INVESTIGATIONS ON THE MANNANS OF IVORY NUT

(Phytelephas macrocarpa)

- by -

IAN R. WILLIAMSON, B.Sc.

Thesis for the degree of Doctor of Philosophy

University of Edinburgh

October, 1952

CONTENTS

Page

MANNAN A

Experimental

MANNAN B

Experimental

Contents

Summary

-----oOo-----

INTRODUCTION

Mannans (or mannosans) are polysaccharides which on hydrolysis give chiefly D-mannose.

Considerable interest attaches to the function of mannose polysaccharides in plant metabolism, and to their relationship to the universally distributed glucose polysaccharides. Mannan serves as a reserve food supply to a large number of different plants and occurs alone or united to glucose, galactose, pentose, etc. Carbohydrate material may be stored in plants in the form of greatly thickened cell walls, when the storage-tissue often acquires a bonelike consistency as is found with the endosperm of *Phytelephas macrocarpa*, and such carbohydrates may be included in the comprehensive category of reserve-cellulose, but chemically they are classified as hemicelluloses, a term which was used by Schulze (1) for those alkali-soluble cell wall constituents which are hydrolysed by hot dilute mineral acids. He used the term "hemicelluloses" since he considered that the substances might be intermediate in the formation of cellulose, but since on hydrolysis they give mannose, galactose, xylose, etc. the term is inapt but it is, nevertheless, commonly used. It is probable that in most cases such hemicelluloses occur in the cell wall not as free compounds, but as constituent units of more complex compounds or molecular aggregates which are decomposed during extraction of the non-cellulosic material from the cell wall (2, 3, 4).

The idea that mannose is a transitory substance is supported by the work of Hérissey (5) who found that seminase is present in the seeds of lucerne, which contain a galactomannan, and during germination sucrose is relatively abundant but mannose is not found, at any rate in any quantity. Thus, D-mannose, while widely distributed in plants apparently occurs, if at all, in the free state in active plant cells only to the extent of its formation during the synthesis of the more complex carbohydrates of which it is a constituent. The mannans in general may be considered terminal products of certain phases of carbohydrate metabolism in higher plants since they are found chiefly in cell walls. However, there are a number of exceptions, e.g., the mannans of Phytelephas macrocarpa may serve as food reserves.

Mannans may be classified according to the type of linking of mannose residues, or to the type of sugar residues present. The former method distinguishes those with 1:4- and 1:6-polymeric linkages, members of the first group appearing to be structurally analogous to cellulose and, on the basis of comparison of optical rotations, to have β-glycosidic linkages. The second method of classification recognises simple mannans, in which the polysaccharide chain is built only of mannose molecules, and mixed mannans, which on total hydrolysis give mannose and another sugar. The second type, which must be distinguished from mixtures of polysaccharides, are designated gluco-, galacto-, etc. mannans.

Miscellaneous Mannans

(excluding ivory nut mannans)

Konjak Mannan (6,7):

By means of superheated water a glucomannan can be extracted from the corms of Conophallus konjak and the same product is obtained by the action of aqueous pancreas preparations on konjak meal under toluene at 35° C. On total hydrolysis the polysaccharide gives mannose and glucose in the ratio 2:1, and acetolysis followed by saponification produces a trisaccharide consisting of two mannose and one glucose units. Methylation of konjak mannan gives a trimethyl product which on hydrolysis gives 2:3:4- and 2:3:6-trimethyl mannose and 2:3:4-trimethyl glucose. Thus, the molecule appears to have polymeric linkages of the 1:6- and 1:4- type.

Salep Mannan:

Orchid tubers (orchis sp.) when extracted with cold water give an alcohol-precipitable mannan, which has been studied by various workers (8, 9, 10, 11, 12, 13). Methylation, followed by hydrolysis gives 2:3:6-trimethyl mannose and end-group assay shows the molecule to have a chain length of 70-80 mannose residues.

Wood Mannans:

A marked difference between the conifers (*Gymnospermae*) and hardwoods (*Angiospermae*) occurs not only in structure but also in the chemical composition of the wood. At present, carbohydrates giving mannose on hydrolysis have been found in only one hardwood, since Fromherz (14) has isolated mannose from *Populus tremula* L. but not from other species of hardwoods, including *Populus tremuloides* Michx. Schorger (15) has shown that mannan is present in a large number of conifers, sometimes in considerable quantity, while Bertrand (16) considered that in them mannan filled the place occupied by xylan in the hardwoods. Tollens (17) found mannose in sulphite pulp, while Bertrand (16) showed the presence of mannan in the wood of other conifers. Kimoto (18) found mannan in *Cryptomeria japonica* Don., and Storer (19) studied several American species.

According to Schorger (15) the mannan content of the sapwood is generally greater than that of the heartwood, and decreases from the base upwards but remains uniform throughout the heartwood in a radial direction. It was found by Nowotnowna (20) that the mannan of softwoods is associated with the cellulose and that there is a large variation in the mannan-xylan ratio occurring in the cellulose.

Alkaline extraction following a pre-treatment with chlorine dioxide is used to extract the mannans from gymnosperms, and osmotic pressure and viscosity studies give results similar to those obtained with salep mannan, which indicates that pine mannans are possibly linear polymers closely related to cellulose, but the purity of these products is doubtful.

The presence of mannan in woods is of technical significance, and Schwalbe (21) stated that waste sulphite liquors contain sufficient fermentable sugars to give 60 litres of ethanol per "tonne" (2200 lbs.) of pulp, while Krause (22) claimed that mannose constitutes about 60% of the total fermentable sugars in sulphite liquors. Although exceptions occur, yeasts which ferment glucose have been found usually to ferment mannose equally well.

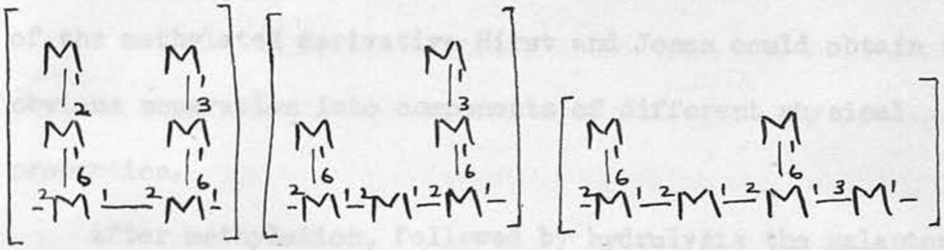
Yeast Mannan:

By the extraction of yeast with hot water and precipitation with alcohol, Schutzenberger (23) obtained a polysaccharide which, because of its resemblance to gum arabic, he called yeast gum. Salkowski (24) and Oshima (25) isolated the mannan by extraction with hot alkali and precipitation with Fehling's solution, and Kraut and Eichhorn (26) obtained it by alkali extraction. The yeast mannans obtained in various ways have different properties, and their optical rotations are compared in table 1.

Table I.

Name of Investigator	Mode of Separation of Mannan	$[\alpha]^{20}_{D}$	Reference
Béchamp.	Aqueous Extraction	+ 60	27
Nagelin and Lowe.		+ 78	28
Meigen and Spreng.		+ 58.5	29
" " "	Boiling $Ca(OH)_2$	+ 47.5	
" " "	Boiling 3% KOH	+ 89.5	
Hessenland.	Boiling $Ca(OH)_2$	+ 98	30
Salkowski.	Boiling 3-6% KOH	+ 90	24
Daoud and Ling.	Boiling 2% NaOH under pressure.	+ 70	31
Haworth, Hirst and Isherwood.	Boiling 6% NaOH	+ 87	32
Harden and Young.	Boiling water.	+ 78	33
Garzuly-Janke.	Treatment with cold 75% H_2SO_4	+ 67	34

Acetylated and methylated products have been obtained by Haworth, Hirst, and Isherwood (32) who, on hydrolysis of the fully methylated polysaccharide, identified 2:3:4:6-tetramethyl D-mannose, 2:3:4-trimethyl D-mannose, and 3:4-dimethyl D-mannose. More recent work by Haworth, Heath and Peat (35) confirmed that tetra-, tri-, and dimethyl mannose were produced in equimolecular proportions, and that the tetramethyl fraction consisted of 2:3:4:6-tetramethyl mannose and the dimethyl fraction of 3:4-dimethyl mannose. However, the trimethyl fraction was shown not to be exclusively 2:3:4-trimethyl mannose (which was present only to the extent of 10% of the trimethyl fraction) but mainly 3:4:6- and 2:4:6-trimethyl mannose in equimolecular proportions. Thus they proposed that one of the following formulae represented the structure of the repeating unit of the mannan molecule:



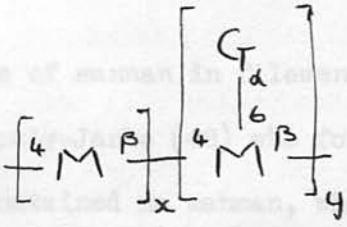
where, M = a D-mannopyranose unit.

Lindstedt (36) studied the hydrolysis of yeast mannan and its oxidation by the periodate ion and obtained results which agreed with the formulae given above. He also prepared a trityl mannan which, assuming that only primary hydroxyl groups reacted, had a trityl content agreeing with formulae of the type shown above.

Galactomannans:

A large number of investigations have been made of these polysaccharides, in which the ratio of D-galactose to D-mannose present varies greatly, and, therefore, it appears that some of these polysaccharides may be mixtures, e.g., of mannan and galactomannan. In their paper on the galactomannan of carob gum Hirst and Jones (37) stated that other workers (38, 39, 40) on the same polysaccharide found that it contained different ratios of galactose to mannose, and Hirst and Jones suggested that galactose may be stored in the plant by being attached randomwise to the main chain of mannose residues; or that the polysaccharides might have been mixtures, although by fractionation of the methylated derivative Hirst and Jones could obtain no obvious separation into components of different physical properties.

After methylation, followed by hydrolysis the galactomannans of carob seed (37, 41) and of guar seed (42, 43) give 2:3:4:6-tetramethyl D-galactopyranose, 2:3:6-trimethyl D-mannopyranose, and 2:3-dimethyl D-mannopyranose, all the galactose present being accounted for as non-reducing end group and no mannose non-reducing end group being detected. Therefore, Andrews, Hough, and Jones (44) suggested that most galactomannans could be represented by the basic formulation:



$$-\left[{}^{4}M^{\beta}\right]_{x}-\left[\begin{array}{c} G \\ |_{\alpha} \\ |_{6} \\ {}^{4}M^{\beta} \end{array}\right]_{y}-$$

where M and G represent D-mannopyranose and D-galactopyranose units, respectively.

Except for the lucerne seed galactomannan studied by Hirst, Jones, and Walder (45) (in which the ratio of galactose to mannose is 2:1) and the galactomannan from fenugreek seed (in which the ratio is 1:1) (44, 46) all the galactomannans studied contain more mannose than galactose. Hirst, Jones, and Walder (45) methylated the galactomannan of lucerne seed and after hydrolysis obtained a mixture of sugars among which 2:3:4:6-tetramethyl D-galactose, 2:4:6-trimethyl D-galactose, and 3:4-dimethyl D-mannose were detected, and they proposed that the polysaccharide had a structure in which half the galactose residues were present as non-reducing end group and other galactose residues were linked through positions 1 and 3, while at least one-third of the mannose residues were linked through positions 1, 2, and 6. Recently Andrews, Hough, and Jones (47) studied a galactomannan obtained from lucerne seed and found that its structure could be represented by the basic formulation given on page 8.

Other Mannans:

The occurrence of mannan in filamentous and budding fungi was studied by Garzuly-Janke (48) who found that fungi with a typical mycelium contained no mannan, whereas the budding fungi did, except Nadsonia and Rhodotorula (the latter yeast is, however, exceptional since it produces carotenoid pigments). He also found that there was an apparent antagonism between mannan and chitin production by fungi.

A polysaccharide mixture is produced when Penicillium Charlesii G. Smith grows on glucose solutions, and after fractional precipitation with alcohol two polysaccharides can be obtained, one giving galactose on hydrolysis and the other, named mannocarolose, giving mannose. After an earlier investigation of mannocarolose by Haworth, Raistrick, and Stacey (49) the polysaccharide was re-examined by Stacey (50) who found that after methylation and hydrolysis equimolecular amounts of 2:3:4- and 3:4:6-trimethyl mannose together with 2:3-dimethyl mannose were obtained.

The capsular substance of Bacillus krzemieniewski has been identified as a mannan (51) and the polysaccharide from M. tuberculosis (human strain) which contains mannose has also been studied (52).

The horny material which constitutes the sugar reserve of the albumin of iris seeds is an arabomannan (53), and Dubat (54) obtained mannose and glucose by hydrolysis from the seeds of several Liliaceae. Takahashi (55, 56) obtained a glucomannan, named "yurl mannan", from lily bulbs, and Curl and Nelson (57) obtained a water-soluble mannan from the seeds of Daubentonia drummondii (a shrub of the pea family), and from the roots of Bletilla striata Otsuki (58) separated a glucomannan. The last-named investigator also identified the characteristic polysaccharide from Cremastra variabilis as a glucomannan (59). Recently Jones (60) has studied a mannan from the seaweed Porphyra umbilicalis and proposed that it was composed of β -D-mannopyranose units linked through carbon atoms one and four, and had a repeating unit of twelve.

The Mannans of Ivory Nut

Ivory nuts are the fruit of the palm *Phytelephas macrocarpa* (Ruiz and Pav.) which grows in the tropics of South America. They are known also as vegetable ivory, tumako, corozo (or corosso) nuts, and tagua nuts.

Industrial Uses of Ivory Nuts

Because of their hardness ivory nuts are used extensively in the manufacture of buttons, and a brief description of the process has been given by Replat (61). Dyeing of vegetable ivory has been described (62, 63), as has the manufacture of buttons from ivory nut waste (64). Ivory nuts, ground and heated to 100° F can be pressed into moulds, binding agents such as nitrocellulose of low nitrogen content, and colouring matter being added if required (65).

Ivory nut waste has been used for the adulteration of foodstuffs, e.g., it has been mixed with bran and cheap flours, although it can easily be detected microscopically (66, 67), and patents have been issued for coffee substitutes containing ivory nuts (68).

The high mannose content of the ivory nut makes it valuable for the preparation of mannose. Hudson and Sawyer (69) hydrolysed ivory nut shavings with sulphuric acid and obtained the sugar in a yield of 40% of the weight of vegetable ivory used, and various modifications of Hudson's method have been described (70, 71, 72, 73).

Mezzadroli (74) isolated a number of micro-organisms with pronounced fermentative action on the mannose obtained from vegetable ivory waste, and obtained 15 litres of alcohol per 100 Kg. of vegetable ivory. The production of alcohol from ivory nuts has been discussed by a number of other workers (75), and the preparation of lactic acid by hydrolysis of vegetable ivory and innoculation with a lactic ferment has been described by Saxe (76).

Biological Decomposition of Mannans

During an investigation into the action of malt diastase on a suspension of ivory nut shavings Paton, Nanji, and Ling (77) observed that, whereas diastase had no action if the suspension had been boiled, when it was added to the unboiled suspension a considerable amount of reducing sugar was formed, and it was shown that the shavings contained an enzyme capable of hydrolysing the mannan present. However, Melnick and Cowgill (78) could detect no mannanase in vegetable ivory, which was found to contain an albumin and globulin, and a reducing substance. The last-named compound gave mannose on acid hydrolysis, but was not identified with any carbohydrate which had previously been reported. Pringsheim (79) isolated a trisaccharide (mannotriose) by fermenting ivory nut mannan with a bacterium obtained from an earth culture, and Pringsheim and Genin (80) studied the fermentative cleavage of salep mannan and postulated that two enzymes, a mannanase and a mannobiase, were present in malt extract, but Klages and Kircher (81) were unable to separate the enzymes of barley malt into di- and polysaccharase and found that essentially the same results were obtained using salep or ivory nut mannan as the substrate, which indicated that the polysaccharides were similar.

Bierry and Giaja (82) found that the digestive juices of molluscs and crustaceans exhibited a more or less marked action on mannans. They also showed (83) that the hepato-pancreatic juice of <u>Helix pomatia</u> attacked mannans and that the intestinal juice of <u>Astacus fluviatilis</u> liberated the galactose of the mannogalactan of lucerne, mannose being found only partially in a degree dependent on the concentration of enzyme present. They considered the differentiation indicated that the "mannogalactan" was a mixture of mannan(s) and galactan(s). The juice attacked ivory nut mannan more readily than lucerne mannan. An α-mannosidase was found in snail enzyme extracts also by other workers (84), and another mannan-hydrolysing enzyme has been described by Tadokoro (85). Bierry and Giaja (82) found no evidence that mannans were hydrolysed by the digestive juices of mammals, and the feeding value to cattle of vegetable ivory meal has been discussed by several investigators (86, 87). Gatin and Gatin (88) found that the mannans present in Japanese edible algae were not digested by man or the higher animals, and postulated that their value in dietetics might be attributed to their stimulating effect on peristaltic action. An enzymic solution extracted with acetone-ether from cow pancreas (89) showed no action on elephant root mannan, but the mannanase prepared from Bacterium aroideae and Bacillus mesentericus saccharised the mannan to mannose and glucose.

Historical Introduction to The Chemistry of Ivory Nut Mannans

Since microscopic methods afforded little information on the nature of the "cellulose" stored as reserve food material in seeds, Reiss (90) studied the action of hydrolytic agents on the "cellulose" forming the thick-walled cells of the endosperm of ivory nuts. In contrast to earlier authors, who referred almost exclusively to cellulose, Reiss showed that in the cell wall cellulose does not always occupy a prevalent place, and that sometimes it is completely absent. There existed a series of substances which were similar to cellulose, e.g., in microchemical reactions, and which on hydrolysis gave various sugars. Reiss extracted ivory nut shavings with cold 75% sulphuric acid and obtained a carbohydrate, which he called seminin, which swelled up and partially dissolved in water to give a laevo-rotatory solution which reduced Fehling's solution. On hydrolysis of seminin he obtained a thick non-crystallisable syrup which he named seminose, and which was shown by Fischer and

Hirschberger (91) to be identical with mannose.

Before the discovery of seminin it was known that the cell wall contained substances other than cellulose and that on hydrolysis galactose and xylose could be obtained, but the nature of these substances was not investigated and they were considered to be indefinite incrusting substances. Schulze (1) proposed the division of cell wall constituents into two groups, the hemicelluloses, which on warming with mineral acids easily changed to sugar, and cellulose, which was hydrolysed with difficulty.

Johnson (92) extracted ivory nut shavings with 10% potassium hydroxide and after precipitation with alcohol and purification of the polysaccharide preparation, found it gave mannose on hydrolysis and had an analysis corresponding to $C_6H_{10}O_5$.

Later, Baker and Pope (93) extracted a carbohydrate from ivory nuts with 5% sodium hydroxide, precipitated it as the copper complex with Fehling's solution, and decomposed the complex with hydrochloric acid to obtain a product which on hydrolysis gave a syrup, 90% of which could be converted to mannose phenylhydrazone. Although they did not isolate it in a crystalline condition, they considered the remainder to be chiefly fructose, since it was laevo-rotatory, had a very sweet taste, and gave phenylglucosazone. Therefore, using the nomenclature of Schulze (94) they termed the polysaccharide a laevulo-mannan.

Studying the carbohydrate constituents of the cell wall of the seed of Phytelephas macrocarpa, Ivanow (95) extracted the ivory nuts with ether and 0.3% potassium hydroxide to free them from fats and protein, and found that the residue contained 2.2% pentosan and 1.6% methyl pentosan, while in addition to glucose mannose was the only hexose found in appreciable amount (37%); only traces of galactose were detected. He identified the pentose as arabinose, which was considered to be a constituent of the cell wall, and mannose was shown to be present in two modifications, as hemicellulose and manno-cellulose.

Taking advantage of the general method of Schmidt and Graumann (96) of using chlorine dioxide to remove lignin from plant products without attacking the carbohydrates present, Pringsheim and Seifert (97) isolated mannan by extracting the "cleaned" ivory nuts with 5% sodium hydroxide, which removed the mannan present as hemicellulose and left the mannocellulose as a residue. Contrary to the findings of Baker and Pope (93), no fructose was detected in the mannan, or in the mannocellulose (which contained, besides mannose, 5-10% of glucose).

Patterson (98) obtained a product different from that of Pringsheim and Seifert. He removed nitrogenous and resinous matter and pentosans from ivory nut meal by treatment with hot

10% sodium hydroxide, then extracted the "cleaned" material with 20% alkali. The polysaccharide-sodium hydroxide complex was isolated as a light gelatinous precipitate on treatment of the extract with alcohol, and was decomposed by boiling with dilute acetic acid to give the polysaccharide (yield 10%). The preliminary sodium hydroxide treatment removed most or all of the mannan which had been studied by Pringsheim and Seifert, and by Baker and Pope. The compound studied by these workers was the modification described by Ivanow as hemicellulose, while Patterson studied the mannocellulose (which he referred to as mannan). He concluded that the mannan was built up entirely from mannose residues since no other sugar derivative was isolated by direct hydrolysis of the polysaccharide, methanolysis of the polysaccharide or triacetate, or hydrolysis of the methylated mannan.

After ten treatments of the polysaccharide with dimethyl sulphate and sodium hydroxide Patterson obtained a product having a methoxyl content of 42.5% (theoretical for a trimethyl hexosan = 45.6%). The product was a slightly yellow, amorphous powder, insoluble in ether, soluble in chloroform, ethyl acetate, acetone, and benzene and showing a marked tendency to form a gel in these solvents. The methylated mannan, on treatment with acid methanol did not give a quantitative yield of the corresponding methyl mannosides and hydrolysis of the resulting syrup with aqueous acid gave a viscous syrup consisting almost entirely of trimethyl mannose. Since the trimethyl mannose could be converted almost quantitatively to tetramethyl α -methyl mannoside, Patterson concluded that all the mannose residues were of the ordinary or stable type. He could not, however, decide whether only one trimethyl mannose was present and, because he did not know the position of the methoxyl groups in the product, he could not determine the position of linking of the mannose units in the mannan.

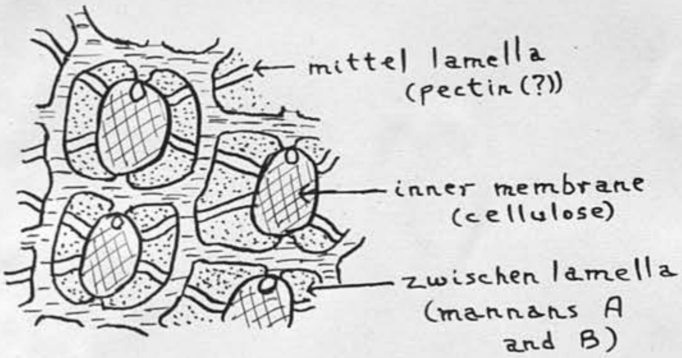
Lüdtke (99) found that ivory nut shavings cleaned with chlorine dioxide and sodium sulphite were completely soluble in Schweizer's solution after ammonium carbonate had been added, and that two different mannans could be separated in the form of their copper-alkali complexes by treating the solution with 2N sodium hydroxide until the mixture was 0.2N with respect to sodium hydroxide, whereas a third component remained in solution. This third substance was precipitated by adding acetic acid and was identified as cellulose by means of its rotatory power in cuprammonium solution, rotatory power of the triacetate, and isolation of cellobiose octaacetate. The mannan mixture was separated into a fraction soluble in 4% alkali (named "mannan A") and one insoluble therein (named "mannan B"). Mannan A was the product investigated by Baker and Pope (93) and its properties were found to agree with those given by the earlier investigators.

The two mannans were distinguished by their optical rotatory power and colour reactions, and these are given in the table below (for comparison the values for cellulose are also given):

	Mannan A	Mannan B	Cellulose
$[\alpha]^{20}_{D}$ in N-NaOH	- 45	Insoluble	Insoluble
$[\alpha]^{18}$ in Schweizer's solution after addition of NH_4HCO_3	+ 0.61	+ 0.52	- 2.05
$[\alpha]^{20}_{D}$ of acetate in $CHCl_3$	- 29	- 25	- 19
Colour with:			
ClZnI	None	Violet	Violet
$I.KI.H_2SO_4$	None	None	Violet

That the colour given by mannan B with chlor-zinc-iodide was not due to admixed cellulose was shown by hydrolysis of the mannan to mannose in a yield proving that the polysaccharide consisted solely of mannose residues. Mannose was also obtained in equal yield from mannan A. The mannans were shown by fractional precipitation from different media to be homogeneous, and gave analyses agreeing with $C_6H_{10}O_5$.

Before the work of Lüdtke the carbohydrates of ivory nuts had been divided into mannan and mannocellulose. He had shown chemically that mannans A and B contained no cellulose and to obtain further evidence he studied ivory nuts microscopically. Gilson (100) had observed that on treating ivory nut slices with chlor-zinc-iodide three layers were visible under the microscope - a highly coloured "inner" layer, a very weakly coloured "zwischen" layer, and a third or "mittel" layer which was uncoloured. Lüdtke found that the mittel layer was dissolved by chlorine dioxide-sodium sulphite treatment, and a microscopic section of such a tissue after treatment with chlor-zinc-iodide as seen in polarised light is shown diagrammatically below:



This diagram showed the distribution of the separate carbohydrates in the ivory nut. The inner membrane, coloured strongly, contained cellulose and the weakly coloured zwischen layer was a mixture of mannans A and B, the latter being responsible for the colour reaction. The nature of the mittel layer remained unknown but Lüdtke suggested that it might contain the pentosan and methyl pentosan detected by Ivanow (95). It was not possible to decide whether or not mannans A and B were chemically bound together, but it was apparent that neither the unknown substance contained in the mittel layer nor the cellulose of the inner layer was chemically combined with the mannan complex.

Ivory nut tissue after delignification was found by Lüdtke to be doubly refracting and after removal of mannan A by extraction with sodium hydroxide the residue was anisotropic, as was the residue from which mannan B had been removed by stronger alkali. According to Herzog and Gonell (101) the insoluble reserve carbohydrates in plant seeds, including ivory nut mannan, are crystalline whereas more readily available energy reserves are amorphous. Mannan preparations from ivory nut and spruce pulp, although amorphous to outward appearances, have been shown to give a moderately sharp X-ray diffraction pattern (102), and Yundt (103) was able to form supersaturated solutions of ivory nut mannan A and by maintaining them at 60-70° C obtained mannan crystals which, when first visible, appeared to be rod-shaped but later assumed a dumbbell shape.

Klages (104) treated ivory nut shavings with chlorine dioxide and extracted mannan A by shaking the residue with 5% sodium hydroxide. Acidification of the extract and addition of methanol gave the polysaccharide as a white powder (yield 40% of the weight of ivory nuts used $[\alpha]^{20}_{D}$ -45° (in N NaOH)).

In an attempt to obtain a fully methylated product, Klages added benzene to the dimethyl sulphate-sodium hydroxide reaction mixture and carried out the reaction at 80° C. (These conditions are somewhat drastic compared with modern methylation technique and might have led to serious degradation of the mannan molecule.) Thus, after two treatments he obtained a product with a methoxyl content of 45.8%. The trimethyl mannan was a yellowish, horny solid and, in contrast to trimethyl cellulose, was fairly soluble in water and produced solutions stable on heating; and it had a small solubility in chloroform, separating out overnight as a gelatinous mass from solutions of concentration greater than 2%. The so-called "high-molecular" behaviour was less strong than that of cellulose derivatives, and corresponded to that of cellodextrins, e.g., concentrated solutions showed no great viscosity and on evaporation of the solution on a glass plate no film was formed. The trimethyl mannose obtained by hydrolysis of the methylated mannan could not be crystallised, but gave a crystalline anilide which was shown by mixed melting point to be identical with the anilide of 2:3:6-trimethyl mannose obtained by Haworth, Hirst, and Streight (105). Therefore, Klages concluded that the mannan consisted either of 1:4-linked mannopyranose units or of 1:5-linked mannofuranose units. To decide which was the case he partially hydrolysed the methylated mannan, methylated the oligosaccharides produced and hydrolysed them. From the end groups a tetramethyl mannose having the ring structure of the mannose units in the original polysaccharide was obtained, and since this was found to be 2:3:4:6-tetramethyl mannose he concluded that the mannan must be built from 1:4-linked mannopyranose units. He found that the trimethyl mannose obtained by hydrolysis of the methylated mannan gave the corresponding anilide in a yield of only 65% and the corresponding yield for the tetramethyl sugar was 50%, whereas, under the same experimental conditions 2:3:4:6-tetramethyl glucose could be quantitatively converted to the anilide. Therefore, he considered his tri- and tetramethyl fractions to be impure and studied the anilide formation from pure tri- and tetra-methyl mannose (obtained by hydrolysis of the corresponding crystalline anilides) but obtained the same low results. He concluded, however, from his investigations that mannan A was built up to an extent of at least 90% in the specified linking of mannose units.

Klages attempted to determine whether the linkages in the mannan were of the α- or β- type and applied several methods to the solution of this problem. Comparison of the rotatory power of the methylated mannan with that of the corresponding methyl mannosides gave results which varied with the solvent used, but which indicated that the polysaccharide was not sterically homogeneous. He also studied the optical rotation of the oligosaccharides produced by partial hydrolysis of the trimethyl mannan but obtained no conclusive results. He assumed, however, that mannan A consisted of a mixture of α- and β- mannans which formed an addition compound, and that the two components were present possibly in the ratio 1:1.

In a later paper Klages and Maurenbrecher (107) re-examined the question. They prepared a mannobiosazone from the mannan and by studying it they concluded that a purely β- mannosidic structure must be assigned to mannan A.

Klages determined the chain length of mannan A by two methods. On one end of the fully methylated polysaccharide a tetramethyl mannose residue is present and after hydrolysis of methylated mannan he separated this from trimethyl mannose by extracting the aqueous solution with chloroform and identified it as the anilide. The weight obtained gave a chain length of about 80 mannose residues for the methylated mannan. For the determination of the reducing group present in the molecule he used the iodine number method. This method had been used by Bergmann and Machemer (108) for the determination of the molecular weight of cellulose, but had been rejected for this purpose by Hess (109) since the oxidation was found not to cease at the limit given by Bergmann but could be increased by repeated action of hypoiodite. Klages found that hypoiodite oxidation gave a value of <u>ca.</u> 70 for the degree of polymerisation of the methylated mannan, and <u>ca.</u> 30 or less for the unmethylated polysaccharide.

More recently, Ward (110) has studied mannan A. He prepared the polysaccharide by three methods, that used by Lüdtke (99) and Klages (104) by extracting the ivory nuts with 5% sodium hydroxide after treatment with chlorine dioxide, by extraction with sodium hydroxide and purification by means of the copper complex as used by Baker and Pope (93), and extraction with 5% sodium hydroxide after treatment of the ivory nuts with sodium chlorite and phosphoric acid. Using the method of Hirst, Jones, and Woods (111) he estimated the mannose content of the hydrolysed polysaccharide by forming mannose phenylhydrazone, and obtained values of 90, 90, and 40%, respectively, for the mannose content of the three preparations.

After hydrolysis of the mannan, followed by neutralisation, Ward fermented the product with yeast to destroy mannose. Addition of phenylmethylhydrazine in alcohol (111) gave no precipitate, nor was it possible to isolate mucic acid after treatment of the hydrolysate with 25% nitric acid by the method of Schorger (112) and, therefore, Ward concluded that the mannan contained no galactose.

Methylation of the mannan with dimethyl sulphate and sodium hydroxide gave, after three treatments, a product which was purified by being dissolved in acetone followed by portionwise addition of light petroleum. The main fraction (yield 60% of the theoretical) was used in the later investigations and had a methoxyl content of 44.8%, ash content of 2.6%, and $[\alpha]_D^{11}$ -22° (c , 1.0, in chloroform). This was hydrolysed by the method described by Haworth, Heath, and Peat (35) using glacial acetic acid and 5% hydrochloric acid and the methyl mannosides were formed, and separated by the solvent extraction method of Brown and Jones (113). 2:3:4:6-tetramethyl mannose, identified by conversion to the anilide, was obtained in a yield which gave a value of 15 for the repeating unit of the polysaccharide. The trimethyl methyl mannoside fraction was divided into two by distillation under reduced pressure and after hydrolysis the first fraction was identified as 2:3:6-trimethyl mannose, while the second, which was not characterised, was considered to contain 2:3:6- and possibly 2:3:4-trimethyl mannose.

Periodate oxidation of the mannan by the method of Halsall, Hirst, and Jones (114) showed that one mole of formic acid was liberated by every five or six mannose units, and on an average each mannose residue consumed 0.97 moles of periodate. To explain his results, Ward postulated that the mannan A had a repeating unit of 15 and contained, besides 1:4-linkages, possibly some 1:6- and perhaps 1:3- linkages (although he did not isolate 2:3:4- or 2:4:6-trimethyl mannose from the products of hydrolysis of the methylated mannan).

Using essentially the same methods that he used to elucidate the structure of mannan A, Klages studied the constitution of mannan B (115). He isolated mannan B by the method used by Lüdtke (99) and found it to have the constants given by that author. The product proved more difficult to methylate than mannan A and the derivative examined had a methoxyl content of 43%. After complete hydrolysis, besides 2:3:6-trimethyl mannose Klages obtained 2:3:4:6-tetramethyl mannose, which could be converted to the anilide only in very poor yield. However, he concluded that the mannan was composed of 1:4-linked mannopyranose units.

Despite their similar constitutions Klages considered that the two mannans were not built up identically since their derivatives showed differences in rotatory power, as given in the following table:

	Mannan A	Mannan B
$[\alpha]_{435.8}$ of mannan in cuprammonium.	+ 0.63	+ 0.50
$[\alpha]_D$ of mannan in :		
2N NaOH	- 44	-
15% NaOH	- 44.5	- 38
$[\alpha]_D$ of triacetyl mannan in $CHCl_3$	- 29.5	- 26
$[\alpha]_D$ of trimethyl mannan in:		
$CHCl_3$	- 21	- 17
C_6H_6	- 60	- 56.5
H_2O	- 51.5	- 49

Thus the mannans might be constructed differently, or one or both might be contaminated with another carbohydrate. However, Klages considered the second suggestion to be unlikely since the difference of rotatory power of all the derivatives of the two mannans was almost constant. It was possible that, as postulated for mannan A, mannan B consisted of α- and β-mannans which, if present in different amounts in each mannan, might explain the difference in rotation.

The kinetics of hydrolysis of methylated mannan B agreed with those of methylated mannan A and, by studying the oligosaccharides produced by partial cleavage of the methylated polysaccharide Klages found that mannan B contained 87% α- and 13% β- linkages (compared with 80 and 20%, respectively, obtained in a similar series of experiments on methylated mannan A). As for mannan A, he assumed that the α- linkages were more readily hydrolysed than the β- linkages and were, therefore, first to appear in the oligosaccharide fractions.

The above summary has reviewed the work on ivory nut mannans carried out before that undertaken in the present investigation, the purpose of which was to decide whether mannans A and B were similarly constituted and to discover the fine structure of each. In particular, in the case of mannan A it was necessary to decide whether the comparatively long unbranched structure postulated by Klages or the short repeating unit structure indicated by the work of Ward represented the true form of the molecule. In the present investigation frequent use was made of the modern methods of chromatography, which proved of great value in the detection of small amounts of material, and which had not previously been applied to the study of ivory nut mannans.

MANNAN A

Discussion

Ivory nut shavings, cleaned by extraction with benzene and with methanol, were delignified under mild conditions with sodium chlorite and acetic acid at pH 4.0. Mannan A was extracted by shaking the delignified material with 7% potassium hydroxide and was precipitated by acidifying the extract with acetic acid and adding methylated spirits. Using the general method of purification which was introduced by Salkowski (116) in his work on yeast mannan, the mannan A was purified as the copper complex by precipitation from alkaline solution by the addition of Fehling's solution. After a second precipitation as the copper complex, the purified mannan A had $[\alpha]_D^{15} - 46^{\circ}$ (c, 0.7 in N sodium hydroxide) compared with the value $[\alpha]_D^{20} - 45^{\circ}$ (in N sodium hydroxide) given by Klages (104) for mannan A.

After hydrolysis and examination on a paper chromatogram the purified mannan was found to yield, in addition to mannose, traces of glucose and galactose. In an attempt to remove these sugars the mannan was fractionally precipitated from slightly acid solution by the gradual addition of methylated spirits. Four fractions were obtained and they were hydrolysed and examined by paper chromatography but there was no apparent reduction in the amount of glucose or galactose present in any fraction.

Anhydrous formic acid has been used by Jones (60) for the hydrolysis of mannan and this reagent was found to be suitable for the hydrolysis of ivory nut mannans. The hydrolysate from mannan A was separated into its component sugars by paper partition chromatography and the amounts of sugars present were estimated, mannose being determined by oxidation with sodium periodate, using the method described by Hirst and Jones (117). The hydrolysate had been spotted onto the chromatogram papers from a micropipette and, since the amount of sugar was the same on each chromatogram, for the estimation of the trace constituents several papers were combined and the sugars estimated by the colorimetric method of Nelson (118). Thus it was found that the amounts of sugars present in the mannan hydrolysate were: mannose 97.6%, galactose 1.8%, and glucose 0.8%.

To determine the total amount of mannose present in the mannan a weighed sample of a reference sugar (xylose) was added to the hydrolysate after treatment with formic acid, the solution neutralised, and the ratio of mannose to xylose estimated after separation on a paper chromatogram. A control experiment was also run in which a sample of mannose was treated with formic acid to determine the amount of mannose destroyed during hydrolysis. The results obtained showed that from the mannan 96% mannose was recovered, and from pure mannose the recovery was 95%.

Because of the difficulty of achieving complete separation of the small quantities of glucose and galactose present a separate estimation of these two sugars was carried out in the absence of a reference sugar, as described above.

The mannan was acetylated by the method of Pacsu and Mullen (119). After dispersal in pyridine and treatment with acetic anhydride an acetate was obtained which had $[\alpha]^{14}_{D}$ -25° (c, 1.0 in chloroform) and an acetyl content of 43.8% (calculated for a triacetate, 44.8%). The molecular weight of the acetate, determined by Barger's method, corresponded to a degree of polymerisation of 10 - 13.

A value for the molecular size of a polysaccharide can be obtained by determining its reducing power by oxidation of the potential aldehydic group to a carboxyl group and measurement of the consumption of oxidising agent. The values for chain length obtained by this method are of doubtful value since, first, previous oxidation of some of the reducing groups may have taken place, and, secondly, the presence of small amounts of highly reducing substances would give a higher value for the reducing power. Bearing these facts in mind, two methods of estimation of the reducing power of the polysaccharide were carried out since they might give some value of the order of molecular weight of mannan A and might be used to compare mannans A and B.

In the first method, the mannan was oxidised with alkaline hypoiodite in a buffered medium (pH 11.4) and the results indicated that one reducing group was present for every 100 mannose units. The second method, introduced by Meyer, Noelting, and Bernfeld (120), depends on the reduction in hot (65° C) alkaline solution of 3:5-dinitro-salicylic acid to a highly coloured compound. Using known weights of mannose a graph of the absorption (measured photometrically) against concentration was drawn and by comparing the absorption of a known weight of polysaccharide with the curve the chain length of the mannan was calculated and found to be 28. Instead of mannose, it would have been better to use as a standard a mannobiose having the same linkage as that of the mannan, but unfortunately no such biose was available. It has been found that the reducing power per mole of disaccharide is greater than that of the corresponding monosaccharide and, therefore, the degree of polymerisation determined using mannose as a standard is low. However, the results obtained from measurements of the reducing power of mannan A do not agree with values for the molecular size determined by other methods (e.g., from the molecular weights of the acetate and trimethyl derivative) and doubt is cast on their validity.

The classical technique of methylation, followed by hydrolysis and identification and estimation of the methylated monoses, gives a valuable insight into the structure of a polysaccharide and this method, combined with recent chromatographic methods, has proved very useful in the study of ivory nut mannans. Mannan A was methylated eleven times by the method of Haworth (121) using dimethyl sulphate and sodium hydroxide and twice by Purdie's method (106) using methyl iodide and silver oxide. The crude methylated material was fractionated by dissolution in light petroleum-chloroform mixtures of increasing chloroform content, and in this way two major fractions were obtained. They had, respectively, $[\alpha]_D^{17}$ -22°, -23° (c,1.0 in chloroform) and methoxyl contents of 44.4 and 44.2%, and were combined.

A sample of fully methylated mannan was hydrolysed with formic acid and the hydrolysate neutralised and examined on a long paper chromatogram. This indicated the presence of tetramethyl and trimethyl mannoses, and smaller quantities of tetramethyl galactose and dimethyl mannose.

The methylated mannan was hydrolysed by heating with anhydrous formic acid and then with dilute sulphuric acid. The hydrolysate was neutralised and separated on a cellulose column, the recovery being 95%.

The fractions obtained from the column were examined:

Fraction I was identified as 2:3:4:6-tetramethyl D-mannose by formation of the anilide by heating under reflux with ethanolic aniline. A sample was converted to the lactone by oxidation with bromine water and from this the phenylhydrazide was formed by treating the lactone in dry benzene with phenylhydrazine. Fraction I was obtained partly crystalline and needle shaped crystals were obtained which melted at 109-111°C. Tetramethyl mannose has usually been obtained as a syrup, but it has been obtained crystalline by Greene and Lewis (122) and by Hendricks and Rundle (123). The crystalline material melted at 49-50°C and had $[\alpha]_D^{25}$ + 6° falling to an equilibrium value of 0°. Although it was impossible to obtain tetramethyl mannose in a completely crystalline form in the present investigation, a mixture of crystals and syrup had $[\alpha]_D^{20}$ + 23° (c, 0.7 in water) which fell to an equilibrium value of + 10°, indicating at least a predominance of the α - form.

Fraction II was identified as 2:3:4:6-tetramethyl D-galactose by conversion to the anilide, which had the correct rotation and melting point (which was undepressed on admixture with a sample of 2:3:4:6-tetramethyl galactose anilide).

Fraction III consisted of 2:3:6-trimethyl D-mannose, which was identified by conversion to the anilide. The fraction was also oxidised to the lactone, which was converted to the phenylhydrazide of 2:3:6-trimethyl D-mannonic acid.

Fraction IV was partly crystalline and was separated on thick paper chromatograms into two fractions. Fraction IVa (206 mg.) crystallised and fraction IVb (34 mg.) remained as a syrup.

Fraction IVa:

Initially this fraction was partially crystalline and was reducing, as shown by its reaction with aniline oxalate, and travelled on a paper chromatogram at a speed corresponding to that of a trimethyl hexose but different from 2:3:6- and 3:4:6-trimethyl mannoses. After several weeks the fraction crystallised almost completely and failed to react with aniline oxalate, and it was also non-reducing to Fehling's solution and to alkaline hypoiodite. Failure to react with aniline oxalate containing 5% phosphoric acid on a chromatogram at 100° C indicated that the compound was not readily hydrolysed. The crystalline material (X) was analysed and found to contain:

C, 50.5%: H, 7.9%; OMe, 42.9%.

These figures corresponded to those required for a hexamethyl disaccharide.

The substance (X) had the following constants:

melting point, 148-150°C;

$[\alpha]_D^{18}$ + 55° (c, 1.4 in water, unchanged in 48 hours);

$[\alpha]_D^{18}$ + 65° (c, 0.7 in 1% methanolic hydrogen chloride, unchanged in 100 hours).

Colour tests showed that the substance (X) was not a ketose derivative, and demethylation followed by examination on a paper chromatogram showed it to be a derivative of mannose. The molecular weight of the substance (X) was determined by Barger's method and found to correspond to that of a disaccharide.

From the previous evidence the crystalline material (X) appeared to be a non-reducing disaccharide of the trehalose type, i.e., having a 1:1 linkage. On hydrolysis only one sugar (Y) was produced since the hydrolysate gave only one spot on paper chromatograms run in different solvents.

Methylation of the disaccharide (X) followed by hydrolysis gave a substance which ran on paper chromatograms at the same speed as 2:3:4:6-tetramethyl mannose, and which was identified as the latter by conversion to the anilide (yield, 90%).

The previous evidence indicated that the disaccharide (X) was composed of two trimethyl mannopyranose units (Y) which were the same and, since they had been shown to be different from the 2:3:6- and 3:4:6-isomers, must be either 2:3:4- or 2:4:6-trimethyl mannose.

Oldham and Rutherford (124) showed that when a sugar derivative having a *p*-toluenesulphonic ester residue on a primary, but not on a secondary alcoholic group is heated with sodium iodide in acetone solution, iodine replaces the tosyl residue. The disaccharide was tosylated and the tosyl derivative heated with acetonic sodium iodide and although only 7% iodine was introduced (theoretical, 39%) the experiment showed the presence of some material containing a free primary hydroxyl group.

Oxidation of the unknown trimethyl mannose (Y) with lead tetraacetate showed the presence of two adjacent hydroxyl groups, and oxidation with periodate liberated formaldehyde, showing that the free hydroxyl groups were attached to carbon atoms five and six.

These facts showed that the monosaccharide (Y) was 2:3:4-trimethyl D-mannose and, therefore, the disaccharide (X) was 1- [2:3:4-trimethyl D-mannopyranosido] -2:3:4-trimethyl D-mannopyranose.

When fraction IVa was obtained from the column it was dried *in vacuo* at 30°C. The product was shown by treatment with aniline oxalate to be reducing, and formation of the disaccharide took place on standing at room temperature. The formation of disaccharides by condensation of two monosaccharide units under more drastic conditions has been reported, and the pyrolysis of ivory nuts has been found to give

1:6-anhydro-β-D-mannopyranose (125), but this appears to be the first occasion on which the condensation of two aldose units to form a disaccharide has taken place at room temperature.

Fraction IVb:

This syrupy fraction was shown by demethylation to be a mannose derivative. It ran on a chromatogram at the same speed as 2:3-dimethyl mannose and a speed different from that of 3:4-dimethyl mannose and was, therefore, assumed to be the former. Since the amount of dimethyl sugar was less than the amount of tetramethyl galactose the 2:3-dimethyl mannose appeared to have no structural significance and had arisen from under- and/or demethylation.

Fraction V was the water wash from the column and contained colouring matter and a trace of glucose.

From the yields of the fractions obtained from the column it was possible to calculate the molar ratios of the methylated sugars present. The results are collected in the table given below:

Fraction No.		Contents of Fraction	Yield (g.)	Moles $x10^3$	Molar Ratios		
I		2:3:4:6-Tetramethyl mannose.	0.242	1.02	1	4.3	1
II		2:3:4:6-Tetramethyl galactose.	0.058	0.24	0.2	1	
III		2:3:6-Trimethyl mannose.	2.623	11.8	11.6	49	10
IV	IVa	2:3:4-Trimethyl mannose (isolated as disaccharide).	0.206	0.97	0.95	4.0	
	IVb	2:3-Dimethyl mannose.	0.034	0.16	0.16	0.7	
V		Colouring matter and trace (5 mg.) of glucose.	0.074				

While it was impossible to separate completely on paper chromatograms tetramethyl mannose from tetramethyl galactose and 2:3:4- from 2:3:6-trimethyl mannose, the ratio of tetramethyl to trimethyl sugar was determined. The ratio of 1 part tetramethyl sugar to 9 parts trimethyl sugar agreed with the results obtained from the cellulose column.

Estimations on mannan A had shown it to yield after hydrolysis 1.8% galactose, while the amount of tetramethyl galactose obtained from the cellulose column was 1.7%. Combined with the absence of any other methylated galactose derivatives in the hydrolysis products of methylated mannan A the complete recovery of galactose as end group showed that the mannan was not contaminated by a galactan and the galactose must be present as the non-reducing end group of a galactomannan.

As no methylated derivatives of glucose were isolated from the cellulose column, the small amount (0.8%) of glucose present in the original polysaccharide must have arisen from a small quantity of glucosan, which was removed during the methylation procedure.

Because of the stability of the polysaccharide to acid hydrolysis it must be composed of pyranose units, and since the main component of the hydrolysate from methylated mannan was 2:3:6-trimethyl mannose the mannan must be built mainly of 1:4-linked anhydromannose units. The optical rotation of the mannan in sodium hydroxide $[\alpha]_D^{15} - 46^\circ$ (c, 0.7) and in anhydrous formic acid $[\alpha]_D^{15} -28^\circ$ (c, 0.8, unchanged in 70 hours), and of the derived acetate in chloroform $[\alpha]_D^{14} -25^\circ$ (c, 1.0), indicated that the majority of the linkages in the polysaccharide were of the β -type.

The molecular weight of methylated mannan A was determined by Barger's method (126) which gave a value of 9-11 for the chain length. Determination of the viscosity of a sample of methylated mannan and application of the Staudinger constant (127) gave a value of 9 for the degree of polymerisation; but too much value could not be attached to this latter determination since the method is not accurate at the low value of viscosity shown by the methylated polysaccharide.

From the foregoing evidence it is obvious that not all the molecules present in mannan A possess identical structures and it is impossible to suggest unambiguous structures for the molecular types present in the mixture. However it is possible to suggest two structures for mannan A. The mannan may consist of a mixture of two molecular types:

(a) a linear molecule of 10 anhydro-D-mannopyranose units linked through carbon atoms 1 and 4, and one residue with a 1:6-linkage; and,

(b) a linear molecule of 10 anhydro-D-mannopyranose units linked through carbon atoms one and four, terminated at the non-reducing end by a D-galactopyranose residue.

Determination of the relative amounts of tetramethyl mannose and tetramethyl galactose required that the molecular types (a) and (b) should be present in the ratio of 4:1. The amount of 2:3:4-trimethyl mannose obtained from the column appeared insufficient to indicate the presence of a 1:6-linkage in all members of both molecular types, but was sufficient to contribute four such linkages to each five molecules. These investigations did not show whether the 1:6-linked mannose residues were situated at the end of or near the middle of the molecules, nor whether all such linkages were present in the mannan molecules (type (a)) and none in the galactomannan molecules (type (b)), or distributed at random between the two types. Another possible structure which would agree with methylation and molecular weight results is a mixture of two molecular types:

(c) A linear molecule of ca. 13 mannose residues linked through carbon atoms 1 and 4, terminated at the non-reducing end by a mannose residue; and,

(d) A linear molecule of 4 mannose residues linked through carbon atoms 1 and 6, terminated at the non-reducing end by a galactose residue.

The types (c) and (d) would be present in the ratio 4:1 and would give a mixture having a mean degree of polymerisation of approximately 13.

The structures suggested above would give, after methylation and hydrolysis, the sugars obtained from methylated mannan A and in the same yields. The two different molecular species have sufficiently similar physical properties not to be separated either in the initial extraction and purification of mannan A or during fractionation of the methyl ether.

Malaprade (128) showed that in a compound containing hydroxyl groups on adjacent carbon atoms oxidation could take place with periodic acid, as shown below:

$$R.CH(OH).CH(OH).R' + HIO_4 = R.CHO + R'.CHO + HIO_3 + H_2O,$$

whereas if two hydroxyl groups or a hydroxyl and an amino group are not attached to contiguous carbon atoms no oxidation occurs (129, 130). Normally the reaction is quantitative and one mole of periodate is consumed for each C-C bond broken.

When more than two adjacent hydroxyl groups are present oxidation may proceed further with the production of formic acid from -CH(OH).CH(OH).CH(OH)- groups and formaldehyde from -CH(OH).CH_2OH groups. The amounts of formic acid and formaldehyde liberated, as well as the amount of periodate consumed, may be determined.

The results of experiments utilising sodium periodate as a reagent specific for adjacent hydroxyl groups are valid only if it can be shown that no other reactions take place during reaction with sodium periodate and that the formic acid and formaldehyde produced are not appreciably attacked by the reagent. It has been shown that the effect of side reactions may be minimised by carrying out the reaction in the dark (131, 132) and by careful control of the pH of the reaction medium (133, 134, 135, 136). In some cases it has been shown that the primary products of reaction of a carbohydrate with the periodate ion are further attacked by this reagent (133, 137).

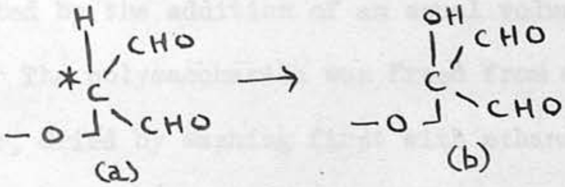
The results of studies on the oxidation of mannan A by periodate showed that:

(a) The amount of sodium metaperiodate consumed during oxidation of the mannan, which was measured against sodium arsenite, showed a point of inflexion at approximately 1.7 moles of periodate per anhydrohexose unit, but oxidation did not cease at this stage.

(b) Carrying out the reaction in a buffered solution at pH 3.8, the periodate uptake became constant at a value of 1.6 moles of periodate per anhydrohexose unit.

(c) Oxidation with aqueous potassium metaperiodate showed that one mole of formic acid was liberated from each 4.0 anhydrohexose units. During this experiment iodine was liberated and the amount of formic acid present diminished, a phenomenon which had been noticed previously by Halsall, Hirst, and Jones (137) and which indicated that secondary oxidation had occurred.

From these results it appeared that the reaction taking place was not the simple one outlined above but that complications due to over-oxidation had arisen. Potter and Hassid (138), following a scheme proposed by Halsall, Hirst, and Jones (137), explained the over-oxidation of amylose by sodium metaperiodate as being due to attack at the reducing end of the molecule. Initial oxidation of this group would give a compound of structure (a) which contained an active carbon atom (marked with an asterisk in the diagram) and further oxidation could ensue to give a compound of structure (b) which is readily oxidised by periodate.



Over-oxidation of the type described above may have occurred during periodate oxidation of mannan A.

EXPERIMENTAL

Extraction of mannan A.

Ivory nut shavings were freed from wax and colouring matter by extraction for twenty four hours in a Soxhlet extractor with benzene and then with methanol. The residue (100 g.) was delignified by the method of Wise (139) as modified by Chanda, Hirst, Jones, and Percival (140), by treating it with water (51.), glacial acetic acid (500 c.c.), and sodium chlorite (500 g.). The mixture was heated to 60°C and, after addition of sodium acetate (20 g.), maintained at 30°C in a water bath for 20 hours with occasional stirring. The white residue was filtered through muslin, washed with ice water then water to remove acid, and dried by suction on a Buchner funnel.

For the extraction of mannan A the cleaned ivory nut shavings were shaken for 16 hours with potassium hydroxide solution (7%), and centrifuged. The solution was slightly brown, due possibly to the presence of traces of lignin, and was acidified with glacial acetic acid and the mannan precipitated by the addition of an equal volume of methylated spirits. The polysaccharide was freed from acid by washing with water, dried by washing first with ethanol and then ether, and allowed to dry in air. The yield of white powder was 26% of the weight of the original ivory nut shavings. Further treatment of the residue from the above potassium hydroxide extraction gave: second extraction, mannan A in a yield of 12% of the weight of ivory nuts used; third extraction 4%. Total yield, 42%. The crude mannan had $[\alpha]_D^{13}$ -39° (c, 1.0 in N sodium hydroxide).

Purification of mannan A

The crude mannan was allowed to swell in water and then dissolved by the addition of aqueous potassium hydroxide, the final concentration of potassium hydroxide in the solution being 7%. The solution was filtered through glass wool to remove traces of insoluble material. Fresh Fehling's solution was added to the polysaccharide solution until the supernatant liquid was deep blue, and the bulky gelatinous complex was removed at the centrifuge and washed with water, care being taken not to remove all the alkali, as the complex becomes soluble in water when the alkalinity is reduced below a certain limit. The complex was suspended in water by vigorous stirring and decomposed by careful acidification with hydrochloric acid (2N), and the slightly turbid solution was filtered through glass wool. An equal volume of methylated spirits was added and the mannan precipitated, washed with hydrochloric acid (N) to remove copper and then with water to remove acid. The product was dried as usual by washing with ethanol and ether. Yield, 82% of the weight of crude mannan. Paper chromatographic examination of the products of hydrolysis of this material showed, in addition to mannose, traces of glucose, galactose, and a pentose (possibly arabinose).

The polysaccharide was purified further by a second treatment as above, and the product shown by hydrolysis and examination of the hydrolysate on a paper chromatogram to contain mostly mannose, with traces of glucose and galactose. This material will be referred to as "purified mannan A" and had $[\alpha]_D^{15}$ -46° (c, 0.7 in N sodium hydroxide) and $[\alpha]_D^{15}$ -28° (c, 0.8 in anhydrous formic acid, unchanged after 70 hours).

Attempted removal of galactose from purified mannan A.

A sample of the purified mannan was dissolved in 7% aqueous potassium hydroxide, the solution acidified feebly with glacial acetic acid, and fractionally precipitated by the gradual addition of methylated spirits. In this way four precipitates were obtained and after hydrolysis they were examined on paper chromatograms, but there was no apparent reduction in the amount of glucose or galactose present.

Quantitative estimation of the sugars present in purified mannan A.

Preliminary experiments had shown that the purified mannan gave on hydrolysis, in addition to mannose, traces of glucose and galactose and the amounts of these three sugars were determined.

(1) Estimation of the ratios of the sugars present.

A sample of the polysaccharide (ca. 200 mg.) was heated in a sealed tube with formic acid (2 c.c., A.R., 98-100%) at 100°C for seven hours and the solution evaporated to dryness. Water was added and evaporation repeated to remove excess formic acid, and the residue was heated in a sealed tube with sulphuric acid (1 c.c., N) at 100°C for three hours to hydrolyse formyl esters. The solution was allowed to cool and acid was removed with Amberlite resin 1 R-4B(OH). Using a micropipette to ensure that the same amount of sugar was present on each paper, the solution was spotted onto a chromatogram paper which had, as usual for quantitative paper estimations, two reference strips one at each edge of the paper. The solvent used for separation of the sugars was the upper phase of a mixture of n-butanol-pyridine-water-benzene (5:3:3:1, v/v) (141). The chromatograms were run for 50 hours then allowed to dry in air, the positions of the sugars were detected as usual by spraying the reference strips with a saturated solution of aniline oxalate in water and developing for a few minutes at 100°C, and the chromatograms cut into sections, each containing one sugar. For the determination of glucose and galactose several papers were combined and the sugars estimated by the arsenomolybdate - Somogyi reagent (118) as described later. Mannose was determined by periodate oxidation by the method of Hirst and Jones (117).

Estimation of glucose and galactose

No pre-treatment of the paper strips was necessary, and the sugars were eluted with cold water by the method of Laidlaw and Reid (142). Standard solutions of glucose and galactose of the same order of concentration as the unknown solutions were treated simultaneously with the unknown solutions and with water and paper blanks.

Reagents:

Copper reagent A:

Dissolved in 1l. water
25 g. anhydrous sodium carbonate.
25 g. sodium potassium tartrate.
20 g. sodium bicarbonate.
200 g. anhydrous sodium sulphate.

Copper reagent B:

15% copper sulphate pentahydrate containing 1 or 2 drops of concentrated sulphuric acid per 100 c.c.

25 c.c. A and 1 c.c. B mixed on day of use.

Arsenomolybdate reagent:

25 g. ammonium molybdate in 450 c.c. water, 21 c.c. concentrated sulphuric acid, mixed with 3 g. $NaHAsO_4.7H_2O$ dissolved in 25 c.c. water.

The solutions were heated at 100°C for 15 minutes to minimise the amount of dissolved oxygen in each, and after cooling copper reagent (2 c.c.) was added and the solutions heated at 100°C for 20 minutes then cooled. Arsenomolybdate reagent (2 c.c.) was then added, followed by water to make the solutions up to 25 c.c., and the absorptions of the solutions were measured colorimetrically in a Spekker photoelectric absorptiometer using spectrum-green 604 filters, the standard solutions being measured against the water blanks and the unknown sugar solutions against the paper blanks. From the graphs constructed using the known solutions, the amounts of glucose and galactose in the unknowns were obtained.

Estimation of mannose.

To remove the solvent completely the paper strips were suspended over water in an evacuated vacuum desiccator overnight. The mannose was then eluted with cold water and the solutions heated with sodium metaperiodate solution (2 c.c., 0.25M) for 30 minutes in a boiling water bath in tubes sealed with B 24 ground glass stoppers. To minimise loss of formic acid by evaporation the upper parts of the tubes were surrounded by cooling coils. After cooling ethylene glycol was added to destroy excess periodate and the solutions were titrated with carbonate-free sodium hydroxide (0.01N) using methyl red-methylene blue indicator (143). Blanks were also run on paper strips of the same area as the strips containing the sugar and the titration figures given below have been corrected for blanks.

Results:

From the calibration curves, four chromatograms contained

2.8×10^{-7} moles galactose and 1.3×10^{-7} moles glucose.

One chromatogram contained mannose which liberated formic acid on periodate oxidation requiring 1.95 c.c. of 0.00980 N sodium hydroxide for neutralisation, therefore, since 1 mole of hexose gives 5 moles of formic acid on periodate oxidation, the above titration figure corresponds to 3.82×10^{-6} moles of mannose.

Thus the mannan gave on hydrolysis the following sugars:

mannose, 97.6%;
galactose, 1.8%;
glucose, 0.8%.

(2) Estimation of total amount of mannose present.

A weighed sample of purified mannan A (31.02 mg.) was hydrolysed as before using formic acid and, after cooling, a reference sugar, in this case xylose (31.90 mg.), was weighed in and the acid removed with Amberlite resin 1R-4B(OH). The sugar mixture was separated on a paper chromatogram as before and the xylose and mannose estimated by periodate oxidation. An estimate was also made of the destruction of mannose during hydrolysis by treating a sample of mannose (37.11 mg.) with formic acid as before, adding xylose (32.67 mg.) and estimating the sugars after chromatographic separation.

The titration figures (c.c. of 0.00980 N NaOH) are given below:

Mannan A.

Mannose ..... 1.34 c.c.
Xylose ...... 1.23 c.c.

Mannose Standard.

Mannose ..... 2.28 c.c.
Xylose ...... 2.03 c.c.

These figures showed that the amount of mannose recovered from the mannan hydrolysate was 96%, and from the mannose standard, 95%.

Preparation of the acetate of mannan A.

The method used was that of Pacsu and Mullen (119). The purified mannan (1 g.) was allowed to swell in water (10 c.c.) for two hours at room temperature, then at 40°C for one hour. Purified pyridine (25 c.c.) was added and the water-pyridine azeotrope removed by distillation until 25 c.c. of solution remained. Addition of pyridine and distillation was repeated twice and the final mannan suspension in pyridine was heated at 100°C with acetic anhydride (15 c.c.) for 48 hours. The dark liquid was centrifuged and the solution on pouring into ice water gave a brownish solid which was washed with water until free from acid, and dried.

The acetyl content was determined by treating a weighed amount of the compound (<u>ca.</u> 0.2 g.) with acetone (40 c.c., A.R.) and sodium hydroxide (25 c.c., 0.1N). After allowing the mixture, and a blank in which the polysaccharide was omitted, to stand at room temperature for 16 hours they were titrated with sulphuric acid using phenolphthalein as indicator. The acetate contained 43.8% CH_3CO; calculated for a triacetate, 44.8% CH_3CO; and had $[\alpha]_D^{14}$ -25° (c, 1.0 in chloroform).

Molecular weight of mannan acetate

The molecular weight of mannan A acetate was determined by Barger's method (126). A solution of the acetate in chloroform (0.2600%) was compared with a series of standard solutions of sucrose octaacetate in chloroform. The determination was carried out in capillary tubes with a bore of 1 - 2 mm. and about 10 cm. long. Seven drops, (the second, fourth, and sixth being mannan acetate solution and the others standard solution) were introduced into each tube, one tube for each standard solution, and the tubes were sealed in a micro-bunsen flame. The first and seventh drops were larger to allow for evaporation and their lengths were not measured. The tubes, fastened by plasticine to microscope slides, were immersed in water in a thermostatically controlled room (20°C) for one hour and the lengths of the drops, excluding the larger end drops, were measured with a travelling microscope having a vernier reading to 0.02 mm. While their lengths were being measured the drops were illuminated by an electric lamp, placed behind a water cell to remove heat radiation. After 24 hours the lengths of the drops were again measured and the change in size with respect to the mannan acetate noted. The isopiestic condition was that at which the drops did not change in size and at which the molarity of the unknown and standard solutions were the same.

If x = the degree of polymerisation of the acetate, then the acetate solution contained $\frac{26}{288.2 \text{ X x X } 10}$ moles/l., and at the isopiestic condition this was equal to the molarity of the standard solution.

The results obtained are given in the table given below:

Molarity of sucrose octa-acetate solution X10^4	Corresponding value of x for equilibrium	Sum of changes of sizes of drops with respect to solution of acetate X10^2 mm.
6	15	+ 16
7	13	+ 10
8	11	0
9	10	- 7
10	9	- 6
11	8	- 7

Thus, the degree of polymerisation of the acetate was 10-13.

Reducing power of purified mannan A.

(a) Hypoiodite oxidation.

The method used was that described by Chanda, Hirst, Jones, and Percival (140).

A sample of the polysaccharide (ca. 50 mg.) in a boiling tube fitted with a B24 ground glass joint was allowed to swell in water (5 c.c.), then iodine (1 c.c., 0.1N) was added followed by 2 c.c. of the buffer solution (25 c.c. 0.1N disodium hydrogen phosphate and 8.67 c.c. 0.1N sodium hydroxide, diluted to 50 c.c. with water). The glass stopper was moistened with fresh potassium iodide solution (10%) and inserted, and the tube allowed to stand in a cool dark place overnight. The stopper was rinsed into the boiling tube, the solution acidified with sulphuric acid (2 c.c., 2N) and titrated with sodium thiosulphate (0.01N). Blanks in which the polysaccharide was omitted were also run.

21. N iodine are equivalent to -CHO.

Normality of sodium thiosulphate = 0.01048.

Weight of mannan A: 50.98 mg.; 52.88 mg.
Titre : 8.70 c.c.; 8.84 c.c.
Blank : 9.34 c.c., 9.36 c.c.
Mean Blank : 9.35 c.c.

Whence, number of anhydrohexose units per reducing group = 92; 122.

(b) Colorimetric method (120).

Standard solutions of mannose (5 c.c.) were treated with 3:5-dinitrosalycylic acid (1 c.c., 1.5%) and sodium hydroxide (1 c.c., 6N) at 65°C for 30 minutes. The solutions were cooled, diluted to 25 c.c. and compared in a Spekker photoelectric absorptiometer, using spectrum-green 604 filters, with a blank treated under the same conditions. Thus a standard graph was prepared.

A weighed sample of mannan A (ca. 10 mg.) was treated as above with 5 c.c. of water and 1 c.c. of each reagent. A blank using approximately the same weight of polysaccharide was run in which the dinitrosalicylic acid was added only after the solution had been cooled. The absorption of the mannan solution was measured against the mannan blank, and from the graph the weight of mannose having the same absorption was obtained and the apparent degree of polymerisation of the mannan calculated.

Standard solutions:

Moles of Mannose X10^7	Spekker reading	Moles of Mannose X10^7	Spekker reading
7.11	0.004	42.7	0.083
14.2	0.008	49.7	0.111
21.3	0.017	56.9	0.133
28.4	0.033		

Mannan A:

Weight of sample:	11.42 mg.,	12.52 mg.
Moles of anhydromannose units:	7.04 X 10^{-5},	7.72 X 10^{-5}
Spekker reading:	0.027,	0,028.
From graph, moles of reducing group:	26.0 X 10^{-7},	26.5 X 10^{-7}.

Thus, the mannan contained one reducing group per 27, 29 anhydrohexose units.

The values of the chain length of mannan A obtained by the above two methods were different and disagreed with the value obtained by other methods. Values of the chain length of the polysaccharide which are derived from measurement of the reducing power are, therefore, of doubtful validity.

Methylation of mannan A

Two batches of purified mannan A (each of 10 g.) were methylated, each being treated as follows:

The mannan (10 g.) was placed in a flask and allowed to swell overnight in water (60 c.c.) and then, after the air in the flask had been displaced by nitrogen which had been freed from oxygen by passage through alkaline pyrogallol solution (a gentle stream of nitrogen being maintained throughout the methylations), sodium hydroxide solution (100 c.c., 40% by weight) was added and the mixture stirred vigorously for four hours. The flask was cooled in water, and dimethyl sulphate (100 c.c.) was added from a dropping funnel over the next six hours and the reaction completed by stirring overnight. Four more methylations were carried out using sodium hydroxide solution (170 c.c., 40% by weight) and dimethyl sulphate (100 c.c.).

After the fifth methylation the solution was carefully adjusted to pH 8 using glacial acetic acid and cooling, and dialysed in a cellophane bag against running tap water until nearly free from sulphate ions (tested with barium chloride solution). The solution was then evaporated to dryness under diminished pressure at 40°C and the residue methylated as before. After the eleventh methylation the solution was adjusted to pH 8, dialysed, and evaporated to dryness. The residue was extracted repeatedly with hot chloroform, filtered hot, and the extracts evaporated to dryness. Yield of slightly brown powder, 11 g.; OMe, 40.4%; ash, 0.9%. The low yield of methylated mannan may be due to loss of polysaccharide during dialysis.

The crude methylated mannan was further methylated by Purdie's method. The methylated polysaccharide (11 g.) was dissolved in methyl iodide (150 c.c.) and heated under reflux on a water bath. Silver oxide (75 g.) was added slowly with shaking and the mixture heated overnight. The methylated mannan was recovered by repeatedly extracting the solid residue with hot chloroform, and the extracts were combined, evaporated to dryness, and dried. Yield of slightly yellow solid, 9 g.; OMe, 43.5%.

Fractionation of methylated mannan A.

The methylated mannan (9 g.) was heated under reflux with occasional shaking on a water bath with solvent (150 c.c.) for 30 minutes and filtered hot. The residue was extracted again with the same solvent (150 c.c.), filtered hot, and the extracts combined, evaporated to dryness and dried on the hyvac. The solvent used was light petroleum (b.p. 60 - 65°C, purified by treatment with concentrated sulphuric acid), and then mixtures of light petroleum and alcohol-free chloroform of increasing chloroform content.

The fractions obtained are summarised in the table given below:

Fraction Number	Solvent Chloroform %	Solvent Petroleum %	Yield g.
1	0	100	Very Small Fractions
2	2	98	
3	4	96	
4	6	94	
5	10	90	2.66
6	15	85	4.80
7	20	80	0.27
8	100	0	0.09

Fractions 5 and 6 had, respectively, $[\alpha]_D^{17}$ -22°, -23° (c, 1.0 in chloroform) and OMe, 44.4, 44.2% and were combined and used in the subsequent work on the methylated mannan.

Hydrolysis of methylated mannan A.

A sample of fractions 5 and 6 (4 g.) was dissolved in formic acid (25 c.c., A.R., 98 - 100%) and heated under reflux on a boiling water bath for 8 hours. The solution became too dark to follow the course of the reaction polarimetrically, but a preliminary small-scale hydrolysis had shown that these conditions gave complete hydrolysis of the polysaccharide. The solution was evaporated under reduced pressure at 100°C to remove formic acid, water was added and the water-formic acid azeotrope removed by distillation. Addition of water and distillation was repeated several times. To hydrolyse formyl esters, the thick, dark brown syrup was heated under reflux on a boiling water bath for 6 hours with sulphuric acid (10c.c., N). The cool solution was treated with Amberlite resin 1R-4B(OH) to remove acid, the resin removed by filtration, and the solution evaporated to a syrup and dried.

Paper chromatographic examination of the sugars obtained by hydrolysis of methylated mannan A.

The hydrolysate was examined on a long paper chromatogram, (50 cm.), using as mobile phase the upper layer of a mixture of n-butanol - ethanol - water (4:1:5, v/v). After development with aniline oxalate the following sugars were detected:

(a) the largest component gave a pink spot of R_G 0.81 corresponding to 2:3:6-trimethyl mannose;

(b) a smaller amount of a sugar of R_G 0.98 giving a pink spot, corresponding to 2:3:4:6-tetramethyl mannose;

(c) a very small amount of sugar giving a brown spot of R_G 0.58 indicating the presence of a dimethyl mannose; and

(d) a substance giving a pink spot of R_G 0.88, corresponding to 2:3:4:6-tetramethyl galactose.

Examination of the mixture on a paper chromatogram using as mobile phase the upper layer of a mixture of benzene-ethanol-water (167: 47: 15, v/v) showed the same sugars to be present.

Separation of hydrolysis products on a cellulose column.

A column of powdered cellulose (90 X 3 cm.) was prepared as described by Hough, Jones, and Wadman (144). The column was washed with water until the eluate was non-reducing to Fehling's solution, then with n-butanol (500 c.c.) and solvent (500 c.c.). The solvent used was a mixture of light petroleum (b.p. 100-120°C, purified by treatment with concentrated sulphuric acid) and n-butanol (purified by distillation over sodium hydroxide) in the ratio 60:40 by volume, saturated with water.

The syrup (3.43 g.) obtained on hydrolysis of methylated mannan A was dissolved in n-butanol and light petroleum was added until there were signs of separation of the solution into two liquid layers. Solvent was allowed to flow from the cellulose column until about one inch at the top was dry, and then the sugar solution was added slowly and allowed to soak in. When all the solution had been added a pad of cotton wool was placed on top of the cellulose, the constant-level reservoir was filled with solvent and inverted over the top of the column, and the column allowed to run. The first 50 c.c. of eluate were collected in a flask since they were not expected to contain sugar, and the rest was collected in 4 c.c. fractions in test-tubes which were changed by the automatic device described by Hough, Jones, and Wadman (144).

The contents of each tenth tube were evaporated to dryness at 100°C in a current of air, one drop of water was added to the residue, and this solution was examined on a paper chromatogram beside standards of known R_G values. Control of the concentration of spotting onto the chromatograms gave an indication of the variation in concentration of the sugars being eluted from the column. The chromatograms were allowed to run for 16 hours using as mobile phase the upper layer of a mixture of n-butanol-ethanol-water (4:1:5, v/v), dried in an oven, sprayed with aniline oxalate and developed by heating at 100°C for a few minutes. Thus the distribution of sugars among the tubes was found and the contents of tubes with the same sugar (or mixture of sugars, where separation had been incomplete) were combined, the tubes rinsed with acetone, and the fractions and rinsings evaporated to dryness under diminished pressure at 40°C. The fractions were purified by dissolution in a little distilled water, treated with a little animal charcoal and filter cel, warmed, cooled, and filtered through a washed bed of filter cel. The filtrate was evaporated to a syrup at 40°C under diminished pressure, and dried. The fractions were examined on paper chromatograms to detect traces of sugar other than the main component.

Since a preliminary paper chromatogram on the hydrolysis products of the methylated mannan had shown very little sugar of R_G less than that of dimethyl mannose to be present, when the latter had been eluted the solvent was changed to water. Some yellow colouring matter from the resin IR-4B(OH), which had been retained at the top of the column then moved down and was eluted. The recovery from the column was 95%.

Examination of the fractions obtained from the cellulose column.

Fraction I. (2:3:4:6-tetramethyl D-mannose).

This fraction was contained in tubes up to 160 and was shown by paper chromatography to contain tetramethyl mannose. The syrup was dried and gave at once some needle-shaped crystals in a golden syrup. Yield, 0.242 g. The syrup had n_D^{20} 1.4600, and $[\alpha]_D^{19}$ + 26° (c, 4.9 in methanol) which agrees with the value $[\alpha]_D^{25}$ + 24° (c, 1.1 in methanol) given by Smith (41) for 2:3:4:6-tetramethyl mannose. The purity of the fraction as determined by oxidation with alkaline hypoiodite was 82%, and by methoxyl determination, 96%.

After re-crystallisation from dry ether crystals were obtained which melted at 109-111°C. It was not possible to obtain sufficient crystals free from syrup to determine their specific rotation, but a mixture of syrup and crystals showed $[\alpha]_D^{20}$ + 23° (c, 0.7 in water) falling to an equilibrium value of + 10°.

Preparation of anilide.

A sample of the fraction (100 mg.) was heated under reflux with freshly distilled aniline (0.04 c.c.) in dry ethanol (2 c.c.) for 3 hours, then evaporated to dryness. The product crystallised and after re-crystallisation from light petroleum gave colourless needles which melted at 144-145°C, undepressed on admixture with an authentic sample of 2:3:4:6-tetramethyl mannose anilide. The anilide had $[\alpha]_D^{18}$ -94° (c, 0.8 in acetone) which, on addition of a trace of hydrochloric acid, changed to -42°, which agreed with the value $[\alpha]_D^{20}$ $-95.5^\circ \longrightarrow$ -39° (c, 2.0 in acetone) given by Irvine and McNicoll (145) for 2:3:4:6-tetramethyl mannose anilide.

Analysis:

Found: C, 61.79%; H, 8.04%.

Calculated for $C_{16}H_{25}O_5N$: C, 61.74%; H, 8.04%.

Preparation of lactone and phenylhydrazide.

A sample of the fraction was oxidised with bromine water at 35°C until the solution was non-reducing to Fehling's solution. The solution was aerated to remove bromine, neutralised with silver carbonate, and treated with hydrogen sulphide. It was then filtered and evaporated to dryness and the residue extracted with chloroform. The extracts were evaporated to dryness, "lactonised" by heating for 2 hours at 95°C on the water pump, and the lactone distilled at 120-140°C (bath temperature)/0.01 mm. The product was a syrup which showed no tendency to crystallise and which had $[\alpha]_D^{15}$ + 148° (initial value, c, 1.0 in water) falling rapidly.

The lactone (80 mg.) was allowed to react at room temperature for 16 hours with phenylhydrazine (0.04 c.c.) in dry benzene (2 c.c.). The resulting crystalline mass, after recrystallisation from benzene, melted at 185-186°C.

The constants obtained agreed with those given by Haworth, Hirst, and Isherwood (32), namely, 2:3:4:6-tetramethyl mannonolactone $[\alpha]^{20}_{D}$ + 150° (initial value, c, 2.0 in water), and 2:3:4:6-tetramethyl mannonic acid phenylhydrazide m.p. 184-185°C.

Fraction II (2:3:4:6-tetramethyl D-galactose).

This syrupy fraction was collected in tubes 161-185 and contained a sugar which ran on a chromatogram at the same speed as an authentic sample of 2:3:4:6-tetramethyl D-galactose. Yield, 0.058 g. The purity of the fraction, as determined by hypoiodite oxidation was 62%, and by methoxyl content, 87%.

Preparation of anilide.

The anilide was prepared by heating a sample of the syrup with aniline in dry ethanol for 3 hours. After removal of the solvent a crystalline mass was obtained, which after recrystallisation from dry ethanol gave colourless needles melting at 195-196°C, undepressed on admixture with an authentic specimen of 2:3:4:6-tetramethyl galactose anilide. The anilide had $[\alpha]^{16}_{D}$ -136° (c, 0.4 in pyridine) and $[\alpha]^{14}_{D}$ -76° (c, 0.3 in acetone). For 2:3:4:6-tetramethyl galactose anilide Smith (41) found $[\alpha]^{18}_{D}$ -140° (c, 0.5 in pyridine, and

Irvine and McNicoll (145) found $[\alpha]_D^{30}$ -77° (c, 1.0 in acetone).

Fraction III (2:3:6-trimethyl D-mannose)

This fraction extended over tubes 184-415 and gave 2.623 g. of syrup having $[\alpha]_D^{13}$ -7° (c, 4.6 in water) which agreed with the value $[\alpha]_D^{18}$ -6.5° (c, 0.6 in water) given by Smith (41) for 2:3:6-trimethyl mannose. The purity of the fraction determined by oxidation with alkaline hypoiodite was 92%, and by methoxyl content, 92%.

Preparation of anilide.

The anilide was prepared in the usual way and after recrystallisation from ether had a melting point of 127-128°C, agreeing with the value 129°C given by Smith (41) for 2:3:6-trimethyl mannose anilide.

Preparation of lactone and phenylhydrazide.

The lactone was prepared in the usual way by oxidation of the syrup with bromine water. The lactone distilled at 150-160°C (bath temperature)/0.03 mm. as a slightly brown syrup which crystallised and after recrystallisation from ether melted at 81-82°C, undepressed on admixture with 2:3:6-trimethyl D-mannono-γ-lactone. The lactone had $[\alpha]_D^{16}$ + 64.5° (c, 3.2 in water) which fell slowly indicating the presence of a γ-lactone. Smith (41) found $[\alpha]_D^{18}$ + 65.5° (initial value, c, 1.0 in water) for 2:3:6-trimethyl mannonolactone.

A sample of the lactone (40 mg.) in ethanol (2 c.c.) was heated with phenylhydrazine (0.02 c.c.) on the water bath at 95°C for 2 hours. After removal of the solvent the residue crystallised and was recrystallised from dry ethanol as colourless elongated prisms. Determination of the melting point under the microscope showed that some change occurred at 135°C, and melting took place at 146-147°C. An authentic specimen of 2:3:6-trimethyl D-mannonic acid phenylhydrazide melted at 132-133°C and was possibly the monohydrate. After melting, cooling, and recrystallisation from dry ethanol the authentic specimen melted at 132-133°C while portions, possibly the anhydrous form, melted at 142-143°C. A sample of the phenylhydrazide prepared from fraction III after recrystallisation from moist ethanol melted at 132-133°C, undepressed on admixture with an authentic specimen of 2:3:6-trimethyl mannonic acid phenylhydrazide. Rafique and Smith (43) obtained 2:3:6-trimethyl mannonic acid phenylhydrazide as the monohydrate m.p. 133°C which could be converted to the anhydrous form having m.p. 144°C.

<u>Fraction IV</u> (a mixture of 2:3:4-trimethyl D-mannose and 2:3-dimethyl D-mannose).

This fraction was contained in tubes 416-954 and was shown by paper chromatography to contain di- and tri-methyl sugars. Yield, 0.240 g.

The fraction tended to crystallise and an attempt was made to separate the components by extraction with ether. In this way a number of fractions were obtained and were examined on paper chromatograms which showed that although complete separation had not been achieved there had been partial separation of di- and tri- methyl sugars. The fractions were spotted separately onto thick paper chromatograms, (Whatman 3 MM paper) which were run using as solvent the upper layer of a mixture of n-butanol - ethanol - water (4:1:5, v/v). The chromatograms had side reference strips, as used with quantitative chromatograms, which were cut off and developed with aniline oxalate to show the position of the sugars in the centre strip. The filter paper sections containing the separated sugars were extracted with acetone in a Soxhlet extractor and the sugars obtained by evaporation of the solutions were purified as usual by dissolution in water and treatment with charcoal and filter cel. The fractions thus obtained were examined on paper chromatograms and found to fall into two classes, fraction IVa having a higher, and fraction IVb a lower, R_G value. After correction for the amount of sugar removed with the side reference strips the yields of the fractions were, respectively, 206 and 34 mg.

Fraction IVa.

This fraction tended to crystallise and gave crystals of a substance (X) which melted at 148-150°C.

A sample of the substance (X) was demethylated by the method described by Hough, Jones, and Wadman (146). The methylated sugar (X) (ca. 5 mg.) was heated at 100°C for 5 minutes in a sealed glass tube with hydrobromic acid (48% w/w, 1 c.c.) then cooled and diluted with water (10 c.c.). Silver carbonate was then added until the solution was neutral, the solution filtered, and hydrogen sulphide passed. The solid was removed by filtration and the solution, concentrated by evaporation, was examined on a paper chromatogram using as mobile phase the upper layer of a mixture of pyridine - n-butanol - water - benzene (3:5:3:1, v/v) and shown to contain mannose.

After standing for several weeks the fraction became almost completely crystalline and failed to give a colour reaction with aniline oxalate or with aniline oxalate containing 5% phosphoric acid. It was also non-reducing to Fehling's solution and to alkaline hypoiodite.

The crystalline material (X) was found to have the following constants:

$[\alpha]_D^{18}$ + 55° (c, 1.4, in water, unchanged after 48 hours);

$[\alpha]_D^{18}$ + 65° (c, 0.7 in 1% methanolic hydrogen chloride, unchanged after 100 hours).

Analysis of the crystalline substance (X):

Found: C, 50.5%; H, 7.9%; OMe, 42.9%.

$C_{18}H_{34}O_{11}$ requires: C, 50.7%; H, 8.0%; OMe, 43.7%; M.W. 426.

The molecular weight of the substance (X) was determined by Barger's method, as described on page 54. The results are collected in the table given below; the substance was dissolved in chloroform to give a solution of concentration 8.76×10^{-3}%.

Molarity of sucrose octaacetate $\times 10^4$ moles/l.	Corresponding value of M.W. of crystalline substance for equilibrium	Sum of changes of sizes of drops with respect to solution of unknown $\times 10^2$ mm.
1	877	+ 36
2	439	+ 22
3	292	- 45
4	219	- 94
5	175	- 15
6	146	- 33

Since the isopiestic condition lay between sucrose octaacetate solutions of molarity 2×10^{-4} and 3×10^{-4} moles/l., the molecular weight of the unknown substance (X) was 292-439.

The crystalline material (X) was hydrolysed with sulphuric acid (N) for 4 hours at 100°C and after neutralisation the hydrolysate was examined by paper chromatography using as solvents the upper layers of mixtures of n-butanol - ethanol - water (4:1:5, v/v) and benzene - ethanol - water (167:47:15, v/v). Only one spot was obtained, which ran at a speed different from those of authentic samples of 2:3:6- and 3:4:6-trimethyl mannose. Spots of the monosaccharide (Y) on filter paper were sprayed with urea oxalate and with naphthoresorcinol and hydrochloric acid and developed by heating at 100°C for a few minutes. No colours were produced, thus showing the absence of ketose derivatives.

Methylation of the crystalline substance (X)

A sample of the material (X) (60 mg.) was methylated by Purdie's method and the methylated compound extracted from the reaction mixture with chloroform. The chloroform extracts were evaporated to a syrup (69 mg.) which was hydrolysed by heating at 100°C for 4 hours with sulphuric acid (N). The solution was neutralised with Amberlite resin 1R-4B(OH) and evaporated to a syrup (60 mg.). The syrup was examined on paper chromatograms run in n-butanol - ethanol - water (4:1:5, v/v) and in benzene - ethanol - water (167:47:15 v/v) and in each case only one spot was detected on development with aniline oxalate, which ran at the same speed as an authentic sample of 2:3:4:6-tetramethyl D-mannose. The syrup was converted to the anilide by heating with dry ethanol (2 c.c.) containing aniline (0.02 c.c.), the solvent removed, and the residue crystallised. After recrystallisation from ether the anilide (70 mg.) melted at 144-145°C, undepressed on admixture with an authentic specimen of 2:3:4:6-tetramethyl D-mannose anilide.

Tosylation and iodination

Tosylation and iodination

The disaccharide (X) was dissolved in dry pyridine and allowed to react with p-toluene sulphonyl chloride for 44 hours at room temperature. The reaction mixture was poured into ice water and the precipitated tosyl compound dissolved in chloroform and washed with water, dilute acetic acid, sodium bicarbonate solution, and then with water. The tosyl compound was obtained on removal of the chloroform and was dissolved in acetone and heated under reflux for 4 hours with its own weight of sodium iodide. The reaction mixture was poured into water and the precipitated iodo-compound dissolved in chloroform and washed with water until free from iodide. The chloroform solution was evaporated to dryness and the residue washed with a little warm water. The washings were acidified with dilute nitric acid and gave a negative test for iodide on addition of silver nitrate.

The compound was analysed for iodine (147) by heating with potassium acetate, glacial acetic acid and bromine on a boiling water bath for 30 minutes, the solution cooled and diluted with aqueous sodium acetate solution. The solution was treated with formic acid to remove bromine and after addition of potassium iodide and sulphuric acid the liberated iodine was titrated with sodium thiosulphate. A blank was also run on the reagents. The compound was found to contain 7% iodine

($C_{18}H_{32}O_9I_2$ requires 39% iodine).

Oxidation of the trimethyl mannose (Y).

The trimethyl mannose (Y) obtained after hydrolysis of the crystalline substance (X) was chromatographically pure and was treated as follows:

(a) Following the method used by Buchanan, Dekker, and Long (148) the monosaccharide (Y) was spotted on filter paper and treated with lead tetraacetate in benzene. After remaining in air at room temperature the sugar zone appeared as a white spot on a brown background, showing that oxidation had occurred.

(b) Following the method described by Chanda, Hirst, Percival, and Ross (149), a solution of the substance (Y) was oxidised for 30 hours at room temperature with sodium periodate solution (0.25M), then potassium chloride was added to reduce the periodate concentration, and the solution centrifuged. To the clear solution were added solutions of phenylhydrazine hydrochloride, potassium ferricyanide, and concentrated hydrochloric acid. The colour produced was the same as that produced by treatment of formaldehyde with the same reagents.

These results showed that the trimethyl mannose (Y) had two adjacent hydroxyl groups, and since formaldehyde had been liberated on periodate oxidation the hydroxyl groups were on carbon atoms 5 and 6. Therefore, the monosaccharide (Y) was 2:3:4-trimethyl mannose and the disaccharide (X) was

1-[2:3:4-trimethyl D-mannopyranosido] - 2:3:4-trimethyl D-mannopyranoside.

Fraction IVb

This fraction showed no tendency to crystallise and was found to contain a sugar which ran on chromatograms at the same speed as 2:3-dimethyl mannose and a speed different from that of 3:4-dimethyl mannose. The sugar was shown by demethylation followed by examination on a paper chromatogram to be a mannose derivative. Therefore, the fraction contained 2:3-dimethyl mannose.

Fraction V

This was the water wash from the column. The fraction (74 mg.) contained mainly colouring matter and this was separated from a trace of sugar by running on a thick paper chromatogram. The sugar (5 mg.) ran on a chromatogram at the same speed as a sample of glucose.

Estimation of the sugars obtained on hydrolysis of methylated mannan A after separation by paper chromatography.

A sample of methylated mannan was hydrolysed with formic acid as described previously. After various solvents had been tried, the upper layer of a mixture of benzene - ethanol - water (167:47:15, v/v) was used as the mobile phase and it was possible to obtain good separation of the tetramethyl sugars from the trimethyl sugars. It was not possible to obtain sufficient separation of the two tetramethyl sugars to estimate them separately, and no attempt was made to estimate the small amount of dimethyl sugar present. After the chromatograms had run they were allowed to dry in air overnight at room temperature, the reference strips were cut off and developed, and the sections containing the sugars to be estimated were cut and the sugars extracted with boiling water for 45 minutes. The sugars were estimated by oxidation with alkaline hypoiodite as described on page 55, oxidation being allowed to proceed overnight. Blanks were run on paper sections equal in area to the sections containing sugar and which had been treated with solvent and allowed to dry in the same manner as the chromatograms.

The results are collected below, the titrations being carried out with 0.01N thiosulphate:

Titre difference from blank (c.c.)		Molar Ratio of tetra-to tri-methyl sugars
Tetramethyl sugars	Trimethyl sugars	
0.16	1.43	1:9.2
0.41	3.68	1:8.9

Molecular weight of methylated mannan A.

The molecular weight of methylated mannan (fraction 5) was determined by Barger's method, which is described on page 54.

If x = degree of polymerisation of the methylated mannan (0.9113% in chloroform) then the solution contained $\frac{9.113}{204.2 \times x}$

moles/l. and at the isopiestic condition this will equal the molarity of the sucrose octaacetate solution. The results obtained are collected in the table given below:

Molarity of sucrose octa-acetate solution X 10^3	Corresponding value of x for equilibrium	Sum of changes of sizes of drops with respect to methylated mannan X 10^2 mm.
2	22	+ 37
3	15	+ 55
4	11	+ 18
5	9	- 15
6	7	- 16

Thus the degree of polymerisation of the polysaccharide was 9-11.

Viscosity of methylated mannan A

A methylated mannan A which had $[\alpha]_D^{17}$ -21.5° (c, 1.0 in chloroform) and a methoxyl content of 44.0% was used. Viscosity determinations were carried out in a thoroughly clean Ostwald viscometer. A solution of the substance (0.0560 g.) was made in freshly distilled m-cresol (10 c.c.) and its time of flow compared with that of the pure solvent in the viscometer maintained at 20°C in a thermostat.

Time of flow of solution (T_1) = 504 seconds.

Time of flow of solvent (T_2) = 474 seconds.

Whence, relative viscosity ($\eta_{rel.}$) = $\frac{504}{474}$ = 1.062,

and the specific viscosity ($\eta_{sp.}$) = $\eta_{rel.} - 1 = 0.062$.

From the Staudinger equation, $\frac{\eta_{sp.}}{c} = K_m.M,$

where c = concentration of solute in gm.mol./l.,

K_m = a constant,

M = molecular weight of solute,

using $K_m = 12 \times 10^{-4}$ (the constant obtained for methylated cellulose (127)) a value of 9 was obtained for the degree of polymerisation of the methylated mannan.

Periodate oxidation of mannan A

(a) Determination of formic acid released

The method of Halsall, Hirst, and Jones (114) as modified by Chanda, Hirst, Jones, and Percival (140) was used. Dry purified mannan A (ca.100 mg.) was weighed into a number of clean, dry bottles and treated with potassium chloride solution (5 c.c., 16%), sodium metaperiodate solution (prepared from sodium paraperiodate by the method of Hill (150)) (10 c.c., 0.083 M), and water (10 c.c.). Blanks were also run. The bottles were stoppered and shaken in the dark and at intervals bottles were removed, the contents allowed to settle, and 20 c.c. of the solution removed, treated with ethylene glycol (1 c.c.) to destroy excess periodate and titrated with carbonate-free sodium hydroxide (0.01018N), using as indicator methyl red-methylene blue (143). Corrections were made for the slight acidity of the polysaccharide by titrating a known weight with alkali, and in the following table the titration figures have been corrected.

Duration of oxidation (hours)	Weight of mannan (mg.)	Titre (c.c.)	Number of $C_6H_{10}O_5$ residues per mole of formic acid
191	109.8	12.74	4.18
290 *	103.0	12.48	4.00
359	104.3	12.60	4.01
455 ø	110.0	8.40	6.35

* This solution was tested with starch but gave a negative reaction for iodine.

ø This solution gave a positive reaction for iodine.

(b) Determination of periodate uptake.

(1) In unbuffered solution

Batches of purified mannan A (ca. 100 mg.) were shaken in the dark with sodium metaperiodate solution (25 c.c., 0.25M) and blanks were also run. Bottles were removed at intervals, 20 c.c. of solution removed and made up to 250 c.c. with water, and 50 c.c. of this solution was treated with sodium bicarbonate (ca.1 g.) and a known excess of sodium arsenite solution followed by potassium iodide (1 g.). After standing for 15 minutes the solution was titrated with iodine (0.1052N). The results are collected below, the titration figures being the difference from the blank.

Duration of oxidation (hours)	Weight of mannan (mg.)	Titre (c.c.)	Periodate uptake (moles/$C_6H_{10}O_5$ residue
191	101.7	3.16	1.66
281	108.9	3.44	1.68
455	105.0	4.40	2.23
502	104.7	4.69	2.39

(2) In buffered solution

The periodate solution was prepared (151) by shaking sodium paraperiodate (17 g.) with water (250 c.c.) and glacial acetic acid (25 c.c.). After filtration the solution was found to have pH 3.8 (which was constant throughout the experiment) and a weighed sample (0.09591 g.) of purified mannan was treated with the periodate solution (100 c.c.) in a glass-stoppered flask and was allowed to stand in the dark with occasional shaking. The polysaccharide dissolved quickly, and portions (10 c.c.) of the solution were withdrawn at intervals and titrated with iodine solution (0.1052N) after addition of sodium bicarbonate, sodium arsenite, and potassium iodide. Blanks were also run and the titration figures given in the table below represent differences from the blank

Duration of oxidation (hours)	Titre (c.c.)	Periodate uptake (moles /$C_6H_{10}O_5$ residue)
70	1.86	1.65
93	1.80	1.60
171	1.80	1.60

MANNAN B

Discussion

After the extraction of mannan A from ivory nut shavings by means of 7% potassium hydroxide solution, the residue was shown by hydrolysis and examination of the hydrolysate on a paper chromatogram to yield a large amount of mannose. Using the nomenclature of Lüdtke (99), the mannan present will be referred to as "mannan B". Mannan A was removed from the residue as completely as possible by treatment with 14% potassium hydroxide solution. Lüdtke (99) and Klages (115) prepared mannan B by treatment of this residue with cuprammonium hydroxide (cuprammonium) and on addition of sodium hydroxide solution until the resulting mixture was 0.2N with respect to sodium hydroxide mannan B was precipitated as its sodium hydroxide complex while cellulose remained in solution and was deposited on acidification. The earlier workers decomposed the mannan complex with acid and precipitated the polysaccharide by the addition of methanol.

In the present investigation the scheme adopted by the earlier authors was modified slightly in an attempt to obtain a purer product. The scheme is shown diagrammatically on page 84. The ivory nut residue after removal of mannan A was dissolved in the dark in cuprammonium containing sucrose to minimise oxidation of the polysaccharide, and sodium hydroxide solution was added to precipitate the mannan complex. The complex was decomposed with acetic acid and the mannan precipitated by the addition of methylated spirits. By extraction of the mannan preparation with 7% potassium hydroxide solution a quantity of soluble material was removed. The residue, freed from alkali by washing, was dissolved in cuprammonium and fractionally precipitated with sodium hydroxide.

The first sodium hydroxide complex was decomposed with acetic acid and precipitate (i) was deposited. After removal of this precipitate, methylated spirits was added to the solution and precipitate (ii) was obtained.

The second sodium hydroxide complex was acidified and precipitate (iii) separated.

These three precipitates were hydrolysed and examined on paper chromatograms and gave the following results:

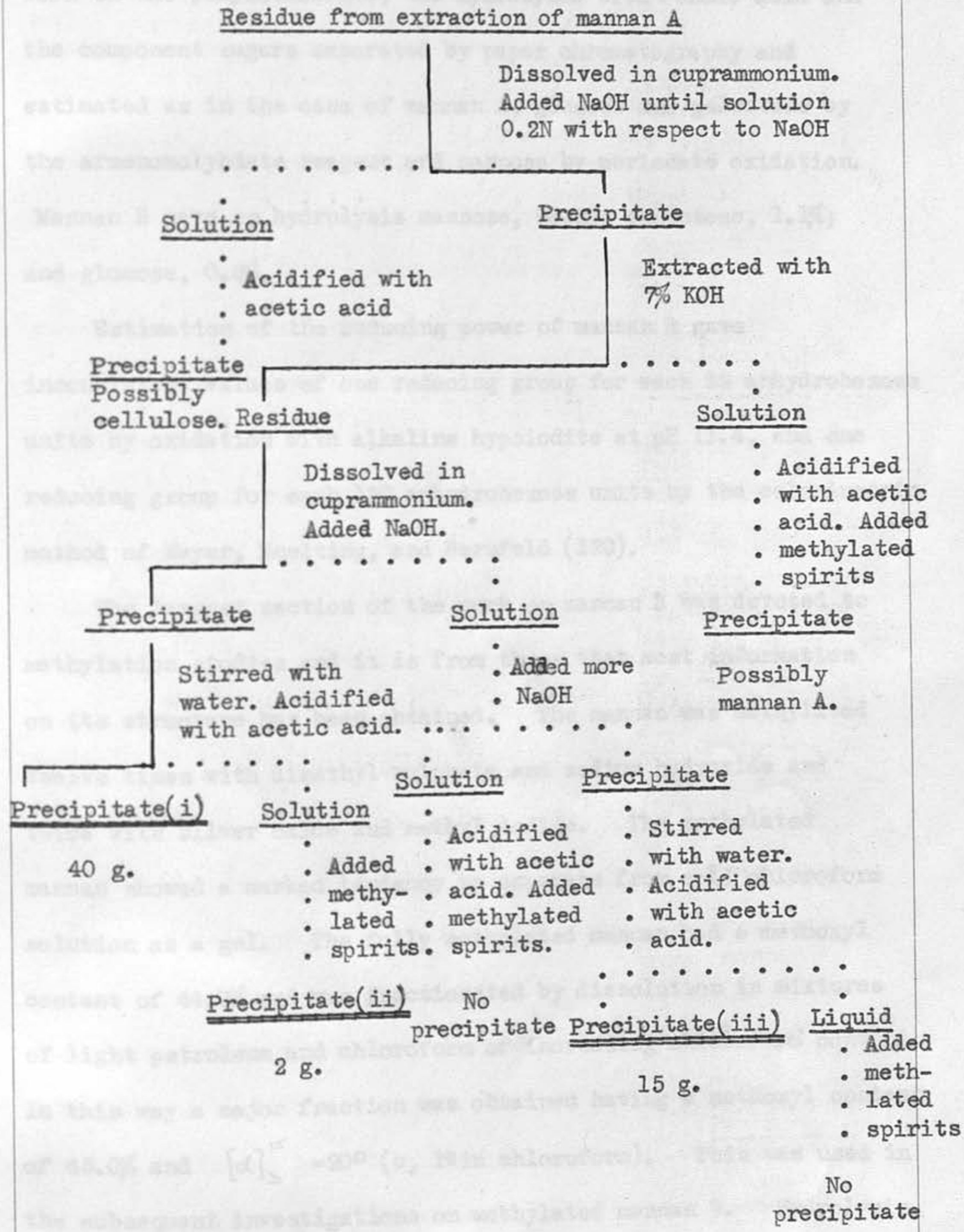
Precipitate (i) (yield, 40 g.) gave after hydrolysis mostly mannose, smaller amounts of glucose and galactose, and a trace of pentose (possibly arabinose).

Precipitate (ii) (yield, 2 g.) gave the same sugars as precipitate (i) but no pentose was present.

Precipitate (iii) (yield, 15 g.) gave mostly glucose, and smaller amounts of mannose and pentose.

Precipitate (i) was considered to contain mannan B and was further purified. It was extracted with 7% potassium hydroxide solution to remove mannan A, washed free from alkali and dried. The preparation after hydrolysis gave mostly mannose together with a considerable quantity of glucose (approximately the same amount as that in precipitate (i)) and a little galactose (less than in precipitate (i)); no pentose was detected. Following a suggestion by Dr. J.K.N. Jones (152) that mannans may be separated from contaminating glucosans by dissolution in anhydrous formic acid, the residue was shaken with anhydrous formic acid and mannan precipitated by the addition of alcohol to the solution. The mannan was hydrolysed and examination of the hydrolysate on a paper chromatogram showed that the glucose content had been considerably reduced by this treatment but a small amount of glucose and galactose still remained. The preparation, which will be referred to as "purified mannan B," had $[\alpha]_D^{15}$ -26° (c, 0.8 in anhydrous formic acid). The residue, insoluble in anhydrous formic acid, was shown by hydrolysis and examination on a paper chromatogram to yield mannose and a considerable quantity of glucose.

Diagrammatic representation of the scheme used for the separation of mannan B.



The purified mannan B, which was used in all subsequent work on the polysaccharide, was hydrolysed with formic acid and the component sugars separated by paper chromatography and estimated as in the case of mannan A, glucose and galactose by the arsenomolybdate reagent and mannose by periodate oxidation. Mannan B gave on hydrolysis mannose, 98.3%; galactose, 1.1%; and glucose, 0.8%.

Estimation of the reducing power of mannan B gave inconsistent values of one reducing group for each 12 anhydrohexose units by oxidation with alkaline hypoiodite at pH 11.4, and one reducing group for each 130 anhydrohexose units by the colorimetric method of Meyer, Noelting, and Bernfeld (120).

The largest section of the work on mannan B was devoted to methylation studies and it is from these that most information on its structure has been obtained. The mannan was methylated twelve times with dimethyl sulphate and sodium hydroxide and twice with silver oxide and methyl iodide. The methylated mannan showed a marked tendency to separate from cold chloroform solution as a gel. The fully methylated mannan had a methoxyl content of 44.9% and was fractionated by dissolution in mixtures of light petroleum and chloroform of increasing chloroform content. In this way a major fraction was obtained having a methoxyl content of 45.0% and $[\alpha]_D^{15}$ -20° (c, 1.0 in chloroform). This was used in the subsequent investigations on methylated mannan B. Hydrolysis was effected with anhydrous formic acid and after neutralisation the hydrolysate was separated on a cellulose column. The fractions were collected and identified as detailed below:

Fraction I contained a mixture of 2:3:4:6-tetramethyl D-mannose and 2:3:4:6-tetramethyl D-galactose. The sugars were separated on a thick paper chromatogram and examined separately. The tetramethyl mannose was identified by conversion to 2:3:4:6-tetramethyl mannose anilide and the tetramethyl galactose by conversion to 2:3:4:6-tetramethyl galactose anilide.

Fraction II was identified as 2:3:6-trimethyl D-mannose by conversion to the anilide, and by oxidation to the lactone followed by formation of the phenylhydrazide.

Fraction III consisted of a mass of crystals with some syrupy component and on a paper chromatogram gave a faint spot running at a speed corresponding to a trimethyl hexose. The crystalline substance melted at 149-151°C.

Fraction IV was similar to fraction III but it was shown by paper chromatography to contain, in addition to a trimethyl hexose, some dimethyl sugar which ran at the same speed as 2:3-dimethyl mannose and gave the same colour on development with aniline oxalate and it was, therefore, concluded that the fraction contained some 2:3-dimethyl mannose. The mixture was heated with dilute sulphuric acid to hydrolyse any disaccharide which might be present and the mixture of di- and trimethyl sugars was separated on thick paper chromatograms, when the trimethyl fraction gave crystals melting at 148-150°C, undepressed on admixture with crystals from fraction III, or with the crystalline material (X) obtained in fraction IV from methylated mannan A. These substances were, therefore, the same as the disaccharide (X) obtained from the hydrolysate of methylated mannan A, namely, 1- [2:3:4-trimethyl D-mannopyranosido] - 2:3:4-trimethyl D-mannopyranoside, and had been formed by the condensation of two molecules of 2:3:4-trimethyl mannose.

Fraction V was a brown syrup and was shown by demethylation to be a mannose derivative. It had an R_G value of 0.29 when run on a paper chromatogram using as the mobile phase the upper layer of a mixture of n-butanol - ethanol - water (4:1:5, v/v), and gave a brown spot on development with aniline oxalate. The syrup was considered to be a monomethyl mannose.

Fraction VI contained a substance giving a positive naphthoresorcinol test for uronic acid and having an R_F value of 0.69 when the solvent used was a mixture of ethyl acetate - acetic acid - water (3:1:3, v/v).

Fraction VII was freed from colouring matter by separation on a paper chromatogram and yielded a small amount of sugar (2 mg.), which was identified on a chromatogram as glucose.

From the yields of the fractions obtained from the column it was possible to calculate the molar ratios of the methylated sugars present. The results are collected in the table given below:

Fraction No.	Contents of Fraction	Yield (g.)	Moles X 10^4	Molar Ratios			
I	2:3:4:6-Tetramethyl D-mannose	0.027	1.2	1	1	1	
	2:3:4:6-Tetramethyl D-galactose	0.028	1.2	1			
II	2:3:6-Trimethyl D-mannose	1.640	73.8	63	6	32	37
III	2:3:4-Trimethyl D-mannose (isolated as disaccharide) (66 mg.)	0.269	12.6	11	1	5.3	
IV	2:3:4-Trimethyl D-mannose (isolated as disaccharide) (203 mg.)						
	2:3-Dimethyl D-mannose	0.029	1.4	1			
V	Monomethyl mannose	0.006					
VI	Uronic acid	0.007					
VII	Colouring matter and trace (2 mg.) of glucose.	0.012					

The mixture of methylated derivatives obtained on hydrolysis of the methylated mannan was separated by paper chromatography and the sugars estimated by alkaline hypoiodite. The results indicated that one non-reducing end group was present for each 39-41 trimethyl mannose residues.

An analysis of purified mannan B had shown it to yield on hydrolysis 1.1% galactose, whereas the amount of tetramethyl galactose obtained from the hydrolysis of fully methylated mannan was 1.3%. Also, since no other methylated galactose derivatives were shown to be present, the galactose must be present in the mannan only as end group and not as a separate galactan. As no methylated derivatives of glucose were isolated from the cellulose column, the small amount of glucose (0.8%) present in the original polysaccharide must have arisen from contamination by glucosan, which was removed during the methylation process.

Because of the stability of the mannan to acid hydrolysis it must be composed of pyranose units, and since the main component of the hydrolysate from methylated mannan was 2:3:6-trimethyl mannose the mannan must be built mainly of 1:4-linked anhydromannopyranose units. The optical rotation of the mannan $[\alpha]_{D}^{15}$ -26° (c, 0.8 in anhydrous formic acid) indicated that the majority of linkages in the polysaccharide were of the β-type.

From methylation studies the following structures could be proposed for mannan B:

(a) an equimolecular mixture of two types of linear molecules, one having a mannose - and the other a galactose non-reducing end group, which would have a mean degree of polymerisation of 38; and

(b) branched structures, containing mannose - and galactose non-reducing end groups in the same molecule, which would have a mean degree of polymerisation of at least 76.

Determination of the degree of polymerisation of methylated mannan B by Barger's method gave a value of 35-43 showing that the mannan had a structure of type (a). Viscosity determination on the methylated polysaccharide gave a value of 9 for the degree of polymerisation, but since the viscosity of the methylated mannan solution was low little value can be attached to this result.

From the results no unambiguous structure could be proposed for mannan B, but one possibility is an equimolecular mixture of two linear types of molecule, each composed of 32 1:4-linked anhydro-D-mannopyranose units and 5 1:6-linked anhydro-D-mannopyranose units, one type having a mannose - and the other a galactose non-reducing end group. It was not possible to determine the distribution of 1:6-linked residues between the two types or the position of such units in the molecule.

An attempt was made to obtain further evidence for the structure of the mannan by studying its oxidation by the periodate ion. The theoretical aspects of this reaction have been discussed on page 43. The experimental results showed that one mole of formic acid was liberated from each 2.4 anhydrohexose units, and that the periodate uptake was 0.98 moles per anhydrohexose unit. Mannan B reacted more slowly than mannan A, reaction not reaching completion until after approximately 600 hours. No definite conclusions could be drawn from the results and the high formic acid release could be due to degradation of the periodate oxidised mannan by prolonged contact with the acid medium. This reaction, which has been postulated by Moe, Miller, and Iwen (133), would produce additional points of attack for the periodate ion leading to the liberation of formic acid.

EXPERIMENTAL

The solid remaining after the extraction of mannan A with 7% potassium hydroxide solution from cleaned ivory nut shavings was shaken with 14% potassium hydroxide solution. After removal of solid at the centrifuge, the clear solution was acidified with glacial acetic acid and an equal volume of methylated spirits added. A small amount of white powder was precipitated and, therefore, the residue was re-extracted with 14% potassium hydroxide solution to remove all mannan A and then freed from alkali by washing with water. It was dried as usual with ethanol and ether. The white, fibrous material obtained served as starting material for the preparation of mannan B.

Extraction of mannan B.

The scheme finally adopted for the separation of mannan B (which is shown diagrammatically on page 84) was that described below. The starting material was allowed to swell in concentrated ammonia to facilitate solution (153), sucrose was added to minimise oxidation of the polysaccharide (154), and the mixture was shaken for 16 hours in the dark with cuprammonium solution prepared as described by Carver (155). To minimise oxidation of the mannan the bottles containing the mixture were almost completely filled with solution. After shaking, the mixture was centrifuged in closed bottles and the solid re-extracted with cuprammonium. Sodium hydroxide solution (2N) was added slowly with stirring to the solution until it was 0.2N with respect to sodium hydroxide. The large, bulky precipitate produced was removed at the centrifuge, stirred with water, and dissolved by acidification with acetic acid. Addition of an equal volume of methylated spirits gave a precipitate of crude mannan B.

The clear solution remaining after removal of the sodium hydroxide-mannan B complex was acidified with acetic acid and gave a precipitate of cellulose. Addition of methylated spirits to the solution after removal of the cellulose gave no further precipitate.

The crude mannan B was freed from copper by washing with water, shaken with 7% potassium hydroxide solution, and centrifuged. The centrifugate was acidified with acetic acid and methylated spirits was added. A large precipitate (possibly mannan A) was obtained and, therefore, the crude mannan B was repeatedly extracted with 7% potassium hydroxide solution until no further solid dissolved. The residual solid was then washed with water until free from alkali.

The residue was dissolved in cuprammonium, filtered through a sintered glass filter to remove traces of solid, and then sodium hydroxide (2N) was added to give a considerable quantity of precipitate, but not to completely precipitate the mannan. The precipitate was removed and sodium hydroxide (2N) was added to the solution to complete precipitation.

The precipitates were each removed at the centrifuge, washed once with sodium hydroxide (2N) - ammonia (0.88) (1:1, v/v), stirred with water, and acidified with acetic acid. In this way the first fraction gave a precipitate (i) which was removed by filtration, and the filtrate treated with an equal volume of methylated spirits to give a precipitate (ii).

The second fraction gave a precipitate (iii) with acetic acid, but addition of methylated spirits gave no further precipitation.

The solution remaining after removal of the second sodium hydroxide-mannan B complex was treated with further sodium hydroxide to ensure complete precipitation, acidified with acetic acid and methylated spirits added, but no further precipitate was obtained.

Precipitate (i) was considered to be mannan B. The hydrolysate was shown by examination on a paper chromatogram, to contain, in addition to mannose, considerable quantities of glucose and galactose and a trace of pentose (possibly arabinose). The precipitate was freed from mannan A by repeated extraction with 7% potassium hydroxide solution. The residue was washed with water until free from alkali and a sample on hydrolysis yielded mainly mannose, but also a considerable quantity of glucose and a trace of galactose; no pentose was detected. A comparison with the hydrolysate from precipitate (i) showed approximately the same quantity of glucose but less galactose to be present.

This preparation was shaken with formic acid (98-100%), centrifuged, and the cloudy solution filtered through filter cel. Mannan was precipitated from the filtrate by the addition of an equal volume of ethanol, separated and washed free from acid. Chromatographic examination of a sample hydrolysate showed mannose to be present in large amount together with a little glucose and galactose. The amount of glucose had been considerably reduced by this treatment with formic acid. The residue, insoluble in formic acid, gave on hydrolysis mannose and a considerable amount of glucose.

The product obtained by the above treatment and which was soluble in formic acid will be referred to as "purified mannan B", and was used in all the subsequent work. It had $[\alpha]_{D}^{15} -26^{\circ}$ (c, 0.8 in anhydrous formic acid, unchanged after 70 hours at room temperature).

Quantitative estimation of the sugars present in purified mannan B.

(1) Estimation of the ratios of the sugars present.

The method used was that described on page 49. A sample of mannan B was hydrolysed with formic acid and the sugars separated on paper chromatograms. Glucose and galactose were estimated by the arsenomolybdate-Somogyi reagent, and mannose by periodate oxidation.

From the calibration curves, four chromatograms contained 1.6×10^{-7} moles galactose and 1.3×10^{-7} moles glucose, and the mannose present on one chromatogram gave after periodate oxidation formic acid equivalent to 1.90 c.c. of 0.00980N sodium hydroxide, i.e., 3.72×10^{-6} moles of mannose.

Thus, the mannan gave on hydrolysis:

mannose, 98.3%;

galactose, 1.1%;

glucose, 0.8%.

(2) Estimation of total amount of mannose present.

This was carried out as described on page 52, using xylose as a reference sugar and estimating the sugars, after separation on a paper chromatogram, by periodate oxidation.

The weight of mannan B hydrolysed was 30.58 mg., and the amount of xylose reference sugar added was 29.15 mg. The titration figures (c.c. of 0.00980N NaOH) were: mannose, 1.44; xylose, 1.30. These results showed that 91% mannose was recovered from the hydrolysate.

Reducing power of purified mannan B.

(a) Hypoiodite oxidation

The method used was that described on page 55.

2I. N iodine are equivalent to -CHO.

Normality of sodium thiosulphate = 0.01048.

Weight of mannan B: 54.13 mg.; 56.11 mg.

Titre : 4.03 c.c.; 3.95 c.c.

Blank : 9.34 c.c., 9.36 c.c.

Mean Blank : 9.35 c.c.

Whence, number of anhydrohexose units per reducing group = 12, 12.

(b) Colorimetric method (120)

The method used was that described on page 56, and the standard mannose curve used for mannan A was also used for mannan B.

Weight of mannan B : 41.66 mg. 37.81 mg.

Moles of anhydro-mannose units : 25.69×10^{-5} 23.31×10^{-5}

Spekker reading : 0.017 0.011

From graph, moles of reducing group : 19.7×10^{-7} 16.0×10^{-7}

Thus, the mannan contained one reducing group per 131; 146 anhydrohexose units.

The values of the chain length of mannan B obtained by the above two methods were different and disagreed with the value obtained by other methods. Values of the chain length of the polysaccharide which are derived from measurement of the reducing power are, therefore, of doubtful validity.

Methylation of mannan B.

Purified mannan B (11 g.) was methylated twelve times by

Haworth's method (121) and twice by Purdie's method (106) and the product isolated as described for mannan A on page 58. Methylated mannan B showed an even greater tendency than did methylated mannan A to separate from solution in chloroform as a gel. The crude methylated mannan B (3.5 g.) had a methoxyl content of 44.9% and was fractionated with light petroleum-chloroform mixtures to give the following fractions:

Fraction	Solvent		Yield
	Chloroform %	Light Petroleum %	g.
A	0	100	0.06
B	5	95	0.07
C	10	90	0.10
D	15	85	0.33
E	20	80	2.83
F	100	0	0.14

Fraction E was an almost white solid having $[\alpha]_D^{15}$ -20° (c, 1.0 in chloroform) and methoxyl content of 45.0% and was used in the subsequent work on methylated mannan B.

Hydrolysis of methylated mannan B.

A sample of methylated mannan B (2.5 g.) was dissolved in formic acid (15 c.c., A.R., 98-100%) and heated under reflux on a boiling water bath for seven hours. The solution became too dark to follow the hydrolysis polarimetrically. Formyl esters were hydrolysed as described for mannan A on page 60, acid removed and the solution concentrated to a syrup.

Paper chromatographic examination of the sugars obtained by hydrolysis of methylated mannan B.

The mixture of sugars was separated on a paper chromatogram using as solvent the upper layer of a mixture of n-butanol - ethanol - water (4:1:5, v/v). The chromatogram after development with aniline oxalate showed spots corresponding to those obtained from methylated mannan A (page 61) except that in mannan B the amounts of tetramethyl mannose and tetramethyl galactose were approximately equal. Examination of the methylated sugars on a chromatogram using as mobile phase the upper layer of a mixture of benzene - ethanol - water (167:47:15, v/v) showed the presence of the same sugars which had been detected using the previous solvent.

Separation of hydrolysis products on a cellulose column.

The syrup (2.01 g.) obtained after hydrolysis of methylated mannan B was dissolved in solvent and separated on a cellulose column in the manner described on page 61. The solvent used was a mixture of light petroleum - n-butanol (60:40, v/v) saturated with water, which was replaced by n-butanol saturated with water when all the trimethyl sugars had been eluted, and by water when all the monomethyl sugar had been eluted.

The fractions were purified as described for mannan A, and were examined.

Examination of the fractions obtained from the cellulose column.

Fraction I. (2:3:4:6-tetramethyl D-mannose and 2:3:4:6-tetramethyl D-galactose).

This fraction extended over tubes 1-264 and was shown by paper chromatography to contain tetramethyl mannose and tetramethyl galactose. Yield, 55 mg. The fraction was spotted onto a long paper chromatogram (80 cm.) with side reference strips and run using as mobile phase the upper layer of a mixture of benzene - ethanol - water (167:47:15, v/v). The reference strips were cut off and developed and the centre section containing the sugars divided and the sugars extracted with acetone in a Soxhlet extractor. The individual sugars were purified as usual and examined, the yields having been corrected for material removed on the side reference strips.

Fraction Ia (2:3:4:6-tetramethyl D-mannose).

Yield, 27 mg. The anilide was prepared in the usual way by heating the syrup in dry ethanol with aniline and after recrystallisation from light petroleum melted at 144-145°C, undepressed on admixture with an authentic sample of 2:3:4:6-tetramethyl mannose anilide. The anilide had $[\alpha]_D^{17}$ -96° (c, 0.1 in acetone) changing to -41° on addition of a trace of acid, which agreed with the value $[\alpha]_D^{20}$ -96° (c, 2.0 in acetone) → -39° given by Irvine and McNicoll (145) for 2:3:4:6-tetramethyl mannose anilide.

Fraction Ib (2:3:4:6-tetramethyl D-galactose).

Yield, 28 mg. The anilide was prepared and after recrystallisation from dry ethanol melted at 195-196°C, undepressed on admixture with a specimen of 2:3:4:6-tetramethyl galactose anilide. The anilide had $[\alpha]_D^{16}$ -137° (c, 0.5 in pyridine), which agreed with the value $[\alpha]_D^{18}$-140° (c, 0.5 in pyridine) given by Smith (41) for 2:3:4:6-tetramethyl galactose anilide.

Fraction II (2:3:6-trimethyl D-mannose).

This fraction was collected in tubes 265-475. Yield, 1.640 g. It was a pale brown syrup having $[\alpha]_D^{17}$-10° (c, 6.0 in water). Smith (41) gave $[\alpha]_D^{18}$ -6.5° (c, 0.6 in water) for 2:3:6-trimethyl mannose. The purity of the fraction determined by hypoiodite oxidation was 95.2%, and by methoxyl content, 94.9%.

Preparation of anilide

The anilide was prepared in the usual way and was recrystallised from dry ether giving long needles which melted at 127-128°C, undepressed on admixture with the anilide prepared from fraction III obtained from methylated mannan A, and had $[\alpha]_D^{16}$ -155° (c, 0.1 in methanol) changing to -40°, which agreed with the values m.p. 129°C and $[\alpha]_D^{15}$ -150° (c, 0.5, in methanol) $\rightarrow$ -38° given by Smith (41) for 2:3:6-trimethyl mannose anilide.

Analysis:

Found: C, 60.9%; H, 7.9%; N, 4.1%.

Calculated for $C_{15}H_{23}O_5N$:

C, 60.6%; H, 7.8%; N, 4.7%.

Preparation of lactone and phenylhydrazide.

The lactone was prepared by oxidising the syrup with bromine water and isolated by the usual procedure. It was recrystallised from dry ether and melted at 80-81°C, undepressed on admixture with a specimen of 2:3:6-trimethyl D-mannonolactone. The lactone had $[\alpha]_D^{20}$ + 69.5° (initial value, c, 0.6 in water) which fell slowly, indicating the presence of a γ -lactone. Smith (41) reported $[\alpha]_D^{18}$ + 65.5° (initial value, c, 1.0 in water) for 2:3:6-trimethyl γ-mannonolactone.

The phenylhydrazide was prepared by heating the lactone in dry ethanol with the theoretical amount of phenylhydrazine. It was recrystallised from moist ether and melted at 130-131°C, undepressed on admixture with a sample of 2:3:6-trimethyl D-mannonic acid phenylhydrazide. The phenylhydrazide had $[\alpha]_D^{16}$ -17° (c, 0.4 in water) which agreed with the value $[\alpha]_D^{18}$ -18° (c, 1.7 in water) given by Smith (41) for 2:3:6-trimethyl mannonic acid phenylhydrazide.

Fraction III

This fraction was contained in tubes 476-870. Yield, 66 mg. The fraction crystallised slowly and was found to contain a substance which ran on a chromatogram with a speed corresponding to that of a trimethyl hexose. After washing with ether the crystals melted at 149-151°C, undepressed on admixture with the substance (X) obtained from fraction IV in the investigation on methylated mannan A.

Fraction IV

This fraction was collected in tubes 871-1044. Yield, 0.232 g. The fraction tended to crystallise and was shown by paper chromatography to contain a substance running at the same speed as the component of fraction III and also a substance running at the same speed as an authentic sample of 2:3-dimethyl mannose and giving the same colour as the latter on development with aniline oxalate.

The mixture was treated at 100°C with sulphuric acid (N) to hydrolyse any disaccharide present, and the hydrolysate was separated on thick paper chromatograms run in n-butanol - ethanol - water (4:1:5, v/v). In this manner two fractions were obtained:

Fraction IVa (yield, 203 mg.) which crystallised and melted at 148-150°C, undepressed on admixture with the crystalline material (X) obtained from fraction IV of methylated mannan A hydrolysate. This fraction was combined with fraction III and its structure determined as described on page 69. The crystalline material (X) was 1 - [2:3:4-trimethyl D-mannopyranosido] - 2:3:4-trimethyl D-mannopyranoside and had been formed by condensation of 2:3:4-trimethyl D-mannose.

Fraction V (monomethyl mannose).

This fraction was collected in tubes 1045-1114. Yield, 6 mg. of brown syrup. Using as mobile phase the upper layer of a mixture of n-butanol - ethanol - water (4:1:5, v/v) the substance had an R_G of 0.29 and gave a brown spot on development with aniline oxalate. Demethylation (146) followed by chromatographic examination showed the substance to be a mannose derivative.

Fraction VI (uronic acid).

Yield of brown syrup, 7 mg. A portion of the syrup was dissolved in a little water and a solution of naphthoresorcinol in alcohol (0.5 c.c., 1%) and concentrated hydrochloric acid (0.5 c.c.) were added. The mixture was boiled, cooled, ether added and the mixture shaken, when the ether layer became coloured violet showing the presence of uronic acid.

Using as mobile phase a mixture of ethyl acetate - acetic acid - water (3:1:3, v/v) the syrup was examined on a paper chromatogram and gave, after development with aniline oxalate, a pink spot with an R_F value of 0.69.

Fraction VII

This was the water wash from the column. The fraction (12 mg.) was dark brown and was separated on a thick paper chromatogram to give a sugar which was separated by cold water elution by the method of Laidlaw and Reid (142). The sugar

(2 mg.) obtained ran on a chromatogram at the same speed as a sample of glucose.

Estimation of the sugars obtained on hydrolysis of methylated mannan B after separation by paper chromatography.

The methylated mannan was hydrolysed with formic acid and the sugars estimated by the method described on page 75. A micropipette was used to control the amount of hydrolysate on each chromatogram and several papers were combined to enable the tetramethyl sugars to be determined; in the table given below this is given as a factor, by which the end group titrations must be divided. The titration figures represent differences from the blank.

Tetramethyl sugars		Trimethyl sugars	Molar Ratio of tetra- to tri-methyl sugars
Titre	Factor	Titre	
0.34	4	3.50	41
0.30	4	2.96	39

Molecular weight of methylated mannan B.

The molecular weight was determined by Barger's method, as described on page 54. The methylated mannan (52.10 mg.) was dissolved in chloroform (10 c.c.) to give a solution containing $\frac{52.10}{204.2 \times 10 \times x}$ moles/l., where, x = degree of polymerisation of the methylated mannan. The results obtained are collected in the table given below:

Molarity of sucrose octa-acetate solution X 10^4	Corresponding value of x for equilibrium	Sum of changes of sizes of drops with respect to methylated mannan X 10^2 mm.
1	255	+ 11
2	128	+ 6
3	85	+ 5
4	64	+ 2
5	51	+ 5
6	43	+ 4
7	35	- 1
8	32	- 3

Thus the degree of polymerisation of the polysaccharide was 35-43.

Viscosity of methylated mannan B.

This was determined by the method described on page 77. A solution of the polysaccharide (87.8 mg.) in m-cresol (10 c.c.) had a time of flow of 287 seconds, compared with 262 seconds for the pure solvent. Thus, from the Staudinger equation, using $K_m = 12 \times 10^{-4}$, a value of 9 was obtained for the degree of polymerisation of methylated mannan B.

Periodate oxidation of mannan B.

(a) Determination of formic acid released

The method used is described on page 78 and the results obtained are given in the table below.

Duration of oxidation (hours)	Weight of mannan (mg.)	Titre (c.c.)	Number of $C_6H_{10}O_5$ residues per mole of formic acid
191	100.9	5.89	8.31
290	102.5	8.05	6.18
354	109.1	11.81	4.48
502	103.6	18.62	2.70
594	103.1	20.64	2.42
643	100.9	20.07	2.44
691	107.2	20.38	2.55

(b) Determination of periodate uptake

This was carried out in an unbuffered medium using the method described on page 79.

The iodine used for titration was 0.1052N, and the titration figures are differences from the blank.

Duration of oxidation (hours)	Weight of mannan (mg.)	Titre (c.c.)	Periodate uptake (moles/$C_6H_{10}O_5$ residue)
191	101.9	0.24	0.25
281	103.6	1.04	0.54
358	109.2	1.36	0.66
643	101.9	1.89	0.99
691	107.9	1.93	0.95

BIBLIOGRAPHY

1. Schulze, Z. physiol. Chem., 16, 387, (1892).

2. Norman, "Biochemistry of Cellulose, The Polyuronides,
Lignin, etc.," 59, (1937).

3. Idem, Ann. Rev. Biochem., 10, 65, (1941).

4. Hassid, ibid., 13, 59,(1944).

5. Hérissey, Rev. gen. Bot., 15, 345, 369, 406, 444, (1903).

6. Nishida and Hashima, J. Dept. Agr. Kyushu Imp. Univ., 2,
277, (1930);
J. Agr. Chem. Soc. Japan, 7, 214;
Bull. Agr. Chem. Soc. Japan, 7, 3, (1931);
ibid., 8, 54, (1932).

7. Nishida and Hatori, J. Agr. Chem. Soc. Japan, 6, 991;
Bull. Agr. Chem. Soc. Japan, 6, 78,
(1930);
J. Agr. Chem. Soc. Japan, 6, 1065;
Bull. Agr. Chem. Soc. Japan, 6, 80,(1930).

8. Gans and Tollens, Ber., 21, 2150, (1880);
Ann., 249, 251, (1888).

9. Pringsheim and Liss, ibid., 460, 326, (1928).

10. Klages and Niemann, ibid., 523, 224, (1936).

11. Husemann, J. prakt. Chem., (2), 155, 237, (1940).

12. Ritthausen, ibid., 102, 32, (1867);
Chem. Zeit., 21, 717, (1897).

13. Effront, Compt. rend., 125, 38, 116, (1897).

14. Fromherz, Z. physiol. Chem., 50, 237, (1906).

15. Schorger, J. Ind. Eng. Chem., 9, 748, (1917).

16. Bertrand, Compt. rend., 129, 1027, (1899).

17. Tollens, Ber., 23, 2990, (1890);
Z. angew. Chem., 5, 155, (1892);
Ann., 267, 349, (1892).

18. Kimoto, Bull. Coll. Agr. Tokio, 5, 254, (1902).

19. Storer, Bussey Inst. Bull., 3, 13, (1902).

20. Nowotnowna, Biochem. J., 30, 2177, (1936).

21. Schwalbe, Z. Angew. Chem., 23, 1540, (1910).

22. Krause, Chem. Ind., 29, 217, (1901).

23. Schutzenberger, Compt. rend., 78, 493, 698, (1874).

24. Salkowski, Ber., 27, 497, 925, (1894).

25. Oshima, Hoppe-Seylers Z. f. physiol. Chem., 37, 42, (1902).

26. Kraut and Eichhorn, Ber., 60, 1639, 1644, (1929).

27. Béchamp, Compt. rend., 78, 645, (1874).

28. Nägelin and Lowe, Ann., 193, 322, (1872).

29. Meigen and Spreng, Hoppe-Seylers Z.f. physiol. Chem., 55, 48, (1908).

30. Hessenland, Z. d. Vereins f. Rubenzuckerindustrie, 42, 671, (1892).

31. Daoud and Ling, J. Soc. Chem. Ind., 365, (1931); Wochenschrift f. Brauer, 537, (1931).

32. Haworth, Hirst, and Isherwood, J.C.S., 784, (1937).

33. Harden and Young, ibid., 1224, (1902).

34. Garzuly-Janke, J. prakt. Chem., 156, 45, (1940).

35. Haworth, Heath, and Peat, J.C.S., 833, (1941).

36 Lindstedt, Arkiv. Kemi, Mineral., Geol., A20, No. 13, (1945).

37. Hirst and Jones, J.C.S., 1278, (1948).

38. Spada, Atti Soc. nat. mat. Modena, 70, 20, (1939).

39. Wise and Appling, Ind. Eng. Chem., Anal., 16, 28, (1944).

40. Lew and Gortner, Arch. Biochem., 1, 32, (1943).

41. Smith, J.A.C.S., 70, 3249, (1948).

42. Ahmed and Whistler, ibid., 72, 2524, (1950).

43. Rafique and Smith, ibid., 72, 4634, (1950).

44. Andrews, Hough, and Jones, J.C.S., 2744, (1952).

45. Hirst, Jones, and Walder, ibid., 1443, (1947).

46. Daoud, Biochem. J., 26, 255, (1932).

47. Andrews, Hough, and Jones, J.A.C.S., in the press.

48. Garzuly-Janke, Zentr. Bakt., 102, II, 361, (1940).

49. Haworth, Raistrick, and Stacey, Biochem. J., 29, 612, (1935).

50. Stacey, J.C.S., 853, (1947).

51. Kleczkowski and Wierzchowski, Soil Science, 49, 193, (1940).

52. Haworth, Kent, and Stacey, J.C.S., 1211, 1220, (1948).

53. Colin and Augem, Bull. soc. chim. biol., 10, 822, (1928).

54. Dubat, Compt. rend., 133, 942, (1901).

55. Takahashi, J. Agr. Chem. Soc. Japan, 6, 791, (1930).

56. Idem, ibid., 7, 219, (1931).

57. Curl and Nelson, J.A.C.S., 66, 1227, (1944).

58. Otsuki, Acta Phytochim (Japan), 10, 29, (1937).

59. Idem, ibid., 10, 1, (1937).

60. Jones, J.C.S., 3292, (1950).

61. Replat, Rev. gén. mat. plastiques, 9, 660, (1933).

62. Hahn, Dyestuffs, 31, 106, (1930).

63. Flemming, Melliand. Textilber., 7, 175, (1926).

64. Grober and Portabella, Brit. Patent 373, 566, May 26, 1932.

65. Long, Brit. Patent 175, 313, Aug. 13, 1920.

66. Bertarelli, Z. Nahr. Genussm., 13, 484.

67. Gerum, ibid., 27, 392.

68. Nottbohm, ibid., 25, 144.

69. Hudson and Sawyer, J.A.C.S., 39, 470, (1917).

70. Horton, J. Ind. Eng. Chem., 13, 1040, (1921).

71. Clark, Bur. Standards Sci. Papers, No. 429, (1922); J. Biol. Chem., 51, 1.

72. Hudson and Jackson, J.A.C.S., 56, 958, (1934).

73. Narayanan, Indian J. Med. Research, 29, 1, (1941).

74. Mezzadroli, Boll. chim. farm., 57, 361, (1918).

75. Meppi, Giorn. chim. ind., 1, 76, (1919).

76. Saxe, U.S. Patent 1, 249, 511, Dec. 11, 1917.

77. Paton, Nanji, and Ling, Biochem. J., 18, 451, (1924).

78. Melnick and Cowgill, ibid., 29, 1515, (1935).

79. Pringsheim, Z. physiol. Chem., 80, 376, (1912).

80. Pringsheim and Genin, ibid., 140, 299, (1924).

81. Klages and Kircher, Ann., 543, 183, (1940).

82. Bierry and Giaja, Compt. rend., 148, 507, (1909).

83. Idem, Biochem. Z., 40, 370, (1912).

84. Utsushi, Huji, Matsumoto, and Nagaoka, Tohoku J. Expl. Med., 50, 175, (1949).

85. Tadokoro and Yoshimura, J. Agr. Chem. Soc. Japan, 9, 254, (1933).

86. Beals, J. Ind. Eng. Chem., 7, 162, (1915).

87. Beals and Lindsey, J. Agr. Research, 7, 301, (1916).

88. Gatin and Gatin, Bull. sci. pharmacol., 14, 447.

89. Norimasa, Hakkô Kôgaku Zasshi, 25, 145, (1947).

90. Reiss, Ber., 22, 609, (1889).

91. Fischer and Hirschberger, ibid., 22, 1155, (1889).

92. Johnson, J.A.C.S., 18, 214, (1896).

93. Baker and Pope, J.C.S., 77, 676, (1900).

94. Schulze, Ber., 24, 2227, (1891).

95. Ivanow, Journ. f. Landw., 56, 217, (1908).

96. Schmidt and Graumann, Ber., 54(B), 1860, (1921).

97. Pringsheim and Seifert, Z. physiol. Chem., 123, 205, (1922).

98. Patterson, J.C.S., 1139, (1923).

99. Lüdtke, Ann., 456, 201, (1927).

100. Gilson, La Cellule, 9, 397, (1893).

101. Herzog and Gonell, Naturwissenschaften, 12, 1153, (1924).

102. Hess and Lüdtke, Ann., 466, 18, (1928).

103. Yundt, T.A.P.P.I., 34, No. 2, 94, (1951).

104. Klages, Ann., 509, 159, (1934).

105. Haworth, Hirst, and Streight, J.C.S., 1349, (1931).

106. Purdie and Irvine, J.C.S., 1021, (1903).

107. Klages and Maurenbrecher, Ann., 535, 175, (1938).

108. Bergmann and Machemer, Ber., 63, 316, (1930).

109. Hess, Dziengel, and Maass, ibid., 63, 1922, (1930).

110. Ward, M. Sc. Thesis, Manchester (1947).

111. Hirst, Jones, and Woods, J.C.S., 1048, (1947).

112. Schorger, "Chemistry of Cellulose and Wood," 538, (1926).
Quoted in "The Methods of Cellulose Chemistry"
by Dorée, 434, (1947).

113. Brown and Jones, J.C.S., 1344, (1947).

114. Halsall, Hirst, and Jones, ibid., 1399, 1427, (1947).

115. Klages, Ann., 512, 185, (1934).

116. Salkowski, Ber., 27, 497, (1894).

117. Hirst and Jones, J.C.S., 1659, (1949).

118. Nelson, J. Biol. Chem., 153, 375, (1944).
Duff and Eastwood, Nat., 165, 848, (1950).

119. Pacsu and Mullen, J.A.C.S., 63, 1487, (1941).

120. Meyer, Noelting, and Bernfeld, Helv. Chim. Acta, 31, 103, (1948).

121. Haworth, J.C.S., 8, (1915).

122. Greene and Lewis, J.A.C.S., 50, 2813, (1928).

123. Hendricks and Rundle, ibid., 60, 2563, (1938).

124. Oldham and Rutherford, ibid., 54, 366, (1932).

125. Knauf, Hann, and Hudson, ibid., 63, 1447, (1941).

126. Barger, J.C.S., 286, (1904).

127. Staudinger and Reinecke, Ann., 535, 47, (1938).

128. Malaprade, Bull. soc. chim., (4), 43, 683, (1928); Compt. rend., 186, 382, (1928).

129. Fleury and Courtois, ibid., 209, 219, (1939); Fleury and Lange, ibid., 195, 1395, (1932). Fleury and Paris, ibid., 196, 1416, (1933); J. pharm. chim., (8), 18, 470, (1933).

130. Bailly, Compt. rend., 206, 1902, (1938).

131. Head, Nat., 165, 236, (1950).

132. Head and Hughes, J.C.S., 2046, (1952).

133. Moe, Miller, and Iwen, J.A.C.S., 69, 2621, (1947).

134. Bell, J.C.S., 992, (1948).

135. Bell, Palmer, and Johns, ibid., 1536, (1949).

136. Greville and Northcote, ibid., 1945, (1952).

137. Halsall, Hirst, and Jones, ibid., 1427, (1947).

138. Potter and Hassid, J.A.C.S., 70, 3488, (1948).

139. Wise, Ind. Eng. Chem., Anal., 17, 63, (1945).

140. Chanda, Hirst, Jones, and Percival, J.C.S., 1289, (1950).

141. de Whalley, Albon, and Gross, Analyst, 76, 287, (1951).

142. Laidlaw and Reid, Nat., 166, 476, (1950).

143. Conway and Byrne, Biochem. J., 27, 419, (1933).

144. Hough, Jones, and Wadman, J.C.S., 2511, (1949).

145. Irvine and McNicoll, *ibid.*, 1449, (1910).

146. Hough, Jones, and Wadman, *ibid.*, 1702, (1950).

147. Heuser, Heath, and Shockley, J.A.C.S., 72, 670, (1950).

148. Buchanan, Dekker, and Long, J.C.S., 3162, (1950).

149. Chanda, Hirst, Percival, and Ross, *ibid.*, 1833, (1952).

150. Hill, J.A.C.S., 50, 2678, (1928).

151. Grangaard, Michell, and Purves, *ibid.*, 61, 1290, (1939).

152. Jones, Private communication.

153. Rich, Paper Trade J., 112, No. 6, 35, (1941).
Mease, J. Research Natl. Bur. Standards, 27, 551, (1941).
Heuser, "The Chemistry of Cellulose," 146, (1946).

154. *Idem*, *ibid.*, 148, (1946).

155. Carver, Bingham, Bradshaw, and Venable, Ind. Eng. Chem., Anal., 1, 49, (1929).

SUMMARY

Mannan A

1. Mannan A was extracted from delignified ivory nut shavings with 7% potassium hydroxide solution, and the polysaccharide precipitated by acidification of the extract and addition of methylated spirits.

2. The mannan, purified by means of the copper complex, gave on hydrolysis 97.6% mannose, 1.8% galactose, and 0.8% glucose.

3. The reducing power of the mannan determined by oxidation with alkaline hypoiodite gave a degree of polymerisation of 100, and by treatment with 3:5-dinitrosalicylic acid, 28. The values of the chain length of mannan A obtained by these two methods were different and disagreed with the value obtained by other methods. Values of the chain length of the polysaccharide which are derived from measurement of the reducing power are, therefore, of doubtful validity.

4. A triacetate was prepared which was found by Barger's method to have a degree of polymerisation of 10-13.

5. The mannan was methylated and the methylated polysaccharide hydrolysed. The hydrolysate was separated on a cellulose column and the following sugars were obtained: 2:3:4:6-tetramethyl D-mannose, 2:3:4:6-tetramethyl D-galactose, 2:3:6-trimethyl D-mannose, 2:3:4-trimethyl D-mannose, and 2:3-dimethyl D-mannose in the molar ratios of, respectively, 1.0: 0.2: 11.6: 1.0: 0.2. All the galactose present in the purified mannan was accounted

for as 2:3:4:6-tetramethyl galactose.

6. The 2:3:4-trimethyl mannose fraction crystallised as a disaccharide, which was shown to be 1 - [2:3:4-trimethyl D-mannopyranosido] - 2:3:4-trimethyl D-mannopyranoside.

7. The degree of polymerisation of the methylated mannan was determined by Barger's method and found to be 9-11.

8. Periodate oxidation experiments on the mannan showed that 1.6 moles of periodate were consumed per anhydrohexose unit, and one mole of formic acid was liberated per 4 anhydrohexose units.

9. On the basis of these results possible structures for the mannan are proposed.

Mannan B.

1. Mannan B was extracted from the residue left after removal of mannan A from ivory nuts. The residue was extracted with cuprammonium hydroxide and the solution treated with sodium hydroxide. Several fractions were obtained and one which was considered to be mannan B was purified by dissolution in anhydrous formic acid and precipitation with ethanol.

2. Purified mannan B gave on hydrolysis 98.3% mannose, 1.1% galactose, and 0.8% glucose.

3. The reducing power of the mannan was determined by hypoiodite oxidation and colorimetrically by means of 3:5-dinitrosalicylic acid. The first method gave an apparent degree of polymerisation of 12, the second, 130. The values of the chain length of mannan B obtained by these two methods were different and disagreed with the value obtained by other methods. Values of the chain length of the polysaccharide which are derived from measurement of the reducing power are, therefore, of doubtful validity.

4. Mannan B was methylated and the methylated polysaccharide hydrolysed. Separation of the mixture of sugars on a cellulose column gave 2:3:4:6-tetramethyl D-mannose, 2:3:4:6-tetramethyl D-galactose, 2:3:6-trimethyl D-mannose, 2:3:4-trimethyl D-mannose, and 2:3-dimethyl D-mannose in the molar ratios of, respectively, 1:1:63:11:1. All the galactose present in purified mannan B was accounted for as 2:3:4:6-tetramethyl galactose.

5. The 2:3:4-trimethyl mannose fraction crystallised as a

disaccharide, which was shown to be 1 - [2:3:4-trimethyl D-mannopyranosido] - 2:3:4-trimethyl D-mannopyranoside.

6. The degree of polymerisation of methylated mannan B was determined by Barger's method and found to be 35-43.

7. Periodate oxidation experiments on the mannan showed that 0.98 moles of periodate were consumed per anhydrohexose unit, and one mole of formic acid was liberated per 2.4 anhydrohexose units.

8. On the basis of these results possible structures for the mannan are proposed.

The author wishes to express his sincere thanks to Professor E.L. Hirst, F.R.S., for his continued interest throughout the course of this work and to Dr. G.O. Aspinall for his friendly advice and encouragement. Acknowledgment must also be made of the valuable assistance given by (the late) Dr. E.G.V. Percival and thanks are expressed to the Department of Scientific and Industrial Research for the award of a maintenance allowance and to Dr. J.K.N. Jones for the gift of specimens.