345. *Quantitative Analysis of Mixtures of Sugars by the Method of Partition Chromatography. Part V. Improved Methods for the Separation and Detection of the Sugars and their Methylated Derivatives on the Paper Chromatogram.*

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The methylated sugars and uronic acids give specific colour reactions with various acidic spray reagents such as *p*-anisidine hydrochloride and aniline trichloroacetate, thus facilitating their detection and identification on the paper chromatogram. Other aids to the separation and identification of the sugars and their derivatives are discussed.

Partition chromatography has found general application in carbohydrate studies as a means of separating mixtures of reducing sugars, sugar alcohols, methylated sugars, and uronic acids

and their methylated derivatives. As a general method for the detection of reducing sugars on the paper chromatogram, Partridge employed an ammoniacal solution of silver nitrate (*Biochem. J.*, 1948, **42**, 238), the reducing sugars giving rise to discrete brown-black spots. Use of this reagent was later extended to the detection on the paper chromatogram of methylated sugars (Hirst, Hough, and Jones, *J.*, 1049, 928), polyhydric alcohols, and methyl glycosides of simple sugars (Hough, *Nature*, 1950, **165**, 400). Various specific spray reagents have been developed for the detection of individual classes of unsubstituted sugars. Their use depends on the formation of furfuraldehyde or a degradation product of furfuraldehyde by the action of an acid upon the sugar, and its subsequent reaction with an aromatic amine or phenol to give a coloured compound (cf. McGowan, *J.*, 1949, 777). For example, naphtharesorcinol and hydrochloric acid (Forsyth, *Nature*, 1948, **161**, 239), and naphtharesorcinol and trichloroacetic acid (Partridge, *loc. cit.*) have been utilised as selective spray reagents for the detection of raffinose, sucrose, and the keto-hexoses. Chargaff, Levine, and Green (*J. Biol. Chem.*, 1948, **175**, 67) have used a solution of *m*-phenylenediamine dihydrochloride in 76% alcohol for the detection of the unsubstituted sugars, which give rise to coloured spots possessing a marked fluorescence in the ultra-violet light. Similarly Partridge (*Nature*, 1949, **164**, 443) records that with aniline phthalate in butanol the pentoses give a characteristic red colour, and that aldohexoses, methylaldopentoses, and hexuronic acids are readily detected as brown spots, ketohexoses giving little or no colour. We find that the methylated derivatives of aldohexoses, pentoses, ketohexoses, and uronic acids give excellent colours with aniline phthalate, trichloroacetate, or phosphate, as little as 1—5 μg. being detectable. Octoses and heptoses also yield brown colours. Partly methylated aldohexoses give rise to brown colours, sometimes with a tint of red, fully methylated aldohexoses giving a characteristic maroon colour. On the other hand, methylated aldopentoses give cherry-red colours. Methylated uronic acids give crimson-red colours of such high brilliance that they are readily distinguished. The rapid rate of formation of colour from methylated uronic acid derivatives indicates that they are easily degraded to furfuraldehyde. Methylated ketoses give green colours. The aniline salt reagent possesses many advantages over the ammoniacal silver reagent for the detection of the methylated sugars. It is equally, if not more, sensitive, giving characteristically coloured spots on a white background, which are stable for some considerable time. A large number of acidic spray reagents containing a variety of phenols and aromatic amines has been examined in order to find a universal reagent which will yield a specific colour with each particular class of sugar or methylated sugar. In this respect we find that solution of *p*-anisidine hydrochloride in *n*-butanol is very satisfactory, since with it aldohexoses give rise to a green-brown colour, ketohexoses a brilliant lemon-yellow colour, methyl aldopentoses an emerald-green colour, and uronic acids a cherry-red colour. Methylated aldohexoses furnish brown colours, sometimes with a tint of red, whereas methylated aldopentose derivatives give intense red colours. 2-Deoxyaldoses give pale-brown colours which appear as characteristic white fluorescent spots in ultra-violet light. The intensity of the coloured spots obtained with the reagent was considerably enhanced in ultra-violet light, thus providing a very sensitive means of detection. Diphenylamine trichloroacetate also serves to distinguish aldohexoses from aldopentoses, yielding with the former a brown and with the latter a purple colour. Furthermore, 2 : 3 : 4-trimethyl and 2 : 4-dimethyl xylose are readily distinguished from 2 : 3 : 5-trimethyl and 2 : 3-dimethyl arabinose since they respectively form an intense purple and a grey or faint purple colour. Methylated fructose derivatives yield a green colour with this reagent. Dimethylaniline is of little value for the detection of the simple sugars, but interesting results were obtained with the more fully methylated sugars. In this case, 2 : 3 : 5-trimethyl and 2 : 3-dimethyl arabinose gave an intense purple colour, whereas 2 : 3 : 4-trimethyl and 2 : 4-dimethyl xylose gave a light-brown colour. Characterisation of methylated arabofuranose derivatives and methylated xylopyranose derivatives on the paper chromatogram is therefore facilitated by the use of acidic sprays of dimethylaniline and diphenylamine. Methylated aldohexoses possessing free hydroxyl groups on $C_{(4)}$ of the sugar molecules, such as 2 : 3 : 5 : 6-tetramethyl, 2 : 3 : 6-trimethyl, and 2 : 3-dimethyl glucose also gave purple colours with dimethyl-aniline, whereas the other methylated aldohexoses gave little or no colour. Methylated fructose derivatives also gave purple colours.

α-Naphthylamine trichloroacetate is yet another general spray reagent, since aldohexoses and their partly methylated derivatives give a brown, fully methylated aldohexoses a red, ketohexoses a yellow, and aldopentoses a green colour. Once more a distinction between the methylated arabinoses and methylated xyloses was noted, since the latter gave intense green colours, whereas the former gave faint green colours; the colours appear purple in ultra-violet light.

Solutions of orcinol or resorcinol in butanol containing a little hydrochloric acid serve as

specific spray reagents for ketoses and their methylated derivatives since on heating only they yield red colours. Solutions of urea hydrochloride or anthraquinone and hydrochloric acid serve a similar purpose since the ketoses and their methylated derivatives yield intense brown-black spots whereas the other sugars yield little or no colour.

In general, the colours obtained with the acidic spray reagents described above improve when kept in a moist atmosphere for a short time or when the paper chromatograms are wetted. The brilliance and the definition of the coloured spots is considerably improved in ultra-violet light, and in some cases a change in colour is noted. The paper chromatograms may be washed with water, dried in a current of warm air, and preserved, but the colours fade somewhat. In practice, it is preferable to run sugars of known constitution alongside "unknowns" on the paper chromatogram, and to compare the rate of movement and the colours obtained with the various spray reagents in order to obtain some indication as to the identities of the "unknowns." We would emphasise that this evidence is not conclusive, however, and wherever possible it is necessary to separate the "unknowns" by partition chromatography on a column of cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511) and identify the sugar by measurement of physical constants and the formation of characteristic derivatives. Methylglycosides of the simple sugars may be detected by the use of the ammoniacal silver nitrate spray reagent (Hough, *Nature, loc. cit.*), which will detect as little as 5 μg. of the sugar glycoside. Methylglycosides may also be detected by the acidic sprays described above, but, in general, the reaction is rather weak and not as sensitive as the silver nitrate reagent. Polyhydric alcohols, which react with the ammoniacal silver nitrate reagent, do not give colours with the above acidic spray reagents and may therefore be distinguished. Colorimetric methods for the quantitative determination of sugars and, in particular, of their methylated derivatives, after separation on paper chromatograms or on columns of cellulose, have been explored, and will be described in an ensuing publication (with Mr. B. Clist).

Jermyn and Isherwood (*Biochem. J.*, 1949, **44**, 402) have recommended the use of solvent mixtures such as acetic acid–ethyl acetate–water, and pyridine–ethyl acetate–water, for the separation of the sugars on paper chromatograms. Hydrolysis of the ethyl acetate occurs, however, and consequently reproducible results are not readily obtained. It is also necessary to prepare fresh solvent for each separation. Rapid and efficient separations of sugars may be obtained, however, by using "miscible solvent mixtures" such as *n*-butanol–ethanol–water (4 : 1·1 : 1·9), and *n*-butanol–pyridine–water (3 : 1 : 1), which give reproducible results and can be used continuously. The R_F values of the carbohydrates and their derivatives can be adjusted by increasing the pyridine, ethanol, or water content of the mixture (thus increasing the R_F values) or by the addition of light petroleum to either solvent mixture (which depresses the R_F values). The separation of sugars is considerably accelerated by the use of Whatman No. 54 filter paper in place of No. 1 filter paper, but the sugars tend to trail on the former paper. No. 54 paper is of great value for the separation of di-, tri-, and oligo-saccharides. Uronic acids and their methylated derivatives may be separated satisfactorily in a miscible solvent mixture composed of *n*-butanol–glacial acetic acid–water (2 : 1 : 1); sugars also show excellent separations in this solvent mixture.

Separation of sugars and their methylated derivatives by partition chromatography on columns of cellulose has been described (Hough, Jones, and Wadman, *loc. cit.*). Although cellulose is best packed as the fine powder, an acid-treated cellulose, marketed as a fine powder, termed "hydrocellulose," forms excellent columns when packed as a slurry in acetone. Separations of sugars and their methylated derivatives on columns of "hydrocellulose" were as successful as those obtained previously on cellulose. This type of column can also be used to separate sugar glycosides and, in the Experimental section, the isolation of α- and β-methyl-L-rhamnopyranosides from the mixture produced on boiling L-rhamnose with methanolic hydrogen chloride is described.

After separation, identification of an unknown sugar may be facilitated by an examination, on the paper chromatogram, of the sugars produced on epimerisation with lime water. For example on epimerisation, either glucose, fructose, or mannose gives rise to a mixture of all three. Demethylation is also a useful aid to the characterisation of the methylated sugars (cf. Hess and Neumann, *Ber.*, 1935, **68**, 1371) since an unknown methylated sugar may be heated with hydrobromic acid at 100° for about 5 minutes, subsequent analysis of the demethylation products on the paper chromatogram giving a pattern by means of which the parent sugar may usually be identified by its R_F value and colour reaction. Tetramethyl glucose is readily converted into partly methylated glucose derivatives and glucose, trimethyl xylose is converted into xylose, and 2 : 6-dimethyl and 3-methyl galactose into galactose. Fructose derivatives, however,

are destroyed. This behaviour may result because of the more ready conversion of ketoses into ω-hydroxymethylfurfuraldehyde (cf. Haworth, Hirst, and Nicholson, *J.*, 1927, 1513).

The separation of sugars and their methylated derivatives on the paper chromatogram shows a marked improvement at elevated temperatures. At 37°, equilibrium is rapidly established in the chromatographic apparatus and, by use of *n*-butanol incompletely saturated with water as the mobile phase, rapid and efficacious separations have been achieved. In contrast to their behaviour on normal paper chromatograms, the sugars travel as small discrete spots of high concentration, which favour better colour formation with the acidic spray reagents described above. A mixture of galactose, arabinose, ribose, rhamnose, 2 : 4-dimethyl galactose, 2 : 3-dimethyl arabinose, 2 : 3 : 6-trimethyl glucose, and 2 : 3 : 4 : 6-tetramethyl glucose was completely separated on the paper chromatogram at 37° in 3 hours. Chromatography at 37° (on No. 54 paper) provides an excellent method for the separation of the di-, tri-, and oligo-saccharides.

Experimental.

Aniline Salts.—Crystalline specimens of aniline acetate, oxalate, phthalate, hydrochloride, phosphate and trichloroacetate were prepared in the usual manner. These were made up into (5·0, 0·5, and 0·1%) solutions in water, in glacial acetic acid, and in *n*-butanol, giving 9 solutions for each salt. Aqueous solutions of a representative selection of sugars and their methylated derivatives (see below), of 2·5 and 5·0% concentration respectively, were prepared. A spot of each was placed on a sheet of Whatman No. 1 filter paper and allowed to dry. A large number of these " test sheets " was prepared. In each case the spot diameter was approx. 5 mm., corresponding to 0·01 ml. of solution or 25 μg. of sugar and 50 μg. of methylated sugar. This may be regarded as an average value for the concentration of sugar or methylated sugar encountered in paper chromatography.

The following sugars and methyl sugars were used. Galactose and its 2 : 3 : 4 : 6-tetramethyl, 2 : 3 : 6-trimethyl, 4 : 6-, 2 : 6-, and 2 : 4-dimethyl, 2-methyl, and 2-deoxy-derivatives; glucose and its 2 : 3 : 4 : 6- and 2 : 3 : 5 : 6-tetramethyl, 2 : 3 : 6-trimethyl, 2 : 3-dimethyl, and 6-methyl derivatives; xylose and its 2 : 3 : 4-trimethyl, 2 : 4-dimethyl, and 2-methyl derivatives; arabinose and its 2 : 3 : 5-trimethyl, 2 : 3-dimethyl, and 2-methyl derivatives; galacturonic acid and its 2 : 3 : 4-trimethyl, 2 : 3-dimethyl, and 2-methyl derivatives; fructose and its 1 : 3 : 4-trimethyl and 1 : 3 : 4 : 5-tetramethyl derivatives; mannose and 4-methyl mannose; altrose, ribose, sorbose, tagatose, 2-deoxyribose, 2-deoxyallose, 2-deoxyrhamnose, glucoheptose, and D-*glucogala*octose. " Test sheets " were sprayed thinly and evenly with the various solution of the aniline salts described above. The papers were then heated at 100° in an electric oven fitted with a glass door and the development of the coloured spots observed. The sugars sprayed with the aniline salts in glacial acetic acid gave rise to colours of optimum intensity after about 3 minutes' heating. On the other hand, the papers sprayed with the aniline salts in *n*-butanol and in water required 10 minutes' heating, and the colours were inferior to those obtained in a medium of glacial acetic acid. Solutions (2·5%) of aniline phosphate, trichloroacetate, or phthalate in glacial acetic acid gave the best results, excellent colours being obtained with both the sugars and the methylated sugars. Any of these three reagents serves as a good general spray for the detection of the sugars or their methyl ethers on the paper chromatogram. Care must be taken to avoid over-heating the sprayed papers, otherwise the specific colours will be destroyed with the formation of brown spots in all cases. An excess of aniline has little effect on the brilliance of the coloured spots.

The above experiments were repeated with a variety of aromatic amines and phenols. The most useful general spray reagents proved to be : a solution (3%) of *p*-anisidine hydrogen chloride in *n*-butanol; a solution (2%) of dimethylaniline in glacial acetic acid containing trichloroacetic acid (5%); a solution (2%) of diphenylamine in *n*-butanol–methanol (1 : 1 v/v) containing trichloroacetic acid (5%); a solution (2%) of α-naphthylamine in *n*-butanol–methanol (1 : 1 v/v) containing trichloroacetic acid (5%). Solutions (3%) of orcinol or resorcinol in alcoholic hydrogen chloride (5%) gave specific red colours with ketoses, and similar solutions of urea and anthraquinone gave specific brown-black colours with the ketoses.

Demethylation.—The methylated sugar (5—10 mg.) in hydrobromic acid (48% w/w; 1 ml.) contained in a sealed glass tube is heated in the boiling water-bath for about 5 minutes. The contents are then immediately diluted with water (10 ml.). Portions of silver carbonate are added until the solution is neutral, whereafter a smaller quantity of decolorising charcoal is stirred in and the solution filtered. The last traces of silver are removed by the passage of hydrogen sulphide and filtration through a charcoal bed. The filtrate is concentrated to a thin syrup which is examined on the paper chromatogram. Typical results are as follows : (*a*) 2 : 3 : 4 : 6-tetramethyl glucose gave spots corresponding to trimethyl glucoses (R_G 0·84, 0·77), dimethyl glucose (R_G 0·50), monomethyl glucose (R_G 0·22), and glucose (R_G 0·10); (*b*) 2 : 3 : 5-trimethyl arabinose gave spots corresponding to dimethyl arabinoses (R_G 0·52, 0·73), monomethyl arabinoses (R_G 0·24, 0·31, 0·41), and arabinose (R_G 0·13); (*c*) 2 : 3 : 4-trimethyl xylose gave dimethyl xylose (R_G 0·67), monomethyl xylose, and xylose (R_G 0·17); (*d*) 3-methyl galactose gave galactose (R_G 0·08); (*e*) 4 : 6-dimethyl galactose gave galactose (R_G 0·08); (*f*) 2 : 3 : 4 : 6-tetramethyl galactose anilide, trimethyl rhamnose anilide, and 1 : 3 : 4 : 6-tetramethyl fructose were destroyed by the above procedure; demethylation products were not detected. The anilides and phenylhydrazones of the sugars, on being warmed with dilute hydrochloric acid, however, yield solutions containing the sugars, which can be separated and detected on the chromatogram in the usual manner.

Epimerisation.—The sugars are epimerised when kept at room temperature for a week or more in saturated solutions of calcium hydroxide. A more rapid epimerisation is achieved by heating the solutions at 100° for 10—15 minutes or at 37° for 24 hours. The solution is then neutralised by the addition of Amberlite resin IR.100. The best results are obtained at low temperatures. Glucose gave spots corresponding to glucose, mannose, and fructose. Galactose gave spots corresponding to galactose,

talose, and tagatose. Arabinose gave spots corresponding to arabinose, ribose, and riboketose. Sorbose gave spots corresponding to sorbose, idose, and gulose. Rhamnose gave spots corresponding to rhamnose rhamnoketose, and glucomethylose. In all cases other degradation products were detected.

Partition Chromatography at 37° [with Mr. J. N. COUNSELL].—The usual type of chromatographic apparatus was used, precautions being taken to ensure that the container (a glass tank fitted with a thick plate-glass lid) was efficiently sealed. The under side of the glass lid and the upper edges of the tank were ground flat and "Apiezon L" grease was applied to the edges. Any leakage in the container results in a serious distortion of the solvent front as it advances over the paper. The whole apparatus was placed in a chamber thermostatically-controlled at 37°. *n*-Butanol saturated with water at room temperature was employed as the mobile phase. The apparatus was charged with solvent and allowed to stand for 24 hours before use. The results (see Table) show that the R_F values of the sugars are considerably increased and that rapid separations may be achieved on both No. 1 and No. 54 Whatman filter papers. A marked improvement in the definition of the spots was also noted. The rate of movement of a number of hexitols and disaccharides on the paper chromatogram was also improved. Chromatography at 37° for 16½ hours on No. 54 paper gave an excellent separation of the sugars; mannose, fructose, and arabinose were clearly distinguished, whereas at room temperature they are very difficult to separate.

Distance (cm.) *from the centre of the sugar spot to the starting point and* R_F *values.**

Whatman filter paper :	No. 1.	No. 1.	No. 54.		No. 54.
Temp. and duration of chromatography :	17°; 16½ hrs.	37°; 6 hrs.	37°; 3 hrs.		37°; 16½ hrs.
Distance from solvent front to the starting line (cm.) :	front 29·1.	front 20·4.	front 30·7.		†
Galactose	1·6 (0·055)	2·35 (0·115)	3·1 (0·102)		12·1 (0·102 ‡)
Glucose	—	—	—		13·3 (0·111)
Mannose	—	—	5·0 (0·163)		18·7 (0·156)
Fructose	—	—	4·9 (0·160)		19·8 (0·165)
Arabinose	2·7 (0·093)	3·9 (0·192)	5·5 (0·178)		20·4 (0·177)
Ribose	4·4 (0·151)	5·7 (0·278)	8·1 (0·265)		28·7 (0·240)
Rhamnose	6·0 (0·206)	8·0 (0·359)	10·5 (0·342)	Sorbitol	15·3 (0·124)
2 : 4-Dimethyl galactose	—	9·35 (0·457)	13·7 (0·446)	Mannitol	15·9 (0·129)
2 : 3-Dimethyl arabinose	11·4 (0·392)	13·1 (0·641)	19·9 (0·650)	Dulcitol	15·3 (0·124)
2 : 3 : 6-Trimethyl glucose	16·4 (0·563)	15·3 (0·748)	23·1 (0·752)	Turanose	6·6 (0·055)
2 : 3 : 4 : 6-Tetramethyl glucose	21·5 (0·738)	17·45 (0·854)	26·3 (0·857)	Sucrose	6·1 (0·051)
				Melibiose	12·8 (0·107)

* R_F values are given in parentheses.
† The solvent front reached the bottom of the paper and was allowed to drip off.
‡ This R_F value was assumed, and the R_F values of the other materials on the chromatogram were calculated by proportion.

Separation of α- and β-Methyl-L-rhamnopyranoside.—L-Rhamnose (10 g.; anhydrous) was boiled with methanolic hydrogen chloride (250 c.c.; 2%) for 12 hours. The cooled solution was neutralised with silver carbonate and filtered, and the filtrate concentrated to a syrup (10·6 g.). The syrup was dissolved in *n*-butanol (7 c.c.) two-thirds saturated with water, and transferred to the top of a column (50 cm. long, 6 cm. diam.) of "hydrocellulose," and the glycosides were separated in the manner described by Hough, Jones, and Wadman (*loc. cit.*), with butanol–water as the mobile phase. The fractions containing the glycosides were examined on the filter-paper chromatogram (Hough, *loc. cit.*). After selection of the appropriate fractions, subsequent concentration gave α- (9·1 g.), $[\alpha]_D^{20}$ −63° (water), R_G 0·53, and β-methyl-L-rhamnopyranoside (0·9 g.), m. p. 139°, $[\alpha]_D^{20}$ +94° (water), R_G 0·42.

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