated triticin was examined by this method, against a sucrose octa-acetate standard, in chloroform solution, the molecular weight was found to lie between 6940 and 10410 (p.140). Since the molecular weight of an acetylated anhydrofructose unit is 288, this corresponds to a molecular size of 24-36 units.

In an attempt to narrow the above range, the concentration of the acetylated triticin solution was increased from 2% to 5%. At this concentration, the results indicated that the isopiestic condition lay at an approximate concentration of 0·006 M. This would indicate a molecular weight of ca.8380, corresponding to 29 acetylated anhydrofructose units.

In studying the methylated polysaccharide, there was, unfortunately, none of Fraction I available. Fraction II, however, which appeared from viscosity measurements to have a lower molecular weight than Fraction I, was investigated by this method, and the molecular weight was found to lie in the region 3753-5252. Since the molecular weight of a methylated anhydrofructose residue is 204, this corresponds to a molecular size of 18-25 units.

When the concentration of the methylated triticin solution was increased, in order to narrow the above range, no reproducible results could be obtained. All the drops of the methylated triticin solution were found to increase in size between the range 0·002 - 0·010 M. This phenomenon has also been noted by Laidlaw and Reid(33) for the methylated fructosan from perennial rye grass (Lolium perenne).

Oxidation of Triticin by the Periodate Ion.

A series of investigations first commenced by Malaprade(40) and continued later by Nicolet and Shinn(41) showed that, if a compound which has 2 hydroxyl groups, or a hydroxyl and an amino group, on adjacent carbon atoms is treated with periodic acid, cleavage of the intermediate C-C bond occurs.

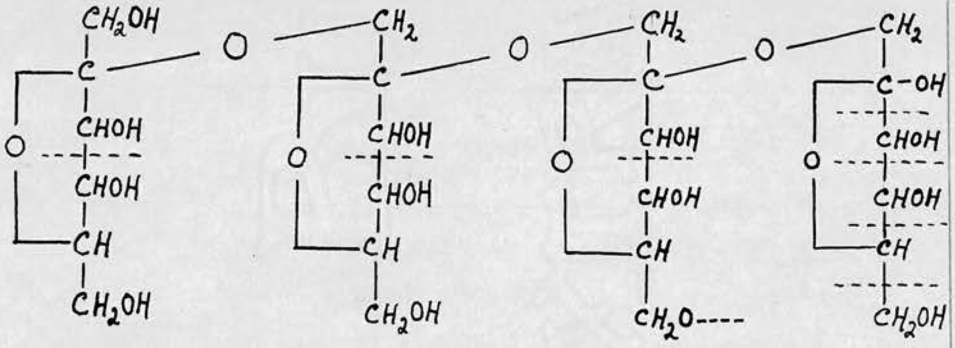
$$R'\text{-CHOH-CHOH-}R + HIO_4 \longrightarrow R'CHO + RCHO + HIO_3 + H_2O$$

$$R'\text{-CHNH}_2\text{-CHOH-}R + HIO_4 \longrightarrow R'CHO + RCHO + HIO_3 + NH_3$$

In the majority of cases this reaction occurs quantitatively, and this factor, together with the specificity of the reagent for such groups, makes it an invaluable reagent in carbohydrate chemistry. One molecule of periodate is consumed for each C-C bond split and, when more than 2 adjacent hydroxyl groups are present, oxidation proceeds through this portion of the molecule, liberating formic acid from -CHOH groups and formaldehyde from $-CH_2OH$ groups. Both of these may be readily estimated.

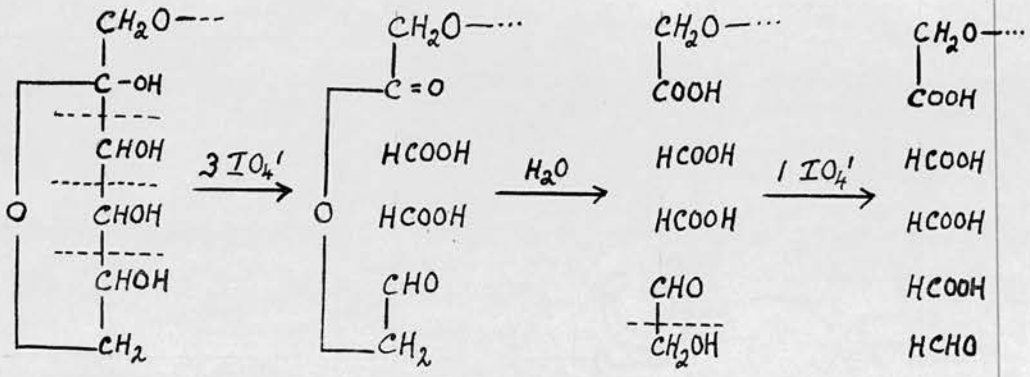
Oxidation by the periodate ion provides a convenient method of estimating the chain length of a polysaccharide molecule, for, if the reducing end group contains 3 adjacent hydroxyl groups, formic acid will be liberated. Provided the non-reducing end group and the non-terminal residues do not produce formic acid on periodate oxidation, then the estimation of the formic acid produced provides a direct measurement of the number of reducing end groups.

From the experimental evidence, the triticin molecule contains a network of 2-6' and 2-1' linkages. As will be shown later (p. 203), the experimental results permit the interchanging of the 2-6' and 2-1' linkages, with the result that the main chain may be either a 2-1' (inulin-type) chain or a 2-6' (phlein-type) chain. It will be seen below, however, that the fact that the 2-1' and 2-6' linkages are interchangeable does not substantially alter the mechanism of the oxidation of the triticin molecule by the periodate ion. Considering a fructosan of the inulin type, containing a main chain of fructose residues linked through C^2 and C^1, with branching through C^6, oxidation by the periodate ion will proceed as follows:-

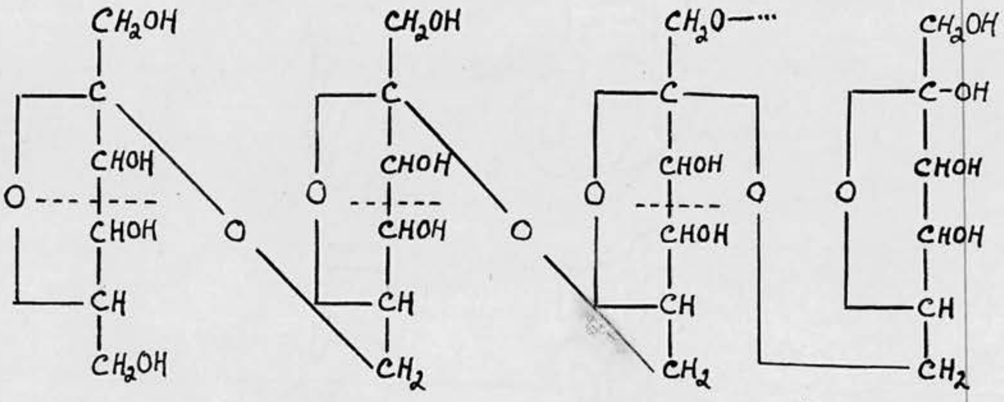


non-reducing end group uptake 1 mole no formic acid	non-terminal residue uptake 1 mole no formic acid	non-terminal residue with branching through C^6 uptake 1 mole no formic acid	reducing end group

The reducing end group will in all probability exist in the pyranose form and will split as follows on periodate oxidation:-



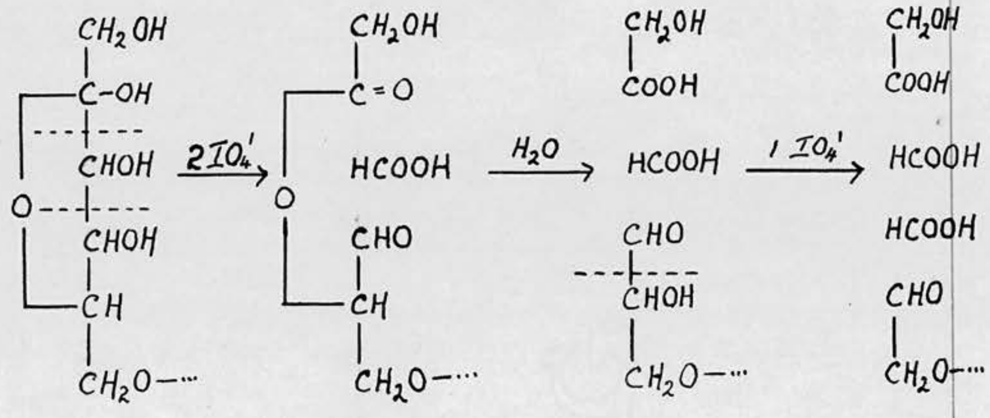
Considering, likewise, a fructosan of the phlein type, containing a main chain of fructose residues linked through C^2 and C^6, with branching through C^1, oxidation by the periodate ion will proceed as follows:-



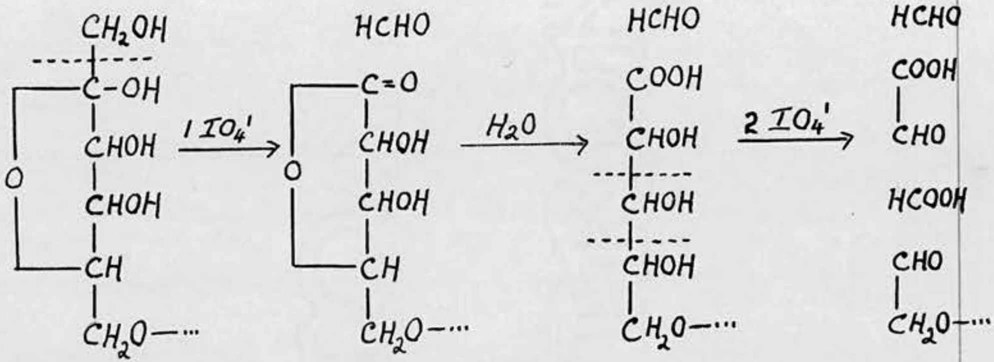
Non-reducing end group uptake 1 mole no formic acid	non-terminal residue uptake 1 mole no formic acid	non-terminal residue with branching through C^1 uptake 1 mole no formic acid	reducing end group

By analogy with fructose, a fructofuranose reducing end group linked through C^6 is capable of splitting in 2 different ways on periodate oxidation.

1)



2)



Cleavage by method (1) will produce 2 moles of formic acid (3 moles of titratable acid), taking up 3 moles of periodate during the cleavage. Cleavage by method (2), however, will produce 1 mole of formaldehyde and 1 mole of formic acid (2 moles of titratable acid), the uptake of periodate again being 3 moles.

According to Khouvine and Arragon,(42) fructose splits in both ways, although cleavage by method (2) appears to occur preferentially. It is reasonable to presume that the reducing end group under consideration will split in both ways also.

It will be seen, therefore, that, no matter whether the main chain contains 2-1' or 2-6' linkages, all the fructose residues except the reducing end group will take up 1 mole of sodium periodate and yield no formic acid.

When the periodate uptake of the purest sample of polysaccharide available ($[\alpha]_D = -48°$) was determined, a constant value corresponding to a periodate uptake of 1·00 moles per fructose residue was obtained (p. 142).

In order to estimate the formic acid liberated, oxidation was performed with potassium periodate, according to Halsall, Hirst and Jones.(23) The sparingly-soluble potassium salt is recommended in preference to the soluble sodium salt in order that over-oxidation may be prevented.

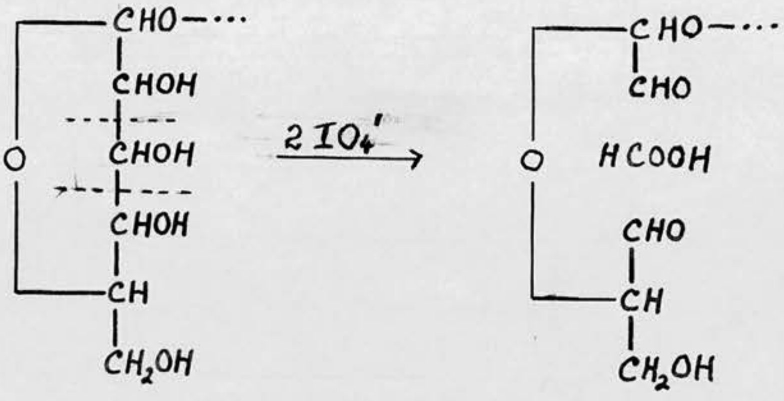
Experimentally it was found that 1 mole of formic acid was liberated for every 29 anhydrofructose residues. The liberation of formic acid might seem to indicate the presence of a fructofuranose reducing end group, since it has been seen that this is the only unit in a true fructosan which can produce formic acid. This feature would exclude a ring structure, as postulated by Schlubach and Peitzner (loc.cit.). Since each fructofuranose reducing end group produces 3 moles of formic acid, if the main chain is of the inulin type, or 2 or 3 moles of titratable acid, dependent upon the method of oxidation, if the main chain is of the phlein type, the above yield of formic acid could only agree with a "chain length" of 58-87 units.

In interpreting the above results, however, certain reservations must be borne in mind.

Firstly, molecular weight determinations indicated that the size of the molecule is not in this region, the "chain length" being much less than 58 units. This would seem to exclude a fructofuranose reducing end group as the source of the formic acid.

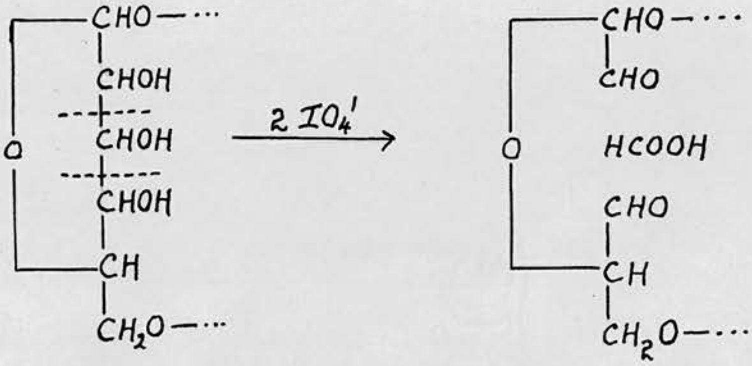
The formic acid may, however, be liberated from a chain glucosan in close association with the fructosan. Methylation studies showed that tetramethyl glucose (2·2%) was produced on hydrolysis of methylated triticin, together with what appeared to be a trimethyl glucose (0·9%) and a dimethyl glucose (1·6%). The high proportion of tetramethyl glucose relative to trimethyl- and dimethyl glucose would not favour the view that the glucose arises from an associated glucosan, but would rather favour the idea of its being an integral part of the structure of the fructosan.

It may be recalled that a glucopyranose non-reducing end group will take up 2 moles of periodate and yield 1 mole of formic acid on periodate oxidation.



The amount of formic acid liberated from the glucopyranose non-reducing end group may be calculated as $3{\cdot}50 \times 10^{-6}$ moles, whereas the amount liberated experimentally was $6{\cdot}12 \times 10^{-6}$ moles; that is, almost twice the calculated value. This may be due either to a discrepancy in the estimation of the aldose content of the tetramethyl fraction isolated on hydrolysis of the methylated triticin, or to incomplete methylation of the non-reducing end group.

An alternative possibility, however, is that a glucose non-terminal residue, which also liberates formic acid on oxidation with periodate, is present in the molecule. It has been mentioned above that alkaline hypoiodite oxidations of the methylated fructose syrups gave evidence for the presence of a di- and a trimethyl aldose, the combined amounts equalling the amount of the tetramethyl aldose. If the dimethyl aldose arises due to undermethylation or demethylation of the trimethyl aldose, then the only possible non-terminal aldose (assumed to be glucose) residue which could liberate formic acid on periodate oxidation would be a 1-6' linked unit. viz.:-



Such a unit would give rise to 2:3:4-trimethyl glucose on methylation and hydrolysis. It was, however, shown on the chromatogram that the trimethyl aldose obtained from trimethyl triticin travelled at exactly the same rate as 2:4:6-trimethyl glucose, and more slowly than 2:3:4-trimethyl glucose. This indicates that the non-terminal glucose unit in the polysaccharide is linked through C^1 and C^3, and such a unit cannot be a source of formic acid on periodate oxidation.

<u>Examination for Formaldehyde</u> (p. 143).

It has already been shown that the reducing end group of a chain of fructofuranose units linked through C^1 and C^2 will liberate formaldehyde on periodate oxidation. Likewise, for a chain of fructofuranose units linked through C^6 and C^2, the reducing end group will liberate formaldehyde on periodate oxidation, if we assume that oxidation proceeds in either of the 2 ways shown previously (p. 193). The presence or absence of formaldehyde in the solution of periodate-oxidised triticin should, therefore, provide good evidence for the presence or absence of a fructofuranose reducing end group.

Application of the phenylhydrazine hydrochloride-potassium ferricyanide colour test for formaldehyde gave a negative reaction, although the test was found to be highly sensitive to a control solution containing 0·001 mg. formaldehyde.

As mentioned previously, formaldehyde may also be readily detected and estimated as the formaldehyde-dimedon complex. The solution of periodate-oxidised fructosan yielded a small precipitate on standing for 48 hours. The formaldehyde-dimedon complex melts at 189° - 190°, whereas the precipitate obtained melted over a range, with decomposition, in the region of 220°. It seems probable that this is another aldehyde-dimedon complex. Each fructose residue in the polysaccharide will, on cleavage of the glycol group by periodate oxidation, yield a di-aldehyde residue, and it is reasonable to presume that the chain of di-aldehyde residues will itself form a complex with dimedon which may tend to precipitate under the experimental conditions employed.

It would seem, however, that formaldehyde is not present in the solution of periodate-oxidised triticin, and this would suggest that there is no fructofuranose reducing end group. If, however, the main chain consists of fructofuranose residues linked through C^6 and C^2, certain reservations must be borne in mind, because, for such a chain, formaldehyde will only be produced when oxidation occurs by one of the two possible ways. It could be that cleavage by the mechanism whereby glycollic acid is produced occurs so much more favourably than the mechanism whereby formaldehyde is produced that the amount of formaldehyde liberated is insufficient to allow detection even by the sensitive colour reaction employed.

General Survey of Results and Proposed Possibilities for the Structure of Triticin.

As a result of methylation studies it has been confirmed that the triticin molecule is built up of multiples of 7 fructose units, giving rise to a tetramethyl-, a trimethyl-, and a dimethyl-fructose in the ratio 3:1:3, on hydrolysis of trimethyl triticin. The trimethyl fructose fraction has been shown to be a mixture of 1:3:4- and 3:4:6-trimethyl fructose, suggesting that triticin may be placed in either the inulin group or the phlein group of fructosans, whereas Schlubach and Peitzner (loc.cit.) stated that it could be placed in neither group. These workers were unable to identify the trimethyl fraction and this is almost certainly due to the fact that they did not recognise that they were dealing, not with a pure compound, but, rather, with a mixture of 2 substitution isomers. The dimethyl fructose fraction has also been conclusively identified as 3:4-dimethyl fructose.

Using the modern techniques dependant upon the principles of partition chromatography, the presence of glucose in hydrolysates of triticin and of methylated glucoses in hydrolysates of methylated triticin has been recognised. Estimations of the glucose contents of both series of hydrolysates gave a value of ca.5%. It must be emphasised that the methylated glucoses were not at any time isolated in a pure state. They were, in all cases, detected by paper chromatography and estimated in the presence of methylated fructoses by oxidation with alkaline hypoiodite. It would appear from the rate of travel on the chromatogram that the trimethyl glucose is 2:4:6-trimethyl glucose but its precise identity is not proved.

No sample of triticin was ever obtained which was completely non-reducing to Fehling's solution. A slight reduction occurred on boiling the solution gently for 15 minutes, but, as a similar result was obtained with sucrose, this provides no trustworthy evidence for the presence or absence of a reducing end group. It has been seen, however, from periodate-oxidation studies that a reducing end group appears to be absent. The formic acid produced on periodate oxidation can be accounted for by the presence of a glucose non-reducing end group. It is highly feasible, therefore, that in triticin, as in inulin, the prospective fructofuranose reducing end group is engaged, in a sucrose-type linkage, with a glucose unit such that the reducing character of the molecule is completely lost.

Molecular weight determinations on the acetate by Barger's method gave a value of approximately 29 units for the molecular size. This value, however, must be accepted with considerable reserve, even although the results of periodate studies indicated also a "chain length" of approximately 29 units if the results were based on the presence of a glucose non-reducing terminal unit in the molecule. Apparatus is not yet available here for the determination of molecular weight by osmotic pressure or vapour pressure measurements, and, until results are obtained by these more refined methods, no accurate statement can be made as to the size of the molecule.

It if is assumed that the molecule is composed of 29 units, then, since 1/7 of the total residues give rise to trimethyl fructose on hydrolysis of trimethyl triticin, 4 moles of trimethyl fructose are produced per mole of methylated fructosan. Moreover, since it would appear that 1:3:4-trimethyl fructose and 3:4:6-trimethyl fructose are produced in the ratio 3:1, then 1 mole of 3:4:6-trimethyl fructose is produced per mole of methylated fructosan.

It will be seen, therefore, that a structure proposed for triticin must account satisfactorily for the following experimental observations.

1) The production of tetramethyl-, trimethyl-, and dimethyl-fructose in the ratio 3:1:3 on hydrolysis of methylated triticin.
2) The presence of both 2-1' and 2-6' linkages.
3) The production of 2 trimethyl fructoses, viz. the

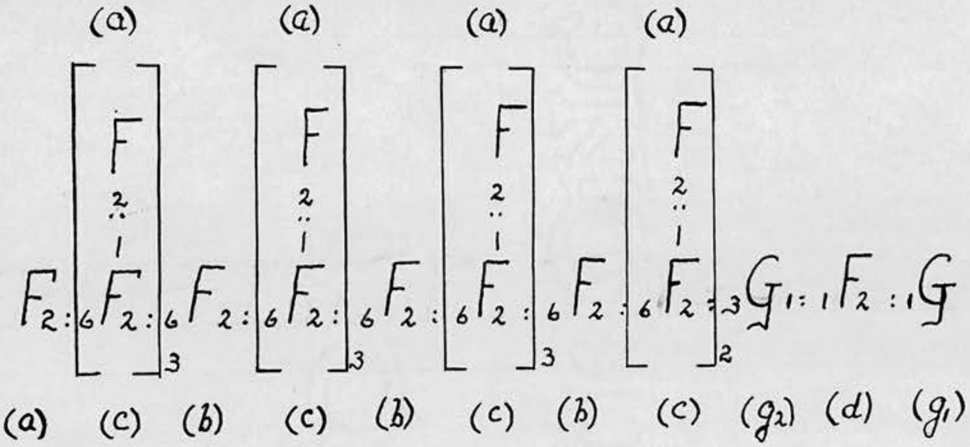
1:3:4- and the 3:4:6-isomers, in the ratio 3:1.

4) A molecular size of approximately 29 units.
5) The apparent absence of a fructofuranose reducing end group.
6) The presence of a glucose non-reducing terminal unit, and also, possibly, a non-terminal glucose unit, which appears to be linked through C^1 and C^3.

It is realised, especially since both 1:3:4- and 3:4:6-trimethyl fructoses are produced, that the 2-6' and 2-1' linkages are interchangeable. On the one hand, the backbone of the molecule may be a chain of fructofuranose units linked through C^2 and C^6, in which case the main chain will have 2-6' linkages, and the side chains 2-1' linkages. On the other hand, the main chain may have 2-1' linkages, and the side chains 2-6' linkages, and these 2 possibilities are ellustrated below. In both chain structures, the potential fructofuranose reducing end group is linked through the reducing group to a glucose unit in a sucrose-type linkage. In both structures also the non-terminal glucose unit occurs at some indeterminate position in the chain and, moreover, no definite information has been obtained as to the actual positions of linkage of this glucose unit and its fructose neighbours. It is believed that the identification of the disaccharide which appears to accompany sucrose in the autohydrolysis of triticin (p.152) would provide some light on this matter.

Proposed Chain Structures for Triticin.

1) With a Phlein-Type "backbone," and Inulin-Type side chains.



$$F_{2}:{}_{6}\left[\begin{array}{c}F\\ 2\\ \vdots\\ 1\\ F_{2}:\end{array}\right]_{3}{}_{6}F_{2}:{}_{6}\left[\begin{array}{c}F\\ 2\\ \vdots\\ 1\\ F_{2}:\end{array}\right]_{3}{}_{6}F_{2}:{}_{6}\left[\begin{array}{c}F\\ 2\\ \vdots\\ 1\\ F_{2}:\end{array}\right]_{3}{}_{6}F_{2}:{}_{6}\left[\begin{array}{c}F\\ 2\\ \vdots\\ 1\\ F_{2}:\end{array}\right]_{2}{}_{3}G_{1}:{}_{1}F_{2}:{}_{1}G$$

where F = a D-fructofuranose unit.

G = a D-glucopyranose unit.

(a) will yield tetramethyl fructofuranose.

(b) " " 1:3:4-trimethyl fructose.

(c) " " 3:4-dimethyl fructose.

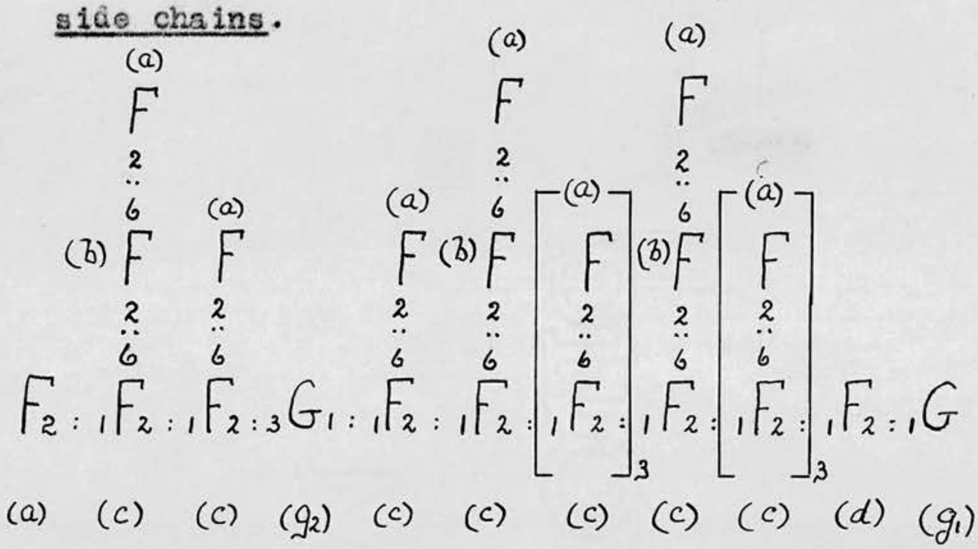
(d) " " 3:4:6-trimethyl fructose.

(g_1) " " tetramethyl glucopyranose.

(g_2) " " 2:4:6-trimethyl glucose.

12a 3b 11c 1d 1g_1 1g_2 Total = 29 units.

2) With an Inulin-Type "backbone" and Phlein-Type side chains.



12a 3b 11c 1d $1g_1$ $1g_2$ Total = 29 units.

For each of the 2 variations given, $(C_6H_{10}O_5)_{29}$, the total weight of methylated sugars liberated on hydrolysis of the methylated polysaccharide will be (222 x 29) = 6438 g.

% isolated as tetramethyl fructose = $\frac{12 \times 23600}{6438}$

= 44·0% (found, 41·6%).

% isolated as trimethyl fructose = $\frac{4 \times 22200}{6438}$

= 13·8% (found, 14·4%).

% isolated as dimethyl fructose = $\frac{11 \times 20800}{6438}$

= 35·6% (found, 39·4%).

% isolated as tetramethyl glucose = $\frac{23600}{6438}$

= 3·7% (found, 2·2%).

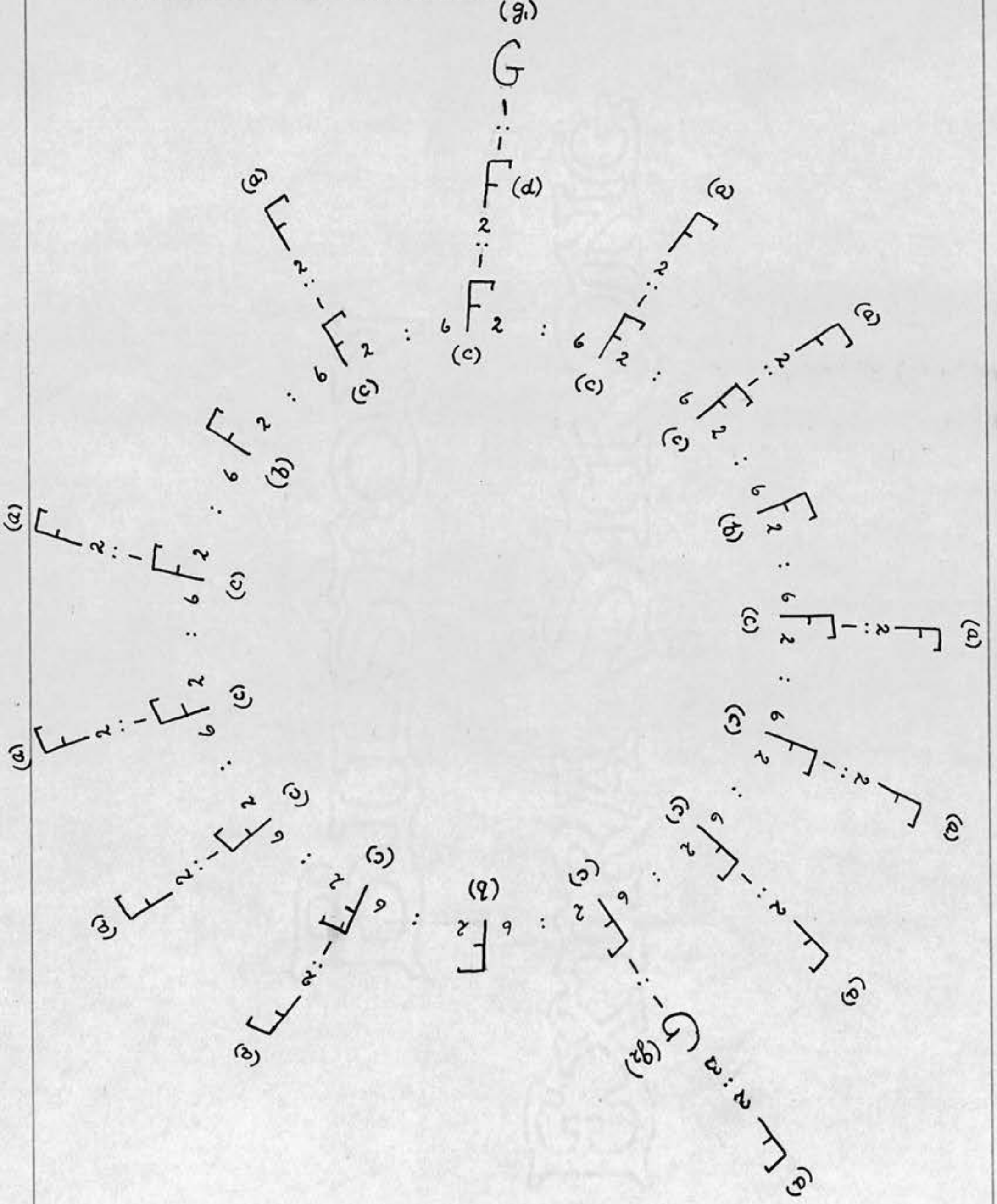
% isolated as trimethyl glucose = $\frac{22200}{6438}$ = 3·5% (found, 2·6%).

The figure quoted for trimethyl glucose (2·6%) represents the total of trimethyl- (0·9%) and dimethyl-glucose (1·7%, expressed as trimethyl), the dimethyl glucose assumed as arising due to undermethylation or demethylation of the trimethyl glucose (p. 196).

The absence of a reducing end group in the polysaccharide molecule may alternatively be accounted for by the presence of a ring system, a feature which was suggested by Schlubach and Peitzner.(24) The D-fructose units may be arranged in a series of closed rings, which are highly ramified, such that each unit ring structure contains 29 hexose residues. Again the 2-1' and 2-6' linkages must be considered as being interchangeable, thus giving rise to the two structures outlined below.

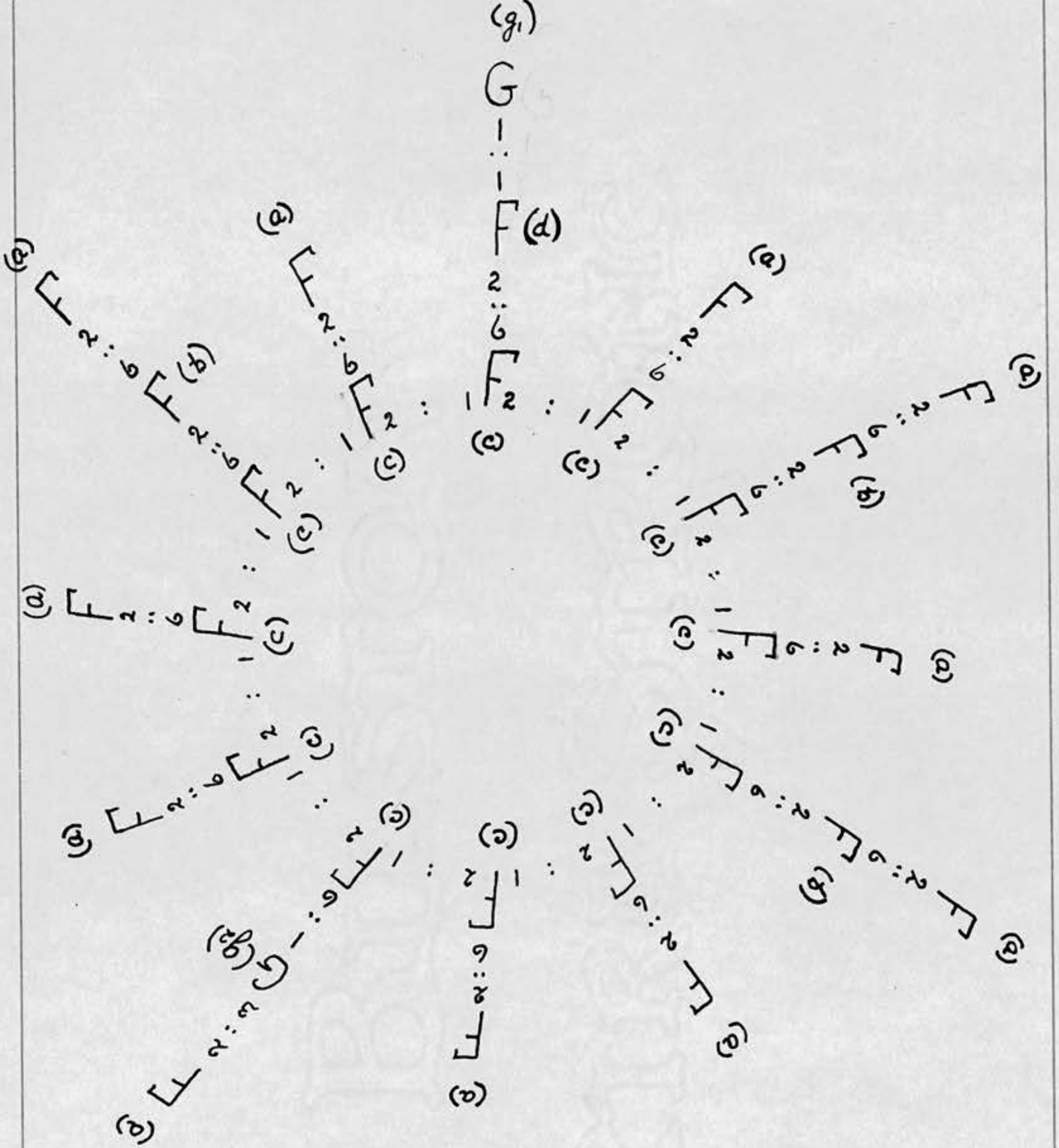
Alternative Ring Structures proposed for Triticin.

1) 15-membered ring, with 2-6' radial linkages and 2-1' side-chain linkages.



11a 3b 12c 1d $1g_1$ $1g_2$ Total = 29 units.

2) <u>12-membered ring, with 2-1' radial linkages and 2-6' side-chain linkages.</u>



11a 3b 12c 1d $1g_1$ $1g_2$ Total = 29 units.

For each of the 2 ring structures shown above, the total weight of methylated sugars produced on hydrolysis of the methylated polysaccharide will again be 6438 g.

% isolated as tetramethyl fructose = $\frac{11 \times 23600}{6438}$

= 40·3% (found, 41·6%).

% isolated as trimethyl fructose = $\frac{4 \times 22200}{6438}$

= 13·8% (found, 14·4%).

% isolated as dimethyl fructose = $\frac{12 \times 20800}{6438}$

= 38·8% (found, 39·4%).

% isolated as tetramethyl glucose = $\frac{23600}{6438}$

= 3·7% (found, 2·2%).

% isolated as trimethyl glucose = $\frac{22200}{6438}$

= 3·5% (found, 2·6%).

It will be seen that the percentage of glucose non-reducing end group demanded is considerably higher than the value observed (compare p. 196).

In the ring structures, the presence of a glucose non-reducing end group, and the production of 1 mole of 3:4:6-trimethyl fructose per mole of methylated fructosan may be accounted for by the substitution of 1 fructose non-reducing end group by a fructose and a glucose unit linked through C^1 and $C^{1'}$, the fructose unit being joined to the ring through the reducing group. In these ring structures, the same points of dubiety exist as were pointed out for the chain structures;

namely, the actual size of the molecule, the position of the glucose unit (g_2) and its mode of linkage. Although, in the ring structures outlined, the glucose unit (g_2) is placed in a side-chain radiating from the loop, it may, in fact, be present in the loop itself.

It is probably artificial to consider the triticin molecule as having either 2-6' or 2-1' linkages in the "backbone" (for the chain structure) or in the loop (for the ring structure). It seems far more likely that mixed 2-1' and 2-6' linkages occur with no preponderant "backbone" or loop, the triticin molecule being considered as a network of 2-1' and 2-6' linkages. As to whether the fundamental structure is a chain, terminated by a sucrose linkage, or a closed ring, there is, as with inulin, no conclusive evidence to allow one to state a preference for either alternative.

Certainly the ring structure accounts more satisfactorily for the quantitative results of the methylation studies, but, on the other hand, a looped structure with a glucopyranose residue attached as a branch to one of the fructofuranose units could not account for the formation of sucrose among the products of autohydrolysis.

Dedonder,(43) however, by studying the controlled hydrolysis of inulin, obtained good evidence for the existence of a chain terminated by a sucrose linkage in the inulin molecule. He was able to detect 7 distinct spots on the chromatogram between fructose and the starting line, all of which disappeared completely on continuing the hydrolysis. The fastest of these travelled at the same rate as sucrose and the next two were found to occur on either sids of raffinose. It would appear that a series of substances representing di-, tri-, tetra- and pentasaccharides etc. are produced. These may be considered as arising from a chain terminated by a sucrose linkage which has been disrupted to various extents.

Continuing this work Dedonder(44) found evidence for precisely the same carbohydrates in the roots of the Jerusalem artichoke, and this would indicate that the oligosaccharides in the roots are, for the most part, derived from a glucosido-fructosan by hydrolysis in the tissues.

Since experimental evidence points to the fact that glucose is also an integral part of the structure of the triticin molecule, it may be that fructosans are, in general, chain glucosido-fructosans. Indeed, recently, Bacon and Edelman(45) have been able to suggest a possible means of biosynthesis of such glucosido-fructosans. They have been able to demonstrate a process of transfructosidation in the

Jerusalem artichoke, whereby fructofuranoside residues are transferred from material of low $\underline{R_F}$ value to short-chain disaccharide or oligosaccharide material of higher $\underline{R_F}$ value. Thus:-

$$\text{Sucrose} + \text{Inulin} \xrightarrow[\text{enzyme}]{\text{carbohydrate-free}} \text{Trisaccharide}$$

F - G F - F - G.

$$\text{Trisaccharide} + \text{Inulin} \longrightarrow \text{Tetrasaccharide}$$

F - F - G F - F - F - G.

In this way one may envisage the building of a chain of fructofuranose residues with a glucose non-reducing terminal unit, although the process does not explain the occurrence of an intermediate glucose unit in the chain as has been suggested for both inulin and triticin.

Further experiments on the nature of the disaccharides obtained during the autohydrolysis of triticin may throw further light on this problem.

SUMMARY

1) An 80% aqueous alcohol extract of the milled couch grass roots was shown to contain glucose, fructose and an oligosaccharide. The glucose and fructose were estimated by quantitative paper chromatography.

2) The polysaccharide was extracted from the grass roots after the removal of the free sugars. Various methods of deproteinisation were employed and the purities of the resulting samples of fructosan compared. The fructosan was purified to a constant specific rotation of -48°.

3) The products of hydrolysis of triticin by 1% aqueous oxalic acid and by boiling water were studied. The fructose liberated was estimated by a colorimetric method, and the glucose by quantitative paper chromatography:- Fructose 91·8%. Glucose 4·8%.

4) Triticin was acetylated and then methylated via the acetate. Three methylations with dimethyl sulphate and sodium hydroxide in an inert atmosphere, followed by 2 methylations with methyl iodide-silver oxide and 2 with methyl iodide-thallous ethoxide yielded the fully-methylated triticin.

5) A sample of under-methylated triticin (OMe = 41·5%) was hydrolysed with aqueous methanolic oxalic acid and the components of the hydrolysate separated on a column

of powdered cellulose. Glycoside material was rehydrolysed and the products of hydrolysis again separated on a column of powdered cellulose. Evidence for the existence of 2 trimethyl fructoses and methylated glucoses among the products of hydrolysis was obtained, but no monomethyl fructose was obtained.

6) Fully-methylated triticin (OMe = 45·0%) was hydrolysed with aqueous methanolic oxalic acid, followed by aqueous oxalic acid, and the products of hydrolysis separated quantitatively on a column of powdered cellulose. The aldose content of each fraction was estimated by alkaline hypoiodite oxidation.

7) A colorimetric method of estimation of tetramethyl fructofuranose was studied.

8) Tetramethyl-, trimethyl- and dimethyl-fructose were obtained in the ratio 3:1:3, and the total methylated glucose recovery estimated, as glucose, as 4·3%.

9) The tetramethyl fraction was identified as tetramethyl fructofuranose, the trimethyl as a mixture of 1:3:4- and 3:4:6-trimethyl fructose, and the dimethyl as 3:4-dimethyl fructose.

10) The molecular weights of the acetylated and methylated triticin were determined by Barger's method. A value of <u>ca</u>.8400 was obtained for the acetate, corresponding to a molecular size of 29 units.

11) The oxidation of triticin by the periodate ion

was studied. The uptake of periodate was estimated as 1·00 moles per fructose residue, and 1 mole of formic acid was liberated for every 29 residues. No formaldehyde was detected in the solution of periodate-oxidised triticin.

12) On the basis of these results, possible alternatives for the structure of triticin are tentatively proposed.

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