313. *Structure of the Dextran Synthesised from Sucrose by a New Strain of* Betacoccus arabinosaceous.

By M. Stacey and G. Swift.

A water-soluble gum-like dextran has been synthesised from sucrose by a new strain of *Betacoccus arabinosaceous* (*Leuconostoc mesenteroides*). The hydrolysis products of the methylated dextran were separated chromatographically and shown to consist of 2 : 3 : 4 : 6-tetramethyl glucose (1 part), 2 : 3 : 4-trimethyl glucose (3 parts), and 2 : 3-dimethyl glucose (1 part). The structure of the repeating unit is thus identical with that described by previous investigators for the dextran from another strain of *L. mesenteroides*. The molecule in these dextrans shows an unusually high degree of branching.

Work carried out in Sweden (Grönwall and Ingelman, *Acta Physiol. Scand.*, 1944, **7**, 97; 1945, 9; Bohmansson, Rosenquist, Thorsen, and Wilander, *Acta Chir. Scand.*, 1946, **94**, 148) and in this country (A. R. Lockwood and his colleagues, unpublished) has revealed that products from the degradation of dextran may find an important use as substitutes for plasma in blood transfusion and in shock treatment. In a study of numerous dextran types we have isolated an organism, a strain of *Betacoccus arabinosaceous* (*Leuconostoc mesenteroides*) (see Experimental section) which has the power of synthesising a dextran at an unusual rate and in relatively large quantities.

The dextran has some physical properties which distinguish it from other dextrans which we have examined over a number of years in these laboratories. These dextrans, notably those produced by various strains of *L. mesenteroides* and *L. dextranicum*, are all retrograded to a water-insoluble form on dehydration or on being kept for long periods. The dextran described herein is unusual and of interest inasmuch as it forms colloidal solutions of high viscosity and it cannot be converted into a water-insoluble form. In its physical properties it closely resembles some of the plant gums. We have deemed it of interest to examine its structure by the usual methylation technique with application of chromatographic separation in order to relate it to other dextrans.

The problem of the constitution of various dextrans has engaged the attention of numerous workers during the past decade. So far it had been established that all these polyglucoses contain a high proportion of repeating units having α-D-glucopyranose units mainly linked through the 1 and 6 positions. In some dextrans, cross linkages of undetermined location also occur.

An investigation of the structure of the dextran synthesised by a strain of *L. mesenteroides* from sucrose was described by Fowler, Buckland, Brauns, and Hibbert (*Canadian J. Res.*, 1937, **15**, 486) who prepared a methylated dextran and hydrolysed it by treatment with methanolic hydrogen chloride. From the fractional distillation of the resulting glucosides they claimed that the dimethyl, trimethyl, and tetramethyl methylglucosides were present in the approximate ratio of 1 : 3 : 1, and these were identified as 2 : 3-di-, 2 : 3 : 4-tri-, and 2 : 3 : 4 : 6-tetra-methyl methylglucoside respectively. A branched chain structure for the dextran based on these results was proposed. These results were subsequently criticised by Brauns (*ibid.*, 1938, **16**, 73) on the following grounds: (*a*) the dextran was incompletely methylated; (*b*) the ratio of tetra- to tri- to di-methyl methylgucosides of 1 : 3 : 1 was not conclusive because of the inefficient fractional distillation employed; and (*c*) a large percentage (18·4%) of material was lost during fractionation. A reinvestigation of this dextran was made by Levi, Hawkins, and Hibbert (*J. Amer. Chem. Soc.*, 1942, **64**, 1959) in order to establish more definitely its structure. In this attempt to reaffirm the claims in the earlier paper these authors pointed out that in complex polysaccharides such as mannan, glycogen, and araban, which contain intricately branched chains, every branching position must yield a dimethyl methylglycoside in the case of hexosans and a monomethyl methylglycoside in the case of pentosans. Consequently, in methylation studies of such polysaccharides a final methoxyl value of 1—2% lower than the theoretical can render the results worthless with respect to the extent of branching and so preclude an accurate structural assignment. These authors treated dextran with methyl sulphate and alkali, and after three such treatments obtained a partly methylated dextran (OMe, 40—41%) which was further methylated by a modified Muskat technique (*ibid.*,1934, **56**, 693) which consisted of suspending the dextran in anhydrous anisole and treating the resulting suspension with liquid ammonia and sodium. The methylation was then carried out by the addition of methyl iodide to this mixture. After six such treatments the dextran had the theoretical methoxyl content of 45·6% as calculated for $C_6H_7O_2(OMe)_3$. The methylated dextran was hydrolysed with

methanolic hydrogen chloride in sealed glass bombs, and the resulting glucoside mixture was fractionated. By this procedure the ratio of 2 : 3 : 4 : 6-tetra- to 2 : 3 : 4-tri- to 2 : 3-di-methyl methylglucoside was again established as being exactly 1 : 3 : 1, thus verifying their earlier results.

On the above evidence, the authors suggested for dextran two tentative branched chain formulæ (cf. Figs. I and II), differing only in the position of attachment of the side-chain. These, however, do not exhaust all the possibilities, and it is conceivable that the side chain may consist of one, two, or three units with a corresponding lengthening of the primary chains. Three of the linkages between the building units are of the 1 : 6 type while the remaining two are either 1 : 4 or 1 : 6. The only evidence available as to whether the linkages are α- or β- is the change in rotation towards the negative side on hydrolysis of the methylated dextran, *e.g.*, $[\alpha]_D + 200° \longrightarrow + 70°$. The importance of these investigations lies in the fact that they indicated that the dextran synthesised by *L. mesenteroides* from sucrose is a highly-branched structure differing from the linear type of structure of the dextran produced from *L. dextranicum* proposed by Peat, Stacey, and Schlüchterer (*J.*, 1939, 581).

Some claims for the confirmation of these results have been obtained by the study of the *L. mesenteroides* dextran molecule with the electron microscope by Ingleman and Seigbahn (*Nature*, 1943, **152**, 154), who claimed that their electron-micrographs and ultra-centrifuge measurements indicated that the molecule possessed an extremely high molecular weight.

Peat, Schlüchterer, and Stacey (*loc. cit.*) and Stacey and Youd (*Biochem. J.*, 1938, **32**, 1943) investigated the dextran synthesised by *L. dextranicum* from sucrose. These authors obtained a methylated dextran, and from its methanolysis products succeeded in isolating a small percentage (0·23%) of an " end group " (identified by conversion into 2 : 3 : 4 : 6-tetramethyl glucopyranose anilide) which indicated a chain length of not more than 550 glucose units, while osmotic-pressure measurements on the methyl ether indicated a chain length of not less than 200 glucose units. The remaining distillation product consisted of approximately 10% of dimethyl methylhexosides. Efforts were made to identify this fraction; proof was obtained that it was not homogeneous and there was some evidence obtained later that it consisted mainly of 2 : 3-dimethyl methylglucoside and an unidentified dimethyl methylglucoside (E. Schlüchterer, 1940, Ph.D. Thesis, University of Birmingham). This investigation was of particular interest in that it indicated that the structure of the dextran synthesised by *L. dextranicum* was essentially of the straight-chain type, though some as yet undetermined branching does occur.

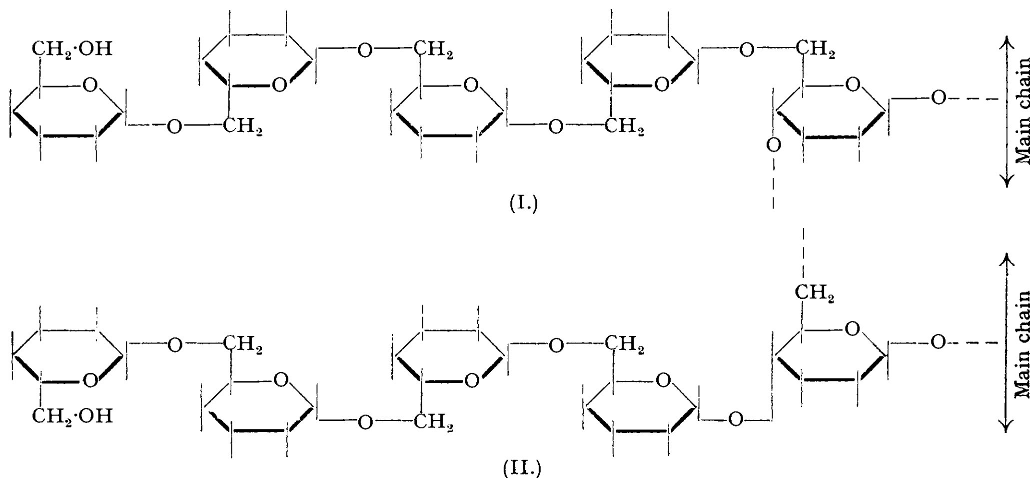
The contemporaneous investigations of Hibbert and his co-workers (*Canadian J. Res.*, 1938, **16**, 151) provided verification of these findings in the *L. dextranicum* dextran. Another interesting dextran is that produced from sucrose by *Betabacterium vermiformê* Ward–Mayer, the production and properties of which were carefully investigated by Mayer (Mayer, " Das Tibi Consortium " Delft, 1938). It was shown (Daker and Stacey, *J.*, 1939, **132**, 585) that this particular dextran consisted of α-glucopyranose units united by 1 : 6-glucosidic linkages in chains which consisted of about 25 glucose units, and the authors suggested that these chains are united to form larger molecular aggregates in possibly the same way as the basal chain units of starch (*i.e.*, amylopectin) are combined.

The new dextran described herein was produced from a sucrose substrate, and it dissolved in water to give clear viscous solutions. It remained gum-like and water-soluble even after being intensively dehydrated over long periods. It was purified by repeated precipitation from aqueous solution with alcohol, and was finally isolated without treatment with acid or alkali as a water-soluble white powder. It was methylated in two 20 g. portions, using methyl sulphate and 30% sodium hydroxide solution, by essentially the method of Peat, Schlüchterer, and Stacey (*loc. cit.*). After two methylations the partly-methylated dextran was purified by precipitation of a chloroform solution with ligroin and was isolated in the form of a pale yellow powder. This product was then thoroughly dried in a vacuum for several days and was then re-methylated by forming the sodio-derivative in liquid ammonia, and treating this with methyl iodide. After 6 additions of sodium with the corresponding quantities of methyl iodide, the product was a white powder.

The fully-methylated dextran was hydrolysed by being heated on a boiling water-bath for 7 hours with glacial glacial acetic acid and 5% aqueous hydrochloric acid (3 : 2 by volume). The acids were neutralised and the solution freed from chloride ions by the addition of lead acetate, the lead then being removed with hydrogen sulphide.

The sugars were separated by essentially the method of Bell (*J.*, 1944, 473; cf. Gilbert, Smith, and Stacey, *J.*, 1946, 622) by use of a partition between organic solvents and water in a column of silica gel.

There were isolated and identified 2 : 3-di-, 2 : 3 : 4-tri-, and 2 : 3 : 4 : 6-tetra-methyl glucose in the proportions of 1 : 3 : 1 (see Experimental section), and thus in this respect the constitution of the repeating unit of the methylated dextran was identical with that described by Hibbert and his co-workers (*locc. cit.*) for the dextran produced by the action of their strain of *L. mesenteroides* on sucrose. The type of structure could be represented by (I) or (II) which are essentially the branched chain formulations of Hibbert and his co-workers (1942, *loc. cit.*).



It appeared, therefore, that the new organism was *L. mesenteroides*, but it was evidently a different strain, because the dextran it produced had physical properties different from those of the dextran from known strains of *L. mesenteroides*.

It is possible that a repeating unit of the type shown in (I) or (II) may be identical with that of the dextrans produced by various strains of *L. mesenteroides*, although it is likely that the branching occurs at different places. Indeed, such small differences as are shown between (I) and (II) and other variations of the type of structure may account for the physical differences between the polysaccharides formed by the various strains. It is likely also that the number of repeating units in each macromolecule is different. It is an interesting finding that even different strains of an organism give dextrans having different physical properties, and it appears that one may eventually be able to identify an organism and its various strains by determining the finer structure of its extracellular polysaccharides.

Experimental.

Characterisation of the Micro-organism (with Mr. A. R. Lockwood).—The organism was originally isolated from an aqueous solution of cane sugar which had become " ropy ". The crude ropy sugar contained yeasts, together with a very small coccus and a larger coccus. The larger coccus was obtained in pure culture, gave good dextran production, and was designated *Betacoccus arabinosaceous* Birmingham.

Morphologically, the organism more closely resembles *Betacoccus arabinosaceous* Orla–Jensen in so far as cultural pleomophism is much less marked than that exhibited by *L. mesenteroides* strains. Elongation of cocci into rod forms in old cultures is much less frequent than with *L. mesenteroides*, and streptococcal chains are encountered continuously even in very vigorously agitated cultures.

Growth on sucrose nutrient agar is characteristically raised and " mucoid " at sucrose concentrations in the region of 10%, but thin-spreading films only are produced on media having lower sucrose concentrations, *i.e.*, 1—3%, under the same cultural conditions. *L. mesenteroides* and *L. dextranicum* produce good raised slimy colonies on these media containing low sucrose contents, and continued sub-culture of this new organism on such media fails to produce the pronounced raised slime. This "osmophilic" character of the strain is further encountered in liquid media containing low sucrose concentrations where growth is slow and sparse with poor dextran production.

Attempts to classify the organism by secondary physiological characters as described by Hucker and Pederson have not been wholly satisfactory, and these investigations are proceeding. Pentoses appear to be weakly fermented, a character in which the organism resembles *L. mesenteroides*, although the good vigorous growth obtained in sucrose media has not been obtained with any other carbohydrate source.

The growth-factor requirements of the organism appear to be identical with those of *L. mesenteroides* and *L. dextranicum*, and, under the most favourable conditions of culture so far developed for these organism as a class, the rate of production of acetic and lactic acids, carbon dioxide, and traces of ethanol exhibited by this new strain is indistinguishable from that of *L. mesenteroides* and other related strains.

Production of Dextran.—The culture medium contained the following : microcosmic salt (4·9 g.),

potassium hydrogen phosphate (1·0 g.), potassium chloride (0·5 g.), *p*-aminobenzoic acid (0·5 g.), hydrated magnesium sulphate (0·5 g.), hydrated ferrous sulphate (0·01 g.), peptone (0·01 g.), sucrose (100 g.), and distilled water (1000 c.c.). The liquid was poured in 200 c.c. quantities into penicillin culture jars and sterilised by being steamed for ½ hour on each of 3 successive days. When cold it was inoculated with a viscous suspension (2 c.c. in each jar) of the *Betacoccus* growing in the same medium. The flasks were incubated at 25°. After 24 hours the medium became opalescent and there was a gradual increase in viscosity which reached a maximum in 5—7 days, at the end of which time the medium lost its opalescence.

By gradual addition of ethanol (1 vol.), the dextran was precipitated as a rubber-like mass which gradually lost its elasticity on being dehydrated. It was fractionated by repeated precipitation by ethanol and acetone from water at different pH values until it was obtained essentially ash-free. Traces of extraneous protein were then removed by chloroform–butanol extraction. The main bulk of dextran (35 g.) was obtained as a reasonably homogeneous white granular powder having $[\alpha]_D^{18°}$ + 180° (*c*, 0·57 in water) (Found: N, 0·02; water, 8·2; ash, 1·9%). For larger scale production the dextran could readily be produced in 6 l. amounts in 10 l. jars.

Despite many attempts at denaturation by dehydration using solvents, vacuum drying, etc., the dextran always remained water-soluble, redissolving to give solutions the high viscosity of which was almost unaffected by addition of electrolytes. Addition of Fehling's solution to the dextran solution rapidly precipitated the dextran as a gelatinous mass.

Hydrolysis of the Dextran.—The dextran (5·0 g.) was hydrolysed by N/10-sulphuric acid at 98°, and the following polarimetric changes were observed: $[\alpha]_D^{20°}$ + 180° (0 hours); + 156° (1¼ hours); + 147° (2½ hours); + 142° (3¼ hours); + 80° (10 hours); + 52° (20 hours, equilibrium value). The sulphuric acid was neutralised with barium carbonate and the filtered solution evaporated in a vacuum to a syrup which crystallised on being kept and was identified as glucose (5·0 g.). Examination of the mother liquor on a paper chromatogram showed that only glucose was present in the hydrolysate.

A comparison was made of some properties of a number of dextrans produced under the same conditions by different organisms which had been kept for a number of years. The dextrans received the same treatment for their purification and the differences are noted in the Table.

Organism.	$[\alpha]_D^{20°}$.	Found, %. Ash.	N.	H_2O.	Behaviour on dehydration.
Mixed culture from beet sugar "slime"	+147° (in dil. NaOH)	8·3	3·5	8·2	Rapidly becomes completely insoluble in water
L. dextranicum (originally obtained from Prof. A. J. Kluyver)	+180° (in dil. NaOH)	6·2	1·8	8·0	Gradually becomes completely insoluble in water
L. mesenteroides (originally obtained from Prof. A. J. Kluyver)	+180° (in dil. NaOH)	3·1	1·2	7·5	Gradually becomes completely insoluble in water
L. citrovorum (originally obtained from Lister Institute)	+179° (in dil. NaOH)	1·5	0·9	6·2	Swells in water but does not dissolve
Betacoccus vermiforme Ward–Mayer (originally obtained from Prof. A. J. Kluyver)	+178° (degraded material in water)	1·5	0·5	5·8	Rapidly becomes completely insoluble in water
Betacoccus arabinosaceous Birmingham	+180° (in water)	1·9	0·98	8·2	Remains completely soluble giving viscous solutions

It will be seen that the only unusual property of the new dextran is its solubility in water, but this difference is quite striking for it relates it more closely to polysaccharides of the gum or mucilage type.

Methylation of Dextran.—The dextran (20 g.) was dissolved in 30% sodium hydroxide (100 c.c.) and treated at room temperature with 30% sodium hydroxide (250 c.c.) and methyl sulphate (180 c.c.) over a period of 2 hours during which 1/10th of the reagents were added drop by drop every 10 minutes. Acetone in portions of 100 c.c. was added at intervals. The reaction was carried out in an atmosphere of nitrogen in a 5-l. bolthead fitted with a mercury seal. After the addition of the reagents the reaction was allowed to proceed for 1 hour at room temperature. Finally, the temperature was slowly raised to 100° in order to destroy sodium methyl sulphate. The solution was then neutralised (hot) with 5N-sulphuric acid; a white flocculent precipitate of partly methylated dextran then came out of solution. This was filtered off and found to be insoluble in all common organic solvents. However, it swelled in dioxan, and this property was used to facilitate further methylation. A further sample (10 g.) was methylated once by this method, and the two batches of partly-methylated dextran were re-methylated by a similar procedure until a chloroform-soluble product was obtained on neutralisation. This property of being soluble in chloroform enabled it to be purified, for it could be precipitated from the solution by ligroin. The precipitate had OMe, 25·2%. The mother liquors from the three methylations were dialysed until free from sodium sulphate and were then evaporated to dryness. Total weight of product, 28·3 g.

Methylation by Liquid Ammonia–Methyl Iodide.—The apparatus and methods were similar to those frequently used for starch methylation, except that, in order to avoid losses due to the sparing solubility of the partly methylated dextran in liquid ammonia, the ammonia was allowed to evaporate instead of the usual syphoning method being used. As the modified method did not suffice to remove any sodium iodide, the product was purified after 4 additions of sodium. Partly methylated dextran (4 g.; OMe, 25%) so obtained was dried in a vacuum for several hours and was then suspended in liquid ammonia (200 c.c.) in a bath at − 80° (solid carbon dioxide–alcohol). Sodium was added and the mixture was then stirred for 6 hours in order to dissolve the metal; methyl iodide was then added dropwise, and the reaction allowed to proceed for ½ hour after which more sodium was added. At the end of this time the ammonia was allowed to evaporate overnight at room temperature, the last traces being removed by placing the tube in a boiling water-bath. The solid was then boiled with water to remove sodium

iodide, filtered, washed several more times with boiling water, and dried (3·8 g.; OMe, 39·4%). The product was suspended in 200 c.c. of liquid ammonia and re-methylated using sodium (2 g.) and methyl iodide (6·5 c.c.), added in 2 portions.

The product was purified as before, but in addition a further purification was carried out by precipitation of a chloroform solution with ligroin. Yield, 3·5 g.; $[\alpha]_D^{20°}$ + 212° (*c*, 1·1 in chloroform) (Found, on three different samples: OMe, 45·5, 45·9, 45·5%). Fractionation by means of solvents did not separate the product into fractions having different methoxyl contents, optical rotation, or viscosity and the product was considered to be essentially homogeneous.

Hydrolysis of the Methylated Dextran.—The methylated dextran (5·0 g.) was dissolved in glacial acetic acid (60 c.c.), and 5% hydrochloric acid (40 c.c.) was added. The solution was then heated on a boiling water-bath for 7 hours. After cooling, a slight excess of lead acetate was added and the precipitate of lead chloride filtered off and washed with a little ice-cold water. Hydrogen sulphide was then passed through the filtrate and washings, and the filtered colourless solution, after being evaporated to small volume with frequent additions of water and freed from chloride ions with silver carbonate, was evaporated to dryness under reduced pressure. Yield, 4·5 g.

Chromatographic Separation of the Methylated Sugars (Gilbert, Smith, and Stacey, *J.*, 1946, 622).—The following precautions were observed: (*a*) the apparatus was freed from grease and all taps were lubricated with graphite; (*b*) organic solvents were distilled from an all-glass apparatus, and the chloroform was thoroughly washed with water before distillation and stored in a large separating funnel under a layer of water.

Silica. This was prepared according to the method of Gordon, Martin, and Synge (*loc. cit.*), but the addition of an indicator to the silica was omitted.

Silica–water column. The dry special silica was ground in a mortar, and ½ its weight of water was stirred into the powder with further grinding to ensure efficient mixing. A glass tube of suitable dimensions (see later) was loosely plugged at one end by cotton-wool resting on a removable, perforated, porcelain dish. The moist silica was made into a paste with chloroform and poured into the tube. The gel rapidly packed into a column on top of the plug. The supernatant chloroform was allowed to drain through the gel, and the column was then freed from traces of grease by running through successively two column-lengths of chloroform. The apparatus was then ready for use.

Separation and identification of sugars. (I) The sugar mixture (4·49 g.) was dissolved in water (40 c.c.), the solution filtered into a graduated separating funnel, and the final concentration brought to approximately 5% by water-washings. The solution was shaken 9 times with its own volume of chloroform, and the latter evaporated, without dehydration, at ordinary pressure. This extract contained all the tetramethyl glucose and, in addition, about 10% of the trimethyl glucose.

(II) The sugars extracted in (I) were dissolved in chloroform and quantitatively transferred by pipette to a column prepared from 25 g. of silica in a tube of 40 mm. diameter. One chloroform-length was then passed through the column, and the eluate taken down to dryness. Weight of sugars isolated, 1·075 g. (fraction *A*).

(III) The aqueous phase from (I) was evaporated to dryness. Weight of remaining sugars, 3·235 g.

(IV) The sugars isolated in (III) were dissolved in acetone and transferred quantitatively by pipette to the column. One column-length of chloroform–butanol (9 : 1) was passed through the column, and the eluate evaporated, without dehydration, at ordinary pressures. A syrup (1·595 g.) remained (fraction *B*). The remaining sugars were eluted from the column by means of acetone and the eluate taken to dryness. A further syrup (1·610 g.) (fraction *C*) remained; this was more viscous than fraction *B*.

Identification of fraction A. This fraction, which was a mixture of crystals and syrup, was extracted with boiling ligroin. The crystals dissolved leaving the syrup. On evaporation of the ligroin, a crystalline product (0·841 g.) was obtained. After being recrystallised twice from ligroin it had m. p. 88° alone or in admixture with 2 : 3 : 4 : 6-tetramethyl glucose (Found: OMe, 52·8. Calc.; OMe, 52·5%).

From the crystals there was prepared an anilide which after 3 recrystallisations from ether–ligroin had m. p. 125°, undepressed in admixture with an authentic specimen of 2 : 3 : 4 : 6-tetramethyl glucose anilide. The syrup which remained after the extraction with ligroin was later combined with fraction *B* to give fraction *D*.

Conversion of the small amount of fraction *D* into the anilides showed that it consisted mainly of 2 : 3 : 4-trimethyl glucose (anilide, m. p. 140°), while by the same method it was shown that fraction *C* consisted mainly of 2 : 3-dimethyl glucose (anilide, m. p. 119°).

From both fractions most of the 2 : 3 : 4-trimethyl derivative was separated in the form of its β-methylglucoside, m. p. 91° (Found: OMe, 53·0%). The residue was converted into the anilides which were quantitatively separated by fractional crystallisation from ether–ligroin and on a silica gel column into 2 : 3 : 4-tri- and 2 : 3-di-methyl glucose. No other constituents could be detected.

The three methylated glucose derivatives were separated from the hydrolysate from further samples of the methylated dextran (3·2 g.), and it was found that there were present 2 : 3 : 4 : 6-tetra-(0·814 g.), 2 : 3 : 4-tri- (2·495 g.), and 2 : 3-di-methyl glucose (0·883 g.). Thus the molecular ratio of these components is 1 : 3 : 1 respectively.

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