THE CHEMICAL STRUCTURE OF DEXTRAN I

by

Irving Levi (M. Sc., Manitoba)

A Thesis Submitted to the Faculty of Graduate Studies and Research of McGill University in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy.

McGill University May, 1942.

ACKNOWLEDGMENTS

The writer desires to express his sincere appreciation to

Dr. Harold Hibbert

whose kind advice and helpful criticism have always been available and of invaluable aid in this investigation; to

Dr. W. L. Hawkins

for his constant help and continued interest; and to

Dr. T. H. Evans

who concurrently investigated the chemical structure of Dextran II.

Acknowledgment is made to the

National Research Council

for the award of two Studentships, and to the

Canadian Pulp and Paper Association

for the award of a Scholarship.

CLAIM TO ORIGINAL RESEARCH

Dextran I, the bacterial polysaccharide produced from sucrose by Leuconostoc mesenteroides, has been investigated by means of methylation studies using improved Haworth and Muskat techniques, and has been shown to possess a comb-like structure. The nature of the oxygen rings and mode of linkages in the main and side chains have been clarified.

An improved and highly efficient fractional distillation technique has been developed for the separation of small amounts of glucosidic mixtures.

The Haworth methylation technique has been modified and adapted generally to the synthesis of partially-methylated glucose derivatives.

2,3,4-Trimethyl glucose has been synthesized by a new method. Improvements have been made in the methods of synthesis of 2,3,4,6-tetramethyl methyl glucoside.

A new crystalline derivative of 2,3-dimethyl glucose is described.

TABLE OF CONTENTS

Identification of the Glucosides

INTRODUCTION

Of recent years, following the elucidation of the constitution and structure of the more common mono- and di-saccharides, the attention of carbohydrate chemists has been focussed more and more strongly on the higher poly-saccharides. For example, among the plant polysaccharides, it has been definitely established that cellulose is a long linear-chain polymer formed from anhydro-glucose units. In the case of inulin and levan, the chain is made up of anhydro-fructose units, while xylan and other pentosans are linear anhydro-pentose polymers.

It is an accepted fact that the polysaccharides occurring naturally in plants are formed primarily from carbon dioxide and water, but the mechanism of this change called "photosynthesis" has not been established. Oxygen is liberated in the process. The algebraic expression summarizing this very complex change is shown by the equation.

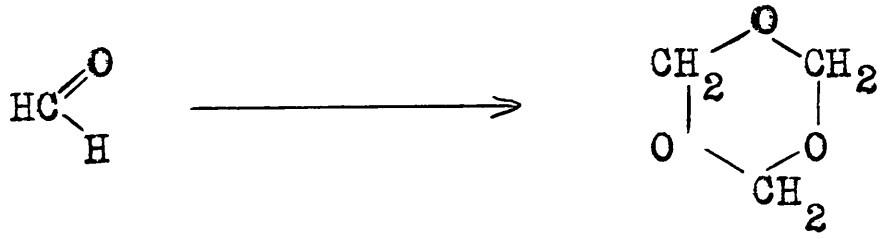
$$6\ CO_2 + 6\ H_2O \xrightarrow[\text{minerals}]{\text{chlorophyll}} C_6H_{12}O_6 + 6\ O_2$$

This must be taken as nothing more than a mere summary, and not a description of the changes involved.

It seems probable that in sugar synthesis at least

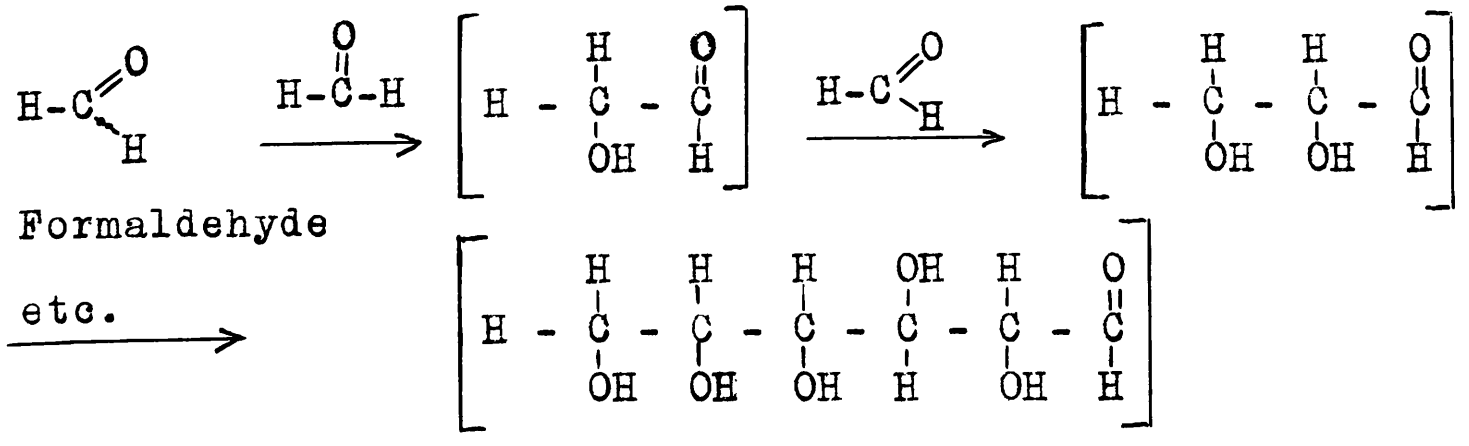
two intermediate steps are involved, (a) an intial reaction to form some simple organic compound, and (b) a condensation of this product to some carbohydrate suitable for translocation and use as a food or a building material.

Since von Baeyer's suggestion in 1870 (1), many workers have assumed, using various mechanisms, that in the initial change, carbon dioxide was converted into formaldehyde. Besides being the simplest compound with the empirical formula for carbohydrates, formaldehyde can be polymerized in the laboratory into complex materials with the general formula $(CH_2O)_n$



Formaldehyde — Trioxymethylene

Trioxymethylene can be formed, and both trioxymethylene and formaldehyde itself can be condensed into hexose sugars under the influence of lime water catalysts (2, 3)



Formaldehyde

etc. →

Glucose

Fischer (4, 5) attained a complete glucose synthesis by developing the experiments of Butlerow (2), who had noted that formaldehyde and alkali produce sugars. Fischer (4) obtained a low yield of racemic glucose phenylosazone from the mixture and then skillfully completed the difficult steps from racemic glucose phenylosazone to d-glucose (5).

The mechanism of the above reaction is not known but the change can be written to correspond with an acyloin reaction. If this type of condensation were stopped at five and six carbon atom units, it is apparent that pentose and hexose sugars capable of forming stable six atom heterocyclic rings would result.

Other simple intermediates have been suggested. In 1843 Liebig (6) with practically no experimental evidence, suggested that oxalic, tartaric, and malic acids were intermediates. Formic acid was suggested by Michael (7) in 1899 with the idea that this substance was later reduced to glyoxal and to glycollic aldehyde. In 1914 Fincke (8) used a similar idea. In 1937 Hibbert (9) showed further possibilities of the formic acid idea in the formation of carbohydrates.

Practically all of these ideas have some measure of feasibility, but all are lacking in experimental verification. Considering the enormous significance of the

reaction and the time and energy which have been expended on its study in the last hundred years, it is astonishing that, for example, it is yet uncertain whether formaldehyde is in reality an intermediate. Although the processes by which formaldehyde, or some closely related substance such as formic acid, are synthesized in the plant are not yet understood, these would seem to represent the most probable intermediates in primary photosynthesis, from which carbohydrates, lignin and other natural products found in the cell sap and plant tissue are formed.

Hibbert (10) has suggested a mechanism for the formation of the polysaccharides from their building-units, through the ethylene-oxide form of the sugars. In any case it seems quite possible that fructose, due to its much more pronounced reactive character is the form of hexose sugar most concerned in the primary changes involved.

Experimental investigations into the nature of plant synthesis present many difficulties due to the impossibility of controlling conditions within the plant. It was therefore considered that much valuable information might be gained from an investigation into the synthesis of polysaccharides by the action of bacteria. Such an investigation was initiated several years ago in these laboratories. Harrison, Tarr and Hibbert (11) studied

the synthesis of levan from sucrose and other carbohydrates by Bacillus subtilis and Bacillus mesentericus, and showed that the essential condition for the formation of the polysaccharide is a terminal fructo-furanose grouping in the substrate molecule. Such a group is present in sucrose and raffinose, and levan was obtained from both these sugars.

Polysaccharide formation by the action of Acetobacter xylinus on many sugars and related compounds was studied by Tarr and Hibbert (12), and the polysaccharide formed by this organism from glucose, fructose, and glycerol respectively, was shown by Hibbert and Barsha (13) to be identical with cotton cellulose.

Finally, the formation of dextran by Leuconostoc mesenteroides was investigated by Tarr and Hibbert (14). They found that polysaccharide formation took place in appreciable amounts only in sucrose solutions, though in the case of two strains of bacteria small amounts were observed in solutions of glucose. These latter results, however, would seem to require confirmation.

The determination of the structure of the dextran synthesized from sucrose by the action of one strain of Leuconostoc mesenteroides, supplied by Dr. A.J. Kluyver, Delft, Holland, is the object of the present investigation. The structure of this polysaccharide is of great interest,

not only to the carbohydrate chemist, but to the immunologist, since there are indications (15, 16, 17, 18), that it may be immunologically specific.

The bacterial polysaccharides have, during the past ten to twenty years, attained a position of great importance in the science of immuno-chemistry. The field has attracted numerous workers, but the pioneers, and those who have contributed more than any others to our knowledge of the immunological role of polysaccharides are Avery and Heidelberger, and their co-workers.

Although a survey of this field is not within the scope of this discussion, it may be noted that Heidelberger has published three reviews on the subject (19, 20, 21). Briefly, the polysaccharides elaborated by many pathogenic bacteria, for example, the pneumococcus and the Friedländer bacillus, are responsible for type specificity. They are recognized immunologically by the precipitin reaction with antisera produced by injection of the homologous organism. There are reports (22, 23, 24, 25, 26) that polysaccharides, other than those produced by bacteria give some indication of being specific. All of these, however, require confirmation.

The present research places dextran in the position of being the only bacterial polysaccharide showing indications

of specific properties, whose structure has been the subject of thorough chemical investigation. Levan may be regarded as a possible exception to this statement in that Zozaya (16) reports a serological study of the polysaccharides of B. subtilis and B. mesentericus. Whether or not these polysaccharides are identical with the levan of Harrison, Tarr and Hibbert (11) is not known.

The determination of the structure of the specific bacterial polysaccharides and a subsequent investigation of the relation of structure to immunological action would undoubtedly be of great interest. Some preliminary work on this relationship has already been carried out (27, 28, 29, 30). However, chemical work on the polysaccharides from the pathogenic bacteria is greatly hampered due to the extremely small yields obtainable from culture media. The building units of some have been ascertained (31, 32, 33, 34, 35, 36, 37, 38), but the positions of linkage and ring structure are still in doubt. On the other hand, dextran is obtainable in large yields, suitable for exhaustive chemical investigation, and would appear to present an excellent starting material for research on the relation of structure to immunological action. This problem has already been attacked in these laboratories where it has been shown that dextran is a haptene (18), that is, the pure polysaccharide gives a precipitin reaction with anti-Leuconostoc sera prepared with the homologous organisms.

SURVEY OF THE LITERATURE

The History of Dextran

The term "dextran" has been applied to carbohydrate slimes originating from sugar syrups, fermenting vegetables and dairy products. The occurrence of dextran has been reported from time to time from the middle of the last century until the present in connection with the wine and sugar industries. In the latter it occurs as one of the components of a slimy mass, blocking the filters and otherwise interfering with the refining processes.

"Dextran" was obtained by Pasteur (39) as a product of the fermentation of glucose, the yield of gummy product amounting to 45%. It is impossible to decide whether or not this dextran was identical with that with which the present investigation deals, since its chemical structure was not investigated. The fact that it was formed from glucose, however, would seem to indicate that it was not, since the strain of L. mesenteroides studied has been found incapable of forming dextran from this sugar under the conditions employed.

Scheibler (40, 41) who undertook the first chemical investigation of these jelly-like substances, regarded dextran

as a constituent of the beet cell. He observed the occurrence of bacteria in the material but maintained that they took no part in its formation.

The substance did not reduce Fehling's solution. Carbon and hydrogen analyses indicated the empirical formula $(C_6H_{10}O_5)_n$ and a rotation of $+223^{\circ}$ was observed. The dextran on hydrolysis by heating with dilute sulphuric acid, yielded glucose. On oxidation of dextran with nitric acid, the only product identified was oxalic acid.

Jubert (42) in 1875 advanced the theory, in contradiction to Scheibler's views, that dextran was a product of fermentation in the sugar vats, and not a constituent of the beet cell. In support of this view he was able to show that the dextran slime was found in pure sucrose solutions, as well as in beet juice. This theory was confirmed in the following year by Durin (43) who identified the viscous material as a product of bacterial action. He also confirmed Scheibler's formula $(C_6H_{10}O_5)_n$ by hydrolyzing the dextran, first to "dextrins" and then to glucose, but drew the erroneous conclusion that the compound was cellulose since he found that it was soluble in Schweizer's reagent. Van Tieghem (44) later found that dextran was insoluble in cuprammonium solution.

Daumichen (45) carried the chemical investigation of dextran somewhat further in 1890, when he formed a tribenzoate and a triacetate. The quantitative hydrolysis to glucose, and formation of glucosazone were confirmed, and in addition to oxalic, saccharic acid was identified among the products of nitric acid oxidation.

Browne (46) who carried out a chemical investigation of the structure of dextran, merely confirmed the work of previous investigators on the hydrolysis to glucose, and the empirical formula $(C_6H_{10}O_5)_n$. However, on the basis of his combustion analyses he advanced the theory that dextran is a hydrated product of variable composition.

The earlier chemical and bacteriological work on dextran has been reviewed elsewhere (47, 48, 49) in greater detail. Early bacteriological studies of the dextrans were complicated by non-systematic nomenclature, and incomplete description of the organisms.

In 1877 van Tieghem (44) published the first description of a dextran-producing organism, to which he gave the name Leuconostoc mesenteroides and which he described as a coccus, appearing either as glassy points, or as larger proliferations. Since the time of van Tieghem numerous organisms have been described as producers of dextran (50, 51). However, the majority of these organisms

were reported in the latter part of the nineteenth century, when the science of bacteriological classification was still in its infancy, and it is probable that many of them are identical.

It is now known that the dextrans are produced by the action on sucrose of certain strains of chain-forming cocci, classified by Hucker and Pederson (47) as the Family Coccaceae, Tribe Streptococceae, Genus Leuconostoc, Species mesenteroides and dextranicus. Leuconostoc mesenteroides (Cienkowski) van Tiegham ferments pentoses (either arabinose or xylose) and sucrose, whereas Leuconostoc dextranicus (Beijerinck) ferments sucrose but not pentoses. Dextran is not produced by the third species of Leuconostoc, citrovorus, and this species ferments neither sucrose nor pentoses. In general, those species of Leuconostoc which ferment sucrose produce in addition to dextran (ca. 25% crude yield on the basis of sucrose) approximately 30% levo-lactic acid, 5% acetic acid, 10% ethanol and 10% mannitol (47).

The structures of three different dextrans have been partially investigated in these laboratories. Dextran I, with which the present investigation is concerned was synthesized by a strain of L. mesenteroides obtained from Dr. A.J. Kluyver of the Technische Hoogeschool Laboritorium,

Delft, Holland; Dextran II, by L. mesenteroides No. 5 (47), obtained from Dr. G.J. Hucker, N.Y. Agric. Exper. Station, Geneva, N.Y.; and Dextran III, by L. dextranicus No. 22 (47) also obtained from Dr. Hucker.

Hibbert and Tarr (14, 48) determined the optimum conditions for the preparation of dextran. Sucrose was found to be the only suitable carbohydrate substrate. The nutrient solution consisted of sucrose (10%), peptone (0.1%), potassium chloride (0.1%) and disodium hydrogen phosphate (0.2%), and incubation was allowed to proceed for ten days at room temperature. The crude dextran was isolated from the concentrated culture by precipitation with methanol, and was purified by electro-dialysis.

More recent preparations of the dextrans have shown the need of supplementing the medium of Tarr and Hibbert (14), especially if the organisms have been cultured for any great periods of time on artificial media. Carruthers and Cooper (52) increased the yield of dextran by adding raw beet sugar or molasses to the media, while Stacey and Youd (53) added commercial maple sugar. Hassid and Barker (54) added yeast extract, magnesium and ammonium sulphates to the medium.

Carruthers and Cooper (52) concluded that dextran was not synthesized by enzymatic action. Quite recently,

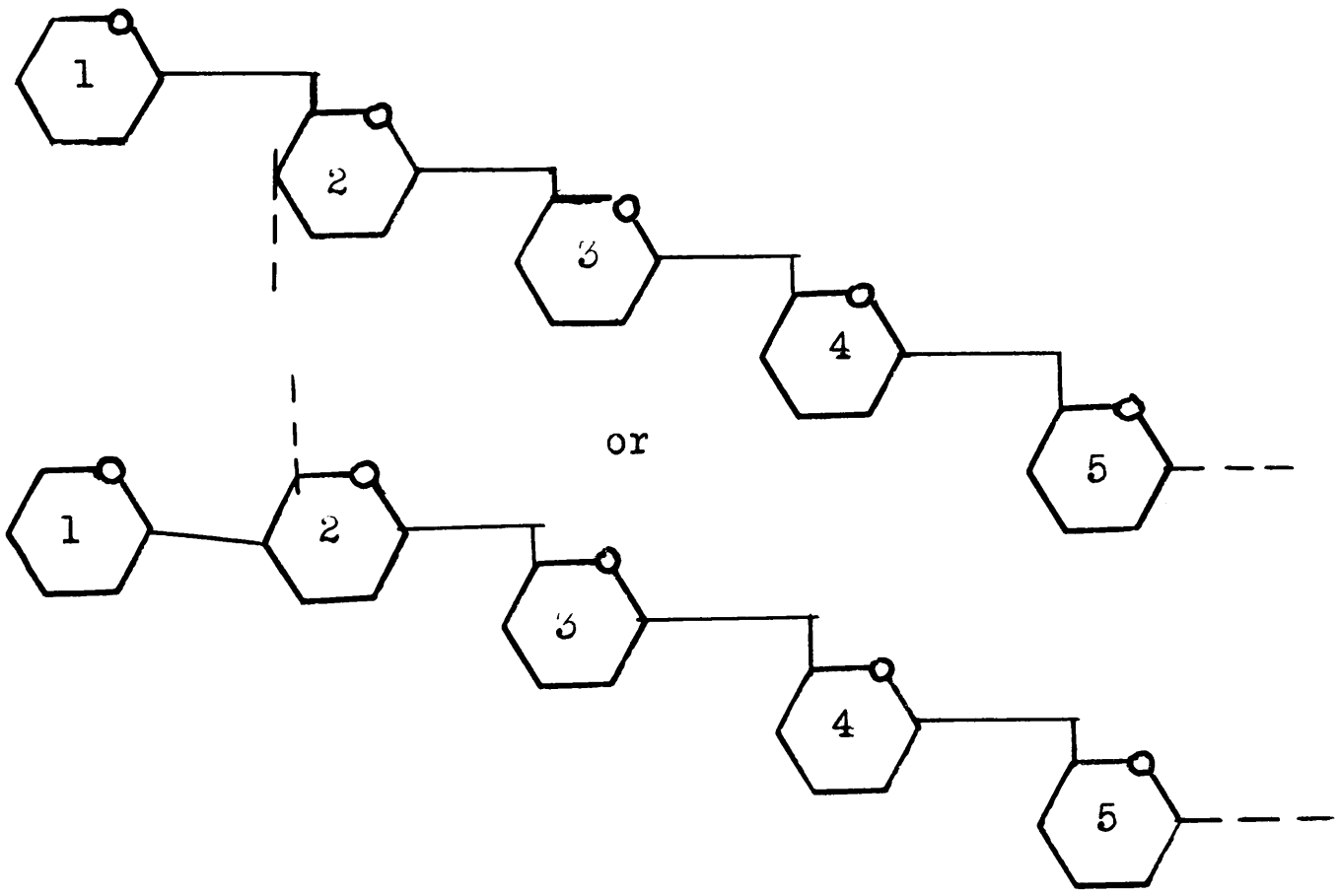
however, Hehre (55) has reported the formation of a dextran-like product from sucrose by the action of a bacteria-free extract of a Leuconostoc culture.

Structure of the Dextrans

Fowler, Buckland, Brauns and Hibbert (56) investigated the structure of Dextran II. On hydrolysis with dilute aqueous sulphuric acid, only glucose, in 90% yield, was obtained. The presence of three hydroxyl groups per glucose anhydride unit was shown by the preparation of a triacetate and a tribenzoate. Numerous (six or seven) Haworth methylations (using dimethyl sulphate and concentrated alkali) of Dextran II did not raise the methoxyl content above 41% (theoretical maximum 45.6%), nor were Purdie methylations (using methyl iodide and silver oxide), or methylation with thallium ethylate and methyl iodide effective in raising the methoxyl content of partially-methylated Dextran II above 43.5%. The maximum theoretical methoxyl content was eventually attained by modified Muskat methylations (57). Partially-methylated dextran, suspended in anisole solution, was treated with sodium in liquid ammonia, and the sodium salt of methylated dextran thus formed was allowed to react with methyl iodide. Three such treatments raised the methoxyl content of partially-methylated dextran from 42% to 45.5%, and five such treatments from 30% to 45.4% (49).

Methylated Dextran II was hydrolyzed by heating with hydrogen chloride (1.5%) in anhydrous methanol in a sealed tube. The mixture of glucosides so obtained was fractionated, first by differential solubilities in chloroform and water, following the method of Macdonald (58). In this treatment the dimethyl methyl glucoside dissolved in the water, the tri-, and tetramethyl methyl glucosides in the chloroform. The chloroform soluble material was fractionated by distillation under reduced pressures. These separations yielded 2,3,4,6-tetramethyl methyl glucoside, 2,3,4-trimethyl methyl glucoside, and a dimethyl methyl glucoside (identified as 2,3-dimethyl-α-methyl glucoside by means of the 6-trityl derivative, the formation of which, however, was not quantitative). Due to this complicated fractionation technique the results indicated that two different ratios of the tetra; tri-, and dimethyl methyl glucosides, namely, 1:2:1 and 1:3:1 were equally possible for the proportions of the three glucosides. However, after consideration of the order of accuracy to be expected from the methods of isolation employed, as well as their accompanying inevitable losses, it was concluded that the three glucosides were present in the ratio of 1:3:1. Nevertheless, it was pointed out (49) that the evidence against a 1:2:1 ratio is of a negative rather than a positive character.

These results indicated a cross-linkage for every repeating unit of five glucose molecules, and 1,6-linkages within each repeating unit. A repeating unit of the following type was suggested as one of the various possibilities for Dextran II (56).



Glucose residue #1 would therefore appear as 2,3,4,6-tetramethyl glucose on hydrolysis of methylated dextran; glucose #2 as 2,3-dimethyl glucose and the other three glucose residues as 2,3,4-trimethyl glucose.

These results (56) were later criticized by Brauns (59), who questioned the completeness of methylation.

of the methylated dextran, and the interpretation of the methyl glucoside fractionation data in view of the large losses (18.4%) of material during fractionation. These same criticisms apply to practically every previous publication dealing with dextran structure.

In a recent re-investigation of the structure of Dextran II (60) it was found that 2,3,4,6-tetramethyl methyl glucosides, 2,3,4-trimethyl methyl glucosides, and dimethyl methyl glucosides were present in the ratio of 0.89:3.00:1.26 respectively. This ratio cannot be accepted without some reservation since Dextran II has been found especially sensitive to degradation during methylation and also during hydrolysis of the methylated derivative (60). The true structure of a polysaccharide can only be determined if the polysaccharide is stable under the conditions used. Nevertheless, the results obtained do appear to indicate a 1:3:1 rather than a 1:2:1 ratio for Dextran II.

Hassid and Barker (54) investigated the structure of the dextran produced by a strain of Leuconostoc mesenteroides. The yields of the methylated dextran (methoxyl content 45.2%) obtained were not stated. Fractionation of the methylated glucosides resulting from hydrolysis of the methylated dextran was attempted by solvent action, and large losses (40%) resulted, in addition

to the loss sustained on hydrolysis (35%). The bulk of the product identified was 2,3,4-trimethyl-β-methyl glucoside, but small amounts of 2,3,4,6-tetramethyl glucose and dimethyl glucose were isolated. The authors attached no particular significance to their values for the molecular weights of dextran and of methylated dextran. Values of 11700 and 14800 respectively were obtained by viscosity measurements, and 2600 $\pm$ 50 and 3275 $\pm$ 50 respectively by means of the McBain air-driven top centrifuge. In the first case an arbitrary viscosity constant, that for cellulose, had to be assumed in the application of the Staudinger viscosity equation. Moreover, the molecular weight values obtained for starch, using this type of centrifuge were only about one-tenth the accepted values.

The structure of Dextran III was investigated by Fairhead, Hunter and Hibbert (61). Fully methylated Dextran III was obtained by Haworth methylations, followed by the use of the Muskat procedure. Hydrolysis of fully methylated Dextran III yielded 90% 2,3,4-trimethyl methyl glucosides, and 10% dimethyl methyl glucoside, but no 2,3,4,6-tetramethyl methyl glucoside was obtained.

Peat, Schlüchterer and Stacey (62) later obtained a small amount (0.25%) of 2,3,4,6-tetramethyl methyl glucoside from the hydrolysis products of a methylated

Dextran III, in addition to 90% 2,3,4-trimethyl methyl glucosides, and on the basis of these results, postulated a minimum chain length of 550 units for Dextran III. The isolation of a considerable quantity of dimethyl methyl glucoside must be regarded as definite proof of the presence of side-chain linkages.

THE STRUCTURES OF POLYSACCHARIDES RELATED TO DEXTRAN

A. Naturally Occurring Polysaccharides

In this discussion, only the naturally occurring polymerized anhydro-hexoses will be considered. Of these, cellulose, starch and glycogen have been the subject of such a prodigous number of researches that it is impossible to give a detailed review of the literature here. Excellent reviews (63, 64, 65) dealing with these subjects have appeared in the last few years. The evidence for the more widely accepted structures for these polysaccharides will be discussed.

Cellulose

Monier-Williams (66) has shown that cellulose on hydrolysis yields glucose quantitatively.

During the period 1920 to 1926 it was believed that cellulose consisted of small structural units held together by some form of residual valence (67). In 1926 Sponsler and Dore (68) showed that X-ray diagrams confirmed the hypothesis most generally held today, that the fibres consisted of long parallel chains of glucose units linked by primary valences.

At present cellulose is believed to consist of a long chain polymer of β-glucose units joined by 1,4-linkages. The spatial arrangement of the units is such that the terminal carbon atoms (C_6) lie first on one side of the chain and then on the other giving, in effect, a repeating unit of the size of cellobiose. It must be assumed that the chain is open, the first unit having a free hydroxyl in the 4-position and the end unit a free hydroxyl in the 1-position. This may be expressed in writing the condensed cellulose formula thus:

$$C_6H_{11}O_6 - \left[C_6H_{10}O_5\right]_n - C_6H_{11}O_5$$

Although there is general agreement regarding the make-up of the cellulose chain, the question of its length is still a contraversial subject. Haworth (69, 70, 71, 72) found by the end group analysis method (determination of tetramethyl glucose) a chain length of from 150 to 250 glucose units. This was apparently in agreement with the length of the crystallites in native cellulose (600 Å) as determined by Meyer and Mark (73, 74).

On the contrary, Staudinger (75, 76) derived from viscosity measurements a degree of polymerization of from 1000 to 2000 glucose units. Hess and Neumann (77a, b, c)

also suggest, on the basis of end group analysis, that cellulose chains consist of thousands of C_6 units.

Starch

Starch yields glucose on complete hydrolysis. When it is broken down by enzymes, maltose and dextrins are obtained (78, 79, 80, 81).

It is generally thought today that a true chemical unit of starch exists. This according to the Birmingham school (82, 83) is an open chain of α-glucopyranose units, joined by 1,4-glucosidic linkages as in maltose. The chains are believed to consist of approximately 24 to 30 glucose units. The same unbranched chain has been obtained from starches of markedly different physical properties, e.g. by Haworth from potatoes (84, 85), maize or waxy maize (85A) and by Hassid and Dore (85A) from canna.

Staudinger (85B) has proposed a formula for starch, in which he pictures a central chain to which is attached, at the CH_2OH of every fourth glucose unit, a lateral chain. In addition, lateral chains are joined to other glucose units of the central chain, through the hydroxyl attached to C_3. Such a formula requires the formation of 2,3- and 2,6-dimethyl-, 2,3,6-trimethyl and 2,3,4,6-tetramethyl glucose from methylated starch. Up to the present, only

2,3,4,6-tetramethyl, 2,3,6-trimethyl and 2,3-dimethyl glucose (86, 87) have been identified with certainty from the hydrolysis products of methylated starch.

The older assumption (88) of a branched molecule for starch was confirmed by Freudenberg and Boppel (89) who showed that the proportion of dimethyl glucose (representing positions of branching) to tetramethyl glucose (end group) was approximately equal for every twenty glucose units.

Glycogen

Haworth (82, 83) and Bell (90, 91) consider glycogen to have an average minimum chain length of twelve to eighteen glucose units, joined by 1,4 linkages. Hydrolysis of methylated glycogen yielded in addition to 2,3,6-trimethyl glucose, approximately equal amount of 2,3,4,6-tetramethyl glucose and dimethyl glucose (a portion of which was identified as the 2,3 derivative (83)). Haworth suggests that the glucose chains are themselves joined by a type of union which links the reducing end of one chain with a hydroxyl in a non-terminal glucose residue of an adjoining chain.

Staudinger and Husemann (92) conclude from their own and Haworth's observations that glycogen consists of a central chain of glycosidically bound units of glucose linked C_1 to C_4, to which are attached at C_2, C_3 and C_6 of

each unit similar side chains of from twelve to eighteen glucose units in length.

Inulin

Of the remaining naturally occurring carbohydrates, inulin has been investigated more thoroughly than any of the others.

Irvine commenced his researches on the polysaccharides with a study of inulin in 1920. Irvine and Steele (93) methylated inulin with dimethyl sulphate and alkali, and found that the reaction stopped at approximately a dimethyl inulin stage (OCH_3 = 35.8%). This figure was confirmed later by Irvine, Steele and Shannon (94), though contradicted by Karrer and Lang (95) who obtained a methoxyl content of 39.4% with these reagents. Irvine, Steele, and Shannon (94) completed the methylation with silver oxide and methyl iodide and obtained a trimethyl inulin with methoxyl content of 44.3%, in the form of a dextrorotatory colorless syrup. Karrer and Lang's trimethyl inulin, on the other hand was laevorotatory and a solid.

Irvine, Steele and Shannon (94) noted that methylation was accompanied by irregular changes in solubility which they attributed to simultaneous depolymerization and polymerization. Two trimethyl inulins were isolated, differing in optical rotation and solubilities, but yielding the

same trimethyl fructose on hydrolysis.

Irvine and Steele (93) on distillation of the hydrolysis products of trimethyl inulin, obtained 76% of a trimethyl-γ-fructose. There was also a preliminary low boiling fraction, which is significant in view of the later work of Haworth (96). On the basis of these results, Irvine concluded that "inulin is an aggregate of γ-fructose residues, each ketose molecule having lost two hydroxyl groups in the formation of the polysaccharide". He suggested two possible linkages, 1,2 or 2,3, but regarded the latter as more probable. Furthermore, the mode of aggregation of the fructose units might be regarded, according to Irvine, either as a condensation through the reducing group and one other hydroxyl, or as a polymerization of actual fructose-anhydride units.

The question as to which carbon atoms are concerned in the condensation, (or dehydration as the case may be) was settled in 1928 by Haworth and Learner (97), who proved, by oxidation methods, that the sugar isolated from the hydrolysis products of trimethyl inulin was 3,4,6-trimethyl-γ-fructose. Therefore the linkage is through the first and second carbon atoms.

This was confirmed by Montgomery (98) in 1934.

He synthesized 3,4,6- and 1,4,6-trimethyl-γ-fructose, and found that the former was identical with the trimethyl fructose obtained from methylated inulin, while the latter was quite different in its properties.

Regarding the actual composition of inulin, there seems still to be some doubt whether fructose is the only sugar present. Schlubach and Elsner (99) claimed to have isolated as high as 8% glucose, while Jackson and Macdonald (100), and later Irvine and Montgomery (101) report up to 3.7% of this sugar in the hydrolysis products of purified inulin. The latter workers ascribed the presence of this sugar to a fructose-glucose conversion during the acid hydrolysis, and stated that glucose is not formed when inulin is hydrolyzed by enzymes. Ohlmeyer (102) reports the isolation of 1.5% of glucose when pure inulin is hydrolyzed by Aspergillus niger at pH 6. From this he calculates a minimum of 70 hexose units in the molecule, based on the presence of one mole of glucose. However, the presence of glucose in the original polysaccharide would still seem to require confirmation.

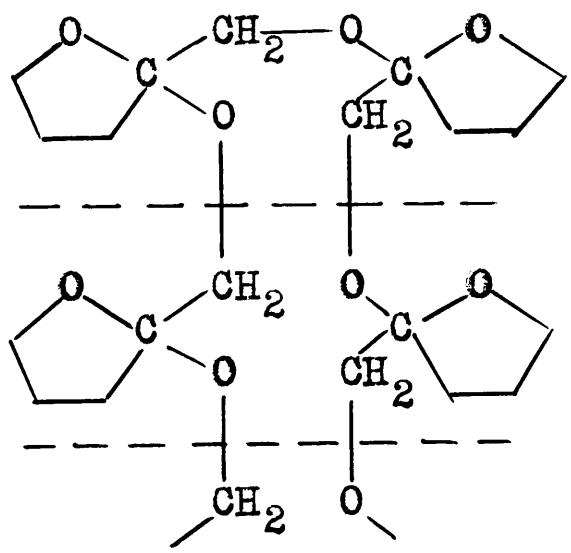
Until recently there has been considerable controversy regarding the molecular complexity of inulin. Many investigators (103, 104, 105, 106, 107, 108) have isolated from inulin, by different treatments, a fructose-

anhydride (in nearly every case a difructose-anhydride). It was therefore concluded that this was present in the polysaccharide as a definite structural unit. Cryoscopic molecular weight determinations of inulin and inulin acetate have, in general, given values corresponding to a difructose-anhydride (109, 110, 111, 112).

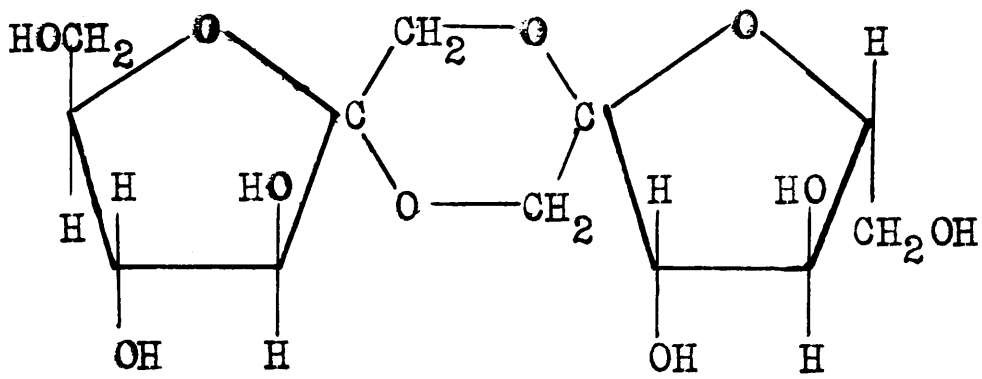
Berner (113) states that in many cases the molecular weight determinations on supposed "depolymerization products" of inulin are valueless, due to contamination with the reagents used. Thus, on heating inulin with acetamide, and precipitation with alcohol, he obtained a product with an apparent molecular weight of 242, which, on purification rose to 3550.. However, Pringsheim, Reilly and Donovan (114) found that when inulin was subjected to this procedure the product was easily soluble in cold water. On standing, the solubility decreased, and the molecular weight increased. It is probable, therefore, that the increase in molecular weight observed by Berner was due more to re-polymerization than to purification.

The extreme ease with which inulin is depolymerized was demonstrated by Drew and Haworth (115). By the ebullioscopic method in water, they obtained a minimum molecular weight of 3200-3600, but showed that this gradually decreased, due to hydrolysis in boiling water. They

postulated a long chain structure for inulin, and in support of this theory showed that partial depolymerization by means of carbon dioxide or traces of hydrochloric acid gives rise to shorter chains, or "levulins". The long chain formula received further confirmation in 1932, when Haworth, Hirst and Percival (96) isolated 3.7% of 1,3,4,6-tetramethyl fructofuranose from the hydrolysis products of methylated inulin, giving a minimum chain length of approximately 30 fructose units. This was confirmed in the following year by Irvine and Montgomery (101). Haworth, Hirst and Percival (96) also isolated 3% of a hexamethyl-difructose-anhydride, which they considered to have been formed by depolymerization and recombination. They suggested a formula of the type:



It is evident that cleavage as indicated by the dotted lines would give rise to a difructose-anhydride such as (116):



This is the structure proposed by Haworth and Streight (117) for the difructose-anhydride originally isolated by Jackson and Goergen (105). In further support of the long-chain formula, Haworth and Streight reported that inulin acetate, on extraction with hot water yields no hexa-acetate of a difructose-anhydride, though they found their inulin hexa-acetate to be readily soluble in hot water.

Since an inulin completely inactive toward Fehling's solution had never been obtained, Haworth, Hirst and Percival suggested that one end of the chain contained a free reducing group (the other having four free hydroxyl groups and giving rise to tetramethyl fructose). This idea has recently been opposed by Schlubach and Schmidt (118), who have prepared an inulin which did not reduce Fehling's solution. They therefore suggest that Haworth's formula must be modified to exclude the terminal reducing group and offer three possible ways in which this may be done:

(i) Presence of a terminal fructose-anhydride group (in which case, it should be possible to isolate a dimethyl fructose, as in the case of graminin (119));

(ii) Presence of a trehalose linkage, which seems unlikely;

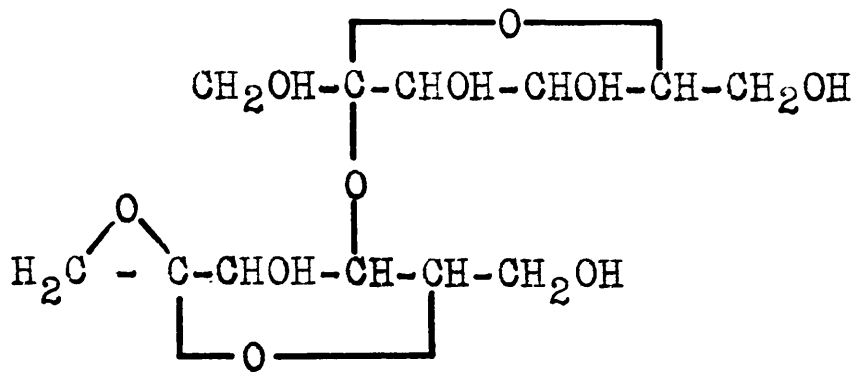
(iii) Formation of a large ring.

In the last case, since the isolation of tetramethyl fructose is an accomplished fact, the ring would have to be closed through the medium of a non-terminal hexose unit. This again would give rise to a dimethyl fructose, the presence of which has not been reported.

In addition to inulin, which has a large molecule, (page 28) several fructosans has been described, most of which give varying amounts of dimethyl fructose when their methyl derivatives are hydrolyzed.

Irisin

In irisin, studied by Schlubach, Knoop and Liu (120, 121), is found the first example of a naturally occurring polysaccharide containing a hexose side-chain. This compound, isolated from Iris pseudoacosus, when methylated and then hydrolyzed yielded equal parts of a di- and a tetra-methyl fructose. The latter, by oxidation methods, was proved to be the 1,3,4,6 derivative. Polymerization was assumed, by analogy with inulin, to be through the 1,2 positions, and the structural unit was postulated as:



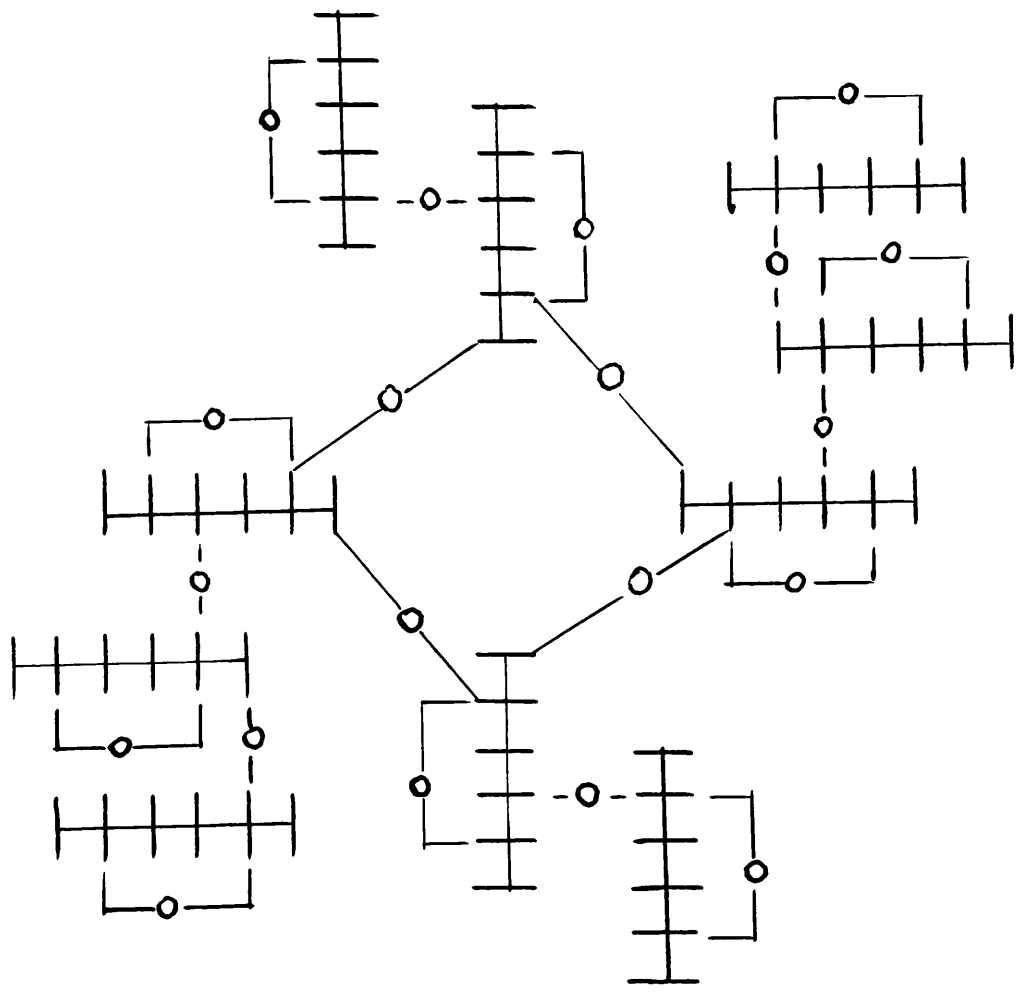
A tetra-fructose- and a di-fructose-anhydride were isolated as intermediate products of hydrolysis. The former, on methylation and hydrolysis, gave the usual mixture of di- and tetra-methyl fructoses. The latter gave only a trimethyl fructose, showing that the side-chain had been split off. The osazone formed from this sugar failed to crystallize on inoculation with the osazone of 3,4,6-trimethyl fructose. This would seem to throw some doubt on the positions assumed for linkage and polymerization, since, if the above formula as suggested by Schlubach be correct, removal of the side chain, followed by methylation and hydrolysis should yield 3,4,6-trimethyl-fructo-furanose.

Graminin

Shortly after the publication of these results on the constitution of irisin, Schlubach and Koenig (119) reported the isolation of a fructose polymer from rye flour. Since it was found in small amounts in other grains, they gave it the name "graminin". It would seem to have a structure resembling

that of dextran, as determined in the present investigation, for on the hydrolysis of the methylated compound, a mixture of di-, tri-, and tetramethyl fructose was obtained, while methylated dextran, hydrolyzed with methanol-hydrogen chloride, yielded di-, tri-, and tetramethyl methyl glucosides. The ratio of the yields of methylated fructoses, from fully-methylated graminin, in the order named, is given either as 1:1:1 or (more probably) 2:1:2. Since separation was effected by conversion to the fructosides, benzoylation, and subsequent distillation of small amounts of material, these authors were uncertain of the conclusions to be drawn from the yields obtained. The tetramethyl fructose fraction was identified as 1,3,4,6, while the trimethyl is regarded as probably 3,4,6. The identity of the dimethyl sugar was not established, but it resembled that obtained from irisin. Cryoscopic molecular weight determinations on the acetylated graminin as well as on the free polysaccharide regenerated from the acetylated compound gave values corresponding to 9-10 fructose units. The molecular weight of the original polysaccharide, as determined by the same method appeared to be somewhat lower, and was found to vary with the concentration used in its determination. In suggesting a formula, these authors assume 10 anhydro-fructose units to be present in the molecule.

Since the compound is non-reducing, there is no terminal reducing group, and, according to the authors, since no mono-methyl fructose was isolated, this reducing group is not masked by an inner-anhydride. Therefore Schlubach and Koenig (119) suggest a large ring as the only possible formula:



Sinistrin

In sinistrin (122) obtained from the squill Scilla maritima it is suggested there are fifteen fructose residues.

Triticin

In triticin, present in couch-grass rhizomes, Schlubach and Peitzner (125) assume a closed ring of fructose residues represented by a multiple of 7, viz., either 14 or 21. In the case of both sinistrin and triticin, as in the case of graminin, tetra-, tri-, and dimethyl methyl fructosides were isolated.

Asparagosin

Murakami (124) describes a fructosan, asparagosin, isolated from the tubers of Asparagus officinalis, which he regards as consisting of only seven fructose units, while Schlubach (125) from molecular weight determinations of the polysaccharide, assigns a molecular configuration of ten fructose anhydride units. The ratios of tetra-, tri-, and dimethyl fructoses obtained by Schlubach and Böe (125) from the hydrolysis of the fully-methylated product, were 1:8:1.

Asphodelin

Asphodelin present in Asphodellus knollen is described by Schlubach and Lendzian (126) as having a 1:5:1 fructose

anhydride ratio, but one of these units is said to be glucose (126), probably originating from an impurity. The different ratios obtained from the various fructosans are shown in Table I.

Table I

Ratios of Di-, Tri-, and Tetramethyl Fructose Found on Hydrolysis of Various Fully-methylated Naturally Occurring Fructosans

Fructosan	Tetra	Tri	Di
Irisin	1	0	1
Graminin	1	1	1
or	2	1	2
Sinistrin	1	3	1
Triticin	3	1	3
Asparagosin	1	8	1

Mannans

With regard to the mannans and galactans, there has been only a relatively small amount of somewhat scattered work carried out on these products. In 1923 Patterson (127) isolated a polysaccharide from ivory nuts which, on hydrolysis, gave 80% of mannose. Repeated methylation gave a trimethyl mannan, which, on hydrolysis, yielded trimethyl mannose.

The latter sugar was identified in 1934 by Klages (128) as 2,3,6-trimethyl mannose. 1.4% Of 2,3,4,6-tetramethyl mannose was also isolated, indicating the polysaccharide consisted of a chain of some 80 manno-pyranose units linked through the 1,4-positions. Klages also isolated from mannan a manno-biose, and a manno-triose, and, on the basis of their optical behaviour concluded that both α and β linkages are present in the original mannan molecule. It is interesting to note that Klages obtained a methoxyl content of 45.8% after only two methylations with dimethyl sulphate and alkali in the presence of benzene.

A similar mannan from salep (129) has been assigned a chain length of sixty units. It should be noted that this mannan is fairly soluble in water, while the ivory nut mannan is insoluble.

A much simpler mannan termed "mannocarolose" (130) is discussed under "bacterial polysaccharides".

Arabo-Galactans

From certain coniferous woods, particularly that of the larch family, water soluble polysaccharides, the arabo-galactans, can be extracted. These have been shown to contain d-galactose and l-arabinose components in a 6:1 molecular ratio (131, 132, 133, 134). Another arabo-

galactan (135) from peanut seeds is stated to be composed of a repeating triose molecule consisting of one galactose and two arabinose units. A proportion of uronic acid may or may not be present as an impurity.

It was assumed that arabo-galactan from larch was a homogeneous polysaccharide, but more recent studies (136, 137, 138) of the acetates, propionates and benzoates of preparations from Eastern, Western, and European larches, tend to discredit this hypothesis.

White (139) has shown that the methyl derivative of the polysaccharide obtained from the western larch Larix occidentalis, when subjected to complete hydrolysis and simultaneous glycoside formation, yields the glycosides of 2,4-dimethyl-d-galactose, 2,3,4-trimethyl-d-galactose, 2,3,4,6-tetramethyl-d-galactose, and 2,3,5-trimethyl-l-arabinose in the approximate molecular ratio 3:1:2:1 respectively. The isolation of a relatively large proportion of the terminal arabo-furanose unit as the crystalline amide of the corresponding acid, strongly suggested a direct linkage of the arabinose fraction to the galactose units of the polysaccharide. The terminal galactose residues were shown (140) to possess a 1,6-linkage, by the isolation of octamethyl-6-d-galactosidogalactose, as well as 2,4-dimethyl-6-tetramethyl-galactosidogalactose, from the partial hydrolysis

products of the methyl ether of arabo-galactan. The formation of the latter compound demonstrated the branched chain linkage of this arabogalactan. The arabinose component of arabo-galactan is joined to a tri-linked galactose residue, the position of such linkage being through the one position of the arabinose component and the 6-position of the galactose residue (140A).

White concluded that the structure of arabo-galactan is best represented by a highly branched chain of galactose residues joined by oxygen linkage through both the 1,3- and 1,6-positions, and that the terminal residues of arabofuranose and galactopyranose are linked to the 6-position of adjacent galactose anhydride units. This is in agreement with Husemann's (141) views that arabo-galactan is a highly branched polysaccharide similar to starch. Owens (142) on the other hand on the basis of viscosity determinations regards it as possessing a spherical molecular form.

Percival (143) states that the main carbohydrate portion of agar-agar consists of β-galactopyranose units linked at positions 1 and 3, since the main products of hydrolysis of the fully-methylated agar-agar is 2,4,6-trimethyl galactose.

B. Bacterial Polysaccharides

The structures of a number of bacterial polysaccharides have been investigated. These include polysaccharides resembling the dextrans, others similar to the pneumococcus polysaccharides, also bacterial cellulose, levans, composed of anhydrofructose units, and polysaccharides of pathogenic organisms, and the very interesting cellulose type of polymer which has recently been reported (144) in which the glucose units are joined in the 1,3 instead of the 1,4 positions.

Vermiformé Dextran

Vermiformé dextran, the polysaccharide produced from sucrose by Betabacterium vermiformé (Ward-Mayer) was shown (145) to consist of basal chains of twenty-five anhydroglucose units joined by 1,6-linkages. Osmotic pressure determinations indicated a molecular weight of approximately 500 units, while a chain length of twenty-seven glucose units was suggested by the iodine number. Hydrolysis of the methylated polysaccharide yielded 2,3,4-trimethyl methyl glucoside (90%) and 2,3,4,6-tetramethyl methyl glucoside (5%).

The polysaccharides produced from sucrose by such nitrogen-fixing organisms as Rhizobium radicicolum (clover

strain) and Azotobacter chroococcum consist of about 90% glucose and 3-4% uronic acid residues (146). Both probably belong to the same class as the specific polysaccharides Pneumococcus Types II and III (page 48).

Bacterial Cellulose

Hibbert and Tarr (12) determined the optimum conditions for the preparation of bacterial cellulose by the action of Acetobacter xylinum on glucose, fructose, glycerol, sucrose, mannitol and galactose. Hibbert and Barsha (13, 147) showed that the bacterial cellulose was identical chemically with cotton cellulose. Bacterial cellulose and cotton cellulose yielded the following derivatives with approximately the same yields and physical constants: cellulose triacetate, α- and β-methyl glucosides from cellulose triacetate, trimethyl cellulose, 2,3,6-trimethyl methyl glucosides, 2,3,6-trimethyl glucose and cellobiose octa-acetate. X-ray diagrams of the two types of cellulose were similar, and the bacterial cellulose acetate could be spun into a thread.

Levans

Harrison, Tarr and Hibbert (11) investigated the production of levan from sucrose by the action of B. subtilis and B. mesentericus (and the corresponding enzyme). The structure of the product synthesized by B. subtilis was

determined by Hibbert and Brauns (148), and that obtained by the action of B. mesentericus and the corresponding enzyme by Hibbert, Tipson and Brauns (149), the two being found identical. Hydrolysis of trimethyl levan gave a trimethyl fructose, which, by oxidation methods, was shown to be 1,3,4-trimethyl fructofuranose. This left the 2 and 6 positions open for linkage between the hexose units, as compared with the fructose polysaccharide, inulin, where the junctions involve positions 1 and 2 (page 25).

The levan synthesized by B. subtilis from raffinose was shown by Mitchell and Hibbert (150) to be identical with that obtained from sucrose.

Challinor, Haworth and Hirst (151) on the basis of the yield of tetramethyl fructose from the hydrolysis products of fully-methylated levan, have determined the chain length of levan as 10-12 fructofuranose units, and confirmed the structure put forward by Hibbert and co-workers.

Other levans produced by widely-different organisms all have similar structures to B. subtilis levan, e.g. the levans obtained from sucrose by B. megaterium, Bact. pruni, Bact. prunicoli (152) and those (153) formed from sucrose or raffinose by Gram-negative milk and soil organisms (Actinomycetes). Certain differences in the properties of levans produced by different organisms are probably due to varying degrees of aggregation of the repeating unit.

Polysaccharides of Pathogenic Bacteria

Few of these polysaccharides are capsular carbohydrates, and very little is known of their chemical structures. In general, they consist of more than one simple sugar. For example, hydrolysis of the polysaccharide from one strain of cholera organism yields glucose and galactose. The polysaccharide of one strain of leprosy bacillus consists of pentosans mostly, composed of arabinose mainly, with a small amount of galactose (154). Varying proportions of mannose and arabinose are obtained on hydrolysis of the polysaccharide of tubercle bacillus strain H-37 (155). The latter polysaccharide differs from those of the phosphatides prepared from the bacterial residues from tuberculin, which consist of inositol and mannose (156).

Polysaccharides of Molds and Yeasts

Mannocarolose

Mannocarolose is produced by the action of the mold Penicillium Charlesii G. Smith (130) on glucose. Hydrolysis of the methylated derivative and conversion to the mannosides was reported (130) to give 2,3,4-trimethyl methyl mannoside (75%), 2,3,4,6-tetramethyl methyl mannoside (13.4%) and dimethyl methyl mannoside. The latter was assumed to be the

result of incomplete methylation, since the methylated polysaccharide had a methoxyl content of only 44.4%. The authors conclude from these results that mannocarolose consists of 8-9 manno-pyranose units linked through the 1-6 positions. However, 2,3,4-trimethyl mannose has since (157) been synthesized and is quite different from the trimethyl mannose isolated from the hydrolysis products of methylated mannocarolose. The structure of the latter is, therefore, still unknown.

Galactocarolose

Galactocarolose produced by the same mold from glucose was investigated by the same authors (158). Mannocarolose and galactocarolose were produced under the same conditions and they were isolated from the polysaccharide mixture of the Czapek-Dox medium (159) in about equal amounts.

Hydrolysis of methylated galactocarolose yielded 2,3,5,6-tetramethyl methyl galactofuranoside (12.4%) and 2,3,6-trimethyl methyl galactoside (80%). These results point to a chain of 9 to 10 units, joined through the 1,5-positions, i.e. a furanose sugar.

Varianose

Varianose, produced by the action of the mold Penicillium varians G. Smith on glucose, was found (160) to

consist of 6 to 8 β-galactopyranose units, (1,4-linkages), with a glucopyranose radical at one end and either l-altrose or d-idose at the reducing end.

Yeast Mannan

Yeast mannan was investigated by Haworth, Hirst and Isherwood (161). The yeast mannan was extracted from the cell wall by the action of boiling dilute alkali on baker's yeast. It was separated from the extracted materials in the form of its copper hydroxide complex, and after removal of the copper was purified by repeated precipitation from aqueous solution by alcohol. Hydrolysis of the methylated mannan was reported to yield 2,3,4,6-tetramethyl methyl mannoside (one part), 2,3,4-trimethyl methyl mannoside (one part), and 3,4-dimethyl methyl mannoside (one part). A structure for the yeast mannan was proposed on the basis of these results. However, the trimethyl methyl mannoside was not the 2,3,4-isomer (157), and therefore the structure of this polysaccharide is also not yet definitely known.

Insoluble Polysaccharide from Yeast

The structure of an insoluble polysaccharide from the yeast Saccharomyces cerevisiae was investigated by Zechmeister and Toth (162), and also more recently by Hassid, Joslyn and McCready (144). The isolation of 2,4,6-trimethyl

glucose as the sole product of the hydrolysis of the methylated polysaccharide indicated a chain of glucopyranose units joined by 1,3-glucosidic linkages. This is the first reported occurrence of such a linkage and stands out in marked contrast to the previously assumed general type of 1;4 linked glucose units found in a wide variety of naturally occurring polysaccharides.

Immunological Significance of Bacterial Polysaccharides

Dextran

Investigations on the immunological character of the dextrans have been carried out mainly from the standpoint of the properties of dextran as an antigen. In a recent investigation (18, 60), however, it has been shown that dextran possesses the properties of a haptene (page 7).

Zozaya (17) reported that nitrogen-free Dextran II was not antigenic, but could be rendered antigenic by adsorption upon a colloidal carrier such as collodion (precipitated from acetone solution into water). Later work indicated that the Dextran II which had been used in this investigation was not nitrogen-free.

Zozaya (17) also reported that Dextran II reacted immunologically with antisera of pneumococci, certain of the Salmonella group, and some types of Streptococcus viridans.

FitzGerald (15) found that the antibody response of rabbits toward 1.5% aqueous dextran solutions varied with the nitrogen (bacterial) content of the dextran. There was no response when the nitrogen content was less than 0.2%.

Hehre (55) mentions briefly precipitin tests with anti-Leuconostoc sera, and dextran, but no details are given.

Pneumococcus Polysaccharides

The polysaccharides of pneumococcus have been the subjects of numerous investigations in the past twenty years (163). The researches of Heidelberger, Avery, Goebel (163) and co-workers have shown that the capsular carbohydrates of pneumococcus are responsible for immunological type specificity, and that these carbohydrates may function not only as haptenes but also as antigens, if the method of isolation has been sufficiently mild so that no functional groups are removed.

The characteristics of the polysaccharides of pneumococcus Types I - XXXII have been studied in some detail (164). All these polysaccharides are optically active, twenty-five being dextro- and seven laevo-rotatory. Nine were uronic acids; seventeen were, partially at least, composed of amino sugars. Types I, IV, V, XII, XXV contained up to 5% nitrogen. Removal of nitrogen with nitrous acid resulted in a loss of specificity. Ten polysaccharides contained no nitrogen. The maximum acetyl content was 16% in the cases of IV and XI. A few of the polysaccharides contained phosphorus, the maximum being 6.4% in the cases of Types XXVIII and XXXII.

Significance of Acetyl Groups

Pneumococcus polysaccharides isolated by early extraction methods contained no acetyl groups. The use of milder extraction methods (165), however, permitted the isolation in certain cases of acetyl-containing polysaccharides. These acetylated carbohydrates are antigens, and were shown by Avery and Goebel (165) to be immunologically different from the deacetylated polysaccharides which function as haptenes only.

Chemical Structure of Pneumococcus Polysaccharides

Acid hydrolysis of pneumococcus polysaccharides has revealed something of their chemical structures. The hydrolysis products are glucose, glycuronic or aldobionic acids, amino sugars or mixtures of these, e.g. (yields on the basis of glucose (37)):

Type I. Polysaccharide yields 28% galacturonic acid, and an amino-sugar derivative. With methanolic hydrogen chloride galacturonic methyl ester is obtained.

Type II. Polysaccharide yields glucose (70%). (Reducing sugars after hydrolysis 95%).

Type III. Polysaccharide yields cellobiuronic acid (166), (reducing sugars after hydrolysis 85%).

Reeves and Goebel (38) have recently observed that hydrolysis of the reduced methylated capsular polysaccharide of Type III pneumococcus yields 2,3,6-trimethyl glucose and 2,4-dimethyl- α- and β -methyl glucosides. The methylated aldobionic acid units are linked through position 3 of the methylated glucuronic acid residue. In the polysaccharide glucose is linked to the third carbon atom of the glucuronic acid which in turn is linked to the fourth carbon atom of the second glucose molecule. The glucuronosidic linkage has the β configuration and the configuration between the aldobionic acid units is assumed to be of the same type. It is of interest to find the linkages of the saccharide units alternating between positions three and four.

Type VIII. Polysaccharide yields cellobiuronic acid and glucose (166). (reducing sugars after hydrolysis 87%).

The numerous researches of Goebel and co-workers have shown that the nature of the sugar in a carbohydrate-protein complex determines the specificity of the complex.

DISCUSSION

The Role of Methylation Studies in the Determination of the Structure of Polysaccharides

Methylation of Dextran

Since dextran possesses a very complicated structure, its complete methylation (which presented unusual difficulties), prior to hydrolysis, is of the utmost importance. The significance of this phase of carbohydrate chemistry, which appears to have been disregarded by many workers, should be pointed out.

The determination of the constitution of a polysaccharide, as Irvine (93) has pointed out, is dependent upon a knowledge of four things:

(a) the nature of the sugars in the polysaccharide;

(b) the stereochemical configuration of these sugars;

(c) the position of linkage or linkages of the individual units;

(d) the position and nature of the internal oxygen ring.

Simple hydrolysis is sufficient to determine the nature of the constituent sugars, while their stereochemical configurations may be established by the action of enzymes,

and also by their optical behaviour (167). A knowledge of the spatial position of the carbon atoms involved in the linkages between the sugar units, and in the formation of the internal oxygen ring, can be arrived at only through substitution of the free hydroxyl groups with unhydrolyzable residues prior to hydrolysis, followed by identification of the hydrolysis products. Irvine (168) in 1909 pointed out the applicability of the methylation reaction for this purpose. His principles were quickly adopted by workers generally in this field and have become more or less standard practice. Haworth and his co-workers (169, 151, 96) have extended the application of the methylation reaction to include the determination of the chain length of polysaccharides.

Methylation of the glycosidic hydroxyl group, with the formation of methyl glycosides, dates from the time of Emil Fischer (170). Methyl alcohol, in the presence of acid catalysts, as used by him, is still employed today for this purpose.

The classical method of methylation of the alcoholic hydroxyl groups in non-reducing carbohydrates is that of Purdie and Irvine (171), and consists of treatment with methyl iodide in the presence of silver oxide. The procedure is, however, slow and expensive, and a distinct advance was made with the introduction of dimethyl sulphate and alkali

as methylating agents. Denham and Woodhouse (172) in a preliminary investigation on cellulose, carried out methylations by treating cellulose, previously steeped in sodium hydroxide solutions, with dimethyl sulphate.

It remained for Haworth (173), working at that time in Irvine's laboratory, to develop the procedure by means of which the reaction, which now bears his name, could be most successfully carried out. He also later introduced the simultaneous deacetylation and methylation of acetyl compounds, in cases where standard type methylation was found to be difficult.

Diazomethane, which finds application in many organic methylations, has been little used in carbohydrate chemistry. It is generally considered to react only with hydroxyl groups of an acidic nature, but in the presence of traces of moisture it is known to methylate polysaccharides (174, 175). Where methylation by means of this reagent is possible, the formation of inorganic salts as by-products of the reaction is avoided.

The use of methyl iodide has been extended by utilizing the preliminary formation of a metallic derivative, followed by treatment with the halide. Fear and Menzies (176), by treating methyl glucoside with thallium ethylate, formed a tri-thallium methyl glucoside (85% yield), and this

on refluxing with methyl iodide gave a trimethyl methyl glucoside in good yield.

Schmid and Becker (109) in 1925 investigated the formation of metallic alkali derivatives of various carbohydrates using liquid ammonia as a solvent. They also suggested the possibility of forming methyl ethers by subsequent treatment of these metallic derivatives with methyl iodide. This, however, was apparently not attempted until 1934, when Muskat (57) reported the methylation of diacetone glucose by treatment of the potassium derivative in ether solution with methyl iodide. By a modification of this method, the complete methylation of dextran finally has been accomplished by the writer.

Most of the complex polysaccharides, such as cellulose (65, 74) mannan (161, 157), glycogen (91, 177, 83), inulin (96), levan (178), graminin (119,121), sinistrin (122), triticin (123) and araban (179), consist of long linear or intricately branched chains. When such a compound is methylated and hydrolyzed, every non-aldehydic end group yields tetramethyl glucose, every branched union yields dimethyl glucose, while trimethyl glucose is obtained from all remaining units. Besides the search for end groups (tetramethyl hexoses and trimethyl pentoses), the location of the positions of branching (dimethyl hexoses and monomethyl

pentoses) is becoming of increasing importance. The presence, therefore, of a dimethyl hexose among the hydrolysis products of a methylated polysaccharide could have a very definite structural significance, so that the importance of complete methylation cannot be over-emphasized. A methoxyl value for the methylated polysaccharide 1 or 2% lower than the theoretical, can render the results worthless with respect to the position of branched-chains and consequently to the structure assigned to the polysaccharide. Complete methylation, as many workers have found, is impossible by the use of dimethyl sulphate and alkali alone. Despite the above rather obvious facts, many papers (145, 130, 162) have reported the results of hydrolysis of incompletely-methylated polysaccharides in which the methoxyl content was as low as 42.5% (162) or 44% (145), (theoretical 45.6%). The English school (62, 145, 130) in particular use only the Haworth methylation technique, and while their final yields may approach 65% of the theoretical (62), a methoxyl content of 44.5% for the methylated polysaccharide (62) seems to be the maximum obtained by this procedure.

In this respect it is of interest to note the difficulty experienced in exhaustively methylating starch to a value of 45.6% methoxyl. Karrer (180), using methyl iodide and silver oxide obtained 32.6% methoxyl. Schmid and

Zentner (175) found only 21% using diazomethane. Irvine and Macdonald (181) by means of dimethyl sulphate and alkali obtained a 61% yield of a product with 27.2% methoxyl; Freudenberg (182) obtained 38% methoxyl by the same method. Hess and Lung (183) increased the methoxyl content by the dimethyl sulphate method to 42-43%, and finally to the theoretical value using the Muskat method.

It was only after the exhaustive methylation of starch was made possible that the proportion of end groups (tetramethyl glucose) was shown to be approximately equal to the proportion of branching positions (dimethyl glucose) for every twenty glucose units (89).

On the other hand, mannocarolose, the mannan produced from glucose by the action of the mold Penicillium charlesii G. Smith (130), on hydrolysis of its methylated derivative, yielded 10% of dimethyl methyl mannoside in addition to tri-, and tetramethyl methyl mannosides (page 42). The presence of the dimethyl methyl mannoside was assumed to be due to incomplete methylation, since the methylated polysaccharide had a methoxyl content of only 44.4%.

Complete methylation of Dextran I to a theoretical value of 45.6% methoxyl (based on three free hydroxyl groups per glucose anhydride unit), has offered almost insuperable difficulties, and much time and effort have been spent in

overcoming these. Previous workers (49; pages 39-40) in these laboratories used a wide variety of methylating agents both known, and not previously used, for this purpose. Thus for example it was early found that by the use of dimethyl sulphate and caustic soda it was possible to raise the methoxyl value after six or seven methylations to about 40-41% (theoretical for fully methylated product 45.6%), but in spite of repeated additional methylations it was not possible to obtain a higher value by the use of this methylating agent.

It may be that the difficulty was due to the initial formation of a dimethyl derivative, which resisted further methylation. It is interesting to note, in this respect, the similar difficulty encountered in the methylation of starch (page 54) where a methoxyl content corresponding approximately to a dimethyl starch was obtained with comparative ease, and which proved resistant to further methylation.

Attempts to increase the methoxyl content of dextran by the use of methyl borate were unsuccessful (49), but the use of thallium ethylate and methyl iodide brought about an increase in value (49). Even with this reagent, however, the final methoxyl value could not be increased beyond 43.3%.

Attempts to effect complete methylation were also tried using the Purdie methylation technique (silver oxide

and methyl iodide) but the results were likewise unsuccessful, the maximum methoxyl content remaining at 43.3% (49).

Complete methylation of Dextran I to the theoretical value of 45.6% OCH_3 now has been achieved by the writer using a combination of the Haworth and Muskat techniques.

Throughout these later investigations, every precaution was taken to obtain yields of material as nearly quantitative as possible, in order that conclusions drawn could be based on results involving the greater part of the original material, and therefore be of real significance. With dextran and its derivatives, this involved reducing experimental manipulations and transfers to a minimum, since these compounds are very difficult to handle on account of their physical properties.

The Dextran I to be methylated had been prepared by previous workers (48). No purification was carried out prior to Haworth methylation, since the impurities (page 99) were removed during the partial purification of the Haworth-methylated dextran.

The Haworth methylation technique was modified (page 99) and better yields (90%) of product with a higher methoxyl content (40.5% methoxyl) were obtained with only three methylations. This can be compared to the five to twelve treatments reported by previous workers who obtained

average methoxyl contents ranging from 43% to 44.5% (49, 56, 61, 62). This more efficient methylation was partly due to the rapid and complete stirring of a fine suspension of the dextran.

To lessen possible degradation of the partially-methylated dextran, an atmosphere of nitrogen was maintained during both the Haworth methylations, and concentrations of solutions, also heating of strongly alkaline solutions for appreciable periods of time was avoided. For example, after a methylation, the reaction mixture was first neutralized and then made just slightly alkaline before being heated to destroy sodium methyl sulphate. Also, all evaporations were carried out at reduced pressures (20 mm.) and at low temperatures (maximum 50°). Excess alkalinity and acidity were avoided at all times.

During the Haworth methylations large quantities of sodium sulphate are formed as a by-product, and dialysis was found to be the best method of eliminating this inorganic material. Various types of dialyzers were tried, but all were found unsuitable either because an exceptionally long time was required to dialyze away the sodium sulphate, or because large amounts of dextran passed through the membrane and were thus lost. The latter in particular was the chief difficulty which many workers in this laboratory had encountered

previously and was one of the main reasons for the reported low yields of methylated dextran (49, 56). The author found that the most suitable type of dialyzer was that described on pages 103-5. It is extremely simple, and very efficient. Cellophane (Du Pont #300, a non-coated product), permitted ready dialysis of sodium sulphate with no appreciable loss of partially-methylated dextran, particularly at temperatures about 35°. The Haworth methylation reaction mixture originally contains large quantities of precipitated sodium sulphate, but this passes into solution and is rapidly removed during dialysis.

The methylated dextran was less soluble in water (alkaline solution) than native dextran, and precipitated out during the Haworth methylation. It was possible therefore to remove most of it from the reaction mixture by centrifugation. Dialysis of the supernatent methylation solution could then be continued without risking the loss of the bulk of material in case the dialysis sack should break. Dialysis was continued until the sodium sulphate no longer crystallized out when the dialyzed solution was concentrated to a volume sufficiently small to be readily remethylated. This usually involved a dialysis period of nine hours.

Partially-methylated water-insoluble dextran, containing approximately 40% methoxyl was obtained by three

successive Haworth methylations and further treatment by this method did not increase the value. Washing with hot water was the method used, at this stage, for the partial purification of methylated dextran. The relatively large amounts of ash (Ca. 9%) which were not removed, apparently did not lessen the efficiency of the Muskat methylation.

Methylations were continued, using various modifications of Muskat's method (57) which have been reported in the literature but none of these was found suitable in this case.

The first variation attempted was that described by Fowler (49). Approximately an additional one percent methoxyl could be introduced by each methylation until a maximum value of 43% methoxyl was reached. This method also required many transfers of material resulting in large losses.

The Muskat procedure was, therefore, slightly modified as described by Freudenberg (184), but in this case the maximum methoxyl value obtainable was even lower than that obtained previously.

The method which finally proved successful was an original modification of the Fowler and Freudenberg procedures. The construction of the apparatus (page 111) (Diagram II) designed for the Muskat methylations of

partially-methylated dextran was such that no removal of product was necessary prior to final purification. Here again approximately an additional one percent was, in general introduced by each methylation, although in several cases it was found that the first Muskat treatment was several times as efficient as the later methylations. Efficient stirring also plays an important role in the progress of the methylation since although partially methylated dextran is soluble to some extent in liquid ammonia, the sodium salt of methylated dextran exists only as a suspension in both ammonia and anisole.

With the apparatus shown it was possible to form the sodium salt, allow it to react with methyl iodide, and then to repeat the whole process several times without removing the material from the reaction flask.

These Muskat methylations were carried out in an atmosphere of nitrogen wherever feasible. This was especially important during vacuum evaporations. Temperatures above 50°, and strongly alkaline or acid solutions were avoided, and the greatest care was taken to exclude moisture during the methylations. Degradation changes resulted when the sodium salt of partially methylated dextran was exposed to heat, air or moisture.

The greatest losses of material occurred during the purification of methylated dextran. These were inevitable

because of the nature of the substance. It was primarily to minimize these, and to save time, that the apparatus shown in Diagram II (page 111) was designed, and in which a series of three Muskat methylations were carried out before attempting purification of the product. The impurities are essentially sodium iodide, and some methyl ammonium iodides, the latter being formed in spite of all precautions taken to remove ammonia before addition of methyl iodide. The methylated dextran is soluble in chloroform leaving the sodium iodide as a residue. The soluble quaternary ammonium salts however are precipitated with the methylated dextran on addition of petroleum ether. Also, methylated Dextran I is insoluble in hot and cold water while sodium iodide and quaternary ammonium salts remain in the aqueous solution. Several treatments in this manner removed most of the impurities.

After the water treatments, the dried methylated dextran was dissolved in chloroform. Usually there was no appreciable residue, but any insoluble material could be readily separated from the chloroform solution by centrifugation and filtration. One to two volumes of petroleum ether (30-50°) were added to the chloroform solution until a noticeable turbidity developed, and this precipitate, which contained most of the remaining impurities, was separated by centrifugation and filtration. The supernatent

solution was then precipitated into sufficient petroleum-ether (30-50°) to make the final ratios of methylated dextran: chloroform: petroleum ether approximately 1:10:200. By use of this procedure and one precipitation only, a powdery product was obtained much purer than that isolated previously after several precipitations, and in which no precautions had been taken to remove ash. This procedure is simpler, and safer than the purification by dialysis previously used (56).

The yield of fully-methylated dextran after six Muskat methylations was 79.3% calculated on the weight of Haworth-methylated (40.5% OCH_3) product taken, and 71.4% calculated on that of the original dextran. This yield is considerably higher than that obtained by previous workers in the dextran field. It is to be noted that earlier investigators (56, 61, 54), with one exception (60), carefully avoided any mention of actual yields of fully methylated dextran obtained by Muskat methylation. It is also apparent that a much higher yield (page 119) of methylated dextran could have been obtained if fewer Muskat methylations had been carried out, but the dextran would not have been fully-methylated, and the isolation of a dimethyl methyl glucoside on hydrolysis of this partially-methylated dextran would not necessarily have had any structural significance (page 54).

The homogeneity of fully-methylated Dextran I was indicated by means of viscosity determinations of the fractionated products (Table V, page 120). Divergencies in these results are of the same order of magnitude as those previously reported by Daker and Stacey (145) for homogeneous methylated vermiformé dextran. The fractions were therefore considered to represent similar degrees of aggregation.

An attempt was similarly made to show the homogeneity of the native Dextran I used for the methylations. This was unsuccessful, however, due to the failure to find a suitable solvent for Dextran I.

Hydrolysis of Methylated Dextran I

Fully methylated Dextran I proved extremely resistant to hydrolysis. This together with the insolubility of native dextran in water and most common organic solvents, indicated it as being the most highly polymerized dextran hitherto investigated.

There are two principal methods used for the hydrolysis of methylated polysaccharides:

(a) Methanolic hydrogen chloride (56), whereby, in one step, the polysaccharide is hydrolyzed to its component sugars and these in turn converted to the corresponding glycosides.

(b) The use of a dilute acid such as hydrochloric acid in 50% aqueous acetic acid (61, 62, 145), followed by isolation of the free sugars and their conversion to the glucosides by means of methanolic hydrogen chloride.

Method (a) was first tried since it was a one step procedure. The methylated Dextran I was insoluble in methanol hydrogen chloride, while the hydrolysis products were soluble. The complete disappearance of solid material was therefore used as an indication of the progress of the hydrolysis. Failure of the hydrolysis solution to reduce Fehling's solution was the test used for the complete

conversion of the free sugars to the glucosides.

In the first experiment, methylated Dextran I was refluxed, at atmospheric pressure and in an atmosphere of nitrogen, with methanol containing 2% hydrogen chloride. After twelve hours, the dextran had still not gone into solution. Similar results were obtained when 8% hydrogen chloride was used and the heating period extended to twenty hours. In this case the dextran was hydrolyzed sufficiently into shorter chains to permit of a partial solution of the material. However, complete hydrolysis to the individual sugars was not obtained.

Further hydrolysis experiments were carried out in sealed glass tubes under pressure. Methylated dextran showed no physical changes after being in contact with methanol containing 2% hydrogen chloride under the above conditions for 48 hours at 95-98°. Here again longer periods of heating, or more concentrated acid solutions merely degraded the dextran.

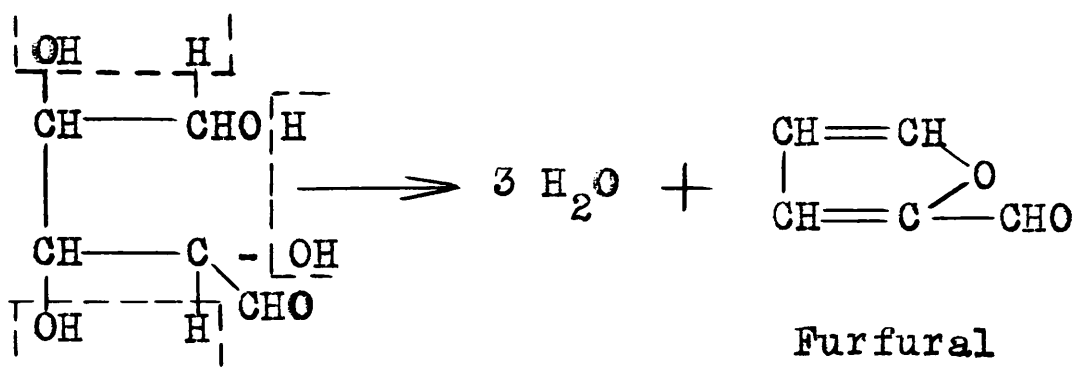
Complete hydrolysis was finally achieved by heating a mixture of methylated dextran and methanol containing 2% hydrogen chloride, to 140-142°, in a tilting electric oven for 60-65 hours.

Under the drastic conditions required for the complete hydrolysis of the methylated dextran, <u>methyl levulinate</u>

(4 %) was formed as a decomposition product. This was not detected until an attempt had been made to fractionate the hydrolysis mixture through an efficient fractionation column, (described in the experimental section (page 130) when it was found in the first fraction collected, the boiling-point being much lower than that of the methylated glucosides. This may explain why previous workers (49, 56) have reported a yield of only about 85% for the hydrolysis of methylated Dextran II, since they used a Widmer flask for the fractionation, and the receivers were not cooled. The methyl levulinate, with other possible decomposition products thus escaped condensation.

Other investigators (185, 186) have also observed the formation of small amounts of methyl levulinate during their investigations on the hydrolysis of methylated polysaccharides. In general, however, this fact has been overlooked in the majority of the investigations reported in the literature. It is the writer's belief that in future investigations, especially of the more complex polysaccharides, now attracting the interest of carbohydrate chemists, and which, because of their complex nature, require drastic conditions for hydrolysis, the detection and determination of by-products such as mentioned above will have to be considered very carefully.

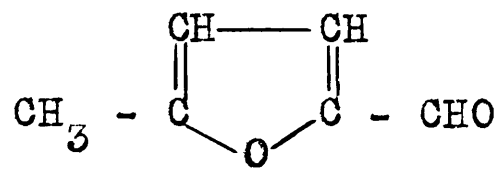
The formation of levulinic acid from glucosides is well known (187). All pentosans, on heating with hydrochloric or sulphuric acid, undergo dehydration with formation of furfural (188).



Pentose

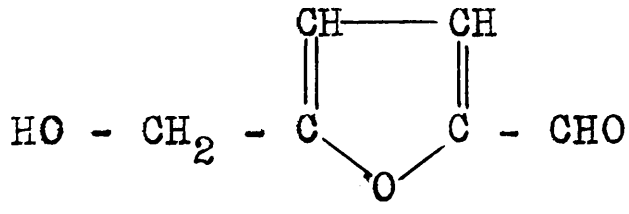
Furfural

In an analogous manner, methyl pentoses yield methylfurfural (189)



Methylfurfural

From hexoses ω -hydroxy methylfurfural is formed.



ω -hydroxy methylfurfural

This latter, however, in presence of acids loses formic acid with formation of levulinic acid (190, 191, 192), the mechanism of the reaction being unknown. A characteristic reaction of hexoses can therefore be formulated, according to Tollens (193), as:

$$C_6H_8O_3 + 2H_2O \longrightarrow H.COOH + CH_3COCH_2CH_2.COOH$$

Levulinic Acid

The detection of methyl levulinate in the hydrolysis mixture raised the question as to what extent the three methylated sugars, 2,3,4,6-tetramethyl glucose, 2,3,4-trimethyl glucose, and 2,3-dimethyl glucose, as well as their corresponding glucosides, decompose to give this product under the conditions used for hydrolysis. It was found that each of the free sugars decomposed in this manner, and to the same extent (about 1.5% methyl levulinate isolated as 2,4-dinitro-phenyl-hydrazone), while the corresponding glucosides were stable under the same conditions. This side reaction, therefore, does not affect the final ratio assigned to the three glucosides (page 78). The decomposition to methyl levulinate therefore takes place during the interval between the hydrolysis of the methylated dextran to its methylated sugars and their subsequent conversion to the glucosides.

It was found necessary to remove the methyl levulinate prior to fractionation by heating an aqueous

solution of the glucosides with barium hydroxide, whereby the levulinate was hydrolyzed and removed as the barium salt.

The overall yield of the hydrolysis products from methylated Dextran I, after removal of the methyl levulinate, was 95%. Of the 5% of the material lost during hydrolysis, 4% of this could be accounted for as methyl levulinate. A methoxyl determination of the glucosidic mixture at this stage gave a value of 52.3% OCH_3, (calculated for $C_6H_8O_2(OCH_3)_4$, OCH_3 = 52.6%). The mixture did not reduce Fehling's solution, showing the absence of free reducing sugars.

The second method (b) was used for the hydrolysis of methylated dextran in an attempt to avoid the undesirable formation of methyl levulinate, but without success. A mixture of 50% aqueous acetic acid and 2% aqueous hydrochloric acid did not completely hydrolyze the methylated dextran after heating to 100° for 25 hours. At the same time a black insoluble residue formed. The long time of contact under the above conditions decomposed rather than hydrolyzed the dextran.

Fractionation of the Hydrolysis Products

The quantitative separation of mixtures of partially-methylated glucosides, obtained on hydrolysis of methylated polysaccharides, has been the object of extensive investigations, and as yet no completely satisfactory solution of this problem has been achieved. Three general methods have been used, but several of these techniques, described in recent years, are extremely complicated and require large amounts of the methylated polysaccharide. As mentioned above, Schlubach and co-workers (119) in their investigation on graminin benzoylated their trimethyl fructose in order to separate it from the corresponding tetramethyl compound, but, due to the unsatisfactory yields, were unable to establish the definite ratio of the sugars. So far no procedure has been reported which permits an effective quantitative separation of mixtures of tetra-, tri-, and dimethyl methyl glucosides. The use of fractional distillation, involving a vacuum-jacketed Widmer flask, or a flask fitted with a vacuum-jacketed fractionating column, has been employed by Haworth and co-workers (169, 194, 186), the course of the fractionation being determined by frequent interruptions of the distillation in order to test the refractive index and rotation of the distillates. In several instances (169, 194)

the mixture of glucosides has first been separated into two major fractions and each of these subjected to a second fractionation. This procedure usually resulted in a relatively high proportion of non-volatile residue. It has been the practice of Haworth and co-workers (169) to allocate this non-volatile residue among the various glycosidic products in the ratio of their equivalent weights, corresponding to the amounts isolated. This assumption can not be justified unless it is verified by control experiments, as has been done in this investigation with respect to the formation of methyl levulinate during hydrolysis. It is the author's belief that this residue is essentially a decomposition product formed as the result of admitting air to the distillation flask during the numerous interruptions in the fractionation procedure.

This general fractionation technique of Haworth has been applied to the determination of the structure of xylan (195, 196, 197), glycogen (194, 83, 144, 198), araban (179), starch (87), and dextran (56, 62, 145).

Hess and Neumann (77), finding the fractional distillation technique as described by Haworth and Machemer (169) inapplicable to the study of cellulose structure, devised a chemical separation for the detection of small amounts of tetramethyl methyl glucoside in the presence of a

large proportion of trimethyl methyl glucoside. Their method, which involved phosphorylation of the trimethyl methyl glucoside, and separation of the resulting ester as its barium salt, has been criticized by Leckzyck (199) and by Hirst and Young (186), who showed by control experiments that only 50% of the tetramethyl methyl glucoside was recoverable by this chemical separation. Furthermore it was demonstrated (185, 186) that the tetramethyl methyl glucoside, isolated in this way, was contaminated with trimethyl methyl glucoside.

In a third type of fractionation, due to Macdonald (58) the separation of tri-, from tetramethyl methyl glucoside was effected by chloroform extraction of the more soluble tetramethyl methyl glucoside from an aqueous solution of the mixture. Macdonald states that a mixture of di-, tri- and tetramethyl methyl glucosides can be separated into its components each having a purity of 97.4% with a total loss of 2.5%. However, this procedure has not found general application in structural carbohydrate chemistry, and Carrington, Haworth and Hirst (200) have pointed out that some trimethyl methyl glucoside must dissolve in the chloroform. They have shown by control experiments that no sharp separation between di-, and trimethyl glucoses can be effected through a water-chloroform partition, since dimethyl methyl glucoside is also extractable by chloroform.

In some cases (194, 49, 56) a combination of this method followed by fractional distillation, according to Haworth and Machemer (169), of the resulting fractions has been used.

Since one of the main objects of this investigation was to employ only strictly quantitative procedures wherever possible (page 57) none of the above methods was considered satisfactory for the fractionation of the glucosidic mixture obtained from methylated Dextran I and a new procedure was developed. This involves the use of a highly efficient fractionation column, its construction being based on the fractionation principles and technique described by Podbielniak (201).

Several samples of synthetic 2,3,4,6-tetramethyl methyl glucoside and of 2,3,4-trimethyl methyl glucoside were distilled through the column and the recovery of distillate was in each case approximately 92%. The samples varied in size from 0.85 to 2.41 grams. During the fractionation of mixtures consisting of the above glucosides with 2,3-dimethyl-α-methyl glucoside, the small amount (less than 0.1 gram) of material held up in the column could also be forced over, permitting an over-all recovery of approximately 95%, even when the total weight of glucosides was only three to four grams.

Several fractionations were carried out with

mixtures of the three glucosides, and in all cases, the bulk of these products was recovered in the three corresponding main fractions, which were analytically pure; intermediate fractions were small. In the case of 2,3,4-trimethyl methyl glucoside, whether alone or in mixtures of glucosides, an actual separation was effected of the solid β - from the liquid α -isomer. There was little if any decomposition of the glucosides. The non-volatile residue was less than one percent, and in many cases could not be detected.

The results of one of these fractionations is summarized in Table II (page 76). Fractions 3, 4 and 5 all came over under identical conditions and actually constitute one fraction, namely, 2,3,4-trimethyl methyl glucoside. In other experiments they were actually collected as one fraction. During this particular fractionation, however, the cuts were made as indicated, since it was desired to keep a close check on the composition of the distillate at all times.

The theoretical methoxyl values for tetra-, tri- and dimethyl methyl glucosides are 62.0, 52.6 and 41.9%, respectively. The amounts of each present in the small intermediate fractions 2 and 6 (Table II) were calculated on this basis.

Table II

Fractionation of Synthetic Mixture of Glucosides

Fraction No.	Weight Grams	OCH_3 %	Tetra	Tri	Di
1	0.746	60.5	0.746		
2	0.151	59.6	0.113	0.038	
3	0.387	52.5		0.387	
4	0.665	52.3		0.665	
5	0.257	52.4		0.257	
6	0.300	50.8		0.249	0.051
7	0.451	42.1			0.451
Total	2.957		0.859	1.596	0.502

Original Mixture (Wt. = 3.048 grams)

Weight of 2,3,4,6-Tetramethyl methyl glucoside	0.872 gr.
Weight of 2,3,4-Trimethyl methyl glucoside	1.660 gr.
Weight of 2,3-Dimethyl methyl glucoside	0.516 gr.
Total	3.048 gr.

Total recovery 97.0%.

The glucosidic mixture obtained from the hydrolysis of completely methylated Dextran I was fractionally distilled (97% recovery) using the apparatus and technique described in the experimental section (page 130) whereby 2,3,4,6-tetramethyl methyl glucoside , 2,3,4-trimethyl methyl glucoside

and 2,3-dimethyl methyl glucoside were obtained in the exact ratio of 1:3:1.

Table III gives a summary of these fractionation results.

Table III

Fractionation of the Hydrolysis Products of Methylated Dextran I

Fraction No.	Weight gr.	% OCH_3	Tetra	Tri	Di	Physical State
1	1.204	61.0	1.204			Colorless syrup
2	0.207	57.8	0.113	0.094		Colorless syrup
3	0.605	52.8		0.605		White crystals
4	2.811	52.4		2.811		White crystals (2,3,4-β-methyl glucoside) and colorless syrup (α-isomer)
5	0.397	46.5		0.172	0.225	Colorless syrup
6	1.013	42.0			1.013	Light yellow syrup
Total	6.237		1.317	3.682	1.238	

Weight of glucosidic mixture fractionated 6.430 gr.

Total weight of recovered glucosides 6.237 gr. (97%).

Calculation of the Ratio

$$\text{Tetra} = \frac{1.317}{250} \times 100 = 0.00527 \text{ moles}$$

$$\text{Tri} = \frac{3.682}{236} \times 100 = 0.01560 \text{ moles}$$

$$\text{Di} = \frac{1.238}{222} \times 100 = 0.00558 \text{ moles}$$

Taking Tri as Standard and equal to 3.00

$$\text{Tetra} = \frac{0.00527}{0.01560} \times 3 = 1.01$$

$$\text{Tri} = \frac{0.01560}{0.01560} \times 3 = 3.00$$

$$\text{Di} = \frac{0.00558}{0.01560} \times 3 = 1.07$$

The ratio of tetra:tri:di is 1.01:3.00:1.07.

IDENTIFICATION OF THE GLUCOSIDES

Identification of the Tetramethyl and Trimethyl Methyl Glucosides

The tetramethyl methyl glucoside was characterized as the crystalline 2,3,4,6-tetramethyl glucose. The trimethyl methyl glucoside was identified through its crystalline isomer as 2,3,4-trimethyl-β-methyl glucoside.

Identification of the Dimethyl Methyl Glucoside

For the final identification of the dimethyl methyl glucoside obtained from methylated Dextran I it was necessary to prepare a crystalline derivative, quantitatively if possible, since this would prove its homogeneity.

Many attempts were made to prepare a crystalline mono-trityl derivative with the object of proving the presence of a free primary hydroxyl group in the 6-position, since Helferich (202) has shown that trityl chloride reacts preferentially with primary hydroxyl groups. Although many workers have assumed that this reagent is specific for the presence of a primary alcoholic group, Hudson and co-workers (202, 203, 204) have shown that this is not the case, and

that in the absence of such a group, reaction with secondary hydroxyl groups may occur. In no case was it possible, in this investigation to obtain a crystalline trityl derivative.

The dimethyl methyl glucoside as obtained from the methylated dextran and subsequent fractionation of the glucosides, is a mixture of α and β isomers. Consequently any derivatives made by reaction with the free hydroxyl groups will also be a mixture of α and β isomers, therefore very difficult to separate.

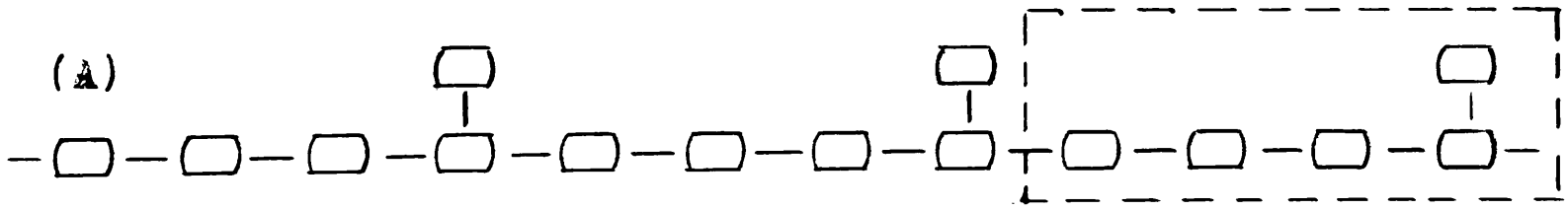
To overcome this difficulty, an attempt was made to prepare a derivative by interaction with the carbonyl group in the free sugar. Anilide formation, mercaptalation (benzyl and ethyl mercaptan), and benzoylation of free dimethyl glucose yielded syrups in each case which could not be crystallized.

The dimethyl methyl glucoside was finally identified as the 2,3-dimethyl derivative by the formation of 2,3-dimethyl gluconophenylhydrazide. The procedure followed for the formation of the 2,3-dimethyl gluconic acid was that of Hudson and Isbell (205, 206). The resulting gluconic acid was obtained in 84% yield as a syrup and was characterized as the crystalline phenylhydrazide which was obtained in 81% yield. This new compound was identical with that similarly obtained from a sample of authentic 2,3-dimethyl α-methyl glucoside.

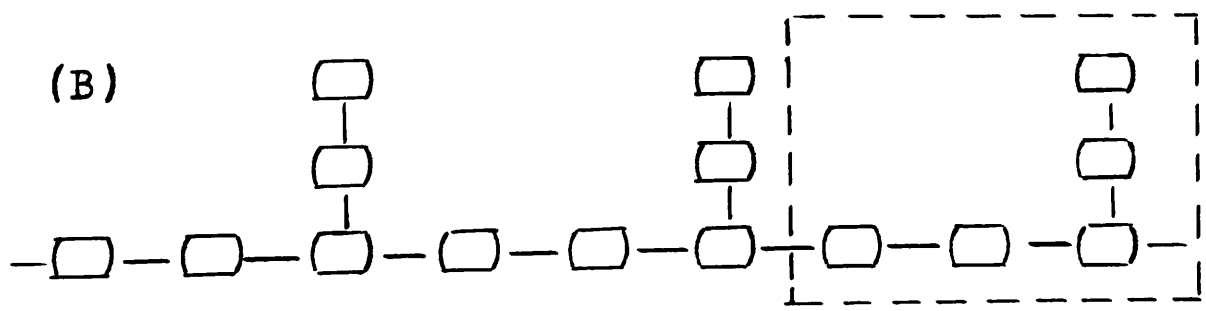
The Structure of Dextran I

The fractionation results shown in Table III (page 77) indicate a 1:3:1 ratio for the three components, isolated as their glucosides from methylated Dextran I, namely 2,3,4,6-tetramethyl glucose, 2,3,4-trimethyl glucose and 2,3-dimethyl glucose respectively. On the basis of this ratio it can be seen that, irrespective of the manner in which the glucose units are linked to one another, there are four possible formations for the gross structure of Dextran I. If we represent each hexose unit by ⬡, these four possibilities may be formulated as follows:

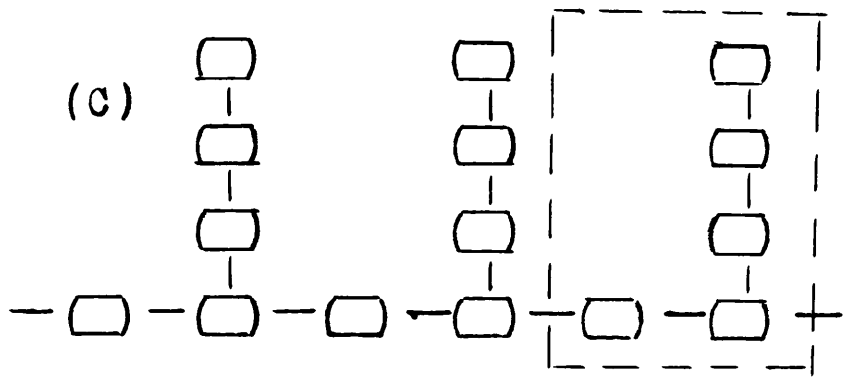
(A)



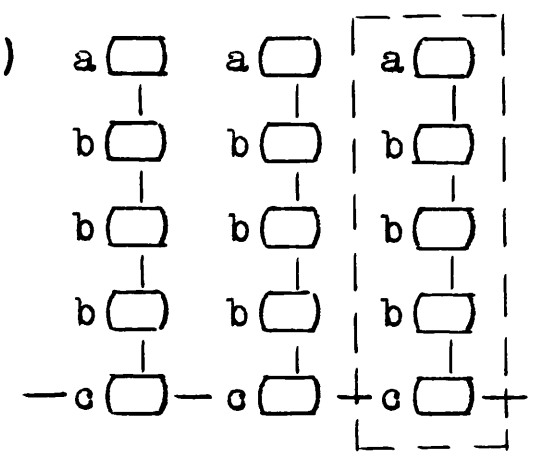
(B)



(C)



(D)



The portion of each structure enclosed by dotted lines represents the repeating unit.

In all of the structures shown above, <u>each</u> singly linked terminal unit (a---a) (D) would yield on hydrolysis of fully methylated dextran, one molecule of tetramethyl methyl glucoside; <u>each</u> doubly linked unit (b---b) (D) one molecule of trimethyl methyl glucoside; and <u>each</u> trebly

linked unit (c---c)(D) a molecule of dimethyl methyl glucoside, and in each case, the ratio of the yields of these three would be 1:3:1.

It is evident that a definite choice between the four formulae given above can be made only after considerable further investigation of the products formed by a graded hydrolysis of methylated dextran. It would be necessary to determine at which glucose anhydride unit, in a typical five unit chain, the cross-linkage is formed. This would involve complete methylation of the dextran, followed by a selective hydrolysis such as might break the glucoside bond of attachment of the side chains, while leaving the main chains themselves intact. This might be accomplished by chemical means, although it is probable that enzyme hydrolysis would prove more satisfactory, especially if the bonds between side and main chains differ in stereochemical configuration from those between the glucopyranose units forming the main chain, since enzymes are known which attack only alpha or beta glucoside links respectively, but not both.

For the purpose of this discussion of the positions of linkage between the individual glucose units, structure (D) may be selected quite arbitrarily.

With respect to the glucose units present at the end of each side chain (a---a) (D), since Dextran I is a

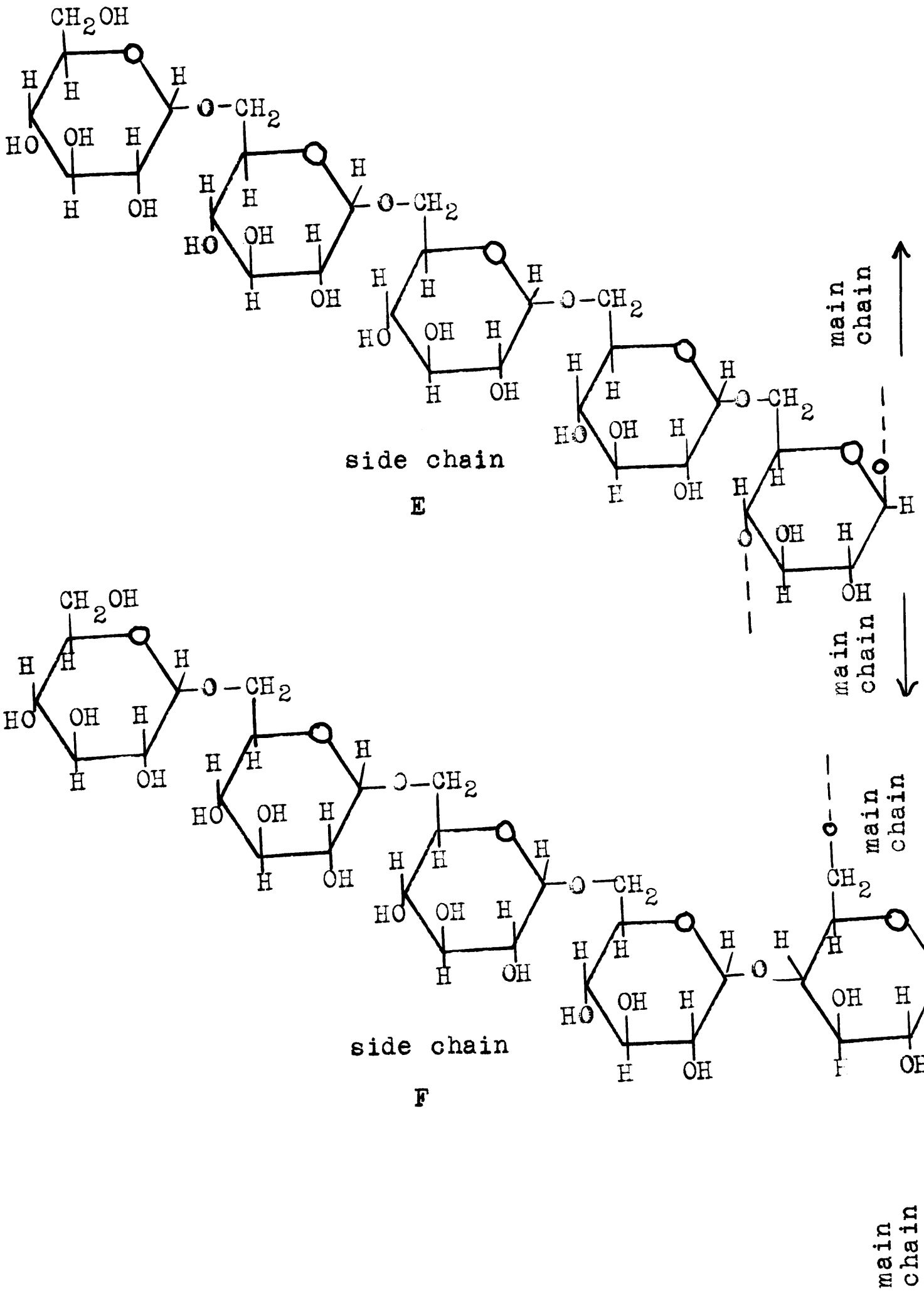
non-reducing polysaccharide, it is obvious that these must be linked through their reducing groups as must also each of the remaining glucose anhydride units present in (D). Furthermore, this mode of linkage of (a) through carbon atom 1, is the only one which can give rise to tetramethyl methyl glucoside on methylation and hydrolysis. The isolation of the latter as 2,3,4,6-tetramethyl glucose proves that this unit is present in the pyranose form in the original polysaccharide and that it is linked to the rest of the side-chain through carbon atom 1.

Also it has been shown (page 79) that the trimethyl glucose units (b---b) (D) are isolated as the 2,3,4-trimethyl methyl glucoside. Therefore, in these side chain units, since the hydroxyl group in the 6 position is the only one not methylated, these linkages in the original polysaccharide are through carbon atoms 1 (the reducing group) and 6. Moreover, since the hydroxyl groups in positions 2,3 and 4 are free in the original polysaccharide side-chains, the original ring structure of this unit must be of the pyranose type.

The dimethyl methyl glucoside (c---c) (D) is obviously formed from the glucose anhydride units through which the methylated side-chains are attached to the main chains, and has been shown conclusively (page 80) to be 2,3-dimethyl methyl glucoside. Assuming a pyranose ring

structure for unit (c), formula (D), there are two possible modes of linkage of this to the main chain, namely through carbon atoms 4 or 6 (see (E) and (F) respectively (page 86). However, the true nature of the oxygen ring in this unit as present in the <u>original</u> dextran cannot be definitely proven from the present experimental data. The dimethyl methyl glucoside isolated on hydrolysis will have the pyranoside structure irrespective of its original presence in either the pyranose or furanose form. The possibility of ring shift during hydrolysis from position 4 to position 5 cannot therefore be overlooked, and this question must remain unsettled until proven by enzymatic or other preferential hydrolysis breakdown procedures. However, in view of the fact that the remaining glucose anhydride units have pyranose structures it may be assumed for the present, that this is true for all the units present.

It also follows that polymerization of the main chain must take place either through carbon atoms 4 or 6 of the units (c) in (D), that of the side chain linkage being through 6 or 4 respectively. As to which position is actually involved in each case nothing definite can as yet be stated. These two possibilities may be represented structurally as follows:


main chain
side chain
main chain
main chain
side chain
main chain

E

F

This investigation has not included determinations of the molecular weight of Dextran I. Dr. R.G. Fordyce (207), in these laboratories, made a determination of the viscosity of Dextrans I (page 12) and II (page 12) and although the actual molecular weights could not be determined due to the inability to calculate the K_m constant of the Staudinger equation, it appears from these results that Dextran I has a molecular weight of the order of ten times that of Dextran II. This observation is borne out by the considerably greater solubility, and ease of hydrolysis of methylated Dextran II (60, 49). Apparently Dextran I differs from Dextran II only in respect to the molecular weight. This is not particularly surprising, since the same polysaccharide in different species of plants does not necessarily have the same molecular weight even though the monosaccharide units and the general pattern in which they are joined may be the same. This appears to be the case for example between glycogens from different sources (177, 194, 91). Such differences will probably be found the rule rather than the exception.

This fact may be of some importance in the immunological phase of this problem, since many of the properties of polysaccharides are more directly the result of a certain molecular architecture rather than that of a function of the constituent sugar units.

The possibility that Dextran I is a simple pentasaccharide, having one terminal glucose anhydride unit is somewhat unlikely, since its general behaviour is that of a higher polysaccharide, so that a simple ring formula, such as that assigned to graminin (page 33) is highly improbable. The fact that end group analyses (reducing power, etc.) of Dextran I show a negative result, just as in the case of cellulose, indicate that the polysaccharide has a high molecular weight. Estimation of the chain length by a quantitative determination of the amount of tetramethyl methyl glucoside formed on hydrolyzing the fully methylated dextran is also inapplicable, since, if there are many side chains present, the tetramethyl methyl glucoside isolated would only be a measure of the total number of glucose anhydride units present in the individual side chains and not of the actual number of such side-chains present in the dextran molecule.

If there were a sufficiently large number of repeating units of the five-membered side-chain type present (formula (D)), any variation in the 1:3:1 ratio for the tetra-:tri-:dimethyl methyl glucoside arising from the presence of the two terminal side-chains (1:4:0), could not be detected. Furthermore, the possibility exists that these two terminal side-chains actually may be united through cross-linkages.

Dextran I does not show a close relationship in chemical structure to Dextran III (page 12). The presence of a tetramethyl methyl glucoside in the hydrolysis products from the fully methylated Dextran III has not been definitely established (61, 62) (page 18) nor has the configuration of the dimethyl methyl glucoside (10% by weight) (61) been determined.

In conclusion it may be stated that Dextran I appears to have a structure more complex than that of any polysaccharide hitherto subjected to critical chemical investigation. The products obtained on methylation and hydrolysis have been identified, and the ratio in which they occur determined. From these data conclusions have been drawn regarding the positions of linkage in the polysaccharide, and the possibilities for its gross structure have been pointed out.

SYNTHESIS OF REFERENCE CARBOHYDRATES

It was found that although most of the reference sugars required in these investigations had been prepared previously, either the details of the procedure were incompletely reported in the literature, or the yields were low. It was the purpose of this phase of the problem to develop a technique for the synthesis of such compounds capable of giving reproducible yields sufficiently high to be applied not only to glucose but to rarer sugars.

The syntheses of 2,3-dimethyl- α-methyl glucoside, 2,3-dimethyl- β-methyl glucoside, 2,3,4-trimethyl- β-methyl glucoside and 2,3,4,6-tetramethyl glucose were undertaken for three principal reasons:

(a) to serve as "controls" for comparison with the methylated sugars isolated from the hydrolysis of methylated dextran,

(b) to permit development of a fractional distillation technique (using these synthetic glucosides) for the later separation of the hydrolysis products of methylated dextran, and

(c) to carry out control experiments, such as the determination of the stability of the methylated sugars and their corresponding methyl glucosides under the conditions used for the hydrolysis of methylated Dextran I.

In addition, 2,3-dimethyl glucose, 2,3,4-trimethyl glucose, 2,3,4-trimethyl- α-methyl glucoside, 2,3,4,6-tetramethyl- α-methyl and β-methyl glucosides, and 2,3-dimethyl gluconophenylhydrazide were prepared. The last named compound was required for comparison with that prepared from the dimethyl methyl glucoside obtained from hydrolyzed methylated Dextran I.

Throughout these syntheses, hydrolyses were carried out using dilute sulfuric acid, rather than dilute hydrochloric acid, the conventional hydrolytic agent, since sulfuric acid is easily removed as barium sulfate. Also, Haworth methylation (in an atmosphere of nitrogen) was substituted for the laborious Purdie technique.

2,3,4,6-Tetramethyl Glucose

The preparation of 2,3,4,6-tetramethyl glucose as hitherto carried out (209) is neither a quantitative nor a one-step procedure. The Haworth methylation of glucose (209) results in low yields of a partially-methylated glucose. One methylation of glucose using the modified procedure outlined in the Experimental Section (page 143) yielded a mixture of tetramethyl, trimethyl, and dimethyl methyl glucosides, as indicated from the refractive indices and methoxyl analyses

of various fractions of the mixture isolated by fractional distillation.

Similar results were obtained after one Haworth methylation of α-methyl glucoside.

Two methylations of α-methyl glucoside as described (page 143) gave a 90% yield of relatively pure 2,3,4,6-tetramethyl-α-methyl glucoside. This yield is considerably higher than obtained previously (210, 211), so that no appreciable degradation of the partially-methylated glucoses could have occurred during the Haworth methylation in an atmosphere of nitrogen. Of the other methods attempted, one (211) involved a modified Haworth methylation followed by Muskat methylation.

Hydrolysis of 2,3,4,6-tetramethyl α-methyl glucoside was carried out, with sulfuric rather than by use of dilute hydrochloric acid, or use of steam and hydrochloric acid (210).

2,3,4-Trimethyl Methyl Glucosides

There are two principal methods described in the literature for the preparation of 2,3,4-trimethyl methyl glucosides. The first (212) may be summarized in the following manner:

α-methyl glucoside $\xrightarrow[\text{pyridine}]{\text{trityl chloride}}$ 6-trityl-α-methyl

glucoside $\xrightarrow[CH_3I]{Ag_2O}$ 2,3,4-trimethyl-6-trityl-α-methyl

glucoside $\xrightarrow[HCl]{CH_3OH}$ 2,3,4-trimethyl-α-methyl glucoside.

The yields obtained by this procedure are generally low, particularly in the first step.

The second method of preparation (213) involves the following steps:

Laevoglucosan $\xrightarrow[Ag_2O]{CH_3I}$ 2,3,4-trimethyl

laevoglucosan $\xrightarrow[HCl]{\text{dilute}}$ 2,3,4-trimethyl

glucose $\xrightarrow[HCl]{CH_3OH}$ 2,3,4-trimethyl-α- and β-methyl glucosides.

A modification of the latter procedure was used to synthesize the 2,3,4-trimethyl methyl glucosides required for these investigations.

It was found that laevoglucosan was too soluble in water to permit of ready recrystallization as reported (213), so this was carried out from ethanol solution with satisfactory results.

The Haworth methylation of laevoglucosan has not previously been reported. However, the 1,6-oxygen bridge in laevoglucosan was found to be quite stable under the conditions used (page 145). In preliminary experiments, lower temperatures and shorter reaction periods were used, but these led to low yields of incompletely-methylated products, and therefore normal conditions for Haworth methylation were employed (page 145).

It was found that the simplest method of extraction of carbohydrates of relatively high methoxyl content from the Haworth methylation reaction mixture was to reflux the latter with chloroform, and separate the chloroform layer. An additional advantage of this method was the fact that higher methylated products were found preferentially soluble in chloroform (58), while those of lower methoxyl content remained in the water layer, which could be concentrated and remethylated.

It was found that part of the chloroform extract of the methylation solution of laevoglucosan could be crystallized, and the remainder remethylated. The crystalline fraction, 2,3,4-trimethyl laevoglucosan, did not require purification by distillation as carried out by Irvine and Oldham (213).

Sulfuric instead of hydrochloric acid (213) was

used in the hydrolysis of trimethyl laevoglucosan and higher yields, practically quantitative, obtained. The conversion of 2,3,4-trimethyl glucose to the corresponding mixture of α- and β-methyl glucosides could also be carried out on a nearly quantitative basis.

Extensive decomposition of 2,3,4-trimethyl glucose resulted when this was distilled, even in an atmosphere of nitrogen, at lower temperatures and pressures than used by Irvine and Oldham (213). Possibly the best method of preparing pure 2,3,4-trimethyl glucose would involve conversion of the crude product to the mixed glucosides, purification of the β-glucoside by recrystallization, and hydrolysis to 2,3,4-trimethyl glucose.

2,3-Dimethyl Methyl Glucosides

2,3-Dimethyl-α-methyl glucoside was prepared[x] using a method only slightly modified from that improved and developed by previous workers in these laboratories (56, 214), and outlined below:

---

[x] 2,3-Dimethyl-α-methyl glucoside and 2,3,-dimethyl-β-methyl glucoside were prepared by Dr. T.H. Evans.

Glucose $\xrightarrow[HCl]{CH_3OH}$ α-methyl glucoside $\xrightarrow[ZnCl_2(anhyd.)]{benzaldehyde}$

4,6-benzylidene-α-methyl glucoside $\xrightarrow[NaOH]{Me_2SO_4}$ 2,3-dimethyl-

4,6-benzylidene-α-methyl glucoside $\xrightarrow[H_2SO_4]{dilute}$ 2,3-dimethyl-

α-methyl glucoside.

An overall yield of α-methyl glucoside amounting to 75% was obtained, that of the benzylidene derivative being 80%.

Syntheses of 2,3-dimethyl-β-methyl glucoside were carried out by two methods (A) and (B).

(A) Glucose $\xrightarrow[NaOAc]{Ac_2O}$ penta-acetyl glucose $\xrightarrow[HOAc]{HBr}$

acetobromoglucose $\xrightarrow[Ag_2O]{CH_3OH}$ tetra-acetyl-β-methyl glucoside

$\xrightarrow{Ba(OH)_2}$ β-methyl glucoside $\xrightarrow[ZnCl_2(anhyd.)]{benzaldehyde}$ 4,6-benzylidene-

β-methyl glucoside $\xrightarrow[NaOH]{Me_2SO_4}$ 2,3-dimethyl-4,6-benzylidene-

β-methyl glucoside $\xrightarrow[H_2SO_4]{dilute}$ 2,3-dimethyl-β-methyl

glucoside.

The final product was crystalline. Oldham's method (215) using dilute hydrochloric acid as the hydrolytic agent in the final step did not yield a crystalline compound.

(B) This was an adaptation of that of Oldham (208).

2,3-Dimethyl-α-methyl glucoside $\xrightarrow[H_2SO_4]{\text{dilute}}$ 2,3-dimethyl glucose $\xrightarrow[\text{pyridine}]{\text{benzoyl chloride}}$ tribenzoyl-2,3-dimethyl glucose $\xrightarrow[HOAc]{HBr}$ dibenzoyl-bromo-2,3-dimethyl glucose $\xrightarrow[Ag_2O]{CH_3OH}$ dibenzoyl-2,3-dimethyl-β-methyl glucoside $\xrightarrow[CH_3OH]{K}$ 2,3-dimethyl-β-methyl glucoside.

The products isolated in the various stages were checked against their properties as recorded in the literature while the final product was subjected to careful purification by repeated recrystallization.

2,3-Dimethyl Gluconophenylhydrazide

2,3-Dimethyl gluconophenylhydrazide was prepared from 2,3-dimethyl glucose. The latter was oxidized by bromine-water, following the method of Hudson and Isbell (205) to the

corresponding gluconic acid in 84% yield, and this with phenylhydrazine gave the desired crystalline end product in 80.4% yield.

EXPERIMENTAL

Methylation of Dextran I

Haworth Methylation

Preliminary methylation of Dextran I was carried out employing the Haworth technique (173). The methylations were carried out in a three-litre, three-necked, round bottom flask, fitted with two dropping funnels, one in either side-neck, and with a motor driven stirrer and nitrogen inlet in the central neck. The flask was surrounded with a water bath kept at 30°.

Thirty grams (ash-free and sucrose-free basis[X]) of Dextran I (for preparation see (14)) were suspended in water (300 cc.) made alkaline by the addition of 25 cc. of 30% sodium hydroxide solution. The air in the flask was removed with nitrogen gas, and a slow stream of the latter maintained throughout the methylations. The mixture was stirred continually at a temperature of 30°.

During approximately one-half hour, sodium hydroxide (50 cc. of a 30% solution) was added dropwise from one funnel

---

X Analyses: % ash 1.6;1.6 average 1.6%
% sucrose 5.4;5.2 average 5.3%
Method for sucrose determination page 151.

and simultaneously dimethyl sulphate (20 cc.) from the other, with stirring. Ten such separate additions were made involving simultaneous addition of a total of 500 cc. sodium hydroxide and 200 cc. dimethyl sulphate. In this way an alkaline reaction was assured throughout the entire methylation. The reaction mixture was then stirred thoroughly for eighteen hours, at the same temperature, partially neutralized with approximately 150 cc. of 50% sulphuric acid, and heated by means of the water bath to 100° for thirty minutes to decompose sodium methyl sulphate. The water bath was removed and the reaction mixture stirred for one hour while cooling to room temperature. It was then completely neutralized (to phenolphthalein) with approximately 50 cc. of 50% sulphuric acid, the temperature being kept below 15° by means of an ice-water bath. Finally 30% sodium hydroxide (10 cc.) was added until slight alkalinity to phenolphthalein.

During the methylation, sodium sulphate was formed in large amounts as a by-product, some of which precipitated out at this stage. The entire reaction mixture was dialyzed against warm (35°C.) circulating (1200 cc. per minute) tap-water, for nine hours using the apparatus and method described on page 103. This removed the greater part of the sodium sulphate so that on concentration to 300 cc. no separation of the latter occurred.

All concentrations of methylated dextran solutions were carried out in a slightly alkaline medium (pH about 7) at reduced pressure (15-20 mm.), in an atmosphere of nitrogen and at a temperature below 50°.

The concentrated dialyzed solution (500 cc.) was remethylated twice by the above procedure (total of three Haworth methylations). In these second and third methylations the greater part of the sodium sulphate settled to the bottom of the flask, while most of the dextran (which became less water soluble as the methoxyl content increased) remained suspended in the liquid and was removed by centrifugation at this stage and set aside while the remainder of the mixture was dialyzed. This was merely a protective measure in case the dialysis membrane should break during dialysis. This dextran was replaced in the flask after the dialyzed solution had been concentrated to 300 cc.

In a typical series of methylations the methoxyl content increased as follows:

First methylation - not determined[x]

Second methylation - 25.7%[xx]

Third methylation - 40.5%.

---

[x] No methoxyl analysis were carried out after the first methylation since it was known beforehand from previous experiments that further methylations were necessary.

[xx] Methoxyl analyses were made according to the method of Viebock and Schwappach (216) as modified by Peniston and Hibbert (217).

In earlier experiments a fourth Haworth methylation was carried out but this did not increase the methoxyl value appreciably. At this stage the methoxyl content was still below that of the calculated value (45.6% OCH_3) for three hydroxyl groups per glucose anhydride unit. Methylations were therefore continued by the Muskat method (page 110).

Haworth Methylations of Dextran I

Weight of crude Dextran I	33.0 gm.
Ash Content	1.6 %
Sucrose Content	5.3 %
Actual weight of Dextran I	30.7 gm.
Weight of crude Dextran I after three Haworth Methylations	37.3 gm.
Ash Content	9.0 % [x]
Actual weight of Dextran I after three Haworth Methylations	34.0 gm.
OCH_3 Content of ash-free[xx] product after three Haworth Methylations	40.5 %

---

[x] It was not necessary to further purify the partially methylated Dextran I at this stage since the inorganic impurities did not interfere with the Muskat methylations. The omission of this step contributed greatly to the final high yield of fully methylated Dextran I obtained (page 118).

[xx] Analytical sample purified until ash-free before analysis (Method page 106).

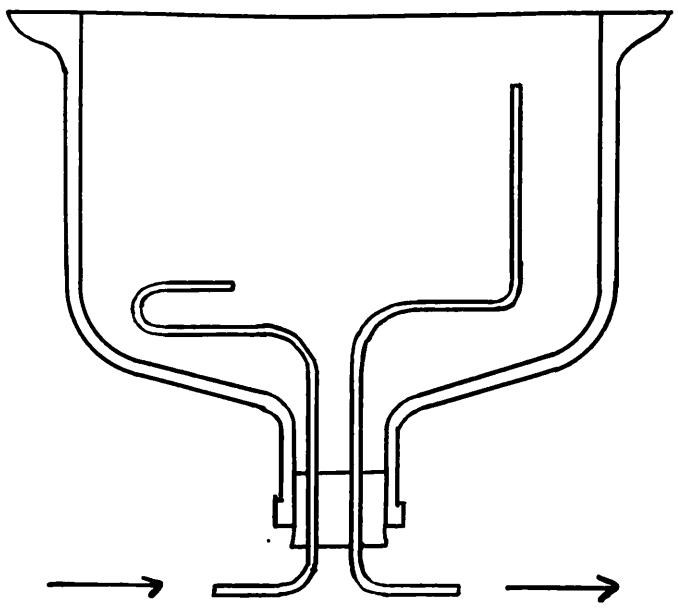
Thus the yield of Haworth methylated Dextran I (on an ash-free basis) was 34.0 grams and the percentage yield calculated on the basis of methylated Dextran I with a methoxyl content of approximately 40.5% (i.e. approximately 2.6 OCH_3 groups per glucose anhydride unit) was approximately 90%.

Dialysis Technique

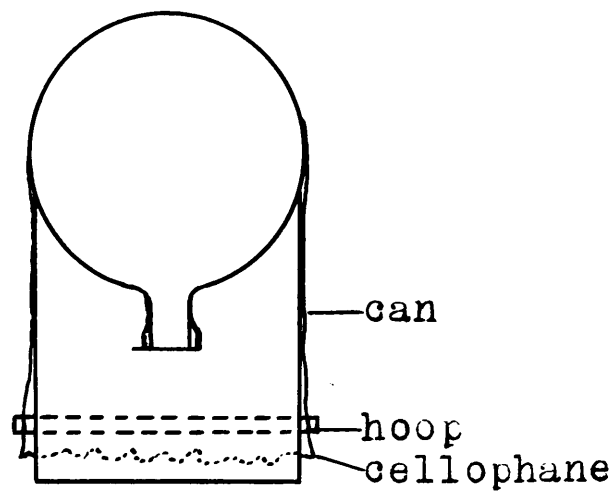
The factors to be considered regarding the dialysis of the methylation mixtures were (1) type of membrane; (2) time required to remove the sodium sulphate; (3) amount of dextran lost by passage through the membrane.

Dialyzer

A dialyzer was assembled in the following manner: The top of a large (diameter 11") vacuum desiccator was stoppered with a two hole rubber stopper, and inverted on a tripod. The water inlet was a glass tube, extending vertically through the rubber stopper, then bent horizontally a few inches, to give a streaming effect to the incoming tap-water (Figure IA). The outlet tube was bent, after passing through the stopper, to conform with the shape of the desiccator top, and extended thereafter vertically almost to the top of the latter.



IA.



IB.

The sack-shaped dialyzing membrane consisted of Dupont #300 Cellophane (a non-coated product) and was made in the following manner: A one litre round-bottom flask was inverted in a tin container as shown in Diagram IB. A wooden embroidery ring (diameter 6") was slipped over the flask and half way down the container. A sheet of cellophane of suitable size (approximately 18" square) was carefully folded over this, avoiding the formation of sharp edges and points, and a second embroidery ring which fitted tightly over the first one was slipped over the cellophane. The sack thus formed was then removed from the "mold" and had a capacity of approximately 3000 cc. The cellophane bag was suspended in the dialyzer by means of copper wires attached to the embroidery hoops. Before use, each bag was tested for leaks, when suspended in, and containing 2000 cc. water. Both hot and cold water-taps were connected with the inlet tube of the dialyzer, and wide variations in the temperature

of the circulating tap water were therefore possible. Solutions were stirred mechanically during dialysis periods.

This dialyzer and procedure employed were very efficient and losses of methylated dextran were small, the sodium sulphate being rapidly removed.

Dialysis Experiments

(a) Dialysis of Sodium Sulphate Solutions

The shortest time in which the greater part of the sodium sulphate could be removed from a solution comparable in volume to that dealt with during methylations of Dextran I was first determined. Solutions containing 450 grams of sodium sulphate per litre were dialyzed for 22, 12, 9 and 6 hours respectively. In each case the volume increased to 2000-2500 cc. during dialysis, and the solution was then concentrated to 300 cc. In none of these experiments, except the last, did any sodium sulphate crystallize out of solution after concentration. Six hours was therefore below the minimum time necessary for a suitable dialysis, while nine hours was the most suitable length of time. During the latter period, approximately 90% of the sodium sulphate was removed. Only 42 grams out of the original 450 grams of sodium sulphate remained after dialysis.

(b) Dialysis of Suspensions of Dextran I

Similar test experiments with suspensions of Dextran I (20 grams in 1000 cc. water) showed that during a nine hour dialysis period approximately 9% of the dextran was lost. For example in a typical experiment 19.1 grams of dextran suspended in 1000 cc. water were dialyzed using the above procedure and 17.5 grams recovered (8.4% loss). During the dialysis of the methylation mixtures of Dextran I these losses were even smaller (approximately 10% overall loss for three dialyses. See page 105.

Suspensions of Haworth methylated dextran were therefore dialyzed for nine hours against tap-water at 35°, because this temperature permitted of a more rapid removal of sodium sulphate with less loss of methylated dextran, the latter being less soluble in hot water than in cold water.

Purification of Haworth Methylated Dextran

A small aliquot (containing approximately 0.4 grams of partially methylated Dextran I) was withdrawn for methoxyl analyses from the concentrated, dialyzed solution after each methylation. This was washed four times, successively, with distilled water (25 cc. each time) and the dextran separated by centrifugation after each washing. The sulphate-free

dextran was then washed with alcohol (15 cc.), followed by ether (15 cc.) and dried in a vacuum oven at 55° for 30-40 hours. The dried white powder was dissolved in anhydrous chloroform (5 cc.) and petroleum ether (30°-50°) added to this solution in a fine stream until a turbidity appeared. This slight precipitate was removed by centrifugation and filtration. The clear filtrate was then added in a fine stream to twenty volumes of petroleum ether (30°-50°), the fluffy-white dextran precipitate removed by centrifugation and dried. The last part of the procedure outlined above (precipitation from chloroform into petroleum ether) was repeated twice (a total of three precipitations) until an analytical sample of partially methylated Dextran I free from ash was obtained.

Muskat Methylation of Partially-Methylated Dextran I (57)

Partially methylated Dextran I (OCH_3 40.5%) was further methylated by a series of Muskat (57) methylations, consisting essentially of sodium salt formation of partially methylated dextran in liquid ammonia and treatment of this with methyl iodide. For a successful methylation by this method, apparatus, reagents and dextran must be absolutely dry.

Method

Anhydrous anisole was prepared by refluxing stock anisole over sodium metal for six hours, and then distilling over sodium b. pt. 152-3°(uncorr.).

Anhydrous Ammonia

Ammonia was withdrawn directly from the pressure tank, passed through a small wash-bottle containing glycerol, to serve as a bubble counter, then through a 30 cm. drying tower containing potassium hydroxide flakes and finally through a 30 cm. tower containing sodium wire.

Drying of the Partially Methylated Dextran I

The dialyzed mixture of partially methylated Dextran I obtained after the third Haworth methylation (page 102) was evaporated to dryness at 40° under reduced pressure in an atmosphere of nitrogen. The residue was digested with hot distilled water and the mixture centrifuged to separate it from any remaining sodium sulphate. This procedure was repeated four times. The dextran was then washed with alcohol, centrifuged, washed with diethyl ether, centrifuged and dried in the vacuum oven at 55°C. for 48 hours. A powdery white product of partially methylated Dextran I was obtained.

Fowler Modification of Muskat Technique (49)

The Muskat (57) technique first employed was that used by Fowler (49) but although satisfactory with Dextran II (49) (page 12) proved unsatisfactory with Dextran I. A small sample (2.5 grams) of carefully purified, ash-free (page 106) partially-methylated Dextran I (40.7% OCH_3) was methylated twice by this procedure without any increase in methoxyl content. Weight of product after two methylations 1.9 grams, ash nil, 40.9% OCH_3.

Freudenberg Modification of Muskat Method (184)

Methylations involving the use of sodium and methyl iodide in liquid ammonia, as described by Freudenberg (184) was applied to native (unmethylated) Dextran I. Two grams (ash 4.1%) were placed in a reaction flask of the type described by Freudenberg (184) and 20 cc. of dry ammonia condensed on the product. A methylation consisted of three successive treatments employing in each of them one gram of sodium and 4 cc. of methyl iodide. Two such complete methylations were carried out. The methoxyl content was raised to 6.0% OCH_3 on the first methylation and to 9.3% on the second methylation, while approximately 50% of the material (0.9 grams) was lost during these procedures mainly during the purification processes. Because of these losses

this method was abandoned.

New Modification of Muskat Method

Further methylations of partially-methylated Dextran I (OCH_3 40.5%) were carried out in an improved type of apparatus (Figure II). The design was a modification of that used by Fowler (49) and by Freudenberg (184) for the Muskat methylation of Dextran II and cellulose respectively.

Apparatus

The apparatus (Figure II) was constructed throughout with standard ground glass joints. The reaction flask (F) was a Pyrex cylinder of approximately 500 cc. capacity fitted with a small side-tube (t). A two-necked adapter (A) which fitted into the flask, carried a condenser (C) fitted in one neck, and a coupling (H) for a mercury seal stirrer in the other.

Procedure

Vacuum-oven dried Haworth methylated dextran was placed in the reaction flask and stirred with dry anisole (10 cc. of anisole per gram of methylated dextran) for six hours in an atmosphere of nitrogen. A thin gel eventually resulted, the solubility of the methylated dextran increasing with methoxyl content.

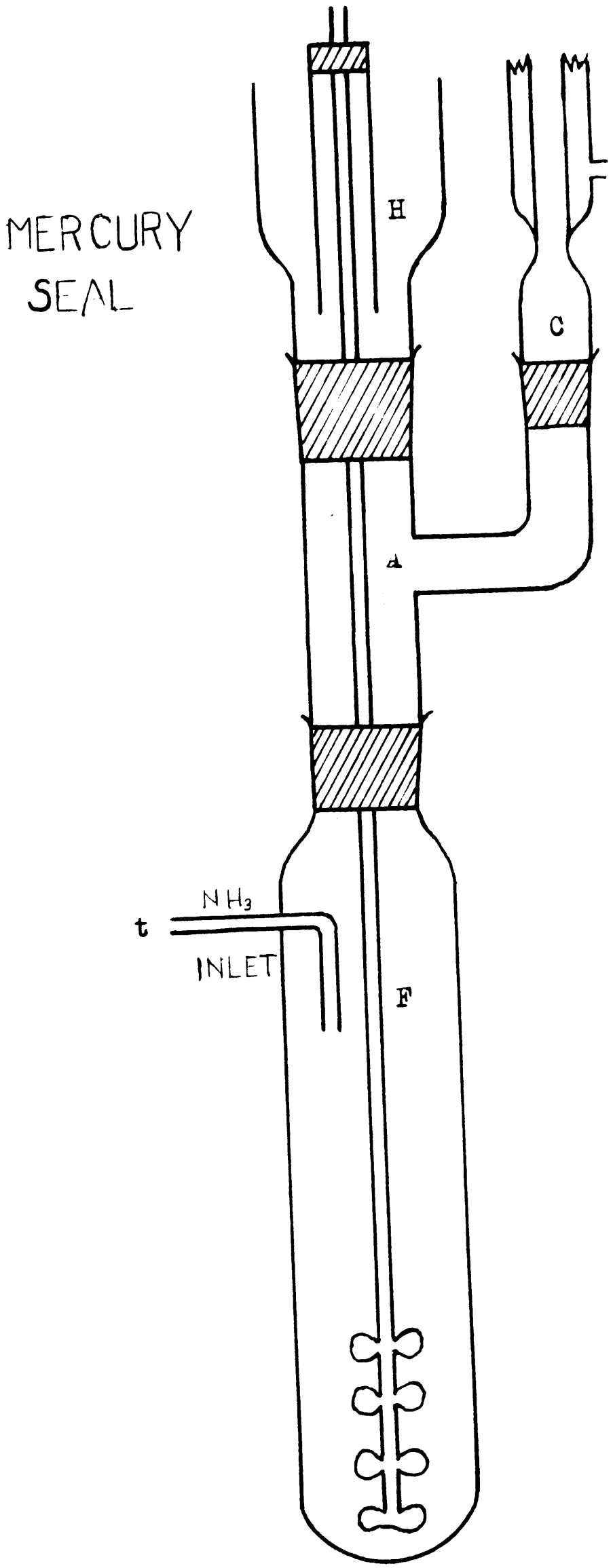


Diagram II.

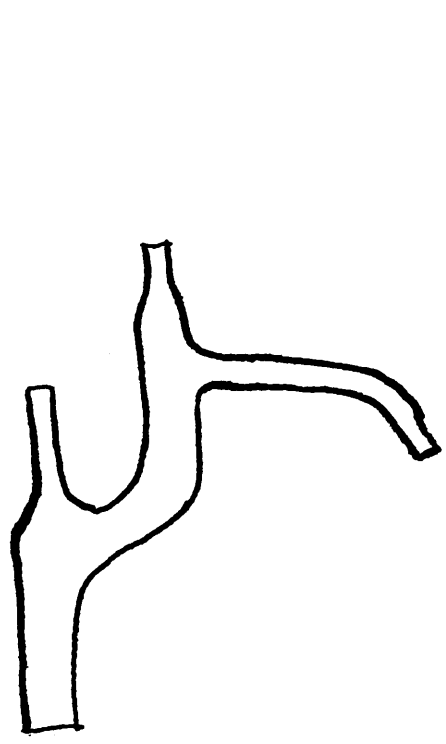


Diagram IIA.

To assure anhydrous conditions, approximately 10% of the anisole was distilled from the reaction mixture at reduced pressure in a nitrogen atmosphere (60° bath temperature). For this purpose a ground-glass connected distilling head (Figure IIA) was inserted in the neck of the reaction flask. This eliminated the necessity of transfering the dextran in any operation thus avoiding subsequent mechanical losses.

The anisole suspension of partially-methylated dextran was frozen by cooling to -70°C. using a dry ice bath of chloroform and carbon tetrachloride. To ensure anhydrous conditions, approximately 0.5-0.9 grams of freshly cut sodium metal was added in small pieces through the neck (C) into which the condenser was fitted, the condenser being raised momentarily for this purpose. Dry ammonia gas was then introduced through the side arm (t) and condensed on the frozen anisole suspension (200 to 300 cc. liquid ammonia). Two to three times the theoretical amount of freshly cut sodium was added in small pieces through C as above.

Theoretical amount of sodium required =

$$\frac{\text{Weight of dextran x (45.6 - } OCH_3 \text{ content of dextran) X 23}}{31 \text{ x } 100}$$

where 45.6 is the theoretical maximum methoxyl content of trimethyl dextran, 23 the atomic weight of sodium and 31 the radical weight of methoxyl.

After sufficient ammonia had been condensed on the frozen anisole and solution of the alkali metal was complete, the Dewar flask surrounding the methylation flask was gradually lowered during 1/2 to 3/4 of an hour, and chloroform-carbon tetrachloride (equal portions) at room temperature, was added to the cooling bath, until the temperature was raised to approximately -55°C. This temperature lies between the melting point of anisole and that of the boiling point of ammonia. When the anisole had melted, the Dewar flask was removed and stirring started to aid in the formation of the sodium derivative of the partially methylated dextran. (The gradual lowering of the Dewar flask was necessary since a methylation flask after use for about ten methylations would occasionally crack due to the marked temperature change when the Dewar flask was lowered suddenly.)

The blue-color of the sodium-ammonia complex formed when stirring was started generally changed to a dark brown color. In some cases the blue color disappeared almost as soon as stirring commenced and additional sodium (0.2 to 0.5 grams) was then added.

The temperature of the reaction mixture was allowed to rise to 18-20° during which the ammonia evaporated spontaneously. The last traces were removed by evaporation at reduced pressure (20 mm) followed by distillation, also

at reduced pressure, of a few cc. (10-15 cc.) of anisole in an atmosphere of nitrogen, the bath temperature being maintained below 60°. Methyl iodide was now added (two equivalents per one equivalent of sodium) and the mixture refluxed at 60° for fifteen hours with stirring.

Sufficient dry anisole was then added to the reaction product, and a portion removed under reduced pressure in order to free the product from any residual methyl iodide and traces of moisture. Subsequent methylations were then carried out in the same manner.

The details of a typical series of Muskat methylations using a partially-methylated Dextran I (page 102) are given below:

Methylated Dextran I, vacuum-oven dried, (40.5% OCH_3) weight on ash-free basis 34.0 grams, (Table IV page 119) was placed in the apparatus shown in Figure II with approximately 300 cc. dry anisole. The Muskat methylations were carried out as described above, using liquid ammonia approximately 200 cc.; sodium 3.5 grams; and methyl iodide 30 cc. for the first methylation.

The quantities of reagents used in the second Muskat methylation were ammonia, approximately 200 cc., sodium 2.5 grams, and methyl iodide 20 cc. and in the third ammonia 200 cc., sodium 2.5 grams, and methyl iodide 20 cc.

The methoxyl content of the partially-methylated Dextran I increased as follows:

After first methylation 41.5% OCH_3

After second methylation 43.3% OCH_3

After third methylation 44.0% OCH_3

(see also pages 101 and 117).

Weight of partially-methylated Dextran I after third Muskat methylation 29.7 grams.

It was found unnecessary to remove the inorganic salts from the partially-methylated dextran after each methylation so that three successive methylations could be carried out prior to purification. At this stage the large amount of inorganic material interfered with efficient stirring and was therefore removed.

Isolation and Purification of Muskat Methylated Dextran I

Most of the anisole in the reaction mixture was removed by evaporation under reduced pressure (15 mm.) at a bath temperature below 60° and in an atmosphere of nitrogen. Water was now added, the resulting mass thoroughly stirred and vacuum evaporation continued, with further additions of water, until only traces of anisole remained in the reaction product. The residual methylated dextran was thoroughly stirred with boiling water (100 cc.) and separated by

centrifuging the hot solution. The residual methylated dextran was further purified by treatment with three additional aliquots of boiling water using 100 cc., 50cc. and 50 cc. respectively, then dried in a vacuum desiccator over calcium chloride. (The first wash liquor was orange in color, the color of the remainder decreasing in intensity.)

The well dried (55°/20 mm. page 108) Muskat methylated Dextran I dissolved readily in cold chloroform (10 cc. chloroform per gram of methylated dextran) and any appreciable residue, e.g. sodium iodide, was removed by centrifuging the chloroform solution followed by filtration. One to two volumes of 30°-50° petroleum ether was added until an appreciable turbidity was apparent. This initial slight precipitate contained much of the residual ash, such as quaternary ammonium compounds and was separated by centrifugation and filtration. The clear filtrate was precipitated into 30°-50° petroleum ether (total volume twenty times that of chloroform used), and the resulting precipitate washed twice with fresh 30°-50° petroleum ether. The insoluble methylated dextran was separated by centrifuging at low speed for five minutes and dried in a vacuum-oven (55° and 20 mm.) for 30 hours.

Fully Methylated Dextran I

The product from the above series of Muskat methylations (29.7 grams, 44.0% OCH_3) was then further methylated three times using in each case approximately 1.5 grams of sodium and 15 cc. methyl iodide, and the product purified as above. Yield 27.6 grams.

The methoxyl content of the partially-methylated Dextran I increased as follows:

After fourth Muskat methylation	44.9% OCH_3
After fifth Muskat methylation	45.3% OCH_3
After sixth Muskat methylation	45.6% OCH_3

(see also page 119).

Analyses of Fully Methylated Dextran I used for Hydrolysis

Carbon and Hydrogen

Ash content nil.

I Weight taken 0.02588 gr; wt. of CO_2 0.04982 gr; wt. of H_2O 0.01845 gr. C = 52.5%; H = 7.92%.

II Weight taken 0.01542 gr; wt. of CO_2 0.02979 gr; wt. of H_2O 0.01120 gr. C = 52.7%; H = 8.07%.

Calculated for $C_6H_7O_2(OCH_3)_3$ C = 52.9% H = 7.85%.

Methoxyl

I Weight taken 0.01221 gr; required 21.46 cc. of 0.0502N sodium thiosulphate solution; OCH_3 = 45.6%.

II Weight taken 0.00828 gr; required 14.55 cc. of 0.0502N sodium thiosulphate solution; OCH_3 = 45.6%.

Calculated for $C_6H_7O_2(OCH_3)_3$, OCH_3 = 45.6%.

Yields of Methylated Dextran I

The total yield of fully-methylated Dextran I, based on 34.0 grams of Haworth methylated dextran (40.5% OCH_3) was 79.3% and based on the starting material (30.7 grams native Dextran I) was 71.4%. Table IV (page 119) gives a summary of the complete series of Haworth and Muskat methylations.

Viscosity Measurements of Fully Methylated Dextran I[x]

Viscosity determinations of various fractions of fully-methylated Dextran I (in one percent chloroform solution) were carried out at 25° using an Ostwald viscometer (218). The fractions were obtained by successive additions of petroleum ether (30°-50°) to a 10% solution of 21 grams of methylated Dextran I in chloroform until a precipitate appeared, centrifuging off the precipitate and repeating the procedure. Fraction I (3.00 grams) was precipitated after the addition of five volumes of petroleum-ether, fraction II

[x] Kindly determined by Dr. E.L. Lovell.

Table IV

Methylation of Dextran I

Crude weight 33.0 gm.
Ash 1.6 %
Sucrose 5.3 %
Weight of Dextran (Ash and Sucrose free) 30.7 gm.

Three Haworth Methylations

- Analytical sample
  Wt. 0.5 gm.
  40.5% OCH_3
- Partially methylated Dextran I (OCH_3 = 40.5%) Wt. 34.0 gm.

  Three Muskat Methylations

  - Analytical sample
    Wt. 0.4 gm.
    44.0% OCH_3
  - Partially methylated Dextran I (OCH_3 = 44.0%)
    Wt. 29.7 gm.

    Three Muskat Methylations

    - Analytical sample
      Wt. 0.5 gm.
      45.6% OCH_3
    - Fully methylated Dextran I (OCH_3 = 45.6%)
      Wt. 27.6 gm.

Yields: Haworth methylated product 90.1 %*: Muskat methylated product based on Haworth methylated product 79.3%; overall yield 71.4%.

* Calculated on the basis of 2.66 OCH_3 groups per glucose anhydride unit.

(17.0 grams) after ten to twelve volumes of petroleum ether and fraction III (0.7 grams) remained as a suspension after the addition of twenty volumes of petroleum ether and was recovered by evaporation of the solvents. These results are summarized in Table V.

Table V

Viscosities of Chloroform Solutions of Fractionated Methylated Dextran I

Fraction	Wt. of fraction gr.	% OCH_3	conc. mg./cc.	η_{sp} x 100	$\eta_{sp/C}$ x 10^5
1	3.0	45.6	19.96	12.45	1230
2	17.0	45.54	19.58	12.71	1385
3	0.7	45.2	20.14	11.68	834

Attempted Fractionation of Unmethylated Dextran I

Unmethylated Dextran I (0.782 grams) was suspended in 200 cc. of distilled water and vigorously stirred for twelve hours at room temperature and the resulting mixture centrifuged. The main portion of the dextran (0.466 grams; fraction I) remained undissolved. The centrifugate (a milky colloidal suspension) was concentrated at 50° to 20 cc. under reduced pressure in an atmosphere of nitrogen

and then 40 cc. (two volumes) of ethanol added in a fine stream with stirring. A finely divided white precipitate formed which was removed by centrifugation as above (0.212 gr; fraction II). On the addition of a further eight volumes of ethanol to the milky centrifuged solution, the turbidity increased but no further precipitate occurred. The mixture was evaporated to dryness under the same conditions as above (0.087 grams; fraction III).

No solvent could be found in which a clear solution of the above fractions could be obtained so that viscosity determinations were impossible. The above three fractions cannot be regarded as conforming to differences in solubility in water but rather to different degrees of dispersion.

Hydrolysis of Methylated Dextran I

(a) Attempted Hydrolysis with Acetic Acid (62)

A suspension of methylated Dextran I (0.30 grams) in a mixture of freshly distilled glacial acetic acid (5 cc.) and 4% aqueous hydrogen chloride (5 cc.) (i.e. 50% acetic acid containing 2% hydrogen chloride) was heated at 100° for thirteen hours under an atomsphere of nitrogen. Only a small portion of the dextran went into solution, which gradually turned dark brown in color.

The insoluble solid material (0.27 grams) was removed by filtration and the filtrate was evaporated under reduced pressure (20 mm.) leaving a non-distillable black residue. The long exposure of methylated Dextran I to the above conditions produced decomposition rather than hydrolysis of the material.

(b) Hydrolysis with Methanolic-Hydrogen Chloride

Fully-methylated Dextran I (7.100 grams) was hydrolyzed in three separate portions of 3.060 grams, 1.520 grams and 2.520 grams respectively.

In a typical hydrolysis of methylated dextran, 3.060 grams were suspended in anhydrous methanol (60 cc.) containing 2% hydrogen chloride, sealed in a glass bomb tube (approximately 60 cm. long and 2 cm. in diameter) and heated at 140-142° in a tilting electric oven for sixty to sixty-five

hours. At the end of this treatment the dextran had dissolved completely and the solution changed from a pale yellow to a clear reddish-brown color. The bomb was cooled slowly to 0°, opened, and the excess hydrogen chloride gas allowed to escape while the solution attained room temperature (one to two hours). The open end of the bomb was fitted during this period with a Kjeldahl trap to avoid loss in case of a too vigorous effervescence.

The solution was transferred to a centrifuge cup and neutralized to litmus with silver carbonate (the theoretical amount was not required since most of the hydrogen chloride had escaped prior to neutralization). The insoluble silver salts, removed by centrifugation and filtration were washed with three 15 cc. portions of anhydrous methanol, the washings being added to the original filtrate. At this stage the methanolic solutions from the three separate hydrolyses were combined and the solvent removed at 40°/20 mm. The residual syrup was taken up in anhydrous ether, filtered to remove a small trace of insoluble inorganic material, and taken to constant weight under reduced pressure (20 mm) (A).

During the fractionation of a hydrolysis mixture obtained from Dextran I in a previous experiment the first fraction was found to be largely methyl levulinate as shown

by its methoxyl content (26.8% OCH_3) and refractive index (1.450 at 24°). The theoretical methoxyl content of methyl levulinate is 23.8% and the refractive index at 15° is 1.424. A quantitative determination of the amount of methyl levulinate present in the above mixture therefore was carried out at this stage.

Quantitative Determination of Methyl Levulinate in the Hydrolysis Mixture

The above syrup (A) was dissolved in exactly 142 cc. of water so that a 10 cc. aliquot contained the glucosides originating from 0.50 grams of methylated Dextran I. To 10 cc. of this solution was added 50 cc. of 2,4-dinitrophenylhydrazine (containing 0.2 grams of reagent), the mixture shaken and allowed to stand twenty minutes at room temperature. The yellow precipitate was removed by filtration, dried under suction and finally in a vacuum desiccator. Yield 0.030 grams of 2,4-dinitrophenylhydrazone of methyl levulinate representing approximately 4.2%[x] decomposition of the methylated Dextran I to methyl levulinate. The product, recrystallized from ethanol, melted at 137-139° (uncorr.) (literature value 141-2°) (see page 125) (219) and a mixed melting point with an authentic sample of

---

[x] Calculated on the assumption that each molecule of methyl levulinate is derived from one molecule of methylated sugar, and taking $C_6H_9O_3(OCH_3)_3$ as the average formula for the mixture of tetra-, tri-, and dimethyl glucose.

2,4-dinitrophenylhydrazone of methyl levulinate showed no depression.

To the filtrate an additional 50 cc. of 2,4-dinitrophenylhydrazine were added and the solution allowed to stand at room temperature for two and one-half hours; no further precipitate formed.

In a series of control experiments it was found that methyl levulinate readily formed a 2,4-dinitrophenylhydrazone in quantitative yield (98%) whether alone or in mixtures of tetra-, tri-, and dimethyl methyl glucosides. After repeated recrystallizations from ethanol the derivative had a constant melting point of 137-139° although the pure derivative is reported (219), perhaps erroneously, to melt at 141-142°.

While the above procedure permitted a quantitative separation of the methyl levulinate, it was inapplicable for its removal from the glucosidic mixture prior to fractionation since the excess 2,4-dinitrophenylhydrazine would contaminate the glucosides. The method of Bell and Synge (206) was therefore employed after a test run using the following synthetic mixture had been found satisfactory.

Quantitative Removal of Methyl Levulinate from Glucosidic Mixtures

(a) Removal of Methyl Levulinate from Synthetic Mixtures

A mixture of 2,3-dimethyl-α-methyl glucoside (2.23 grams) and 2,3,4,6-tetramethyl methyl glucoside (1.83 grams) was dissolved in water (20 cc.) and methyl levulinate (1.00 grams) added to the solution. Aqueous barium hydroxide (10 cc. saturated at 92-93° containing approximately 9 grams of barium hydroxide) was added and the mixture kept at 63° for two hours. The mixture was then evaporated at 60-70°/ 20 mm. and the resulting residue hand extracted with the following successive portions of chloroform: 30, 20, 10, 10 cc.

The combined chloroform solutions were filtered, evaporated to dryness, and the syrup distilled at 110-180° under high vacuum (0.005 mm.). Weight of distilled glucosides, 3.88 grams (95.7% of starting glucosidic mixture).

2,4-Dinitrophenylhydrazine (25 cc. containing 0.1 grams of reagent) was added to several drops of the distillate and the solution allowed to stand several hours. No precipitate was obtained, indicating complete absence of methyl levulinate.

(b) Removal of Methyl Levulinate from the Fully-Methylated Dextran Hydrolysis Mixture

The remainder of the aqueous solution (132 cc.)(page 124) containing the hydrolysis products originating from 6.60 grams of methylated Dextran I was treated with aqueous barium hydroxide (20 cc.) as outlined in the preceding section. Weight of the distilled glucosides (clear pale yellow syrup) 7.24 grams (95% yield). Analysis: Found OCH_3 = 52.3%; calculated for $C_6H_8O_2(OCH_3)_4$, OCH_3 = 52.6%.

Stability of Methylated Sugars and Glucosides under the Conditions Employed for Hydrolysis of Methylated Dextran I

(a) Stability of the Methylated Sugars

2,3-Dimethyl glucose (0.442 grams, 30.3% OCH_3; theoretical 29.8% OCH_3); 2,3,4-trimethyl glucose (0.837 grams, 41.6% OCH_3; theoretical 41.9% OCH_3) and 2,3,4,6-tetramethyl glucose (1.112 grams, 52.4% OCH_3, theoretical 52.6% OCH_3) were each converted to their respective glucosides under conditions similar to those used for the methanolysis of methylated Dextran I.

Method

The methylated sugar was dissolved in anhydrous methanol (25 cc.) containing 2% hydrogen chloride and heated

in a glass bomb tube in a tilting electric oven at 140-143° for fourty-eight hours. The cooled solution was then worked up as described on page 126. The resulting non-reducing (to Fehling's solution), almost colorless syrup was dissolved in water (20 cc.) and treated with 2,4-dinitrophenylhydrazine solution (100 cc. containing approximately 0.8 grams of reagent) as described on page 124. The resulting precipitate, which formed at once, was filtered on a tared sintered glass crucible, dried in a vacuum desiccator for six hours and weighed. Mixed melting points of the recrystallized product (from ethanol) with authentic 2,4-dinitrophenylphenylhydrazone of methyl levulinate showed no depression. The amounts of methyl levulinate formed are shown in Table VI (page 129).

(b) Stability of the Methylated Glucosides

A mixture (1.386 grams) containing 2,3,4,6-tetramethyl methyl glucoside (0.372 grams), 2,3,4-trimethyl methyl glucoside (0.614 grams) and 2,3-dimethyl methyl glucoside (0.400 grams) was similarly treated (method page 122-124) with methanolic hydrogen chloride followed by 2,4-dinitrophenylhydrazine. No precipitate formed indicating the absence of methyl levulinate.

Table VI

Decomposition of Methylated Sugars to Methyl Levulinate

Sugar	Weight of Sugar gr.	Wt. of 2,4-dinitro-phenyl-hydrazone obtained mg.	Corresponding weight of methyl levulinate mg.	Wt. of sugar* corresponding to wt. of methyl levulinate obtained mg.	Decomposition of sugar to methyl levulinate %	M.P. of 2,4-dinitro-phenyl-hydrazone of methyl levulinate obtained
2,3,4,6-tetra-methyl glucose	1.112	27.2	11.4	20.7	1.8	138-139.5°
2,3,4-tri-methyl glucose	0.837	19.2	8.0	13.7	1.6	137-139°
2,3-di-methyl glucose	0.442	10.3	4.3	6.9	1.5	137.5-139°

* See footnote page 124

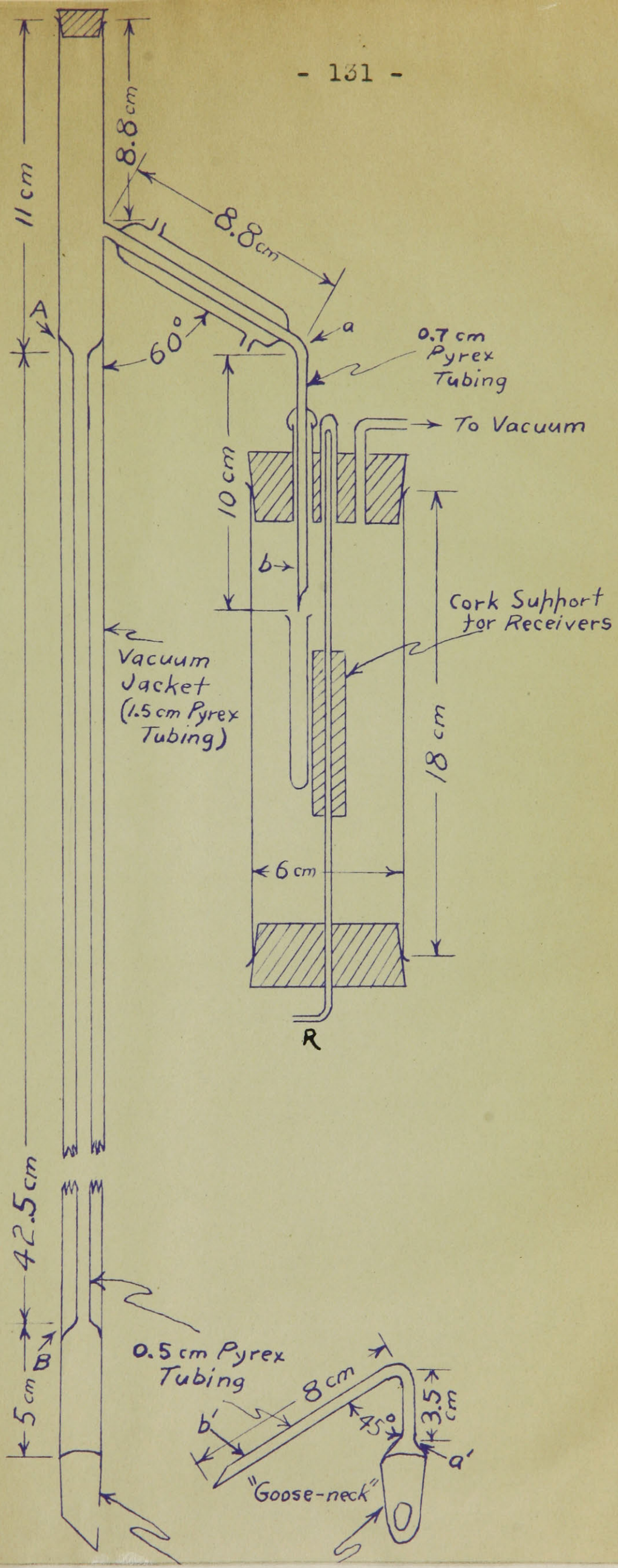


Diagram III.

No. 12/18 Pyrex Ground bevelled to 45° angle and with hole in bottom as shown.

having a resistance of 4.69 ohms per foot in a spiral of approximately 3/16" pitch. The column head, as well as the delivery tube of the small condenser ('a' to 'b' Figure III page 131) was wound similarly in continuous manner, and connected, finally, in series, with a variable resistance. The entire column was shielded from drafts with an 18" length of 40 mm. Pyrex tubing (air-jacket) supported by cork wedges. The exposed area of the column head was covered with a layer of asbestos.

The distillate receiver, constructed from a cylinder of 60 mm. Pyrex tubing, 180 mm. long, and flanged at each end, had its ends closed by means of No. 13 rubber stoppers. The upper one was equipped with three holes, the centre one of which contained a glass bearing (12 mm. tube) to accommodate the end of the glass pivotting rod, while the two off-centre holes provided for vacuum inlet and fractionating column delivery tube respectively. The lower stopper contained one central hole through which a lubricated (glycerol) glass pivot rod passed, and on which was mounted a cork cylinder containing from eight to nine receiving tubes, thus permitting collection of a number of fractions at reduced pressure without stoppage of the distillation. The small condenser was equipped for passage of cold water or steam depending upon the nature of the isolated fraction.

Column Efficiency (220)

The number of theoretical plates in the column was determined according to the method described by Morton (221) using a mixture of pure benzene and carbon tetrachloride, with the following results.

Table VII

Efficiency of Fractionating Column

Rate of distillation (drops/min.)	N_D^{25}		CCl_4 (%)		Theoretical Plates	H.E.T.P (cm.)
	Vapor	Liquid	Vapor	Liquid		
Total reflux	1.4710	1.4789	67.5	47.0	7.3	5.75
0.5	1.4729	1.4791	62.5	46.2	6.1	6.89
2.0	1.4731	1.4786	62.2	47.5	5.2	8.10
2.2	1.4751	1.4815	62.0	41.8	5.8	7.30
3.5	1.4752	1.4810	57.1	42.5	4.9	8.60
14	1.4739	1.4739	59.0	59.0	0	-

The analytical data used in the above Table was that given by Rosanoff and Easley (222) and quoted in the International Critical Tables (223).

It was found that the column was equivalent to 7.3 theoretical plates, the height of each being 5.75 cm. at total reflux. At partial reflux the efficiency fell to six plates at a distillation rate of two to three drops per minute.

At higher rates, for example fourteen drops per minute, straight distillation without fractionation was obtained.

Fractionation Technique

The glucoside mixture to be fractionated was placed in a pear-shaped flask (25 cc. capacity) which had been loosely packed with glass wool to maintain smooth boiling, and fitted to the bottom of the fractionating column by means of a ground glass joint. The system was evacuated to a constant pressure (regulated by a "bleeder" stop cock) and the bath temperature slowly increased until the maximum height of the reflux level was attained (about one-third up the column). Heat was then applied very gradually to the column (but not to the condenser top) while the bath temperature was kept constant, until the liquid refluxed to within 1" of the top of the column. These conditions were then maintained for several hours to establish equilibrium; during this time, the gold-plated wire became completely wetted with the liquid.

When the column temperature was raised slightly (3-4°) distillation of tetramethyl methyl glucoside began and this was collected at the rate of 150 to 200 mg. per hour. Cold water was passed through the condenser during this operation. Due to the slow rate under which the column was operated at reduced pressure the true boiling

points could not be determined, but a thermometer installed in the column head with its bulb opposite the condenser side arm gave the approximate values. The approximate column temperatures were read on a thermometer placed within the column air-jacket.

After a certain number of hours (dependent on the amount of tetramethyl methyl glucoside in the mixture) of constant distillation under these conditions, a dry spot on the previously completely wetted gold-plated spiral wire, became clearly visible, usually at about one-fifth the distance from the base. During the next one to two hours the column dried completely with the exception of several inches at the bottom. The receiver was then changed by turning the glass rod (R), the vacuum being maintained constant. The first fraction was pure tetramethyl methyl glucoside as shown by methoxyl analyses.

The bath temperature was then slowly increased, and simultaneously that of the column, until the spiral wire was again wetted, and equilibrium conditions maintained for four hours. The column temperature was then raised a further 2-3° which assured the same uniform rate of distillation. The first four or five drops of distillate sufficed to remove any residual "tetra" and was retained as an intermediate fraction of "tetra" and "tri". The third fraction

was pure trimethyl methyl glucoside. It was necessary at this point, to shunt the condenser tip (a-b Figure III) into the heating system, and to pass steam instead of cold water, through the condenser to prevent solidification of the β-isomer of 2,3,4-trimethyl methyl glucoside.

After complete distillation of the trimethyl methyl glucoside, the column again became "dry", only the lowest two inches being wetted with dimethyl methyl glucoside. The column was now allowed to cool to room temperature and the vacuum released.

The pear-shaped distilling cup was removed and the column and condenser washed by distilling through, a small same amount of chloroform. The chloroform distillate and washings from the column were combined, taken to dryness, this residue constituting the fraction intermediate between tri-, and dimethyl methyl glucosides.

The dimethyl methyl glucoside could not be removed through the column because of its high boiling point and was distilled out of the flask through a short "goose neck" (Diagram III). This had a glass joint ground to fit the distilling flask and was wound with nichrome ribbon. This heating element was inserted in series with the heating unit of the column thus permitting of temperature control. The temperature of the "goose neck" was kept at 100° during the

distillation and the pressure reduced to 0.010 mm.

Control Distillations of Individual Glucosides and Fractionations of Glucosidic Mixtures

(a) Distillation of Individual Glucosides

Pure samples of 2,3,4,6-tetramethyl methyl glucoside (3.60 grams) and 2,3,4-trimethyl methyl glucoside (3.15 grams) were distilled through the fractionating column and were found to be stable under these conditions. The recovery of each glucoside was approximately 92%.

Pure 2,3-dimethyl methyl glucoside (1.13 grams) was heated at 130-140° for twenty hours at 0.140-0.150 mm. (approximate conditions used during fractional distillation of glucosidic mixtures) and then distilled through the "goose neck" at 180-240°/.005 mm. The recovered distillate represented 95% of the starting material and there was neither charring nor decomposition.

(b) Fractionations of Synthetic Glucosidic Mixtures

A series of control fractionations of glucosidic mixtures of 2,3,4,6-tetramethyl methyl glucoside, 2,3,4-trimethyl methyl glucoside and 2,3-dimethyl methyl glucoside was carried out prior to the fractionation of the glucosidic mixture obtained from the hydrolysis of methylated Dextran I. The

experimental technique was the same as that outlined on page 134 and the results of a typical fractionation are given in Table II (page 76).

Fractionation of the Glucosidic Mixture from Hydrolyzed Methylated Dextran I

The glucosidic mixture (6.43 grams) obtained from hydrolyzed fully methylated Dextran I (page 127) was fractionated through the same column (Diagram III) using the technique described previously (page 134).

The system was evacuated to a pressure of 0.140-0.150 mm., the bath temperature slowly increased to 130-133°C and the column heated to 50-52° (temperature of air jacket), causing the liquid to reflux to within one inch of the top of the column. These conditions were maintained for four hours to attain equilibrium. The column temperature was then raised to 54-55° causing the first fraction of pure tetramethyl methyl glucoside to distill over at the rate of 150-200 mg. per hour. Weight 1,204 grams. Found OCH_3 = 61.0%. Calculated for $C_6H_7O(OCH_3)_5$, OCH_3 = 62.0%.

When distillation ceased and the gold-plated spiral wire had dried (pages 134-136) the bath temperature was slowly increased to 142-145° and that of the column to 60° (temperature of air jacket), and total reflux again maintained for four hours. On raising the column temperature

to 62-63° distillation recommenced at the same rate. An intermediate fraction between tetramethyl methyl glucoside and trimethyl methyl glucoside was collected (weight 0.207 grams OCH_3 = 57.8%) and the receiver then changed to collect pure crystalline 2,3,4-trimethyl-β-methyl glucoside (fraction 3). Weight 0.605 grams, Found OCH_3 = 52.8%. Calculated for $C_6H_8O_2(OCH_3)_4$, OCH_3 = 52.6%.

Fraction 4 obtained at the same temperature was a mixture of α- and β-trimethyl methyl glucosides. Weight 2.811 grams. Found OCH_3 = 52.4%. Calculated for $C_6H_8O_2(OCH_3)_4$, OCH_3 = 52.6%.

The column was now allowed to cool, the distilling cup removed, and the column and condenser washed by distilling through/5 cc. of chloroform. The chloroform distillate and washings from the column were combined, taken to dryness, the residue constituting fraction 5, (intermediate between trimethyl methyl glucoside and dimethyl methyl glucoside). Weight 0.397 grams, OCH_3 = 46.5%.

The dimethyl methyl glucoside was distilled at 170-240°/0.005 mm., through the "goose neck", which was maintained (electrically) at 100-110°. Weight 1.013 grams. Found OCH_3 = 42.0%. Calculated for $C_6H_9O_3(OCH_3)_3$, OCH_3 = 41.9%.

Table III (page 77) gives a summary of the results of this fractionation.

IDENTIFICATION OF THE GLUCOSIDES

(a) Identification of the Tetramethyl Methyl Glucoside

A portion (0.70 grams) of fraction 1 (pages 138, 77) was dissolved in 15 cc. of 5% sulphuric acid and heated on the constant level water bath for eighteen hours after which time the rotation was constant.

The acid solution was neutralized to litmus with solid barium carbonate, filtered, and the filtrate taken to dryness at 55°/20 mm. in an atmosphere of nitrogen. The residue was extracted with four 25 cc. portions of hot anhydrous acetone, the combined acetone solutions were filtered and the solvent evaporated at room temperature leaving a pale yellow syrup to which 10 cc. of anhydrous ether were added. On standing a precipitate of fine white needles was obtained as the ether evaporated. Weight 0.50 grams (75% of theoretical). The product was recrystallized from low-boiling (30°-50°) petroleum-ether containing 5% diethyl ether. Melting point 90-91°. A mixed melting point with an authentic sample of 2,3,4,6-tetramethyl glucose showed no depression.

(b) Identification of the Trimethyl Methyl Glucoside

A portion (0.58 grams) of fraction 3 (pages 139, 77) which had solidified completely in the receiver, during the fractionation, was pressed on a porous plate and recrystallized from low-boiling (30-50°) petroleum-ether containing about 5% diethyl ether. A mass of fine white needles was obtained (0.42 gr. (72% recovery)) melting point 93-94°, which showed no mixed melting point depression with an authentic sample of 2,3,4-trimethyl-β-methyl glucoside (208, 224, 225).

(c) Identification of the Dimethyl Methyl Glucoside

Oxidation to Dimethyl Gluconic Acid (205)

A portion (0.60 grams) of the dimethyl methyl glucoside (fraction 6, page 139) was hydrolyzed to the corresponding dimethyl glucose and this in turn oxidized (205) to the corresponding dimethyl gluconic acid following the procedures outlined on pages 148-150. Weight of dimethyl glucose 0.540 grams (96% of theoretical). Found OCH_3 = 30.2%. Calculated for $C_6H_{10}O_4(OCH_3)_2$, OCH_3 = 29.9%.

0.538 Grams of dimethyl glucose yielded 0.487 grams of dimethyl gluconic acid (84%).

Formation of 2,3-Dimethyl Gluconophenylhydrazide

The dimethyl gluconic acid (0.487 grams) was converted to the corresponding dimethyl gluconophenylhydrazide following the procedure outlined on page 150. Weight of recrystallized product (from ethanol) 0.556 grams (81.4%). Melting point 166.5-167.5°. Mixed melting point with 2,3-dimethyl gluconophenylhydrazide (page 151) showed no depression. Found C = 53.5%, H = 7.1%, N = 9.0%, OCH_3 = 19.5%. Calculated for $C_{14}H_{22}O_6N_2$, C = 53.5%, H = 7.0%, N = 8.9%, OCH_3 = 19.7%.

SYNTHESIS OF REFERENCE COMPOUNDS

I. 2,3,4,6-Tetramethyl Glucose

The methods of Haworth and co-workers (173) were modified as previously discussed (page 91).

(a) 2,3,4,6-Tetramethyl-α-Methyl Glucoside

α-Methyl glucoside[x] (50 grams) was vigorously stirred in a nitrogen atmosphere with water (60 cc.) at 50°. Dimethyl sulphate (50 cc.) was added in one portion, and during the next two hours, sodium hydroxide (100 cc. of a solution of 100 grams of sodium hydroxide in 175 cc. of water) was added dropwise, the temperature being kept at 50°. During a further three hours, sodium hydroxide, (300 cc. of the solution described above) and dimethyl sulphate (160 cc.) were gradually added[xx]. The solution was stirred for sixteen hours in an atmosphere of nitrogen (temperature approximately 60°). The reaction mixture was cooled to 10-15°, partially neutralized with 50% sulphuric acid, heated to 100° for one-half hour to destroy sodium methyl sulphate, then left slightly alkaline to phenolphthalein. The reaction mixture (supersaturated with sodium sulphate) was exhaustively

---

[x] Prepared by T.H. Evans in these laboratories (60).
[xx] Technique page 99.

extracted by refluxing with several portions of chloroform. The combined chloroform extracts were evaporated to dryness, and remethylated as above. The resulting syrup was fractionally distilled through a Widmer column.. Yield 58 grams (90%). Boiling point 91-93°/0.5-0.6 mm. (bath temperature 120-130°). Found OCH_3 = 61.3%. Theoretical 62.0%. $[\alpha]_D^{22}$ 1.4458. (Literature value 1.4460 (20°) (210)).

(b) 2,3,4,6-Tetramethyl Glucose

2,3,4,6-Tetramethyl-α-methyl glucoside (23.2 grams) was hydrolyzed at 100° with 3% sulphuric acid (200 cc.) until constant rotation was reached (about twelve hours). The hydrolysis solution was neutralized to Congo red with barium carbonate, and the filtrate evaporated to dryness at 40°/20 mm. in an atmosphere of nitrogen. The semi-crystalline residue was extracted with dry ether, and the ether solution concentrated in vacuo. This residue was dissolved in a mixture of petroleum ether (30-50°, 150 cc.) and 10 cc. diethyl ether, cooled to 0°. The crystalline product (12 gr.) was removed and the mother liquor rehydrolyzed, and again subjected to recrystallisation. Weight obtained 6.5 grams. Total yield of crystalline material approximately 18.5 gr. (84%). After one additional recrystallization this material melted at 90-91° (90-93° (210)). OCH_3 = 52.4% (Theoretical 52.6%).

II. 2,3,4-Trimethyl Glucose

(a) 2,3,4-Trimethyl Laevoglucosan

Laevoglucosan was recrystallized from ethanol, M.p. 177-8° (uncorr.) (179-180° (226)). Ten grams were dissolved in water (10 cc.) and the solution stirred in an atmosphere of nitrogen. Dimethyl sulphate (10 cc.) was added immediately, and then sodium hydroxide (20 cc. of a solution of 100 gr. in 175 cc. water) added dropwise during two hours. Dimethyl sulphate and sodium hydroxide (20 cc. and 40 cc. respectively) were added[x] during one and one-half hours. The temperature was slowly raised to 60° during the next two hours, then dimethyl sulphate (10 cc.) was added slowly. The temperature was kept at 60° for four hours, and the reaction mixture stirred at 50° for a further twelve hours. It was then about half neutralized with 50% sulphuric acid, heated at 100° for one-half hour, cooled below 20°, and rendered slightly alkaline to phenolphthalein by the addition of 50% sulphuric acid. The reaction mixture was exhaustively extracted by refluxing with several portions of chloroform (100, 75, 75, 50, and 50 cc.) successively. The combined chloroform extracts were dried and evaporated in vacuo. Yield about 10 grams. The aqueous methylation liquors left after chloroform extraction were cooled to -15° and

---

[x] Technique page 99.

the sodium sulphate removed by filtration, washed and discarded. The aqueous solutions were concentrated and remethylated. The main amount of methylated laevoglucosan was not completely crystalline due to incomplete methylation. After two recrystallizations from ether it melted at 61-62° (63-64° (213)). The material recovered from the ether mother liquors was added to the concentrated aqueous methylation solutions, and both were again methylated using dimethyl sulphate and sodium hydroxide as described above. Total yield 10 gr. (approximately 80% of theoretical).

(b) 2,3,4-Trimethyl Glucose

Trimethyl laevoglucosan (8.0 gr. M.p. 61°) dissolved in 3% sulphuric acid (100 cc.) was heated on a constant level boiling water bath. The hydrolysis was followed polarimetrically and after seventeen hours the reaction was apparently complete. The solution was rendered slightly alkaline to Congo red by the addition of a thin aqueous paste of barium carbonate, the solids removed by centrifugence and filtration, and washed twice with water. The combined aqueous solutions were evaporated under reduced pressure (50°) in an atmosphere of nitrogen. Last traces of water were removed by evaporation, after additions of several small volumes of acetone. The syrupy residue was dissolved in acetone, filtered, evaporated

(50°/20 mm, nitrogen atmosphere), and dried to constant weight. Yield 7.9 grams (91% of theoretical) light yellow oil, OCH_3, 41.6%. Theoretical 41.9%.

(c) 2,3,4-Trimethyl- α and β -Methyl Glucosides from 2,3,4-Trimethylglucose

2,3,4-Trimethyl glucose (9.8 grams), dissolved in about 300 cc. anhydrous methanol containing 2% hydrogen chloride, was refluxed for approximately seventeen hours at which time the solution no longer showed a reducing action with Fehling's solution. Sufficient silver carbonate (10 grams) was added to render the solution slightly alkaline to Congo red, the mixture centrifuged, filtered and the residue washed twice with methanol. The combined methanolic solutions were evaporated (50°/20 mm; nitrogen atmosphere), the residual syrup was dissolved in anhydrous methanol, filtered, and the solvent removed (50°/20 mm; nitrogen atmosphere). Yield of crude product 9.8 gr. (94%).

This on distillation (nitrogen atmosphere) yielded 9.2 grams of a mixture of crystalline (Fraction 1) and syrupy (Fraction 2) material.

The former was 2,3,4-trimethyl β-methyl glucoside, and the latter the corresponding α-isomer. A portion of the β-isomer was recrystallized twice from petroleum ether

(30-50°) containing 5% diethyl ether. M.p. 93-4° (94° (49)). The total distillate had a methoxyl content of 52.1% (Theory 52.6%)

III 2,3-Dimethyl-α- and β-Methyl Glucosides

2,3-Dimethyl-α-methyl glucoside and the corresponding β-isomer were prepared according to the procedures outlined by Evans (60) (pages 95-97).

IV 2,3-Dimethyl Gluconophenylhydrazide

(a) 2,3-Dimethyl Glucose

2,3-Dimethyl-α-methyl glucoside (5.4 grams. M.p. 82-83°) dissolved in 200 cc. normal sulphuric acid, was heated at 100° for eighteen hours. The hydrolysis was followed polarimetrically until constant rotation was reached. The faintly yellow colored reaction solution was neutralized to litmus with a paste of barium carbonate and water.

The precipitate was centrifuged, washed with water, and the supernatent sugar solution removed and filtered. Evaporation of the filtrate (40-50°/20 mm.; nitrogen atmosphere) left a residual syrup which was extracted five times with hot 25 cc. portions of anhydrous chloroform. The combined

chloroform solutions were evaporated to constant weight under the same conditions as above, leaving a pale yellow syrup. Weight 4.4 grams (86% of theoretical). Found, OCH_3 = 30.1%. Calculated for $C_6H_{10}O_4(OCH_3)_2$, OCH_3 = 29.8%.

(b) 2,3-Dimethyl Gluconic Acid

This was obtained by use of the excellent method of Hudson and Isbell (205). 2,3-Dimethyl glucose (2.4 grs.) was dissolved in 115 cc. of water in a brown glass bottle and cooled in an ice-water bath to 0°. To this was added 8.2 grs. of barium benzoate and 0.9 cc. of bromine. The reaction flask was shaken for several minutes at 0° until the bromine had dissolved and was then allowed to stand for fifty-one hours at room temperature in absence of light until the mixture no longer reduced Fehling's solution. The excess bromine was removed by passage of a rapid stream of air through the mixture, and the benzoic acid and excess barium benzoate by filtration. Barium in solution was then precipitated quantitatively with 5N sulphuric acid, the barium sulphate removed and the filtrate neutralized with silver carbonate (about 12 grams), finally with one gram of silver benzoate, and again filtered. The filtrate (approximately 300 cc.) was concentrated to approximately 125 cc. (50°/20 mm.) and extracted with three 25 cc. portions of chloroform to remove benzoic acid.

Silver present as soluble salts was removed with hydrogen sulphide. The clear filtrate was taken to dryness at 70°/20 mm., the pale yellow residual syrup extracted with three 20 cc. portions of hot anhydrous dioxane, filtered, and again taken to dryness. The last traces of dioxane were removed by heating the residue to 80°/0.01 mm. for five hours. Yield 2.16 grams (83.6%) of a pale yellow syrup which did not reduce Fehling's solution. Found 27.4% OCH_3. Calculated for $C_6H_{10}C_5(OCH_3)_2$ 27.7%.

(c) 2,3-Dimethyl Gluconophenylhydrazide

2,3-Dimethyl gluconic acid (0.80 grams) was dissolved in 50 cc. of anhydrous ether by refluxing for three hours, freshly distilled phenylhydrazine (2.0 grams) added, and the solution refluxed for seven hours. The solid reaction product which separated out was removed by filtration, an additional 0.2 grams of phenylhydrazine added to the filtrate and this refluxed for a further twelve hours. An additional amount of solid material separated out which was removed by filtration and added to the first portion. This last filtrate was taken to dryness, and on addition of a few ccs. of benzene to the residue gave a further small quantity of crystals. Total crude yield 1.08 grams (96% of theoretical); which, after two recrystallizations from boiling ethanol gave 0.90 grams

(80% yield) of a pure white product consisting of fine short needles. M.p. 166-167°. Found, C = 53.3%; H = 7.1%; N = 9.3%; OCH_3 = 19.6%. Calculated for $C_{14}H_{22}O_6N_2$, C = 53.5%; H = 7.0%; N = 8.9%; OCH_3 = 19.7%.

Sucrose Determination

The sucrose (present as an impurity in the crude Dextran I) was extracted with hot 80% ethanol, the ethanol evaporated, and the resulting aqueous sucrose solution inverted with dilute hydrochloric acid, and the reducing sugars determined using the method of Scales (227). Preliminary experiments with pure glucose and sucrose were carried out in order to acquire the correct technique.

To the dextran (0.100 grams), approximately 25 cc. aqueous ethanol (80% ethanol by volume) was added, and the mixture heated on a steam bath for two hours. A small funnel was inserted in the neck of the flask and alcohol added from time to time as evaporation proceeded. The solution was then filtered, and the alcohol evaporated on a steam-bath. Water was added to the residue to give a volume of approximately 30 cc., 6 cc. of 20.5% hydrochloric acid then added, and inversion allowed to proceed at room temperature for twenty-four hours. The solution was then neutralized with anhydrous sodium carbonate, and the reducing sugars determined in the usual manner.

SUMMARY

Dextran I, the bacterial polysaccharide produced from sucrose by Leuconostoc mesenteroides, was methylated completely using improved Haworth and Muskat techniques, to a trimethyl dextran, OCH_3 45.6%.

Hydrolysis of this trimethyl dextran with methanol-hydrogen chloride gave a mixture of 2,3,4,6-tetra, 2,3,4-tri-, and 2,3-dimethyl methyl glucosides in 95% yield. The mixture of glucosides was fractionated by a new technique using a modified Podbielniak column and an excellent separation of the glucosides effected in a yield of 97%. In this manner the ratio of tetra- : tri- : dimethyl methyl glucoside as 1:3:1 was established and it was shown that Dextran I possessed a comb-like structure. The nature of the oxygen rings and mode of linkages in the main and side chains have been clarified.

The Haworth methylation technique has been modified and adapted generally to the synthesis of partially-methylated glucose derivatives.

2,3,4-Trimethyl glucose has been synthesized by a new method. Improvements have been made in the methods of synthesis of 2,3,4,6-tetramethyl methyl glucoside.

A new crystalline derivative of 2,3-dimethyl glucose has been described.

BIBLIOGRAPHY

1. Baeyer, A.v., Ber., 3: 63, (1870).
2. Butlerow, A.v., Ann., 120: 295, (1861).
3. Ewart, A.J., Proc. Roy. Soc. Victoria, 31: 328, (1919).
4. Fischer, E., and Passmore, F., Ber., 22: 359, (1889).
5. Fischer, E., Ber., 23: 370, 799, (1890).
6. Liebig, J., Ann., 46: 62, (1843).
7. Michael, A., J. prakt. Chem., 60: 485, (1899).
8. Fincke, H., Biochem. Z., 61: 157, (1914).
9. Hibbert, H., Address to Cellulose Division, Am. Chem. Soc., Chapel Hill, N.C., (1937).
10. Hibbert, H., Can. J. Research, 8: 103, (1933).
11. Harrison, F.C., Tarr, H.L.A., and Hibbert, H., ibid, 3: 449, (1930).
12. Tarr, H.L.A., and Hibbert, H., ibid, 4: 372, (1931).
13. Hibbert, H., and Barsha, J., ibid, 5: 580, (1931).
14. Tarr, H.L.A., and Hibbert, H., ibid, 5: 414, (1931).
15. FitzGerald, J.G., Trans. Roy. Soc. Can., Sec. V., 27: 1, (1933).
16. Zozaya, J., J. Exp. Med., 54: 725, (1931).
17. Zozaya, J., ibid, 55: 325, 353, (1932).
18. Evans, T.H., Hawkins, W.L., and Hibbert, H., ibid, 74(6): 511, (1941).
19. Heidelberger, M., Chem. Rev., 3: 403, (1927).
20. Heidelberger, M., Ann. Rev. Biochem., 1: 655, (1932).

21. Heidelberger, M., ibid, 2: 503, (1933).

22. Sordelli, A., and Mayer, E., Compt. Rend. Soc. Biol., 108: 675, (1931).

23. Heidelberger, M., Avery, O.T., and Goebel, W.F., J. Exp. Med., 49: 847, (1929).

24. Mueller, J.H., and Tomcsik, J., ibid, 40: 343, (1924).

25. Kesten, H.D., Cook, D.H., Mott, E., and Jobling, J.W., ibid, 52: 813, (1930).

26. Nishimura, S., ibid, 50: 419, (1929).

27. Avery, O.T., and Goebel, W.F., ibid, 50: 533, (1929).

28. Goebel, W.F., Babers, F.H., and Avery, O.T., ibid, 60: 85, (1934).

29. Goebel, W.F., Babers, F.H., and Avery, O.T., ibid, 60: 599, (1934).

30. Goebel, W.F., J. Biol. Chem., 110: 391, (1935).

31. Heidelberger, M., and Avery, O.T., J. Exp. Med., 38: 73, (1923).

32. Heidelberger, M., and Avery, O.T., ibid, 40: 301, (1924).

33. Heidelberger, M., and Goebel, W.F., J. Biol. Chem., 74: 613, (1927).

34. Heidelberger, M., Goebel, W.F., and Avery, O.T., J. Exp. Med., 42: 727, (1925).

35. Goebel, W.F., J. Biol. Chem., 74: 619, (1927).

36. Goebel, W.F., and Avery, O.T., J. Exp. Med., 46: 601, (1927).

37. Heidelberger, M., Goebel, W.F., and Avery, O.T., ibid, 42: 701, (1925).

38. Reeves, R.E., and Goebel, W.F., J. Biol. Chem., 139: 511, (1941).

39. Pasteur, L., Bull. Soc. Chim., 30, (1861).

40. Scheibler, C., Z. Ver. deut. Zucker-Ind.,19: 472, (1869).

41. Scheibler, C., ibid, 24: 309, (1874).

42. Jubert, P., ibid, 25: 105, (1875).

43. Durin, E., Compt. rend., 83: 128, (1876).

44. van Tieghem, Z. Ver. deut. Zucker-Ind., 27: 464, (1877).

45. Daümichen, P., ibid, 40: 701, (1890).

46. Browne, C.A. Jr., J. Am. Chem. Soc., 28: 453, (1906).

47. Hucker, G.J., and Pederson, C.S., N.Y. State Agric. Exper. Station Tech. Bull., No. 167, (1930).

48. Tarr, H.L.A., Ph.D. Thesis, McGill University, (1931).

49. Fowler, F.L., Ph.D. Thesis, McGill University, (1936).

50. Beijerinck, M.W., Fol. Mikrobiol., 1: 377; Zentr. Biochem. Biophys. 14: 679, C.A. 8: 944, (1914).

51. Buchanan, and Fulmer, Physiology and Biochemistry of Bacteria, Williams and Wilkins, 1928, Vol. 1, pp. 94-95.

52. Carruthers, A., and Cooper, E.A., Biochem. J., 30: 1001, (1936).

53. Stacey, M., and Youd, F.R., ibid, 32: 1943, (1938).

54. Hassid, W.Z., and Barker, H.A., J. Biol. Chem., 134: 163, (1940).

55. Hehre, E.J., Science, 93: 237, (1941).

56. Fowler, F.L., Buckland, I.K., Brauns, F., and Hibbert, H., Can. J. Research, B15: 486, (1937).

57. Muskat, I.E., J. Am. Chem. Soc., 56: 693, 2449, (1934).

58. Macdonald, J.Y., ibid, 57: 771, (1935).

59. Brauns, F.E., Can. J. Research, B16: 73, (1938).

60. Evans, T.H., Ph.D. Thesis, McGill University, (1941).

61. Fairhead, E.C., Hunter, M.J., and Hibbert, H., Can. J. Research, B16: 151, (1938).

62. Peat, S., Schlüchterer, E., and Stacey, M., J. Chem. Soc. 581, (1939).

63. Armstrong, E.F., Ann. Rev. of Biochem., Vol. VII, page 51, (1938).

64. Freudenberg, K., ibid, Vol. VIII, page 81, (1939).

65. Heuser, E., Organic Chemistry, Ed. by H. Gilman, Vol. II. Chapter 18, John Wiley and Sons, New York.

66. Monier-Williams, G.W., J. Chem. Soc., 119: 803, (1921).

67. Hess, K. and Wittelsbach, W., Z. Elektrochem., 26: 232, (1920).

68. Sponsler, O.L., and Dore, W.H., Colloid Symposium Monograph 4; 174, (1926). The Chemical Catalog Co.

69. Haworth, W.N., and Machemer, H., J. Chem. Soc., 2672, (1932).

70. Haworth, W.N., and Machemer, H., Trans. Faraday Soc., 29: 14, (1933).

71. Haworth, W.N., and Machemer, H., Nature, 129: 365, (1932).

72. Haworth, W.N., Monatsh., 69: 314, (1936).

73. Hengstenberg, J., and Mark, H., Z. Krist., 69: 271, (1928).

74. Meyer, K.H., and Mark, H., Z. physik. Chem. B2: 115, (1929).

75. Staudinger, H., Angew. Chem., 49: 804, (1936).

76. Staudinger, H., Naturwissenschaften, 25: 673, (1937).

77. Hess, K., and Neumann, F., (a) Ber., 70B: 710, (1937),
(b) Ber., 70B: 721, (1937),
(c) Ber., 70B: 728, (1937).

78. Hanes, S.H., Can. J. Research, 13: 185, (1935).

79. Hanes, C.S., New Phytologist, 36: 101, 189, (1937).

80. (a). Myrbäck, K., Oertenblad, B., and Ahlborg, K., Compt. rend. trav. lab. Carlsberg, Sér. Chim., 22: 357, (1938).

(b). Myrbäck, K., and Ahlborg, K., Svensk. Kem. Tid., 49: 216, (1937).

81. Haworth, W.N., Hirst, E.L., Kitchen, H., and Peat, S., J. Chem. Soc., 791, (1937).

82. Haworth, W.N., and Percival, E.G.V., ibid, 1342, (1931).

83. Haworth, W.N., Hirst, E.L., and Isherwood, F.A., ibid, 577, (1937).

83A. Haworth, W.N., Hirst, E.L., and Woolgar, M.D., ibid, 177, (1935).

84. Haworth, W.N., Hirst, E.L., and Webb, J.I., ibid, 2681, (1928).

85. Hirst, E.L., Plant, M.M.T., and Wilkinson, M.D., ibid, 2375, (1932).

85A. Hassid, W.Z., and Dore, W.H., J. Am. Chem. Soc., 59: 1503, (1937).

85B. Staudinger, H., and Husemann, E., Ber., 70: 1451, (1937).

86. Hirst, E.L., and Young, G.T., J. Chem. Soc., 1471, (1937).

87. Freudenberg, K., and Boppel, H., Ber., 73B: 609, (1940).

88. Meyer, K.H., and Mark, H., Der. Aufbau der hochpolymeren Naturstoffe, page 212, Leipzig, (1930).

89. Freudenberg, K., and Boppel, H., Ber., 71: 2505, (1938).

90. Bell, D.J., Biochem. J., 29: 2031, (1935).

91. Bell, D.J., ibid, 30: 2144, (1936).

92. Staudinger, H., and Husemann, E., Ann., 530: 1, (1937).

93. Irvine, J.C., and Steele, E.S., J. Chem. Soc., 117: 1474, (1920).

94. Irvine, J.C., Steele, E.S., and Shannon, M.I., ibid, 121: 1060, (1922).

95. Karrer, P., and Lang, L., Helv. Chim. Acta., 4: 249, (1921).

96. Haworth, W.N., Hirst, E.L., and Percival, E.G.V., J. Chem. Soc., 2384, (1932).

97. Haworth, W.N., and Learner, A., J. Chem. Soc., 619, (1928).

98. Montgomery, T.N., J. Am. Chem. Soc., 56: 419, (1934).

99. Schlubach, H.H., and Elsner, H., Ber., 62: 1493, (1929).

100. Jackson, R.F., and McDonald, E., Bur. Standards J. Research, 5: 1151, (1930).

101. Irvine, J.C., and Montgomery, T.N., J. Am. Chem. Soc., 55: 1988, (1933).

102. Ohlmeyer, P., Pharm. Monatsh., 14: 158, (1933).

103. Vogel, H., and Pictet, A., Helv. Chim. Acta., 11: 215, (1928).

104. Pringsheim, H., and Reilly, J., Ber., 61: 2018, (1928).

105. Jackson, R.F., and Goergen, S.M., Bur. Standards, J. Research, 3: 27, (1929).

106. Irvine, J.C., and Stevenson, J.W., J. Am. Chem. Soc., 51: 2197, (1929).

107. Jackson, R.F., and McDonald, E., Bur. Standards J. Research, 6: 709, (1931).

108. Pringsheim, H., and Hensel, W.G., Ber., 64: 1431, (1931).

109. Schmidt, L., and Becker, B., ibid, 58: 1968, (1925).

110. Bergmann, M., and Knehe, E., Ann., 449: 302, (1926).

111. Reilly, J., and Donovan, P.P., Sci. Proc. Roy. Dublin Soc., 19: 409, (1930).

112. Pringsheim, H., and Hensel, W.G., Ber., 63: 1096, (1930).

113. Berner, E., ibid, 63: 1356, (1930).

114. Pringsheim, H., Reilly, J., and Donovan, P.P., ibid, 62: 2378, (1929).

115. Drew, H.D.K., and Haworth, W.N., J. Chem. Soc., 2690, (1928).

116. Haworth, W.N., Brit. Assoc. Advancement Sci. Rept., 31, (1935).

117. Haworth, W.N., and Streight, H.R.L., Helv. Chim. Acta., 15: 693, (1932).

118. Schlubach, H.H., and Schmidt, H., Ann., 520: 43, (1935).

119. Schlubach, H.H., and Koenig, K., ibid, 514: 182, (1934).

120. Schlubach, H.H., Knoop, H., and Liu, M.Y., ibid, 504: 30,, (1933).

121. Schlubach, H.H., Knoop, H., and Liu, M.Y., ibid, 511: 140, (1934).

122. Schlubach, H.H., ibid, 523: 130, (1936).

123. Schlubach, H.H., and Peitzner, H., ibid, 530: 120, (1937).

124. Murakami, S., Acta, Phytochim., 10: 43, (1937).

125. Schlubach, H.H., and Böe, H., Ann., 532: 191,(1937).

126. Schlubach, H.H., and Lendzian, H., ibid, 532: 200, (1937).

127. Patterson, J., J. Chem. Soc., 123: 1139, (1923).

128. Klages, F., Ann., 509: 159, (1934); 512: 185, (1934).

129. Klages, F., and Niemann, R., ibid, 523: 224, (1936).

130. Haworth, W.N., Raistrick, H., and Stacey, M., Biochem. J., 29: 612, (1935).

131. Wise, L.E., and Peterson, F.C., Ind. Eng. Chem., 22: 362, (1930).

132. Wise, L.E., Hamer, P.L., and Peterson, F.C., ibid, 25: 184, (1933).

133. Wise, L.E., and Unkauf, H.C., Cellulosechem., 14: 20, (1933).

134. Peterson, F.C., Maughan, M., and Wise, L.E., ibid, 15: 109, (1934).

135. Miyama, R., J. Dept. Agr. Kyushu Imp. Univ. 4: 195, (1935).

136. Peterson, F.C., Barry, A.J., Unkauf, H., and Wise, L.E., J. Am. Chem. Soc., 62: 2361, (1940).

137. Husemann, E., J. prakt. Chem., 155: 13, (1940).

138. Hirst, Jones, and Campbell, Nature, 147: 25, (1941).

139. White, E.V., J. Am. Chem. Soc., 63: 2871, (1941).

140. White, E.V., ibid, 64: 302, (1942).

140A White, E.V., ibid, Forthcoming publication.

141. Husemann, E., Naturwissenschaften, 27: 595, (1939).

142. Owens, H.S., J. Am. Chem. Soc., 62: 930, (1940).

143. Percival, E.G.V., and Somerville, J.C., J. Chem. Soc., 1615, (1937).

144. Hassid, W.Z., Joslyn, M.A., and McCready, R.M., J. Am. Chem. Soc. 63: 295, (1941).

145. Daker, W.D., and Stacey, M., J. Chem. Soc., 585, (1939).

146. Cooper, E.A., Daker, W.D., and Stacey, M., Biochem. J., 32: 1752, (1938).

147. Barsha, J., and Hibbert, H., Can. J. Research, 10: 170, (1934).

148. Hibbert, H., and Brauns, F., ibid, 4: 596, (1931).

149. Hibbert, H., Tipson, R.S., and Brauns, F., ibid, 4: 221, (1931).

150. Mitchell, W., and Hibbert, H., ibid, 7: 345, (1932).

151. Challinor, S.W., Haworth, W.N., and Hirst, E.L., J. Chem. Soc., 676, (1934).

152. Lyne, R.R., Peat, S., and Stacey, M., ibid, 257, (1940).

153. Veibel, S., Biochem. J., 32: 1949, (1938).

154. Anderson, R.J., and Creighton, M.M., J. Biol. Chem., 131: 549, (1939).

155. Heidelberger, M., and Menzel, A.E.O., ibid, 118: 79, (1937).

156. Anderson, R.J., Peck, R.L., and Creighton, M.M., ibid, 136: 211, (1940).

157. Haworth, W.N., Hirst, E.L., Isherwood, F., and Jones, J.K.N., J. Chem. Soc., 1878, (1939).

158. Haworth, W.N., Raistrick, H., and Stacey, M., Biochem. J., 31: 640, (1937).

159. Clutterbuck, P.W., Haworth, W.N., Raistrick, H., Smith, G., and Stacey, M., ibid, 28: 94, (1934).

160. Haworth, W.N., Raistrick, H., and Stacey, M., ibid, 29: 2668, (1935).

161. Haworth, W.N., Hirst, E.L., and Isherwood, F.A., J. Chem. Soc., 784, (1937).

162. Zechmeister, L., and Toth, G., Biochem. Z., 284: 153, (1936).

163. White, Benjamin, "The Biology of Pneumococcus", (1938), New York, The Commonwealth Fund.

164. Brown, Rochel, J. Immunol., 37: 445, (1939).

165. Avery, O.T., and Goebel, W.F., J. Exp. Med., 58: 731, (1933).

166. Hotchkiss, R.D., and Goebel, W.F., J. Biol. Chem., 121: 195, (1937).

167. Haworth, W.N., Loach, J.V., and Long, C.W., J. Chem. Soc., 3146, L927).

168. Irvine, J.C., Biochem. Zeit., 22: 357, (1909).

169. Haworth, W.N., and Machemer, H., J. Chem. Soc., 2270, (1932).

170. Fischer, E., Ber., 26: 2400, (1893).

171. Purdie, T., and Irvine, J.C., J. Chem. Soc., 83: 1021, (1903).

172. Denham, W.S., and Woodhouse, H., ibid, 103: 1735, (1913).

173. Haworth, W.N., ibid, 107: 8, (1915).

174. Schmid, L., Ber., 58; 1963, (1925).

175. Schmid, L., and Zentner, M., Monatsch., 49: 111, (1928).

176. Fear, C.M., and Menzies, R.C., J. Chem. Soc., 937, (1926).

177. Bell, D., Biochem. J., 31: 1683, (1937).

178. Hibbert, H., Tipson, R.S., and Brauns, F., Can. J. Research, 4: 221, (1931).

179. Hirst, E.L., and Jones, J.K.W., J. Chem. Soc., 496, (1938).

180. Karrer, P., Helv. Chim. Acta., 3: 620, (1920).

181. Irvine, J.C., and Macdonald, J., J. Chem. Soc., 1502, (1926).

182. Freudenberg, K., and Rapp, W., Ber., 69: 2041, (1936).

183. Hess,K.and Lung, K., Ber., 70: 1259, (1937).

184. Freudenberg, K., Plankenhorn, E., and Boppel, H., ibid, 71B: 2435, (1938).

185. Averill, F.J., and Peat, S., J. Chem. Soc., 1244, (1938)

186. Hirst, E.L. and Young, G.T., ibid, 1247, (1938).

187. Pringsheim, H., "Zuckerchemie" Leipzig (1925) page 21.

188. Stone, W.E., and Tollens, B., Ann., 249: 227, (1888); Ber., 21: 2150, (1888).

189. Maquenne, Compt. rend., 109: 571, (1889).

190. Kiermayer, Chemiker-Zeitung, 19: 1004, (1895).

191. Ekenstein, W.A. Van and Blanksma, J.J., Chemisches Zentralblatt I; 1509, (1909); I: 1961, (1910).

192. Pummerer, R., and Gump, W., Ber., 56: 999, (1923).

193. Grote, A.F., Kehrer, E., and Tollens, B., Ann., 206: 207, (1881).

194. Haworth, W.N., and Percival, E.G.V., J. Chem. Soc., 2277, (1932).

195. Haworth, W.N., and Percival, E.G.V., ibid, 2850, (1931).

196. Haworth, W.N., Hirst, E.L., and Oliver, E., ibid, 1917, (1934).

197. Bywater, R.A.S., Haworth, W.N., Hirst, E.L., and Peat, S. ibid, 1985, (1937).

198. Hassid, W.Z., and McCready, R.M., J. Am. Chem. Soc., 63: 1632, (1941).

199. Leckzyk, E., Ber. 71: 829, (1938).

200. Carrington, H.C., Haworth, W.N., and Hirst, E.L., J. Am. Chem. Soc., 55: 1084, (1933).

201. Podbielniak, W.J., Ind. Eng. Chem., Anal. Ed., 3: 177, (1931); 5: 119, (1933).

202. Helferich, B., Z. Angew. Chem., 41: 871, (1928).

202A. Hockett, R.C., and Hudson, C.S., J. Am. Chem. Soc., 53: 4456, (1931).

203. Hockett, R.C., and Hudson, C.S., ibid, 56: 945, (1934).

204. Jackson, E.L., Hockett, R.C., and Hudson, C.S., ibid, 56: 947, (1934).

205. Hudson, C.S., and Isbell, H.S., ibid, 51: 2225, (1929).

206. Bell, D.J., and Synge, R.L.M., J. Chem. Soc., 833, (1938).

207. Fordyce, R.G., Unpublished research. Dept. of Industrial and Cellulose Chemistry, McGill University, (1939).

208. Oldham, J.W.H., J. Am. Chem. Soc., 56: 1360, (1934).

209. (a) Haworth, W.N., and Leitch, G.C., J. Chem. Soc., 113: 188, (1918).

(b) Haworth, W.N., and Sedgwick, W.G., ibid, 2573, (1926)

210. West, E.S., and Holden, R.F., Organic Syntheses, 20: 97, (1940).

211. Hendricks, B.C., and Rundle, R.E., J. Am. Chem. Soc., 60: 2563, (1938).

212. Robertson, A., and Waters, R.B., J. Chem. Soc., 1709, (1931).

213. Irvine, J.C., and Oldham, J.W.H., ibid, 119: 1744, (1921).

214. Godard, H.P., Unpublished research, Dept. of Industrial and Cellulose Chemistry, McGill University, (1937).

215. Oldham, J.W.H., and Rutherford, J.K., J. Am. Chem. Soc., 54: 366, (1932).

216. Viebock, F., and Schwappach, A., Ber., 63: 2818, (1930).

217. Peniston, Q.P., and Hibbert, H., Paper Trade Journal, page 1-5, Oct. 26., (1939).

218. Lovell, E.L., Ph.D. Thesis, McGill University, page 234, (1940).

219. Leger, F.J., Ph.D. Thesis, McGill University, (1938).

220. Ward, C.C. "Review of the Literature on the Construction, Testing and Operation of Laboratory Fractionating Columns." United States Dept. of the Interior, Bureau of Mines, Technical Paper 600, United States Gov't Printing Office, Washington.

221. Morton, A.A., "Laboratory Technique in Organic Chemistry", McGraw Hill, page 74, (1938).

222. Rosanoff, M.A., and Easley, C.W., J. Am. Chem. Soc., 31: 953, (1909).

223. International Critical Tables, Vol. 7, page 77, (1930).

224. Irvine, J.C., and Oldham, J.W.H., J. Chem. Soc., 127: 2729, (1925).

225. Haworth, W.N., Hirst, E.L., and Miller, E.J., ibid, 2436, (1927).

226. Vogel, H., and Georg, A., "Tabellen der Zucker und ihrer Derivative", Julius Springer, (1931).

227. Scales, F.M., "Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists". Third Ed., (1930). XXXIV - 36, page 378.