528. *Quantitative Analysis of Mixtures of Sugars by the Method of Partition Chromatography. Part IV. The Separation of the Sugars and their Methylated Derivatives on Columns of Powdered Cellulose.*

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Separation of mixtures of simple sugars and of their methyl ethers, on the semi-micro scale, has been achieved by partition chromatography on columns of powdered cellulose (cf. Hough, Jones, and Wadman, *Nature*, 1948, **162**, 448). Application of this procedure to the separation of synthetic mixtures of the simple sugars and their methylated derivatives has given pure specimens of the components, which, under favourable conditions, were recovered quantitatively.

The plant gums and mucilages, the polysaccharides present in animal tissues and those synthesised by bacteria and fungi, are characterised by their molecular complexity. They are, in the main, composed of a variety of monosaccharide units linked together in the most intricate fashions. The separation, identification, and estimation of the sugars resulting from the hydrolysis of any one of these complicated polysaccharides is obviously a matter of importance. Until recently, the analysis of a mixture of sugars was a task of great difficulty, depending largely on precipitation by a specific reagent (cf. Hirst, Jones, and Woods, *J.*, 1947, 1048). The development of improved methods for the analysis of a sugar mixture was therefore necessary in order to establish with certainty the constitution of the complex polysaccharides. The recent application of partition chromatography on sheets or strips of filter paper to the analysis of mixtures of sugars has not only facilitated the identification of components (Partridge, *Nature*, 1946, **158**, 270; *Biochem. J.*, 1948, **42**, 238), but has also enabled their quantitiative micro-determination to be performed (Flood, Hirst, and Jones, *Nature*, 1947, **160**, 86; *J.*, 1948, 1679; Hawthorne, *Nature*, 1947, **160**, 714; Fisher, Parsons, and Morrison, *ibid.*, 1948, **161**, 764; Hough, Hirst, and Jones, this vol., p. 928). By this procedure, however, it is not possible to distinguish either between D- and L-stereoisomers of the sugars, or between sugars such as fructose, sorbose, and tagatose, which show similar properties on the paper chromatogram. The final proof of their identity, therefore, still depends on their separation and on their identification by means of physical properties, in particular, their optical rotations.

The application of chromatography to the carbohydrate field was first described by Reich (*Compt. rend.*, 1939, **208**, 748) who observed that a mixture of the *p*-phenylazobenzoates of glucose and fructose yielded two coloured bands when developed on a column of alumina or silica gel. Since this publication, the method of adsorption analysis has been extended by many other workers to the separation of simple mixtures of sugars and their derivatives (cf. Binkley and Wolfrom, Sci. Rep., No. 10, Sugar Research Foundation, Inc., New York, 1948). For example, McNeely, Binkley, and Wolfrom described methods for the separation of sugars and their acetyl derivatives on columns of commercial clays, such as "Celite," "Magnesol," etc. (*J. Amer. Chem. Soc.*, 1945, **67**, 527).

The separation of amino-acids and their acetyl derivatives by partition chromatography on columns of silica gel or potato starch (Martin and Synge, *Biochem. J.*, 1941, **35**, 1358; Elsden and Synge, *ibid.*, 1944, **38**, ix) suggested the application of similar principles to the separation of intimate mixtures of reducing sugars. Columns of cellulose are used in preference to columns of starch, silica gel, or commercial clays, since they are relatively simple to prepare and give highly reproducible results. Furthermore, the mobile phase percolates down the column, without the use of pressure or suction, at a relatively faster rate, and hence a more rapid separation is achieved. The cellulose is tightly packed in the form of a powder, which is readily obtained from Whatman ashless filter tablets. The uniformity of packing and the performance of the cellulose column may be observed by separating a suitable mixture of dyes on the column.

In general, the methods used hitherto for the chromatographic separation of sugars suffer from some inherent disadvantage, such as the need to form coloured derivatives of the sugars, the necessity of extruding and examining the column, or continually testing the

eluate in order to decide when to change the receiver. The most serious disadvantage of all, however, is the inability to decide whether a zone of sugar consists of a single entity or of two closely placed bands of sugar. These difficulties have been overcome by the construction of an automatic apparatus, which changes the receptacle for the eluate at regular and frequent intervals. A similar receiver changer has been designed by Moore and Stein (*J. Biol. Chem.*, 1948, **176**, 337), the control mechanism of which is, however, different from and more complicated than that described in this paper. Fractionation of the eluate in this manner gives a large number of small portions. A subsequent qualitative examination of each portion by the method of partition chromatography on sheets of filter paper gives a picture of the distribution of each component amongst the receivers. From such chromatograms, it is thus possible to decide which portions of the eluate to combine in order to obtain the highest yield of each individual sugar. When the components of the mixture possess widely differing *Rf* values, it is possible to obtain recoveries of the order of 96% of pure material. Should the components have similar *Rf* values, however, with a consequent overlapping of the sugar zones, it is then expedient to divide the contents of the receivers in such a manner as to obtain pure samples of sugar in order to facilitate their identification, although the yield will not be quantitative. This is not a serious disadvantage since, after identification, the composition of the mixture of sugars may be determined quantitatively on the micro-scale by the use of sheet-filter-paper partition chromatography.

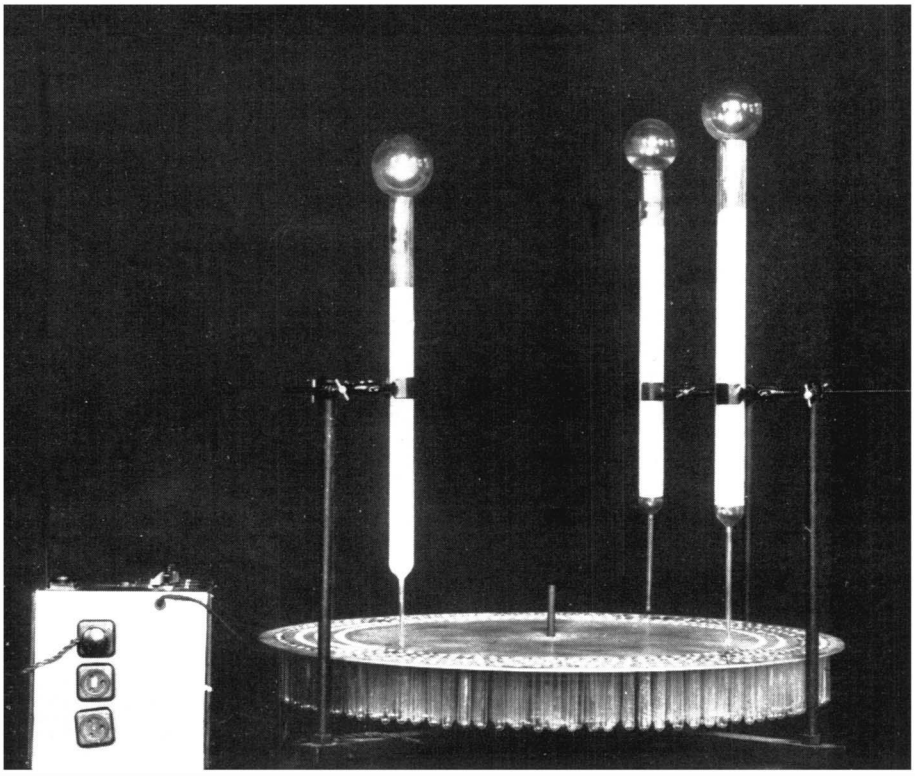
Using this method and employing *n*-butanol, saturated with water, as the mobile phase, we have separated, in the pure state and with individual recoveries of 95—100%, a two-component mixture composed of L-rhamnose and L-arabinose and a four-component mixture composed of L-rhamnose, D-ribose, L-arabinose, and D-galactose. The isolation of pure crystalline specimens of L-rhamnose, D-tagatose, and D-galactose from the hydrolysis products derived from *Sterculia setigera* gum serves to illustrate the utility of the method in the investigation of a complex polysaccharide (Hough, Hirst, and Jones, *Nature*, 1949, **163**, 177).

The columns may be used repeatedly if they are washed with the requisite solvent after each experiment, in order to remove any soluble impurities, and provided that they are never allowed to run dry. The amount of any particular sugar that can be separated on the column is approximately proportional to the *Rf* value, and the time taken for it to emerge is inversely proportional to the *Rf* value. Most of the experiments with the simple sugars were carried out using *n*-butanol, saturated with water, as the mobile phase, since this system gave the high degree of resolution which is necessary for the analysis of a complex mixture. The separation of a sugar such as galactose or glucose which possesses a low *Rf* value in butanol, saturated with water, necessitates the collection and evaporation of a large volume of eluate. Furthermore, the detection of the sugar may be difficult unless the eluate is concentrated. In order to overcome this difficulty, solvents such as *iso*propyl alcohol–water, acetone–water, and ethanol, in which the sugars have a higher solubility, have been investigated. The results show that these solvents are of greater utility than the butanol–water solvent for the separation of a simple mixture of sugars of low *Rf* value, since they pass down the column at a greater rate than butanol–water, the concentration of sugar in the eluate is higher, and they may be evaporated at lower temperatures, thus enabling the separation to be performed more rapidly.

The structure of the polysaccharides has, to a large extent, been investigated by the process of exhaustive methylation, followed by an examination of the methylated sugars produced on hydrolysis of the fully methylated polysaccharide. The separation and quantitative determination of these methylated sugar derivatives by fractional distillation in a high vacuum is attended by many experimental difficulties. Recourse has therefore been had to the chromatographic technique (cf. Jones, *Ann. Repts.*, 1946, **43**, 178); Bell (*J.*, 1944, 473) has achieved the absolute separation of the tetra-, tri-, and di-methyl glucose fractions obtained from methylated glycogen or methylated starch by countercurrent partition between chloroform–butanol, the mobile phase, and water held by a column of silica gel. Complex mixtures of methylated sugars may be separated and determined quantitatively on the micro-scale by use of partition chromatography on sheets or strips of filter paper (Hirst, Hough, and Jones, *loc. cit.*).

The separation of the methylated sugars on columns of cellulose, by use of *n*-butanol, saturated with water, was in the main unsatisfactory. Good separations were, however, obtained by using a solvent composed of light petroleum (b. p. 100—120°) (60% v/v) and *n*-butanol (40% v/v). In contrast with the butanol–water solvent, a wide spatial separation of the methylated sugars is obtained, thus facilitating their separation on the column. This solvent is very suitable for the separation of tetramethyl hexoses and trimethyl pentoses from partly methylated sugars.

FIG. 1.



[*To face p.* 2513.

A mixture of 2 : 3 : 4 : 6-tetra- and 2 : 4 : 6-tri-methyl D-glucose, 2 : 4-dimethyl D-xylose, and 2 : 4-dimethyl D-galactose has been separated on a column of cellulose by countercurrent partition using the petroleum–butanol mixture as the mobile phase. The eluate was fractionated into small portions by the automatic receiver changer, and the degree of separation observed by examining a small quantity of each portion of the eluate on sheet-filter-paper chromatograms. The tetramethyl glucose and dimethyl galactose were completely separated, but the zones of trimethyl glucose and dimethyl xylose overlapped slightly so that their quantitative recovery was not possible. Nevertheless, by careful selection of the portions of eluate, pure crystalline specimens of each of these sugars were obtained on removal of the solvent.

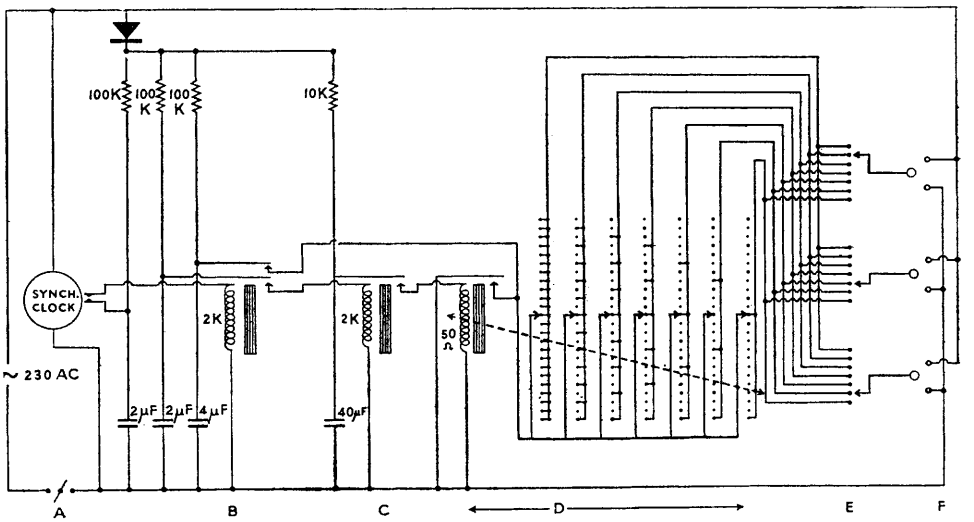
Experimental.

[All *Rg* values were determined using the upper phase of a mixture of *n*-butanol (40 parts), water (50 parts), and ethanol (10 parts), unless otherwise stated; Brown, Hough, Hirst, Jones, and Wadman, *Nature*, 1948, **161**, 720.]

The Automatic Receiver Changer.—The apparatus is illustrated in Fig. 1, which shows the control unit on the left, and the table with the columns above it on the right. The large aluminium disc on the table has four concentric rows of holes around its periphery, each hole acting as a support for the flanged test-tubes (10-ml.). The test-tubes act as receivers for the eluate. The disc is rigidly attached to, and

Fig. 2.

Automatic receiver changer (circuit of control unit).



supported by, a central shaft, which is turned through a small angle at regular intervals, so that each consecutive empty tube is brought into position beneath the column at the necessary time. The control shaft is turned by an electrical mechanism (externally controlled), mounted underneath the disc. The whole assemblage is mounted on a cruciform base plate, each arm of which extends a little beyond the edge of the disc and carries on this extension, a short vertical rod, which serves as a rigid support for the columns. The table is therefore capable of collecting and fractionating the eluate from four columns simultaneously. Three or more tables may be controlled concurrently by the control unit and at the same time, each table may be rotated at different intervals of time by selection of the appropriate switch on the control unit. In this way, subsequent test-tubes are brought underneath each column at predetermined time intervals, which may be of 5-, 10,- 15-, 20-, 30-, 40-, or 60-minutes duration. The time intervals chosen will depend upon the rate of passage of solvent through the column.

The control unit (Fig. 2) consists of a synchronous electric clock (*A*), the hour shaft of which is fitted with a 12-stepped cam that shuts the associated contacts at 5-minute intervals. This causes the relay (*B*) to shut momentarily and accomplishes two subsequent actions. First, it delivers a current pulse to the switch mechanism of the eight-row uniselector (Post Office type) (*D*), and from here the pulse passes to the switches (*E*) along one or more lines. The 8 rows of the uniselector are wired in such a manner that the first row connects in every position with the switches (*E*), the second row connects with the switches (*E*) in alternate positions only, and succeeding rows in every 3rd, 4th, 6th, 8th, and 12th position, respectively. Hence, by connection to the appropriate row of the uniselector by means of switches (*E*), it is possible to obtain pulses at either 5-, 10-, 15-, 20-, 30-, 40-, or 60-minutes interval. The pulse then passes from the switch to the corresponding plug socket (*F*) and thence to the table (Fig. 3). The second action of the relay (*B*) is to close relay (*C*). The action of the latter is delayed by means of a heavy copper band around the core; this leads to an appreciable time lag between the shutting of relay (*B*) and relay (*C*). When relay (*C*) is closed, the selector is moved round one position. If the current pulse

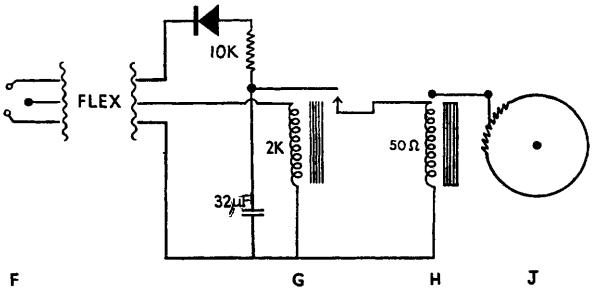
to the tables is unfinished by the time the selector moves, it is shorted to earth by a switch incorporated in the selector and thus the movement of any table connected to the next selector position is prevented.

The power supply for the control unit is derived from A.C. mains; the relays and the selector switch utilise D.C. at 290 v., which is obtained by incorporating a metal rectifier in the circuit. The plug socket (*F*) on the control unit is connected to the tables by a three-core cable. Two of these cores carry A.C. 230 v., whilst the third carries the operating pulse from the control unit. The pulse closes the relay (*G*), which immediately discharges a heavy condenser through the magnet (*H*) (the condenser is recharged from A.C. 230 v. supply by means of a metal rectifier). This magnet operates a pawl and ratchet mechanism (*J*) which moves the ratchet one tooth for each pulse received. The number of teeth on the ratchet is equal to the number of holes in a row around the periphery of the tables, namely 104.

Preparation of the Columns.—A column of powdered cellulose contained in a glass tube (20 inches long and 1½ inches in diameter) drawn out at one extremity is used. A perforated porcelain disc rests upon this constriction. A thin even layer of cotton wool is placed on this disc and above it the cellulose is packed in the form of a fine powder, obtained by rubbing Whatman ashless filter tablets through a 80-mesh sieve. When the column is being packed, great care must be taken to ensure that the packing is uniform, otherwise a subsequent distortion of the zones of sugar will result. The cellulose powder is introduced into the tube in portions sufficient to fill only 1 inch of its length. After each addition, the cellulose powder is packed by tapping the base of the glass tube gently and repeatedly on a piece of wood. It is then further compressed with a ram-rod, the plunger of which has the same diameter as the column. The column is tightly packed in this manner until the cellulose packing is about 4 inches from the top of the containing tube. The surface of the cellulose must be flat and horizontal, in order to avoid distortion of the zones of sugar, and it is covered by a porcelain filter-disc to prevent the surface from being disturbed. Before use, the column is washed with the solvent which is supplied from a constant-head

FIG. 3.

Circuit of a table.



reservoir until all the soluble impurities have been removed. Once the column has been washed, it should always remain in contact with the solvent, since the cellulose recedes from the walls of the tube when the solvent is allowed to evaporate. If the column is not required for immediate use, the outlet should be stoppered and the reservoir left in position.

Testing the Efficiency of the Column.—The performance of the column may be observed visually by placing a suitable mixture of the following dyes on the column and noting the appearance and the separation of the coloured materials as they advance down the column. It has been found that various dyes move at different rates on sheet-filter-paper chromatograms. The *Rg* values of a number of the more suitable of these coloured materials have been determined on sheet-paper chromatograms, using the top layer of a mixture of *n*-butanol (40%), ethanol (10%), water (50%) as the mobile phase. *Rg* values determined were as follows: auramine 1·00, dimethyl-yellow 0·95, bromothymol-blue 0·83, brilliant-cresyl-green 0·73, metanil-yellow 0·48, cresol-red 0·41, methyl-red 0·38, bromocresol-green 0·30, bromophenol-blue 0·26, and methyl-orange 0·23. If the coloured materials travel through the column in the form of regular horizontal bands, the column is regarded as satisfactory for use, the uniformity of packing being thus indicated. These dye can also be used as markers, since a dye that moves slightly faster than the fastest-moving component of the sugar mixture, can be incorporated in the mixture to be resolved, and it is then only necessary to examine the eluate after the coloured material has emerged from the column.

Procedure for the Separation of Simple Sugars.—The mixture of sugars is dissolved in the minimum quantity of water, to give a thin syrup which was introduced on the column with the aid of a small pipette and allowed to soak into the cellulose. A little cellulose powder is then placed on the top of the column and above it the porcelain filter-disc. The constant-head reservoir is then replaced and the solvent allowed to percolate down the column. The eluate is fractionated into approx. 5-ml. portions by the automatic device which changes the receiver after the appropriate intervals of time.

Small spots of eluate from each receiver are placed in chronological order along the starting line of a sheet-filter-paper chromatogram and separated in the usual manner. In some cases, such as when the sugar is weakly reducing or when the concentration of sugar is small, it may be necessary to concentrate small portions of the eluate in order to determine which tubes contain reducing sugar. After spraying the chromatogram with an ammoniacal solution of silver nitrate and heating, a picture of the distribution of the separated sugars amongst the receivers is obtained. Hence, it is possible to decide which portions of the eluate to select and combine, in order to obtain pure specimens of sugar. The solvent is then removed by distillation under reduced pressure at 40°, and the residue dissolved in a little water, filtered, and evaporated.

After the experiment, the column may be washed with the requisite solvent and used again. If the column is not required for immediate use, the outlet should be stoppered. It is convenient to store the test-tubes in aluminium racks, each rack holding 100 tubes.

Typical Separations of Known Mixtures of Simple Sugars.—(i) A mixture of L-rhamnose hydrate (300 mg.; *Rg* 0·30) and L-arabinose (200 mg.; *Rg* 0·13) was separated in the above manner, using *n*-butanol, saturated with water containing a little ammonia, as the mobile phase. The eluate from the column was fractionated into approx. 5-ml. portions by the automatic device which changed the receiver after 20-minute intervals. Examination of the contents of the receivers, by the sheet-filter-paper partition chromatography, indicated that the sugars had been completely separated. Two solutions were thus obtained, each of which was filtered through a No. 4 sintered-glass filter and the solvent evaporated under reduced pressure, leaving (*a*) L-rhamnose (anhydrous) (260 mg.; 96% recovery) and (*b*) L-arabinose (193 mg.; 96% recovery). Each fraction crystallised on removal of the solvent and, after recrystallisation from methanol, pure specimens of L-rhamnose hydrate and L-arabinose were obtained.

(ii) A mixture of D-galactose (125 mg.; *Rg* 0·07), L-arabinose (389 mg.; *Rg* 0·13), D-ribose (192 mg.; *Rg* 0·21), and L-rhamnose hydrate (505 mg.; *Rg* 0·30) was separated in the manner described previously. Examination of portions of the eluate on the paper chromatogram in the usual manner showed that the galactose and arabinose fractions were chromatographically pure, but the zones of rhamnose and ribose overlapped slightly so that their quantitiative recovery was not possible. The eluate was divided up in such a manner as to lead to the highest possible recovery of each individual sugar. Five fractions were therefore obtained, four of which contained a single sugar, and one a mixture of ribose and rhamnose. These gave (*a*) L-rhamnose (anhydrous) (430 mg.; 94·5% recovery), (*b*) L-rhamnose and D-ribose {50 mg.; $[\alpha]_D$ + 12° (*c*, 0·5 in H_2O)}, (*c*) D-ribose (180 mg.; 94% recovery), (*d*) L-arabinose (377 mg.; 97% recovery), and (*e*) D-galactose (138 mg.; 110% recovery). On evaporation, the fractions (*a*), (*c*), (*d*), and (*e*) crystallised; the crystals were extracted with a little ether to remove traces of oily impurities and then recrystallised from methanol, to yield pure specimens of L-rhamnose hydrate, D-ribose, L-arabinose, and D-galactose, respectively.

(iii) A mixture of D-galactose (50 mg.), L-arabinose (50 mg.), D-ribose (50 mg.), and L-rhamnose (50 mg.) was completely separated into its constituents by the above procedure and pure crystalline specimens of each sugar were isolated.

(iv) A mixture of L-rhamnose hydrate (0·5 g.), L-arabinose (0·5 g.), and D-galactose (0·5 g.) was separated as described above, using an *iso*propyl alcohol–water mixture (9 : 1 v/v) as the mobile phase. The eluate was divided into approx. 8-ml. portions by changing the receiver every 10 minutes. Examination of the contents of the receivers in the usual way indicated that a separation had been achieved. The rhamnose was found in the first fraction (140 ml.) of the eluate, the arabinose in the second fraction (170 ml.), and the galactose in the last fraction (280 ml.). Each of these fractions was concentrated, yielding a rhamnose fraction (0·54 g.), an arabinose fraction (0·53 g.), and a galactose fraction (0·41 g.). The rhamnose fraction contained traces of arabinose and readily gave a crystalline specimen of L-rhamnose hydrate (0·39 g.). The arabinose fraction contained a little galactose and yielded a pure crystalline specimen of L-arabinose (0·42 g.) on draining on a tile and recrystallisation from methanol. The galactose fraction contained no other sugars and gave a pure crystalline product on evaporation.

(v) A mixture of sugars consisting of D-galactose (0·5 g.), L-arabinose (0·5 g.), and L-rhamnose hydrate (0·5 g.) was separated as above, using an acetone–water mixture (95 : 5 v/v) as the mobile phase. In this way, pure crystalline L-rhamnose hydrate (0·3 g.) was obtained from the first fraction. The second fraction consisted of a mixture (0·47 g.) of rhamnose and arabinose; it was not further examined. The third fraction gave, on removal of the solvent, pure crystalline L-arabinose (0·27 g.). After all the arabinose had been eluted, the galactose was eluted with methanol. Concentration of the eluate gave pure crystalline D-galactose (0·49 g.).

(vi) A mixture of L-arabinose (0·1 g.) and L-rhamnose hydrate (0·1 g.) was separated in the usual way, using aqueous ethanol (95%) as the mobile phase. Examination of the eluate as described above showed that complete separation of the rhamnose from the arabinose had been achieved.

(vii) A mixture of L-rhamnose hydrate (1 g.), L-arabinose (1 g.), and D-galactose (1 g.) was examined in the usual manner, using methanol as the mobile phase. The eluate was collected in 8-ml. portions by changing the receiver every 5 minutes. The eluate was examined on the paper chromatogram and a slight separation observed, the first few receivers containing only rhamnose and the last few receivers containing only galactose. The separation was so poor, however, that no attempt was made to isolate crystalline sugars.

Separation of Mixtures of Methylated Sugars.—Investigation has shown that usual partition solvents such as butanol–water are unsatisfactory for the separation of mixtures of the more fully methylated sugars. In some cases, the zones of sugar overlapped considerably and hence the isolation of pure specimens of material was difficult. In particular, tetramethyl glucose could not be separated from trimethyl glucose. In order to overcome this difficulty, a solvent composed of light petroleum (b. p. 100—120°) (60% v/v) and *n*-butanol (40% v/v) was employed, since a wide spatial arrangement of the methylated sugars was obtained on sheet-paper chromatograms when this solvent was used in place of the butanol–water solvent (see Table).

	Rg value.	
	Butanol–water.	Butanol–petroleum.
6-Methyl glucose	0·185	0·09
Rhamnose	0·30	0·15
2 : 4-Dimethyl galactose	0·40	0·25
2 : 4-Dimethyl xylose	0·66	0·56
2 : 4 : 6-Trimethyl glucose	0·75	0·71
2 : 3 : 4-Trimethyl xylose	0·94	0·95
2 : 3 : 4 : 6-Tetramethyl glucose	1·00	1·00

Separation of a Known Mixture of Methylated Sugars.—A mixture of 2 : 3 : 4 : 6-tetramethyl D-glucose (53 mg.; *Rg* 1·00), 2 : 4 : 6-trimethyl D-glucose (353 mg.; *Rg* 0·76), 2 : 4-dimethyl D-xylose (135 mg.; *Rg* 0·66), and 2 : 4-dimethyl D-galactose (140 mg.; *Rg* 0·40) was separated by partition chromatography on a column of cellulose, using a solvent composed of light petroleum (b. p. 100—120°) (60% v/v) and *n*-butanol (40% v/v) as the mobile phase. The eluate was fractionated into 5-ml. portions by changing the receiver every 10 minutes. After examination on sheet-paper chromatograms in the usual manner, the eluate was divided in such a manner as to lead to the highest possible recovery of pure samples of each individual sugar. In all, five fractions were obtained, four of which contained a single sugar. After removal of the solvent from each fraction, the resultant syrups crystallised and, after weighing the yield of each fraction, they were recrystallised from ether–light petroleum to yield pure crystalline specimens of the methylated sugars: (*a*) 2 : 3 : 4 : 6-tetramethyl D-glucose (51 mg.), (*b*) 2 : 4 : 6-trimethyl D-glucose (56 mg.), (*c*) 2 : 4 : 6-trimethyl D-glucose and 2 : 4-dimethyl D-xylose {376 mg.; $[\alpha]_D$ +62·6° (*c*, 3·7 in H_2O)} [fraction (*c*) crystallised when kept; the crystals were separated on a tile and recrystallised from ether–light petroleum, to yield 2 : 4 : 6-trimethyl D-glucose], (*d*) 2 : 4-dimethyl D-xylose (57 mg.), and (*e*) 2 : 4-dimethyl D-galactose (140 mg.).

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