149. *The Constitution of Yeast Mannan.*

By W. N. Haworth, R. L. Heath, and S. Peat.

The constitution of the mannan of yeast has been re-examined. The earlier work of Haworth, Hirst, and Isherwood (J., 1937, 784) has been confirmed in regard to the homogeneity of the polysaccharide and of its acetate and methylated derivative and also in regard to the nature of some of the products of hydrolysis of methylated mannan. It is confirmed that tetramethyl mannose, trimethyl mannose, and dimethyl mannose are produced in equimolecular proportions and that the " tetra " fraction consists of tetramethyl mannose, and the " di " fraction exclusively of 3 : 4-dimethyl mannose. It is shown, however, that the trimethyl mannose fraction is not constituted exclusively of 2 : 3 : 4-trimethyl mannose and that this sugar does not, in fact, constitute more than 10% of the " tri " fraction. The main constituents of this fraction are 3 : 4 : 6- and 2 : 4 : 6-*trimethyl mannose*, which are present in equimolecular proportion and together constitute 90% of the fraction.

The probable structure of yeast mannan is discussed in the light of these observations.

In an earlier paper (Haworth, Hirst, and Isherwood, J., 1937, 784) a preliminary investigation of the mannan of yeast was reported. It was then shown that this polysaccharide, which is extracted by alkali from baker's yeast, is essentially homogeneous and composed exclusively of mannose residues. The structural arrangement of the mannose units was investigated by a procedure involving methylation of the mannan, hydrolysis of the methylated polysaccharide, and examination of the constituent partly methylated mannose fragments. Tetramethyl mannose, trimethyl mannose, and dimethyl mannose were found to be present in equimolecular proportions and it appeared that these fragments were tetramethyl mannopyranose, 2 : 3 : 4-trimethyl mannose and 3 : 4-dimethyl mannose respectively. The constitution assigned to the trimethyl mannose fraction was based on a study of its oxidation products and principally on the observation that its oxidation with nitric acid gave a hydroxytrimethoxyadipic acid (characterised as the crystalline diamide, m. p. 191°), which was therefore reasonably described as 2 : 3 : 4-trimethyl mannosaccharic acid. Nevertheless, when 2 : 3 : 4-trimethyl mannosaccharamide was later prepared (Haworth, Hirst, Isherwood, and J. K. N. Jones, J., 1939, 1878) by a method which left no doubt as to its constitution, it was shown not to be identical with that prepared by the oxidation of the trimethyl mannose from methylated mannan. Moreover, discrepancies were apparent between the properties of authentic 2 : 3 : 4-trimethyl mannono-δ-lactone monohydrate (m. p. 73°; $[\alpha]_D^{20°}$ + 138° ⟶ + 81° in water) on the one hand and the trimethyl mannonolactone (m. p. 91°; $[\alpha]_D^{20°}$ + 138° ⟶ + 44° in water) from mannan on the other.

Having regard to these observations, it was deemed advisable to re-examine the tri-

methyl mannose fraction from mannan. The procedure described in the earlier paper was adopted for the extraction of mannan from yeast and for the preparation of the acetylated derivative. The mannan itself and the acetate had the same physical properties as those already recorded. Moreover, the methylated mannan, which was prepared by direct methylation, was not to be distinguished from the methylated mannan prepared previously.

The homogeneity of the methylated mannan was established by its precipitation from chloroform solution in several fractions (see Table I). The main fractions did not materially differ from each other in respect of methoxyl content, rotation and viscosity in chloroform solution, and particle weight as determined by osmotic pressure measurement.

The methylated mannan gave solutions of low viscosity when compared with similar solutions of linear polymers such as methylated cellulose. Nevertheless the particle weight, determined osmotically by Dr. Chambers in this laboratory, was large and corresponded to aggregates of 200 to 400 mannose units.

Methylated mannan is resistant to weak hydrolytic agents and its hydrolysis was achieved in the earlier investigation by the use of methyl-alcoholic hydrogen chloride at 150° under pressure. We have now found it more convenient to use the less drastic method of heating the methylated mannan at 100° with a mixture of equal parts of glacial acetic acid and 5% hydrochloric acid. This procedure gave a mixture of partly methylated mannoses, which were converted into the methyl glycosides in a separate operation. The glycosides were then separated by fractional distillation (see Table II).

We have confirmed in every particular the earlier findings as to the composition of the tetramethyl methylmannoside and dimethyl methylmannoside fractions. The former consists exclusively of tetramethyl methylmannopyranoside and the latter of 3 : 4-dimethyl methylmannopyranoside. Two crystalline derivatives of the dimethyl mannose are reported for the first time, namely, 3 : 4-*dimethyl α-methylmannoside* and 3 : 4-*dimethyl* 1 : 2-*monoacetone mannose.*

It was also confirmed that the " tetra," " tri," and " di " fractions were present in very nearly equimolecular proportions.

The Trimethyl Mannose Fraction.—It became apparent that the " tri " fraction did not consist exclusively of 2 : 3 : 4-trimethyl mannose. In fact this sugar constitutes not more than 10% of the mixture, the main constituents of which are 2 : 4 : 6-trimethyl mannose and 3 : 4 : 6-trimethyl mannose in equimolecular proportion.

The trimethyl methylmannoside fraction yielded on hydrolysis with aqueous acid a syrup which crystallised in part. The crystalline part (85% of the whole) was separated into a number of fractions by crystallisation from suitable solvents and it became evident that more than one trimethyl mannose was present in the crystalline material. Furthermore, adequate separation of the constituents was not being achieved by fractional crystallisation. One fraction had m. p. 102° and proved to be pure 3 : 4 : 6-trimethyl α-mannose, identical with that synthesised by Bott, Haworth, and Hirst (J., 1930, 1395). The other fractions had melting points 10—20° lower than that of 3 : 4 : 6-trimethyl mannose and none of them consisted of a single compound. Evidence that two trimethyl mannoses were present was obtained when, during the course of recrystallisation of one fraction, two forms of crystals appeared together and it was possible to separate these mechanically. One form melted at 102° and was identified as 3 : 4 : 6-trimethyl α-mannose. The other form had m. p. 90° and the melting point of a mixture of the two forms was depressed to 70°. Inasmuch as the two substances mutarotated in aqueous solution in the same direction it was clear that they did not represent the α- and the β-form of the same sugar.

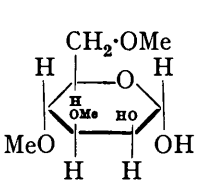
Analysis of the substance of m. p. 90° required its formulation as the monohydrate of a trimethyl hexose, and this view found confirmation in dehydration experiments. The possibility that the compound was a monohydrate of 3 : 4 : 6-trimethyl mannose was next explored. The product of its dehydration was a viscid syrup which crystallised in part when treated with acetone. Every precaution was taken to avoid the re-formation of the hydrate, but recrystallisation yielded only the hydrate of m. p. 90° and no trace of 3 : 4 : 6-trimethyl mannose was found. Similarly, all attempts to form a hydrate from authentic 3 : 4 : 6-trimethyl α-mannose were without success.

There was thus a strong indication that the substance of m. p. 90° was a trimethyl

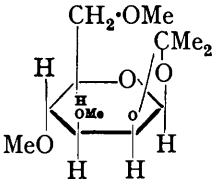
mannose other than 3 : 4 : 6-trimethyl mannose. The field of search was narrowed by the fact that the still unidentified trimethyl mannose must belong to the pyranose series. This followed from an observation that the methylation of the trimethyl methylmannoside fraction gave crystalline tetramethyl α-methylmannopyranoside in 91% yield.

The possible presence of furanose sugars being thus excluded, it is evident that the substance of m. p. 90° can only be the hydrate of one of the following: 3 : 4 : 6-, 2 : 3 : 4-, 2 : 3 : 6-, or 2 : 4 : 6-trimethyl mannose. 3 : 4 : 6-Trimethyl mannose had already been excluded; 2 : 3 : 4-trimethyl mannose and 2 : 3 : 6-trimethyl mannose are known as syrups which do not form hydrates and it was therefore assumed, as a working hypothesis, that the substance of m. p. 90° was the monohydrate of the hitherto unknown 2 : 4 : 6-trimethyl mannopyranose. Furthermore, from the direction of mutarotation it appeared to be an α-form.

Investigation of the substance of m. p. 90° was hampered by the difficulty, already referred to, of separating it from 3 : 4 : 6-trimethyl mannose. This difficulty was eventually overcome by the use of a chemical method of separation, based upon the assumed constitution of the substance, which involved the condensation of the mixture of trimethyl sugars with acetone. It is possible for 3 : 4 : 6-trimethyl mannose (I) to yield, with acetone,



(I.)



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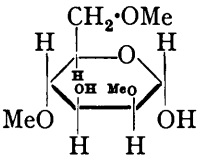


(III.)

the 1 : 2-monoacetone derivative (II), whereas 2 : 4 : 6-trimethyl mannopyranose (III), not having two hydroxyl groups available on adjacent carbon atoms, is unlikely to condense with acetone. It is further to be expected that the solubility differences of the acetone compound and the unchanged trimethyl mannose would suffice for the separation desired.

This argument has been substantiated in practice. The crystalline mixture was treated repeatedly with acetone containing a little sulphuric acid. Crystallisation of the product from ether effected a clean separation of the unchanged hydrate of m. p. 90° and 1 : 2-monoacetone 3 : 4 : 6-trimethyl mannose. The latter is a syrup and from it was prepared by hydrolysis pure 3 : 4 : 6-trimethyl mannose (m. p. 102°). This method of separation made possible the isolation of a sufficient quantity of the hydrate for closer examination whereby its constitution was established.

The hydrate, on being boiled with alcoholic aniline, yielded an *anilide* (m. p. 134°) which was not identical with either 3 : 4 : 6-*trimethyl mannose anilide* (m. p. 143°) or 2 : 3 : 6-trimethyl mannose anilide (m. p. 127°; Haworth, Hirst, and Streight, J., 1931, 1349). The compound must be, therefore, either 2 : 3 : 4-trimethyl mannose or 2 : 4 : 6-trimethyl mannose. That it was, in fact, the latter compound followed from the observation that its oxidation with bromine water yielded a crystalline lactone (m. p. 98°) which was not identical with 2 : 3 : 4-trimethyl mannono-δ-lactone (m. p. 73°) or with 3 : 4 : 6-trimethyl mannono-δ-lactone (m. p. 97°), both of which were synthesised by published methods for the purpose of this comparison. Furthermore, the *amide* (m. p. 145°) prepared from the lactone was not identical with the known amides of either 2 : 3 : 4- or 3 : 4 : 6-trimethyl mannonic acid.

The amide (m. p. 145°) gave a negative Weerman test for α-hydroxy-amides, a confirmation of the allocation of a methoxyl group to position 2, and confirmatory evidence of the presence of a methoxyl group on C_4 was forthcoming from the result of the methylation of the lactone (m. p. 98°). The product was tetramethyl mannono-δ-lactone, from which was also prepared the known phenylhydrazide of 2 : 3 : 4 : 6-tetramethyl mannonic acid. Had a hydroxyl group been available on C_4 of the trimethyl mannonic acid, methylation would have yielded tetramethyl mannono-γ-lactone.

The evidence accumulated, therefore, leaves no reasonable doubt that the substance of m. p. 90° is 2 : 4 : 6-*trimethyl* d-*mannopyranose monohydrate*. It can also be stated that this product is the α-form, for it was possible to prepare from it, in small yield, the crystalline β-form. The latter was not a hydrate.

The 3 : 4 : 6-trimethyl mannose constituent was further characterised by its conversion into a crystalline *anilide*, by its oxidation to the crystalline δ-lactone, and by the conversion of the lactone into the crystalline *amide* and phenylhydrazide.

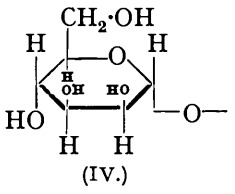
Having established that the crystalline part (85%) of the trimethyl mannose fraction from methylated mannan was a mixture of 3 : 4 : 6-trimethyl mannose and 2 : 4 : 6-trimethyl mannose monohydrate, it was possible on the basis of quantitative dehydration experiments to calculate that the relative proportion of these two sugars was roughly 1 : 1.

The non-crystalline part (15%) of the trimethyl mannose fraction was oxidised with bromine water and, in addition to 3 : 4 : 6-trimethyl mannono-δ-lactone, there was obtained crystalline 2 : 3 : 4-trimethyl mannono-δ-lactone. The yield of each crystalline lactone represented only 2% of the syrupy trimethyl mannose. Nevertheless, the non-crystallisable part still contained 2 : 3 : 4-trimethyl mannonolactone inasmuch as its treatment with alcoholic ammonia gave crystalline 2 : 3 : 4-trimethyl mannonamide, again in small yield (2%). No evidence could be adduced of the presence of any other lactone or amide, but the difficulties involved in accurate quantitative separation made impossible a decision between the extremes of 1 and 10% as the proportion of 2 : 3 : 4-trimethyl mannose in the " tri " fraction.

Discussion.

Acid hydrolysis of methylated mannan resolves it into its constituent methylated mannose units, which are shown to be tetramethyl mannopyranose, 3 : 4 : 6-trimethyl mannose, 2 : 4 : 6-trimethyl mannose, 2 : 3 : 4-trimethyl mannose and 3 : 4-dimethyl mannose. No other derivative of mannose or of any other sugar has been found. If the three trimethyl mannoses are grouped together as the " tri " fraction, it is found that the " tetra," " tri," and " di " fractions are present in equimolecular proportions. The greater part (90%) of the " tri " fraction consists of 3 : 4 : 6- and 2 : 4 : 6-trimethyl mannose, present in approximately equimolecular proportion. 2 : 3 : 4-Trimethyl mannose is also present but in a quantity which cannot be greater than 10% of the " tri " fraction or 3% of the methylated mannan. The possible significance of the presence of this sugar will be discussed below.

The repeating unit of yeast mannan would appear to be composed of six mannose residues linked in such a way that two residues, represented by (IV), give rise to tetramethyl mannose; two (V) yield 3 : 4-dimethyl mannose; one (VI) gives 3 : 4 : 6-trimethyl mannose and one (VII), 2 : 4 : 6-trimethyl mannose.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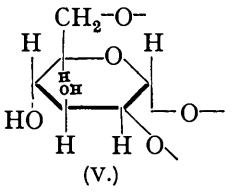
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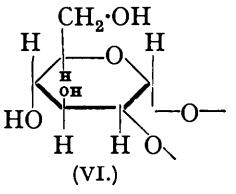
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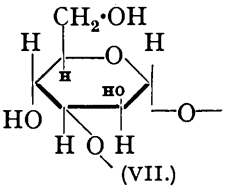
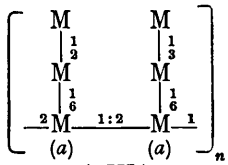

(VII.)

Although the stereochemical form is represented as that of α-mannose, it must be understood that this is based on nothing more definite than the comparatively high dextrorotation (+ 88°) of the polysaccharide and its diminution during hydrolysis. The possible presence of some β-linkages is not excluded.

It is obviously not possible to envisage the complete pattern of the structure of yeast mannan on the facts at present available, but certain general detailed features may be made out. Although the mannose residues under consideration are derived from one and the same polysaccharide, it is still possible to represent the repeating unit by a number of alternative formulæ. Some of these possibilities are given in (VIII) to (X). In these

formulæ, M represents a mannose residue, and the numerals, the carbon atoms of each residue involved in the glycosidic linkages.



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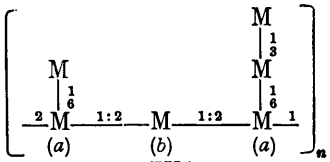

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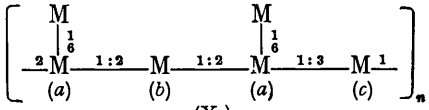

(X.)

The general arrangement is clearly pictured as a central chain of mannose units (glycosidically linked) with side chains radiating from this main chain. The number of mannose units in each side chain may be 1, as in (X), or 2, as in (VIII), or some side chains may consist of one mannose member and others (an equal number) of two. This is shown in (IX).

In these formulæ, the attachment of the side chain is represented as taking place at C_6 of the " di " unit (*a*), rather than at the alternative C_2, and there are some grounds for this preference. It seems probable that mannan consists of a terminated chain of these repeating units, where *n* is of the order of 30—60 (the particle size corresponding to 200—400 mannose residues). If the non-reducing terminal residue of the main chain is represented by the " di " unit (*a*), then this terminal unit in methylated mannan will give rise to 2 : 3 : 4-trimethyl mannose and no other likely combination of mannose residues in the repeating unit would yield 2 : 3 : 4-trimethyl mannose. This trimethyl mannose is in fact obtained in small yield from methylated mannan, an observation that lends support to the view expressed and to the formulation of the side chain junction as being 1 : 6- and not 1 : 2-glycosidic. It is to be observed that the termination of the main chain at any other point than that shown at (*a*), say at (*b*) or (*c*), means that the terminal mannose unit gives rise to tetramethyl mannose and no 2 : 3 : 4-trimethyl mannose would be formed.

Experimental.

The mannan was extracted from baker's yeast by following the procedure of Haworth, Hirst, and Isherwood (*loc. cit.*). After purification by the method of these authors, the mannan showed $[\alpha]_D^{17°}$ + 88° in water and + 90° in 5% sodium hydroxide solution (Found: ash, 0·6%).

A sample of mannan (6 g.) was fractionated by solution in water (100 c.c.) containing acetic acid (5 c.c.) and precipitation by the addition of alcohol. The fractions showed $[\alpha]_D^{16°}$ + 87·5°, + 88·5°, and + 88·0° in water.

A sample of the mannan was treated with acetic anhydride and pyridine and the acetate, obtained in 80% yield, showed $[\alpha]_D^{20°}$ + 59° (*c*, 0·835 in chloroform) (Found: $CH_3{\cdot}CO$, 44·4. Calc. for $C_{12}H_{16}O_8$: $CH_3{\cdot}CO$, 44·8%). Haworth, Hirst, and Isherwood record $[\alpha]_D^{24°}$ + 62° for the acetate.

Methylation of Mannan.—Mannan (20 g.), dissolved in water (100 c.c.) and 30% sodium hydroxide solution (50 c.c.), was methylated at 45—50° with methyl sulphate (250 c.c.) and 30% sodium hydroxide solution (650 c.c.). Methylated mannan separated at the end of the methylation when the temperature was raised to 100°. The methylation treatment was repeated eight times, in the last seven of which acetone was used as an additional solvent. The crude product was dissolved in chloroform, and the solution dried with anhydrous magnesium sulphate, concentrated to small bulk, and treated with light petroleum until it became turbid. The small fraction so precipitated, which contained most of the ash, was removed in a centrifuge and the clear solution was run into a large volume of petroleum. The methylated mannan so precipitated was dried in a vacuum first at 60° and finally at 100°. It was obtained in 70% yield and showed $[\alpha]_D^{18°}$ + 88° in chloroform (Found: OMe, 44·1; ash, 0·3%).

Fractionation of Methylated Mannan.—Methylated mannan (108 g.) was dissolved in chloroform, and the solution concentrated until it became viscous. Light petroleum was added in small quantities with shaking until about 20 g. of the methylated mannan had separated (fraction A). Subsequent fractions were obtained by the further addition of petroleum. Each fraction was purified by reprecipitation from chloroform solution. Some properties of the fractions are given in Table I. The osmotic pressure and viscosity measurements in chloroform solution were carried out by Dr. W. T. Chambers in this laboratory.

TABLE I.

Fraction.	Wt., g.	Ash, %.	OMe, % (ash corr.).	$[\alpha]_D^{17°}$ in $CHCl_3$.	$\eta_{sp.}/c.$ (c = g./100 c.c.).	M from osmotic pressure.	M (hexose units).
A	23·5	0·51	43·5	+88·1°	0·212	76,000	380
B	20·5	0·38	44·1	+88·3	0·211	76,000	380
C	24·0	0·32	44·3	+89·1	0·195	72,000	360
D	23·0	0·35	44·3	+87·9	0·175	59,000	295
E	8·5	0·22	44·4	+88·7	0·121	35,000	175
F	0·5	0·33	41·8	+88·4	0·062	18,000	90

Hydrolysis of Methylated Mannan.—Methylated mannan, fraction A (21·3 g.) was dissolved in glacial acetic acid (213 c.c.), and 5% hydrochloric acid (213 c.c.) added. The solution was heated on a boiling water-bath until its rotation was constant (α_D + 2·04° ⟶ + 0·40° in 12 hours). The hydrochloric acid was neutralised (to Congo-red) with barium carbonate (30 g.) and the acetic acid solution was taken to dryness at 45—50°. The residue was exhaustively extracted with boiling chloroform, the combined extracts dried over anhydrous magnesium sulphate, and the chloroform evaporated. The hydrolysate (21·4 g.) was converted into the methyl glycosides by being boiled with 2% methyl-alcoholic hydrogen chloride (200 c.c.) until the rotation was constant (12 hours). The solution was neutralised with silver carbonate, and the product isolated in the usual way. The yield of the mixture of glycosides was 23·3 g.

Fractions B, C, D and E of the methylated mannan were combined (71·6 g.) and submitted to the same hydrolytic procedure. The total yield from all the fractions except the last (*i.e.*, from 92·9 g. of methylated mannan) was 97·5 g. of the mixture of glycosides.

Fractional Distillation of the Glycoside Mixture.—The distillation was carried out in a high vacuum from a Widmer flask with a vacuum-jacketed column. The undistillable residue was rehydrolysed, and the product distilled. Ultimately the fractionation shown in Table II was achieved. Fraction 1 contained 0·92 g. of methyl lævulate.

TABLE II.

Fraction.	Wt., g.	OMe, %.	$[\alpha]_D^{17°}$ in $CHCl_3$.	$n_D^{20°}$.	Fraction.	Wt., g.	OMe, %.	$[\alpha]_D^{17°}$ in $CHCl_3$.	$n_D^{20°}$.
1	1·02	—	—	1·4258	9	3·32	48·0	+60·4°	1·4602
2	13·72	58·9	+50·9°	1·4465	10	10·42	45·4	+72·3	1·4660
3	9·59	58·9	+50·9	1·4472	11	12·56	39·2	+88·6	1·4700
4	6·65	59·6	+46·2	1·4480	12	4·76	39·1	+87·8	1·4708
5	10·51	54·2	+45·8	1·4540	13	1·49	45·1	+52·7	1·4735
6	2·64	51·4	+55·0	1·4572	14	0·61	51·6	+57·5	1·4540
7	12·18	51·5	+60·0	1·4572	15	0·64	44·7	+78·3	1·4690
8	4·64	51·5	+59·7	1·4580	Residue	0·11	—	—	—

It was possible to calculate on the basis of refractive index and methoxyl content the proportions, in the distilled glycosides, of tetramethyl methylmannoside ($n_D^{20°}$, 1·4475; OMe, 62·0%), trimethyl methylmannoside ($n_D^{20°}$, 1·4575; OMe, 52·6%), and dimethyl methylmannoside ($n_D^{20°}$, 1·4705; OMe, 41·9%) as follows:

TABLE III.

Fraction.	Wt., g.	"Tetra," g.	"Tri," g.	"Di," g.	Fraction.	Wt., g.	"Tetra," g.	"Tri," g.	"Di," g.
1	1·02	0·10	—	—	9	3·32	—	2·63	0·69
2	13·72	13·72	—	—	10	10·42	—	3·62	6·80
3	9·59	9·59	—	—	11	12·56	—	—	12·56
4	6·65	6·65	—	—	12	4·76	—	—	4·76
5	10·51	3·88	6·63	—	13	1·49	—	—	1·49
6	2·64	—	2·64	—	14	0·61	0·23	0·38	—
7	12·18	—	12·18	—	15	0·64	—	0·08	0·56
8	4·64	—	4·37	0·17	Totals	94·75	34·17	32·53	27·03

When allowance is made for the weights removed for refractive index determination, the weight ratio is tetra : tri : di = 34·44 : 33·26 : 27·57, which corresponds to a molecular ratio of 1·00 : 1·02 : 0·90.

Examination of the Tetramethyl Methylmannoside Fractions.—Fraction 1 (Table II) contained methyl lævulate, which was removed by heating with barium hydroxide solution at 90° for 2 hours. It was thus shown that 90% of the fraction (1·02 g.) consisted of this ester.

Fractions 2 and 3 crystallised almost completely. Each showed $[\alpha]_D$ + 51° in chloroform and therefore consisted of 95% of tetramethyl α-methylmannopyranoside ($[\alpha]_D$ + 57°). The β-form has $[\alpha]_D$ − 87° (Haworth, Raistrick, and Stacey, *Biochem. J.*, 1935, **29**, 612)..

Fraction 2 (13·25 g.) was recrystallised from light petroleum, and 12·03 g. (92%) of crystalline tetramethyl α-methylmannopyranoside obtained showing m. p. 38—40° and $n_D^{15°}$ 1·4480 (superfused crystals). The crystalline fraction 2 (8·0 g.) was hydrolysed with hot 2N-sulphuric acid ($[\alpha]_D$ + 43° ⟶ + 4° in 5½ hours) and the syrupy product was boiled with aniline (1 mol.) in alcohol. Tetramethyl mannopyranose anilide (m. p. 144—145°) was obtained in quantitative yield. The anilide underwent mutoratation in dry methyl alcohol: $[\alpha]_D^{15°}$ − 84·0° ⟶ − 7·5° in 11 hours (Found: C, 61·8; H, 7·9; N, 4·9; OMe, 39·6. Calc. for $C_{16}H_{25}O_5N$: C, 61·7; H, 8·0; N, 4·5; OMe, 39·9%).

Examination of the Trimethyl Methylmannoside Fractions.—Fractions 6, 7, and 8 did not crystallise.

Methylation of fraction 7. A portion of fraction 7 (0·5 g.) was methylated three times with methyl iodide and silver oxide; the product, after crystallisation from light petroleum, showed m. p. 39—40° and $[\alpha]_D^{22°}$ + 60·0° (*c*, 1·0 in chloroform). In admixture with tetramethyl α-methylmannopyranoside it showed no depression of m. p. The yield was 91% and it is concluded that fraction 7 consists entirely of trimethyl methylmannoside.

Hydrolysis of fraction 7. (*a*) A small-scale hydrolysis was carried out with weak acid with a view to the detection of any methyl furanoside. Fraction 7 (0·25 g.) was dissolved in N/20-sulphuric acid (30 c.c.), and the solution kept at room temperature for 16 hours. The rotation remained constant at $[\alpha]_D$ + 45·6°. Similarly no hydrolysis occurred when the solution was heated on a boiling water-bath for 4 hours, or when the acid strength was increased to N/10 under the same conditions. It is probable, therefore, that a furanose sugar is not present. When the acid strength was increased to N, hydrolysis occurred at 100° and was complete in 15 hours.

(*b*) The bulk of fraction 7 (11·25 g.) was hydrolysed by heating at 100° with 2N-sulphuric acid for 5 hours ($[\alpha]_D$ + 52·0° ⟶ + 10·4°). The acid was neutralised with barium carbonate, the solution evaporated, and the product extracted from the residue with ether. The yield of trimethyl sugar obtained under (*a*) and (*b*) was 10·71 g., *i.e.*, 99%. This trimethyl mannose will be referred to as substance (A).

When the syrup A was nucleated with 3 : 4 : 6-trimethyl mannose (m. p. 102°), crystallisation ensued. Subsequent recrystallisation indicated the presence of more than one substance and a laborious fractionation was carried out as follows:

TABLE IV.

Crop	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
Wt., g.	2·3	0·4	0·9	0·5	0·3	0·2	2·1	0·6	0·2	0·8	0·6	0·3
M. p.	102°	100°	78°	90°	90°	88°	88°	85°	82°	92°	78°	95°

The total weight of crystalline product was 9·2 g. (86%) and the non-crystalline residue, 1·87 g. The latter will be referred to as (B). Fraction A8 was visually heterogeneous and it was possible to separate from it, mechanically, two crystalline forms (*a*) and (*b*), which were present in equal quantities. The first crop (*a*) had m. p. 102° and was 3 : 4 : 6-trimethyl mannose. The second (*b*) melted at 90° and in admixture with (*a*), at 72°.

The identification of the trimethyl mannose (b). The substance (*b*) mutarotated in aqueous solution in the same direction ($[\alpha]_D^{18°}$ + 21° ⟶ + 14° in 2 hours) as 3 : 4 : 6-trimethyl α-mannose and could, therefore, not be the β-form of this sugar. Its analysis corresponded to its being a monohydrate (Found: C, 45·2, 45·3; H, 8·2, 8·3; OMe, 39·0, 39·2. $C_9H_{18}O_6,H_2O$ requires C, 45·0; H, 8·3; OMe, 38·75%). This was confirmed when the crystals (164 mg.) were heated at 100° in a vacuum to constant weight. The loss in weight was 6·9% ($C_9H_{18}O_6,H_2O$ requires H_2O, 7·5%). The dehydrated material was a syrup which on contact with any ordinary solvent (*e.g.*, acetone, ether) reverted to the crystalline hydrate, m. p. 90°. All attempts to prepare a hydrate from authentic 3 : 4 : 6-trimethyl mannose were without success and it was concluded that (*b*) was not a hydrate of 3 : 4 : 6-trimethyl mannose. A similar lack of success attended attempts to prepare a hydrate of 2 : 3 : 4-trimethyl mannose (itself a syrup).

For reasons given later, substance (*b*) was recognised as 2 : 4 : 6-*trimethyl mannose monohydrate* and the crystalline substance (A) is a mixture of this hydrate with 3 : 4 : 6-trimethyl mannose.

The separation of (A) into its constituents was achieved by its condensation with acetone. The crystalline crops A3, 7, 9, 10 and 11 were combined (3·87 g.) and dehydrated at 100°. The loss in weight corresponded to the presence of 65% of the monohydrate. The dehydrated product (a syrup, 3·68 g.) was dissolved in dry acetone (200 c.c.) to which was added concentrated sulphuric acid (1·5 c.c.), and the solution kept at room temperature for 24 hours. After neutralisation with anhydrous sodium carbonate, the solution was evaporated at 40—50° in the presence of a little barium carbonate. The residue was extracted with warm ether; the extract on cooling deposited large crystals (1·30 g.) of 2 : 4 : 6-trimethyl mannose monohydrate (m. p. 89—90°).

The ethereal mother-liquor was evaporated, and the residue extracted several times with small volumes of warm light petroleum. The extract, which contained 3 : 4 : 6-trimethyl 1 : 2-monoacetone mannose, was evaporated. The residue insoluble in petroleum was submitted to a second treatment with acetone and sulphuric acid and further quantities of 2 : 4 : 6-trimethyl mannose monohydrate and 3 : 4 : 6-trimethyl 1 : 2-monoacetone mannose were obtained. The total weight of the hydrate separated was 1·8 g., corresponding to 70% of the hydrate in the mixture (Found: C, 45·1; H, 8·3; OMe, 39·7. Calc. for $C_9H_{18}O_6,H_2O$: C, 45·0; H, 8·3; OMe, 38·75%).

It was shown that the material soluble in light petroleum was the acetone derivative of 3 : 4 : 6-trimethyl mannose inasmuch as its hydrolysis with hot N-sulphuric acid yielded crystalline 3 : 4 : 6-trimethyl α-mannose (m. p. and mixed m. p. 102°) in 80% yield.

The two trimethyl mannoses mutarotated in the same direction, thus: 3 : 4 : 6-Trimethyl α-mannose: $[\alpha]_D^{16°}$ + 27·5° ⟶ 10°, equilibrium value in water (*c*, 1·0). 2 : 4 : 6-Trimethyl α-mannose monohydrate: $[\alpha]_D^{18°}$ + 23° ⟶ + 16°, equilibrium value in water (*c*, 1·0), calculated on basis of anhydrous material.

Fractions A4, A5, and A6 were combined (1·0 g.) and heated to constant weight at 100°. The loss in weight was 7·5% and it was therefore concluded that this material contained no 3 : 4 : 6-trimethyl mannose (Calc. for $C_9H_{18}O_6,H_2O$: H_2O,7·5%). It was fractionally crystallised from either and two crops of crystals, 1 and 2, were separated.

Crop 1 (0·72 g.) was 2 : 4 : 6-trimethyl α-mannose monohydrate, m. p. 89—90°.

Crop 2 (48 mg.), m. p. 104—107°, proved to be the anhydrous β-*form* of this sugar (Found: C, 48·3; H, 8·1; OMe, 42·6. $C_9H_{18}O_6$ requires C, 48·7; H, 8·1; OMe, 41·9%). It mutarotated upwards, $[\alpha]_D^{20°}$ − 5·7° ⟶ + 19·0°, equilibrium value in water (*c*, 2·1), and was converted into the hydrate of the α-form by solution in water containing a trace of hydrochloric acid (0·1%). The substance clearly is 2 : 4 : 6-trimethyl β-mannose.

The non-crystalline residue (*B*). This syrup (1·87 g.) contained OMe, 42·1% ($C_9H_{18}O_6$ requires 41·9%) and showed $[\alpha]_D^{18°}$ + 9·4° in water (*c*, 1·27) and + 26·5° in methyl alcohol (*c*, 1·66). A portion of the syrup (0·54 g.) was converted into the glycoside by being boiled with 2% methyl-alcoholic hydrogen chloride for 9 hours ($[\alpha]_D$ + 30° ⟶ + 83·5°). The glycoside (0·57 g.; 98% of the theoretical) was methylated by three treatments with methyl iodide and silver oxide. The product (0·56 g.; 92%) crystallised completely and on recrystallisation from light petroleum gave pure tetramethyl α-methylmannopyranoside (0·50 g., m. p. 38—40°, $[\alpha]_D^{19°}$ + 60° in chloroform) in 90% yield. Clearly (B) contains only mannose derivatives.

Oxidation of (*B*). The remainder of the syrup B (1·3 g.) was dissolved in water (12 c.c.) and treated with bromine (1·5 c.c.). After 2 days at room temperature, the solution, which was no longer reducing, was aerated and neutralised with silver carbonate. The silver salt in solution was decomposed with hydrogen sulphide, and the solution filtered and evaporated at 35—40°. The syrupy product was "lactonised" by being heated at 90° in a vacuum for ½ hour. Yield, 0·85 g. (65% of the theoretical). The syrup was dissolved in a little ether and, on being cooled, the solution deposited a small quantity of crystals. Two crystalline forms were present and it was possible to separate them mechanically into fractions 1 (80 mg.; plates) and 2 (120 mg.; fine needles). Fraction 1, after recrystallisation, had m. p. 97° alone and in admixture with authentic 3 : 4 : 6-trimethyl δ-mannonolactone (m. p. 97°). In admixture, however, with 2 : 4 : 6-trimethyl δ-mannonolactone (m. p. 97—98°), the m. p. was depressed to 74°. Fraction 2, after recrystallisation from ether, had m. p. 72—73° and showed no depression of m. p. in admixture with 2 : 3 : 4-trimethyl δ-mannonolactone (m. p. 73·5°; specimen synthesised by P. I. Wilson in this laboratory: cf. Haworth, Hirst, Isherwood, and J. K. N. Jones, *loc. cit.*).

Further quantities of the two lactones were obtained from the mother-liquors, which were ultimately evaporated to dryness to give a syrup (0·5 g.). This syrup was dissolved in methyl-alcoholic ammonia. After 2 days at 0°, the ammonia and methyl alcohol were removed in a

vacuum desiccator, and the residue crystallised. Several crops of crystals were obtained from solution in a number of solvents, but each crop consisted of a mixture of amides which could not be satisfactorily separated. A small specimen (12 mg.) was obtained with m. p. 130° and this was shown to consist almost entirely of 2 : 3 : 4-trimethyl mannonamide, since in admixture with an authentic specimen (m. p. 138°) it showed no depression of m. p. The amide gave a negative Weerman test for α-hydroxy-amides.

The Identification of 2 : 4 : 6-*Trimethyl Mannose.*—2 : 4 : 6-Trimethyl mannose anilide was prepared in 65% yield by boiling dehydrated 2 : 4 : 6-trimethyl mannose monohydrate (0·1 g.) with aniline (42 mg.) in dried alcohol (6 c.c.). The anilide crystallised from ether in fine needles, m. p. 134°. It displayed mutarotation in dry methyl-alcoholic solution : $[\alpha]_D^{20°}$ − 150° ⟶ + 8° in 13 hours. Prepared similarly, 3 : 4 : 6-*trimethyl mannose anilide* had m. p. 140—143° and $[\alpha]_D^{18°}$ + 154·5° ⟶ − 55·5° in methyl alcohol (24 hours) (Found : C, 60·6; H, 8·0; N, 4·7; OMe, 31·7. $C_{15}H_{23}O_5N$ requires C, 60·6; H, 7·8; N, 4·7; OMe, 31·3%). The m. p. of the mixture of the two anilides was depressed.

2 : 4 : 6-*Trimethyl δ-mannonolactone* was prepared from the monohydrate by oxidation with bromine water in the usual way. The product crystallised after distillation in a high vacuum (Found : OMe, 42·3. $C_9H_{16}O_6$ requires OMe, 42·3%). It melted at 97—98° and in admixture with 3 : 4 : 6-trimethyl δ-mannonolactone (m. p. 97°), the m. p. was depressed to 72°. Similarly, a mixture with 2 : 3 : 4-trimethyl δ-mannonolactone (m. p. 73°) showed a depressed m. p.

The rate of hydration in aqueous solution indicated that all three lactones were of the δ-series, thus :

2 : 4 : 6-Trimethyl *d*-mannono-δ-lactone, $[\alpha]_D^{20°}$ + 141° ⟶ + 30° in 103 hours.

3 : 4 : 6-Trimethyl *d*-mannono-δ-lactone, $[\alpha]_D^{20°}$ + 169° ⟶ + 113° in 71 hours (cf. Bott, Haworth, and Hirst, J., 1930, 1404).

2 : 3 : 4-Trimethyl *d*-mannono-δ-lactone, $[\alpha]_D^{18°}$ + 129·2° ⟶ + 78·4° in 72 hours (cf. Haworth, Hirst, Isherwood, and Jones, *loc. cit.*).

2 : 4 : 6-*Trimethyl* d-*mannonamide* was prepared in the usual way from the lactone. It had m. p. 145° and $[\alpha]_D^{20°}$ + 7·0° in water (*c*, 2·03) (Found : C, 45·7; H, 7·9. $C_9H_{19}O_6N$ requires C, 45·6; H, 8·0%). The amide gave a negative Weerman test for α-hydroxy-amides.

3 : 4 : 6-Trimethyl *d*-mannonamide was also prepared in quantitative yield from the corresponding lactone. After recrystallisation from acetone–light petroleum it showed m. p. 143° and $[\alpha]_D^{18°}$ + 28° in water (*c*, 1·0).

Haworth, Hirst, Isherwood, and Jones (*loc. cit.*) record for 2 : 3 : 4-trimethyl *d*-mannonamide, m. p. 143° and $[\alpha]_D^{20°}$ + 5° in water (*c*, 0·8).

2 : 4 : 6-*Trimethyl α-Methylmannoside.*—This was prepared by the treatment of 2 : 4 : 6-trimethyl mannose monohydrate (0·5 g.) with 2% methyl-alcoholic hydrogen chloride (50 c.c.). At room temperature, $[\alpha]_D^{20°}$ changed from + 32° to + 58° in 151 hours. Thereafter the solution was boiled until the rotation became constant at + 70° (4 hours). The product, which was a syrup (0·46 g.; 94% of the theoretical), was methylated by three treatments with methyl iodide and silver oxide, and crystalline tetramethyl α-methylmannopyranoside (m. p. 38—39°; $[\alpha]_D^{20°}$ + 62° in chloroform) obtained in 94% yield. It showed no depression of m. p. in admixture with an authentic specimen.

Methylation of 2 : 4 : 6-*Trimethyl Mannonolactone.*—The crystalline lactone (m. p. 97—98°; 0·35 g.) was dissolved in the minimum volume of dry acetone and methylated twice with methyl iodide (5 c.c.) and dry silver oxide (0·5 g.). The product, extracted with chloroform, was a syrup which contained silver tetramethyl mannonate, methyl tetramethyl mannonate, and small amounts of methyl pentamethyl mannonate. Accordingly, it was dissolved in water, and the silver salts decomposed with hydrogen sulphide. The esters were hydrolysed by treatment of the product with a slight excess of N/10-sodium hydroxide. Thereafter the solution was made just acid with N/10-sulphuric acid and finally neutralised with silver carbonate. The soluble silver salts were decomposed with hydrogen sulphide and the syrup obtained by evaporation was purified by solution in ether, filtration and the evaporation of the solution. The product (0·24 g.) was distilled in a high vacuum. The small first fraction was discarded; the main fraction (0·175 g.), boiling at bath temperature 140°/0·1 mm., had $n_D^{20°}$ 1·4620. In aqueous solution (*c*, 1·0) it showed $[\alpha]_D^{18°}$ + 120° ⟶ + 54° in 100 hours. This fraction was shown to consist mainly of tetramethyl *d*-mannono-δ-lactone inasmuch as its treatment in ethereal solution with phenylhydrazine gave, in 70% yield, 2 : 3 : 4 : 6-tetramethyl mannonic acid phenylhydrazide (m. p. and mixed m. p. 186—187°). Proof is thus afforded that the original trimethyl mannonolactone belongs to the δ-series.

Examination of the Dimethyl Methylmannoside Fractions.—After being kept for some weeks, fractions 11 and 12 (Table II) crystallised to hard masses, m. p. 80°.

Fraction 11 was shown to be composed entirely of 3 : 4-dimethyl α-methylmannoside. A portion of fraction 11 (0·48 g.) was methylated by four treatments with methyl iodide and silver oxide. The product (0·47 g.; 90% of the theoretical yield) crystallised completely. It was recrystallised from light petroleum and was recognised as tetramethyl α-methylmannopyranoside (m. p. 39—40°; $[\alpha]_D^{22°}$ + 59° in chloroform). The remainder of fraction 11 (11·86 g.) was now fractionally crystallised from acetone–ether. The main crops (10·30 g.) were identical (m. p. 87°; $[\alpha]_D^{18°}$ + 67° in water; + 107° in chloroform; $n_D^{15°}$ 1·4730 on superfused crystals). In addition, a non-crystallisable residue (R) (1·35 g.) was obtained.

The crystalline dimethyl α-methylmannoside (8·0 g.) was hydrolysed by heating at 100° with 2N-sulphuric acid (400 c.c.). The following changes in $[\alpha]_D$ were observed: + 65° ⟶ ± 0° (constant value) in 5 hours. The product of hydrolysis was a crystalline solid (7·19 g.; 95% of the theoretical yield). After recrystallisation from acetone–ether it showed m. p. 114° and $[\alpha]_D^{20°}$ + 30·0° in dry methyl alcohol (*c*, 1·0) (Found: C, 42·7; H, 8·1; OMe, 27·2; H_2O of hydration, 8·3. Calc. for $C_8H_{16}O_6,H_2O$: C, 42·5; H, 7·9; OMe, 27·4; H_2O of hydration, 8·0%). It was identified as 3 : 4-dimethyl α-mannose monohydrate (cf. Haworth, Hirst, and Isherwood, *loc. cit.*). In aqueous solution the monohydrate displayed mutarotation: $[\alpha]_D^{18°}$ + 22° (initial value); + 14° (10 mins.); + 10° (20 mins.); + 6° (40 mins.); + 4° (60 mins.); + 4° (80 mins.).

The syrup residue (R), which showed $[\alpha]_D^{18°}$ + 35° in chloroform, was shown to be 3 : 4-dimethyl αβ-methylmannoside inasmuch as it gave, on hydrolysis with 2N-sulphuric acid, crystalline 3 : 4-dimethyl mannose monohydrate (m. p. 112—113°) in 90% yield.

3 : 4-*Dimethyl* δ-*Mannonolactone.*—The conclusions of Haworth, Hirst, and Isherwood (*loc. cit.*) on the constitution of the dimethyl mannose are confirmed. Oxidation of the dimethyl mannose monohydrate (1·5 g.) with bromine water yielded the crystalline lactone, m. p. 159—160°; $[\alpha]_D^{15°}$ in water, + 178° ⟶ + 131° (constant) in 120 hours (Found: C, 46·6; H, 6·6; OMe, 30·1. Calc. for $C_8H_{14}O_6$: C, 46·6; H, 6·8; OMe, 30·1%). Treatment of the lactone with methyl-alcoholic ammonia gave 3 : 4-dimethyl mannonamide (m. p. 141°; $[\alpha]_D^{19°}$ + 25·7° in water) (Found: C, 43·4; H, 7·8; N, 6·4; OMe, 27·6. Calc. for $C_8H_{17}O_6N$: C, 43·1; H, 7·6; N, 6·3; OMe, 27·7%). The amide gave a strongly positive Weerman test for an α-hydroxyamide.

Conversion of 3 : 4-*Dimethyl Mannose into* 3 : 4 : 6-*Trimethyl Mannose.*—3 : 4-Dimethyl mannose monohydrate (3·0 g.) was dissolved in acetone (120 c.c.) to which concentrated sulphuric acid (0·9 c.c.) was added. The reaction at room temperature was complete in 24 hours and thereafter the solution was neutralised with sodium carbonate, filtered, and evaporated at 35° in the presence of a little barium carbonate. The residue was exhaustively extracted with acetone, and a syrup recovered from the extract. The syrup was then repeatedly extracted with ether, in which the greater part dissolved. The small ether-insoluble residue was shown to be unchanged 3 : 4-dimethyl mannose. The ethereal extract was evaporated, and the residue extracted with warm light petroleum. On being concentrated, the extracts deposited 3 : 4-*dimethyl mannose* 1 : 2-*monoacetone* in large plates (0·40 g., *i.e.*, 18% yield). The compound had m. p. 94° and $[\alpha]_D^{16°}$ − 17° in water (*c*, 1·0; no mutarotation). It was non-reducing to Fehling's solution (Found: C, 53·3; H, 8·1; OMe, 24·8. $C_{11}H_{20}O_6$ requires C, 53·2; H, 8·1; OMe, 25·0%). It was hydrolysed in the cold by N/100-sulphuric acid ($[\alpha]_D^{20°}$ − 17° ⟶ + 3° in 380 hours).

The monoacetone derivative (0·1 g.) was now methylated by two treatments with methyl iodide and silver oxide and yielded a mobile syrup (Found: OMe, 35·3. $C_{12}H_{22}O_6$ requires OMe, 35·5%). The syrup (0·1 g.) was hydrolysed by being treated at 100° with N-sulphuric acid (10 c.c.): $[\alpha]_D^{16°}$ + 10° ⟶ + 4° in 2 hours. The product was 3 : 4 : 6-trimethyl mannose (m. p. and mixed m. p. 102—103°) and was obtained in quantitative yield.

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