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Filter-paper Partition Chromatography of Sugars

1. GENERAL DESCRIPTION AND APPLICATION TO THE QUALITATIVE ANALYSIS OF SUGARS IN APPLE JUICE, EGG WHITE AND FOETAL BLOOD OF SHEEP

BY S. M. PARTRIDGE, WITH A NOTE BY R. G. WESTALL
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge

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The development by Consden, Gordon & Martin (1944) of a partition method of chromatography, in which sheets or strips of filter paper are used instead of the familiar packed column, has already shown its usefulness as a method for the qualitative analysis of protein or peptide hydrolysates for amino-acids, especially in circumstances where the amount of material available is small. Although the method is not suitable for obtaining accurate quantitative results, it has the special advantage that use can be made of very small differences in partition coefficient, with the result that useful separations may be obtained with as many as nineteen or twenty amino-acids on the same chromatogram. The present communication describes an extension of the method to the qualitative analysis of sugars, either in biological fluids or in the hydrolysis products of polysaccharides. Preliminary results have already appeared (Partridge, 1946).

The essentials of the method are as follows: A small quantity of the solution to be analyzed is introduced in the form of a circular spot near the top of a sheet or strip of filter paper. The paper is then hung vertically from a trough containing a solvent which has previously been saturated with water, so that the top edge of the paper is immersed in the solvent. The trough and the filter-paper strips are then suspended inside a closed vessel, the atmosphere in which is kept in a condition of saturation both with respect to water and to the solvent. A sharp horizontal liquid front forms, which advances down the paper at a speed dependent upon the physical properties of the solvent used. To irrigate a paper strip some 35 cm. long, 18–24 hr. are usually sufficient, after which the strip may be dried and the new positions of the components of the solution rendered visible by spraying the paper with an appropriate reagent.

Under these conditions, partition of the solute takes place between water bound by the cellulose and the solvent which is moving over the surface of the cellulose fibres. The distance moved by any solute in the direction of flow is then a function of (1) the partition coefficient of the solute between water and the wet solvent, (2) the volume of water bound by unit area of the filter paper, and (3) the volume of wet solvent held by unit area of the filter paper after irrigation. In any single experiment, factors (2) and (3) are substantially constant provided the filter paper is uniform in texture, thickness and water content, so that under ideal conditions the relative distance (R_F) moved by each

solute is dependent upon its partition coefficient only.

The theory of the method has been dealt with in some detail by Consden *et al.* (1944), who give the definition

$$R_F = \frac{\text{distance moved by solute}}{\text{distance moved by advancing front of liquid}}.$$

These authors have shown experimentally that the values of R_F given by amino-acids on cellulose may be correlated fairly accurately with their partition coefficients as measured by direct methods, and the conclusion is drawn that true adsorption by the cellulose does not play a significant part.

In the work described below the reagent used to reveal the position of the spots formed by the reducing sugars has been almost exclusively a solution containing silver nitrate and a large excess of ammonia. This was sprayed on to the chromatogram after removal of the solvent used to irrigate it, and the filter-paper strip was then heated. In this way metallic silver was precipitated in the region occupied by reducing sugars, giving rise to brown or black spots. The $AgNO_3$-ammonia reagent has the advantage of being effective for all reducing sugars, and is therefore of general application to these, but for the non-reducing disaccharides or trisaccharides, other methods must be employed. For those containing a ketose, an acidic spraying reagent based on naphthoresorcinol is suitable, and details of its use are given in the text. In specialized cases other reagents were found to be of advantage, a particular instance being the use of *p*-dimethylaminobenzaldehyde as a reagent for the *N*-acetylhexosamines.

EXPERIMENTAL

With certain minor modifications, the experimental conditions that allow of separation of the amino-acids are also suitable for the qualitative analysis of sugars. Whatman no. 1 filter paper has been used exclusively, and for preparing strip chromatograms, the paper was cut into pieces 43 cm. long × about 12 cm. wide. The sugar solutions used were roughly 1% (w/v) with respect to each individual sugar, and 3–4 μl. containing 30–40 μg. of each sugar was introduced as a circular spot on a horizontal line ruled 7·5 cm. from the top of the paper strip. When a number of different sugar solutions was run on the same strip of paper, the spots were introduced at intervals of 1·5 cm. along the horizontal line. The strips were usually irrigated with the solvent for 18 hr. overnight. The solvent was dried off in an oven at 100–105°, the position of the solvent boundary being marked in ink before transfer to the oven. In order to reveal the position of the sugars the paper was sprayed rapidly and evenly with a mixture containing equal parts of silver nitrate solution (0·1 N) and ammonia solution (5 N), and the strip replaced in the oven (105°) for 5–10 min.; the sugars appeared as dark brown spots on a white or light brown ground. In runs carried out in phenol the lower part of the paper was usually uniformly coloured light brown, the upper part being white or light grey. The developed strips began to darken owing to the presence of silver salts a few hours after they had been removed from the oven, but if a permanent record was desired, the strips were washed first in distilled water and afterwards in running tap water for about 1 hr., followed by drying.

The apparatus used was that described by Consden *et al.* (1944). It was found, however, that the glass troughs could be replaced with advantage by troughs of a similar pattern made from stainless steel. For drying off the solvent and heating the papers a special oven was constructed. This had a large glass window in the front in order to allow the development of the spots to be kept under observation as the temperature increased after introducing a sprayed chromatogram. This feature was of value, since when using $AgNO_3$-ammonia as a spraying reagent it was found that certain sugars (such as glucosamine and chondrosamine) react at lower temperatures, and this effect often served as a useful confirmation of an identification based on R_F value. The oven was equipped with a small centrifugal fan which caused air heated by a bank of electrical strip heaters to be drawn slowly through the drying chamber and then to be discharged outside the building.

Use of naphthoresorcinol as a reagent for di- and trisaccharides containing ketoses

For many non-reducing oligosaccharides or glycosides the use of an acidic reagent is necessary in order to secure hydrolysis. It was found that after spraying a filter-paper chromatogram with a dilute solution of an acid and heating to 80–100°, concentration of the acid took place to a point at which dehydration of the sugars occurred, with consequent liberation of substituted furfurals. The position of the sugars could therefore be revealed by making use of the well known colour reactions of furfurals with phenolic reagents. Under certain conditions the reaction was found to be highly specific for the ketoses. A number of phenolic substances were tested for suitability as spraying reagents, and of those examined naphthoresorcinol appeared most satisfactory, although other common substances of this type, such as α-naphthol or resorcinol, were also effective. The reaction took place with phenol itself, and in chromatograms irrigated with this solvent there was usually sufficient phenol left on the paper to develop a colour with fructose on spraying with an acid and heating in the oven. Collidine inhibited the reaction to some extent, and this solvent was avoided where phenolic reagents were to be used.

A spraying reagent based on hydrochloric acid (0·25 N) and containing naphthoresorcinol (0·1% w/v) was found to be effective in producing strong red colours with the ketoses at 100–105°, but since the mineral acid tended to break down the cellulose of the filter paper it was found preferable to replace it by trichloroacetic acid. The reagent finally adopted was as follows: *Solution* (*a*) naphthoresorcinol, 0·2% (w/v) in ethanol; *solution* (*b*) trichloroacetic acid, 2·0% (w/v) in water. Equal volumes of the two solutions were mixed immediately before use, and the paper sprayed evenly with the mixture. The paper was then allowed to dry off partially at room temperature before being introduced into the oven at 100–105° for 5–10 min. Fructose, sorbose, sucrose and raffinose gave very strong red spots, the colour of which was stable for at least 12 hr.

Under the conditions described, the reaction was very selective for the ketoses, other sugars giving no more than a trace of colour at temperatures above 100°. However, on

allowing the heated chromatograms to stand for a few hours in the open air, the pentoses and uronic acids developed strong blue colorations. The presence of moisture appeared to be necessary for the development of the blue colour, and in cases where it was desired to reveal the positions of the pentoses and hexuronic acids the sprayed chromatogram was heated in a humid atmosphere at 70–80°. For this purpose, open baths of water were inserted in the oven above the heaters. At 70–80° in humid air, development of the deep blue or purple colours required 10–15 min., and under these conditions the colour due to the ketoses was orange-brown. The blue or purple colour was very stable, and was particularly intense in the case of the hexuronic acids.

Use of the Morgan & Elson reaction for hexosamine

The reagents devised by Elson & Morgan (1933) and Morgan & Elson (1934) for the colorimetric estimation of hexosamines and *N*-acetylhexosamines were adapted for use with the filter-paper technique as a second method of revealing the presence of the amino sugars. The method is of particular value for *N*-acetylglucosamine, since this substance is only slowly oxidized by the ammoniacal silver nitrate reagent, but gives an excellent reaction with *p*-dimethylaminobenzaldehyde.

The reagents were as follows:

(*a*) *Acetylacetone reagent.* Solution (1): Acetylacetone (0·5 ml.) dissolved in butanol (50 ml.). Solution (2): 50% (w/v) aqueous KOH (5 ml.) and ethanol (20 ml.). 0·5 ml. of solution (2) were added to 10 ml. of solution (1) just before the reagent was required. Crystals occasionally appeared in the mixed solution, but they readily redissolved on addition of a few drops of 50% (v/v) aqueous ethanol. The reagent was not stable and was made up daily from fresh acetylacetone.

(*b*) *p-Dimethylaminobenzaldehyde reagent.* *p*-Dimethylaminobenzaldehyde (1 g.) after recrystallization from aqueous ethanol was dissolved in ethanol (30 ml.) and 30 ml. of conc. HCl added. The solution was then diluted with 180 ml. of redistilled butanol. The reagent was stable in ethanol-HCl solution and a stock solution in the solvent could be stored for several weeks.

After evaporating off the solvent, the chromatograms were sprayed with solution (*a*) and heated in the oven for 5 min. at 105°. The dry paper strips were then sprayed with solution (*b*) and returned to the oven for a further short heat treatment (5 min.) at 90°. Under these conditions the free hexosamines gave cherry-red colorations which were stable for several days. *N*-Acetylglucosamine gave a strong purple-violet colour which was also stable. However, unlike the free hexosamines, the *N*-acetyl derivatives gave strong violet colorations with the *p*-dimethylaminobenzaldehyde reagent alone, without any previous treatment with acetylacetone, and this reaction could be utilized to serve as a confirmatory test for the presence of the *N*-acetylated derivatives.

Other reducing sugars also gave colours with these reagents under the conditions described above, but the colorations were usually very light and faded rapidly, particularly on raising the oven temperature. The colours given by various neutral reducing sugars ranged from blue to pink-violet, each sugar giving a characteristic shade, but it was considered doubtful if the method would be of value for revealing the presence of the neutral sugars since the reaction was not sufficiently sensitive.

Solvents

Phenol. It was found convenient to prepare a stock solution containing phenol (detached crystals, B.P.) (900 g.) and water (100 g.). This mixture remained liquid at room temperature, and to prepare a saturated solution, the stock solution was gently shaken with an excess of water, and after separation the lower phenol layer was run off into the trough of the chromatographic chamber. The aqueous layer was run into the bottom of the chamber in order to maintain saturated conditions in the air above it. Some batches of phenol were not suitable for use as purchased, since they gave rise to a heavy black discoloration with the $AgNO_3$-ammonia reagent. Such samples could be improved considerably by steam distillation, but in one case this treatment was insufficient and the sample was further treated by shaking at intervals for several days in a large bottle with 1% (w/v) ammonia solution until the mixture acquired a deep blue colour. After steam distillation the solvent was then found to be satisfactory.

When amino sugars were under investigation, it was necessary to carry out irrigation with phenol in an atmosphere containing ammonia, since otherwise these basic sugars gave rise to elliptical spots or trails. For this purpose, the aqueous layer in the bottom of the chamber was brought to a known concentration with NH_3 solution. However, when ammonia was used a few crystals of KCN were also added to the aqueous liquid, since catalytic oxidation processes take place under the influence of traces of copper (Consden *et al.* 1944), and, in the absence of a small concentration of HCN, the advancing phenol front on the paper becomes deeply stained with dark-coloured oxidation products.

s-Collidine. This solvent was purchased as a dark-coloured liquid. Bromine (5–10 ml.) was cautiously added to the solvent (1 l.) in a large unstoppered flask with frequent vigorous shaking; after removal of the precipitate by filtration crushed sodium thiosulphate was added with further shaking, and the liquid was again filtered. The solvent was then allowed to stand 24 hr. over solid NaOH and after filtration was distilled, a middle fraction boiling at 172° being retained. Prepared in this way, the liquid remained colourless for several weeks, and after saturation with water at room temperature was used for developing the chromatograms without further additions. (This method of purification was communicated privately by Dr A. J. P. Martin.)

n-Butanol-acetic acid mixture. The butanol was purified by distillation and the dry solvent (40 ml.) was mixed with glacial acetic acid (10 ml.) and water (50 ml.). After shaking the mixture, the aqueous layer was run into the bottom of a dry chamber, and the solvent layer used to fill the trough. Since the mixture is a three-component system the volume of water contained in it is important.

RESULTS

Table 1 shows the R_F values obtained with a number of neutral sugars in certain of the more suitable solvents and solvent mixtures. In general, it has been found that only those solvents that give rise to two phases with water lead to useful separations, and further that R_F values are generally higher the

Table 1. *R_F values of neutral sugars in various solvents on Whatman no. 1 paper (corrected to 20°)*

Solvent ...	Phenol	*s*-Collidine	*n*-Butanol, 40%* Acetic acid, 10% Water, 50%	*n*-Butanol, 45%* Ethanol, 5% Water, 49%	*n*-Butanol	*iso*Butyric acid	Methyl ethyl ketone
Additions	NH_3 (1% w/v), HCN	None	None	NH_3 (1% w/v)	NH_3 (1% w/v)	None	NH_3 (1% w/v)
D-Glucose	0·39	0·39	0·18	0·105	0·070	0·13	0·025
D-Galactose	0·44	0·34	0·16	0·090	0·060	0·14	0·015
D-Mannose	0·45	0·46	0·20	0·130	0·100	0·15	0·050
L-Sorbose	0·42	0·40	0·20	0·120	0·085	0·16	0·050
D-Fructose	0·51	0·42	0·23	0·135	0·100	0·18	0·045
D-Xylose	0·44	0·50	0·28	0·170	0·125	0·19	0·090
D-Arabinose	0·54	0·43	0·21	0·145	0·100	0·21	0·075
D-Ribose	0·59	0·56	0·31	0·210	0·180	0·22	0·165
L-Rhamnose	0·59	0·59	0·37	0·285	0·220	0·30	0·180
D-Deoxyribose	0·73	0·60	—	—	—	0·32	—
L-Fucose	0·63	0·44	0·27	—	—	—	0·095
Lactose	0·38	0·24	0·09	0·0	0·0	0·070	0·0
Maltose	0·36	0·32	0·11	0·15	0·01	0·085	0·0
Sucrose	0·39	0·40	0·14	—	—	—	—
Raffinose	0·27	0·20	0·05	—	—	—	—

* All percentages are v/v.

greater the water content of the solvent phase. Since the amount of water carried by the solvent phase is dependent upon temperature, mobilities are higher the nearer the temperature of the chamber approaches the critical solution temperature of the solvent. Thus when phenol is used, an increase in temperature gives rise to increasing R_F values, while in *s*-collidine the effect is reversed. The temperature effect becomes less marked at working temperatures well below the critical point, and when phenol or collidine is used normal variations in the temperature of the room during a run can be tolerated. It is, however, advisable to lag the chamber in order to reduce such temperature disturbances to a minimum.

Since the differences in temperature between summer and winter working affect the R_F values of all the sugars to approximately the same degree, a standard sugar (glucose) was included in every chromatogram, and all R_F values obtained at temperature $t°$ were then multiplied by a factor a_t, where

$$a_t = \frac{R_F \text{ value of glucose at } 20°}{R_F \text{ value of glucose at } t°}.$$

Applying this correction it has been found that R_F values obtained on the same batch of filter paper are remarkably constant in spite of the uncontrollable variations in other factors met with in day-to-day working.

The behaviour of a solvent may be influenced by the addition to it of a small quantity of a solvent more readily soluble in water. This increases the solubility of water in the new solvent phase, and thus effects a general increase in R_F values. Thus the addition of acetic acid to *n*-butanol leads to an increase in R_F values and, in this case, to an improvement in the separations obtained with some of the sugars.

The behaviour of sugar mixtures

The R_F value given by a neutral sugar is not substantially affected by the presence of other sugars at comparable concentration in the same solution, although small deviations have sometimes been noted in the chromatograms given by solutions containing four or five different sugars. Pl. 2 (*a*) shows a 40 hr. chromatogram obtained in collidine with a solution containing ribose, mannose, fructose, galactose, lactose and galacturonic acid, the concentrations being 1% (w/v) with respect to each sugar. For the purpose of reference the individual sugars were run alongside the mixture. The photograph in Pl. 2 (*a*) should be compared with Fig. 1 (p. 242), which illustrates the degree of separation obtainable by use of two solvents, phenol-1% (w/v) NH_3 and collidine, and has been obtained by plotting the R_F values of the sugars in phenol as ordinates and the values in *s*-collidine as abscissae.

Sensitivity. Pl. 2 (*b*) shows a chromatogram obtained in collidine with a solution containing glucose (1% w/v) and xylose (1% w/v). The solution was applied in amounts varying from 4 μl. to 0·5 μl. The R_F values obtained by measuring the centre of the spots show no systematic variation with the amount of sugar present and this has been found to be the general rule. The amount of sugar applied (5–40 μg.) covers the range over which the method is useful for general purposes, but with some sugars (particularly glucose) much smaller amounts may be detected, and for special purposes considerably

larger amounts of sugar have been applied without causing serious distortion of the spots.

Interfering substances. Substances other than sugars present in the test solution may interfere in a variety of ways, and for the purpose of clarity they may be classified into groups as follows:

(*a*) Reducing substances which react with the ammoniacal silver nitrate reagent.

(*b*) Substances which may have mobilities close to those of the sugars it is desired to identify and which may affect the R_F values of the sugars by 'salting out'.

togrums. Purines, creatine, creatinine and lactic acid and the common amino-acids were inactive, but inositol gave a weak brown spot developing at 110°, or at a rather lower temperature if a small concentration of NaOH was added to the spraying reagent. Ascorbic acid, gallic acid and tannic acid gave an immediate black coloration in the cold, and could readily be distinguished from the sugars by this means.

Interfering effects of class (*b*) were noticed in attempts to identify reducing sugars occurring in complex biological mixtures such as fruit juices or

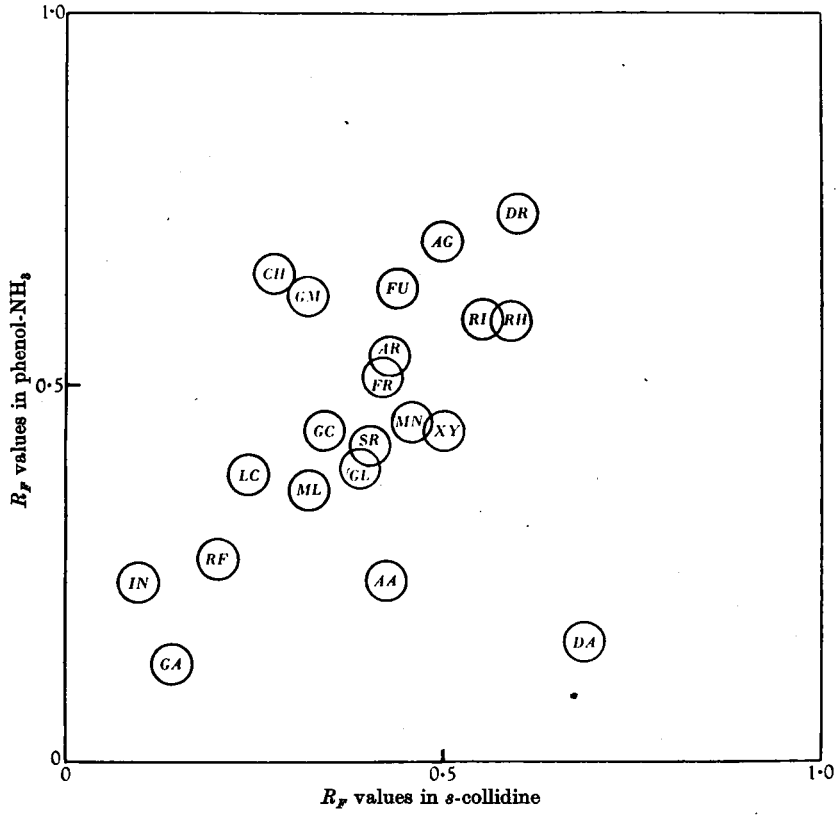


Fig. 1. Illustrating the separation obtainable by use of two solvents. The R_F values in phenol-1% NH_3 and *s*-collidine are plotted at right angles. *AA*, ascorbic acid; *AG*, acetylglucosamine; *AR*, arabinose; *CH*, chondrosamine; *DA*, dehydroascorbic acid; *DR*, deoxyribose; *FR*, fructose; *FU*, fucose; *GA*, galacturonic acid; *GC*, galactose; *GL*, glucose; *GM*, glucosamine; *IN*, inositol; *LC*, lactose; *ML*, maltose; *MN*, mannose; *RF*, raffinose; *RH*, rhamnose; *RI*, ribose; *SR*, sorbose; *XY*, xylose.

(*c*) Inorganic salts, which in addition to causing effects as under (*b*), may also be involved in reactions of the ion-exchange type leading to the localization of strong acids or bases which give rise to silver-reacting spots by modification of the cellulose of the filter paper.

Numerous substances occurring in tissue extracts were tested for their reaction with $AgNO_3$-ammonia under the conditions used in developing the chromatograms. In chromatograms obtained with the untreated extracts it was not uncommon to find that the spot due to a reducing sugar was badly distorted, or displaced by substances which revealed themselves as white spots or trails on the cream or buff-coloured ground of the chromatograms. As described below, reliable results could be obtained with such extracts only if steps were taken to remove the electrolytes, preferably by

muscle press juice.

means of ion-exchange materials. However, the presence of such substances as the amino-acids could usually be tolerated provided their concentrations were not much greater than those of the sugars under test; this was particularly so if the non-sugar substances had R_F values either above or below the range occupied by the sugars.

The action of the paper chromatogram in separating the ions of inorganic salts is described in more detail by Westall in the note attached to this communication. When sufficient of the test solution was available the effect could be eliminated by making use of ion-exchange materials, and, in fact, the preliminary removal of all but small concentrations of electrolytes proved to be a necessary step before commencing the analysis of any unknown solution. With the hydrolysate of a pure carbohydrate, where the mineral acid may be removed quantitatively, the presence of interfering substances was not to be expected, and, in fact, in the example given in the second part of this communication (this vol. p. 251), the analysis of such a carbohydrate was uncomplicated by interference effects.

Reducing sugars with acidic or basic properties

The behaviour of the neutral reducing sugars on the filter-paper chromatograms was apparently uncomplicated by the various tautomeric equilibria known to occur in their aqueous solutions, and the spots revealed by the $AgNO_3$-ammonia reagent showed no tendency to trail. However, a few of the more common sugar derivatives with basic or acidic properties were investigated, and with some of these substances the products of reversible reactions taking place at or near room temperature showed some separation on the chromatograms. The results obtained with the sugar acids and amino sugars are given below; R_F values in the more useful solvents are shown in Table 2.

Glucuronic acid and its lactone. The source of glucuronic acid was L-menthol-D-glucuronide prepared biosynthetically by the method of Williams (1939). A sample of crystalline glucurone, $[\alpha]_D + 19{\cdot}7$, m.p. 178–180°, was prepared from this substance by hydrolysis with 0·2N-sulphuric acid according to the method of Quick (1927). The first hydrolysis product is a mixture containing glucuronic acid and its lactone in the proportion of 7 : 3, but crystallization from hot glacial acetic acid favours lactone formation and the product after two recrystallizations is the pure lactone.

The mixture of glucurone and glucuronic acid formed by first hydrolysis when applied to the filter-paper chromatogram gave rise to two well separated spots after development with the solvents (Pl. 3 (*a*) and (*b*)). The slower moving spot was due to glucuronic acid and was usually heart-shaped or elliptical, sometimes appearing with a marked forward trail. The spot due to glucurone had a high R_F value in all solvents and was invariably circular. The glucurone spot, however, developed more slowly than glucose after spraying with the silver reagent, and for this reason might easily be missed in the examination of the chromatograms.

Crystalline glucurone gave a single circular spot of high R_F in acidic solvents, but in basic solvents the spot was often accompanied by a backward trail, indicating conversion to glucuronic acid during the course of the run (Pl. 3 (*b*)). In the presence of ammonia vapour from a 1 % (w/v) aqueous ammonia solution, phenol behaves as a basic solvent, and the ammonia concentration is sufficient to complete the conversion of glucurone to the salt of glucuronic acid. Under these conditions the spot due to glucurone was entirely absent, the application of solutions of crystalline glucurone giving rise to the glucuronic acid spot accompanied by a forward trail.

Glucuronic acid reduced the $AgNO_3$-ammonia reagent very rapidly at 100°, but the lactone reduced only slowly at 110°. For this reason, when the lactone was suspected to be present, NaOH solution was added to the $AgNO_3$-ammonia reagent to bring the concentration of the NaOH to 0·05N. Glucurone was thereby converted on the filter paper to the sodium salt of glucuronic acid which rapidly reduced the silver reagent. Development of the colour was carried out at 110°, but the paper could not be heated longer than 3–5 min. because of excessive darkening. Butanol-acetic acid mixture was found the most suitable solvent for mixtures containing glucurone; the silver reagent containing sodium hydroxide was not suitable for use with phenol as the chromatographic solvent.

Galacturonic acid. D-Galacturonic acid does not readily form a lactone, and the crystalline acid gave rise to a single spot of low mobility which, like that due to glucuronic acid, was usually heart-shaped and often showed a short forward trail. Glucuronic acid and galacturonic acid may be differentiated by the formation of the glucurone spot by the former. R_F values are given in Table 2.

Glucosamine and chondrosamine. In basic solvents (phenol-NH_3 and collidine) the hexosamine hydrochlorides travelled as well defined circular spots which rapidly reduced the silver reagent at low temperatures (90°). In neutral or acidic solvents, however (phenol, butyric acid, butanol-acetic acid), the hexosamines appeared after development as brown spots with black forward trails. The trails reacted with the silver reagent at low temperatures and appeared much more rapidly than the spots during development. It appears, therefore, that the hexosamines travel as the free base under alkaline conditions, but in neutral or slightly acidic solvents give rise to equilibria between free base and cation which

result in partial separation, the free base travelling faster than the ion.

The Morgan & Elson reagents described above may be used to show the position of the spots due to the hexosamines, and in cases where the hexosamine spot is in close proximity to that due to another sugar, the specific reaction for the former is of considerable value.

N-Acetylglucosamine. This substance had a high mobility in most solvents, but gave a rather weak reaction with the ammoniacal silver nitrate reagent. However, the reaction with *p*-dimethylaminobenzaldehyde described above was very sensitive and also specific for the *N*-acetylhexosamines, and could be used with advantage wherever the *N*-acetyl derivatives were suspected.

Ascorbic acid. Freshly prepared aqueous solutions (1 % w/v) of synthetic L-ascorbic acid were used. In phenol-ammonia, collidine and *n*-butanol-acetic acid, ascorbic acid gave well characterized spots which produced an immediate black colour on spraying with the $AgNO_3$-ammonia reagent in the cold. The presence of HCN was necessary when using phenol-ammonia as the chromatographic solvent in order to suppress catalytic oxidation of the ascorbic acid. The values for R_F found are given in Table 2.

solution had $[\alpha]_D + 53°$ (*l*, 2·0) (calculated on the weight of ascorbic acid taken) 30 min. after preparation. The solution was then titrated with N-NaOH to the temporary end point (phenolphthalein) in order to neutralize the mineral acid formed during the oxidation but to avoid salt formation with dehydroascorbic acid and consequent rupture of the lactone ring.

(*c*) A neutralized solution of dehydroascorbic lactone was prepared as in method (*b*), but the addition of N-NaOH was carried past the temporary end point until a permanent colour with phenolphthalein was obtained. The addition was carried out at room temperature. The solution contained the sodium salt of dehydroascorbic acid and was yellow in colour.

When freshly prepared the solutions (*a* or *b*) containing the lactone and that (*b*) containing the sodium salt of the free acid gave the same chromatogram, all three solutions yielding a single purple-brown spot which developed rapidly with the $AgNO_3$-ammonia reagent at 80–90°. This result was unexpected since glucuronic acid-glucurone mixtures gave rise to two distinct spots, widely separated in R_F value. However, inspection of Table 2 shows that the R_F values of dehydroascorbic acid in different solvents are markedly anomalous, since mobility is

Table 2. *R_F values of a number of substances related to the sugars on Whatman no. 1 paper (corrected to 20°)*

Solvent	Phenol	*s*-Collidine	*n*-Butanol, 40%* Acetic acid, 10% Water, 50%	*iso*Butyric acid
Additions	1% NH_3, HCN	None	None	None
D-Galacturonic acid	0·13	0·14	0·14	0·09
D-Glucuronic acid	0·12	0·16 [0·72]†	0·12 [0·32]†	0·08 [0·22]†
D-Glucurone	0·12	— [0·72]†	— [0·33]†	— [0·22]†
D-Glucosamine hydrochloride	0·62	0·32	0·13 [0·17]‡	0·05 [0·20]‡
Chondrosamine hydrochloride	0·65	0·28	0·12 [0·16]‡	— [0·19]‡
N-Acetylglucosamine	0·69	0·50	0·26	0·25
L-Ascorbic acid	0·24	0·42	0·38	0·19
Dehydroascorbic acid	0·16	0·68	0·27	0·16
i-Inositol	0·23	0·10	0·09	—

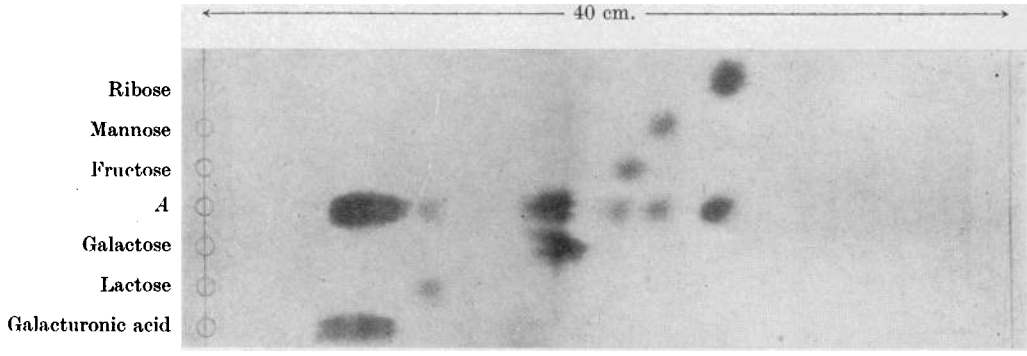
* All percentages are v/v.
† Values in brackets [ ] due to the lactone.
‡ Values in brackets [ ] due to free base; the two spots were connected by a trail.

Dehydroascorbic acid. Dehydroascorbic acid was prepared by oxidation of ascorbic acid with iodine according to the methods given by Herbert, Hirst, Percival, Reynolds & Smith (1933).

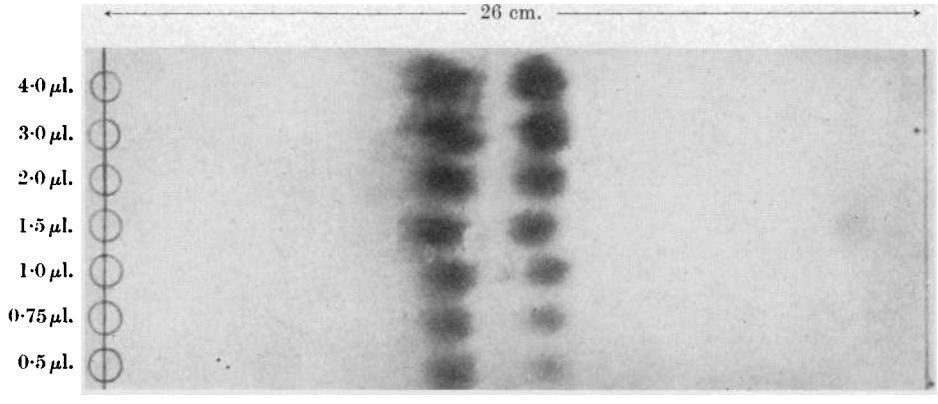
(*a*) An aqueous solution of ascorbic acid was oxidized with the calculated amount of iodine dissolved in ethanol, the addition being made at room temperature.

(*b*) An aqueous solution of ascorbic acid (10 ml. of 0·2M) was titrated with 0·2N-iodine in aqueous KI solution until the end point was reached. The

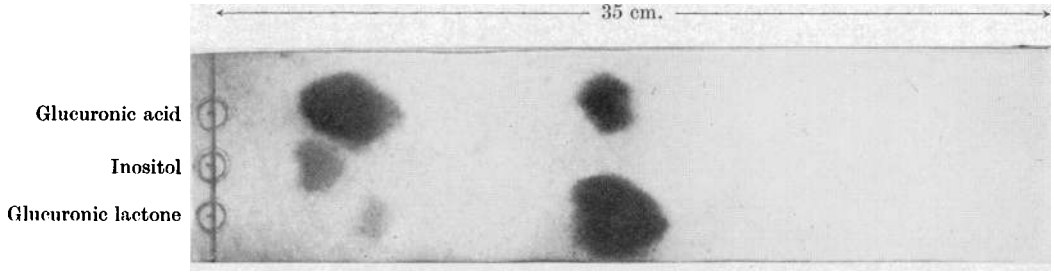
very high in collidine, but low in phenol-NH_3, and it is possible that the components of the system are too rapidly interconvertible to allow of the complete separation of either during the course of the run. In such a system the R_F value of the mixture would be expected to lie near that of the predominant component, and would therefore be markedly affected by any alteration in the solvent likely to disturb the equilibrium between the two components either in the solvent phase or in the water phase. The spots due to dehydroascorbic acid were usually



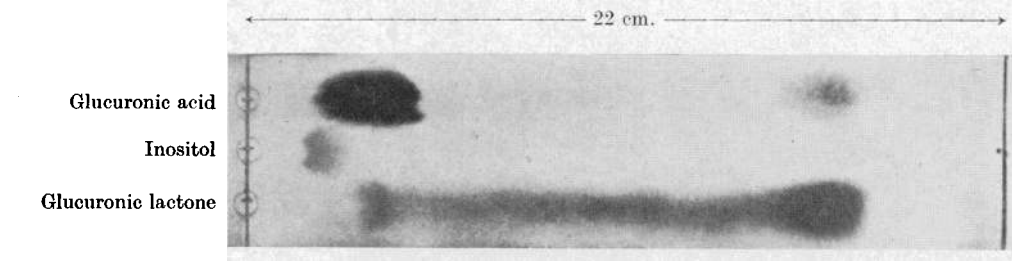
(*a*) Filter-paper chromatogram of a mixture containing six sugars. The mixture was introduced in the circle marked '*A*' and the individual sugars were placed in the other circles as indicated. The chromatogram was run 40 hr. in *s*-collidine.



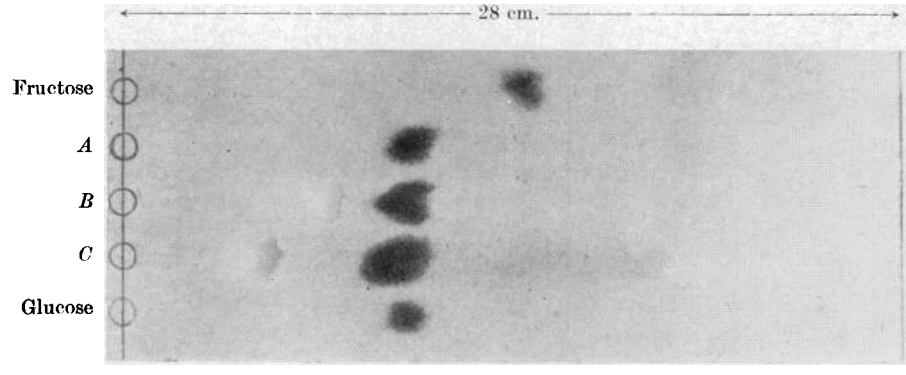
(*b*) Filter-paper chromatogram of a solution containing glucose (1% w/v) and xylose (1% w/v). The volume of solution applied to each circle is indicated. The chromatogram was run 20 hr. in *s*-collidine.



(*a*) Chromatogram run in butanol-acetic acid mixture. The chromatogram was sprayed with ammoniacal silver nitrate solution containing NaOH to a concentration of 0·05 N.



(*b*) Chromatogram run in *s*-collidine and sprayed with ammoniacal silver nitrate solution containing NaOH to a concentration of 0·05 N.



(*c*) Chromatogram of an extract of egg white in aqueous ethanol. *A*, extract treated with 'Zeo Karb 215' and 'Deacidite'. *B*, extract treated with 'Zeo Karb' only. *C*, untreated extract. The chromatogram was run 18 hr. in phenol-1 % NH_3.

elliptical and showed marked forward trailing in collidine, but they were well separated from those due to ascorbic acid in most solvents.

Solutions (*a*) and (*c*) were unstable, and after keeping for 24 hr. gave traces of a second reducing substance yielding a slow-moving spot in collidine and butanol-acetic acid. This spot gave a brown colour with the silver reagent, but required heating to 100–110° for colour development. Solution (*a*) regenerated ascorbic acid on keeping for a few days at +2°, the solution giving rise to a third, small, but well defined spot which oxidized the silver reagent in the cold, and had an R_F value identical with that of ascorbic acid.

Inositol. The sample of *i*-inositol gave no reduction with alkaline ferricyanide (Hagedorn & Jensen, 1923) and was nitrogen-free. It was observed, however, that the substance gave a slow reduction with the $AgNO_3$-ammonia reagent under the ordinary conditions used in developing the paper chromatograms. The rate at which reduction took place was increased when NaOH was added to the $AgNO_3$-ammonia reagent to a concentration of 0·05N, but even under these conditions the brown spot due to inositol was much less intense than that due to an equivalent concentration of a reducing sugar.

The mobility of inositol was low in all solvents (R_F values are given in Table 2), and consequently it was readily distinguished from aldoses and ketoses; but since the colour obtained with the silver reagent was not strong it was easily missed in the examination of chromatograms, particularly if present in the test solution in low concentration.

Application of method to extracts of biological origin

The object of this investigation was to establish a method for the qualitative analysis of polysaccharide hydrolysates. In such liquids the problem is relatively simple, since once the free mineral acid has been removed the solution is likely to be relatively free from salts, organic acids and other substances which might interfere. It was, however, considered of interest to explore the chromatographic analysis of a few examples of extracts of biological origin known to contain reducing sugars. The results of three of these investigations are given below.

Detection of glucose, fructose and sucrose in apple juice. An apple-juice extract was prepared from Bramley's Seedling apples and ethanol was added to a concentration of 80% (v/v). After standing a few days at +2°, the solution was filtered, and the clear supernatant (500 ml.) retained for the analytical experiments. Standard methods of analysis showed: fructose, 0·34%; glucose, 0·25%; sucrose, 0·07%; malic acid, 0·40%; total dry solids, 2·45%.

Part of the ethanolic solution was evaporated to small bulk under reduced pressure and made up with distilled water so that the resulting concentration was five times the original. This solution was clarified by centrifugation and used for the chromatographic experiments. In the description of the results which follows, the runs are classified according to the solvent used. This was usually neutral or alkaline phenol, since the best separation between glucose and fructose is obtained with phenol as the solvent.

(*a*) *Solvent, phenol; additions, none.* Brown spots of correct mobility for glucose and fructose could be distinguished readily, but they were much distorted in shape by the presence of other material of similar R_F value. Among the interfering substances observed were two well defined spots due to pigments of R_F 0·59 and 0·49, which gave transitory green or brown colours in the cold on spraying with $AgNO_3$-ammonia. In addition to the pigments there were a number of substances of low mobility which gave an immediate black colour in the cold on spraying with the $AgNO_3$-ammonia reagent. These were afterward shown to be tannins. There were further substances giving spots or trails which appeared colourless against the pale grey or cream colour of the paper background.

(*b*) *Solvent, phenol; additions,* NH_3 1% (*w/v*), KCN. The appearance of the chromatograms was much improved in this solvent. Most of the acidic interfering substances had now very low mobilities due to the presence of the ammonia, and therefore did not interfere with the development of the spots due to the reducing sugars. Glucose and fructose gave well defined spots of correct R_F value, and the black colour due to the tannins was confined to a short trail springing from the position originally occupied by the test solution. However, since it was desired to test for the presence of other reducing sugars possibly present in small amounts, further runs were carried out with higher concentrations of the test solution. These were rather unsatisfactory, due to the simultaneous increase in concentration of interfering substances. For this reason an attempt was made to utilize ion-exchange reagents to remove acids, bases and salts while leaving the non-electrolytes in solution.

Use of ion-exchange columns. 'Zeo-Karb 215'* was used for removal of bases and cations and 'Deacidite'* for removal of acids. A portion (100 ml.) of the 80% ethanolic apple-juice extract was evaporated to small bulk, and made up again to its original volume with distilled water in readiness to pass through the columns. In order to minimize the possibility of adsorption of sugars by the ion-exchange resins, sufficient of the test solution was

* Both these reagents may be obtained from Permutit Co. London.

passed to saturate the deacidite column with respect to anions, and the first runnings from both columns were rejected.

The columns were prepared by taking sufficient of the wet ion-exchange material to make up a volume of 22 ml. This gave a column 16 cm. in height when the material was packed into the filtration tubes. The deacidite was activated with 2N-NaOH solution, followed by thorough washing, while 2N-HCl solution was used to activate the zeo-karb. The test solution was passed slowly through the zeo-karb column and immediately transferred to the deacidite column. The pH of the solution flowing from the second column was followed by means of indicators, and the experiment was stopped when the reaction fell to pH 6·5, indicating approaching saturation of the deacidite. In all, 90 ml. of the solution were passed through both columns in this way, the first 15 ml. run from either column being rejected.

A portion (50 ml.) of the effluent was concentrated to 5 ml., and the small precipitate which formed was removed by centrifugation. The concentrated effluent solutions were tested by the filter-paper technique using phenol-NH_3, butanol-acetic acid mixture and collidine as solvents. The developed papers indicated freedom from interfering substances, and the R_F values given by glucose and fructose in the concentrates were not detectably different from those given by authentic glucose and fructose solutions run on the same chromatogram.

In order to ascertain if adsorption of reducing sugars by the columns was likely to be a serious consideration in further applications of the technique, the experiment was repeated under identical conditions except that 10 ml. of the test solution instead of 90 ml. were passed down the columns, and no part of the effluent was rejected. The relative intensity of the glucose and fructose spots was unaffected in the resulting chromatograms, and from this it was concluded that for qualitative purposes the method was satisfactory as far as glucose and fructose were concerned.

Detection of sucrose. Chromatograms were set up with the concentrated effluent from the ion-exchange columns used for the detection of glucose and fructose. Phenol and butanol-acetic acid were used as solvents and fructose, glucose and sucrose were included as reference sugars. After removal of the solvent by evaporation the chromatograms were sprayed with the naphthoresorcinol-trichloroacetic acid reagent as described in the experimental section. The spots due to fructose and sucrose in the mixture gave strong red colorations and had R_F values identical with those of the reference sugars. Glucose gave no colour with this reagent.

Detection of glucose in the white of egg. The white was separated from a hen's egg taken a few hours after laying. After dilution with water to 100 ml., absolute ethanol (400 ml.) was added with stirring and the precipitate allowed to settle. The coagulated proteins were filtered off and washed with a small volume of 80 % (v/v) aqueous ethanol. The ethanolic extract was then allowed to stand for a few days at +2°, when it was refiltered and evaporated to 60 ml. under reduced pressure. Part of the concentrated solution (20 ml.) was further evaporated under reduced pressure to 2 ml. (solution (*a*)). A second part (40 ml.) was passed through a column packed with activated 'Zeo-Karb 215' (wet volume 9 ml.) and the first 5 ml. of the effluent rejected. The effluent solution was then divided into two equal parts and one portion concentrated to 2 ml. (solution (*b*)). The remainder of the solution was passed through a column packed with prepared 'Deacidite' (wet volume 10 ml.), the first 6 ml. of the effluent being rejected and the remainder concentrated to 2 ml. (solution (*c*)). Passage through the zeo-karb column removed a yellow pigment from the solution and CO_2 was evolved; the effluent from the deacidite column was clear and colourless.

Chromatograms were prepared using phenol-NH_3 and butanol-acetic acid mixture as solvents, the three solutions being run side by side on the same paper strip. All three solutions showed well characterized spots corresponding to glucose (Pl. 3 (*c*)); fructose was not found. Grey-brown irregular spots due to interfering substances were visible with (*a*) and with (*b*) but were absent from (*c*).

Detection of fructose, glucose and inositol in the foetal blood of the sheep. The presence of fructose as well as glucose in the foetal blood of the sheep was demonstrated by Bacon & Bell (1946) who isolated the 2 : 3, 4 : 5 diacetone derivative of fructose (Bell, 1947). A laevorotatory sugar had previously been reported by Paton, Watson & Kerr (1907). An extract of whole foetal blood that had been deproteinized by ethanol-chloroform treatment was kindly supplied by Dr J. S. D. Bacon. This was prepared from 50 ml. of blood and contained, in addition, water (150 ml.), ethanol (100 ml.) and chloroform (50 ml.). The solvents were removed by evaporation and the solution made up to 40 ml. A portion of the aqueous solution (20 ml.) was passed through a column containing activated zeo-karb (3 g.) and then through deacidite (5 g.), following the procedure described above for white of egg. The salt-free effluent from the columns was concentrated under reduced pressure to a volume of 2–3 ml., after which it was evaporated to dryness under vacuum at room temperature and the dry residue made up to 0·25 ml. with distilled water.

Chromatograms in three solvents, phenol, collidine and butanol-acetic acid, showed the presence of glucose, fructose and inositol when sprayed with the ammoniacal silver nitrate reagent. Since the

spot due to inositol was light in colour, further chromatograms were sprayed with ammoniacal silver nitrate containing NaOH to a concentration of 0·05N, and in these the inositol showed strongly in the mixture and had R_F values identical with the values given by authentic specimens. Further chromatograms run in phenol and butanol-acetic acid were sprayed with naphthoresorcinol-trichloroacetic acid mixture. These showed the presence of fructose as red spots of R_F value identical with those due to authentic specimens of the sugar.

The procedure given above was repeated with samples of blood from three normal adult sheep (not pregnant), and in each case glucose only was found; fructose and inositol appeared to be absent or in quantities too small to be detected.

DISCUSSION

In Fig. 1 the R_F values of the reducing sugars in phenol-NH_3 have been plotted as ordinates and the values in *s*-collidine as abscissae. The circles drawn around each point give an approximate indication of the relative size of the coloured spot normally encountered, so that the figure gives a general indication of the degree of separation obtainable with the two solvents.

With two solvents only, complete separation between several pairs of sugars is not obtainable, but other solvents and solvent mixtures allow of further separation. A useful third solvent is *n*-butanol-acetic acid mixture, and experience has shown that it is usually necessary to run chromatograms with at least three solvents if unambiguous identifications are to be ensured.

With none of the solvents tested has complete separation been obtained on 18–20 hr. chromatograms between sorbose and glucose, arabinose and fructose or chondrosamine and glucosamine, so that clear identification is not yet possible when both sugars of any of these pairs appear in a solution together. However, when only one sugar of such a pair is present the separation is usually sufficient for successful identification by reference to the R_F values in several solvents.

Some inference as to the effect of chemical constitution on R_F values may be drawn from the spread of the sugar spots as displayed in Fig. 1. In general, as would be expected, the more strongly hydrophilic substances are the more slowly transported. The carboxylic acid group is particularly effective in giving rise to a low R_F value as indicated by the slow movement in both solvents of the uronic acids. When an —OH group is replaced by —NH_2 as in the hexosamines, a higher R_F value results with phenol but not with collidine. Still higher values with both solvents may be obtained by replacement of —OH by H as in the deoxysugars.

The aldohexoses occupy roughly the centre of the scale, but overlap with the ketoses. The pentoses appear to be rather faster and the disaccharides maltose and lactose are slower. However, it is characteristic of the results that the sugars most difficult to separate are not necessarily members of the same group in the usual chemical classification. The behaviour of dehydroascorbic acid appears to be anomalous, since it is slow in phenol, but has a very high mobility in collidine. This may be due to the intervention of the lactone structure.

The bearing of various external conditions upon the reproducibility of the R_F values of the amino-acids has been discussed by Consden *et al.* (1944), and since the same considerations apply with equal force to the behaviour of reducing sugars the subject need not be considered in detail here. It is sufficient to point out that variations in R_F value due to changes in temperature and other factors may be considerable, and in the analyses described in the experimental section identification of the components of a mixture was always based on the R_F values of authentic specimens of the sugars when run side by side on the same filter-paper strip. In order to compensate for temperature variations, a standard sugar (glucose) was put up on every chromatogram, and all R_F values were corrected to 20° by reference to the standard sugar.

It is apparent from inspection of Fig. 1 that where more than a small number of reducing sugars appear in the same solution there would be an advantage in carrying out two-dimensional chromatograms, following the technique described by Consden *et al.* (1944). A few such chromatograms have been prepared, the spot containing the mixed sugars being developed in one direction with phenol and afterwards in a direction at right angles with collidine. The neutral reducing sugars gave encouraging results, but it was found that in the case of amino sugars too much spreading of the spot took place, with the result that the sugars could not be identified accurately by reference to their R_F values. The two-dimensional technique appears to be less reliable than the filter-paper strip method where the test solution contains a small concentration of one component, and since more than two or three different sugars are rarely found in natural mixtures, it has generally been considered preferable to apply the filter-paper strip method rather than the more time-consuming two-dimensional technique.

The R_F values given by the sugars are little affected by the presence of other sugars even though they may appear in the test solution in much larger quantities; in one experiment, glucose showed its normal R_F value in the presence of a concentration of lactose 50 times greater. Interference effects by electrolytes however are serious, and in carrying out the analysis of solutions of biological origin con-

taining sugars it is almost always necessary to effect the prior removal of salts, acids and bases. A convenient way in which this may be done is by making use of ion-exchange reagents. For this purpose two synthetic ion-exchange resins were found to be suitable. Cations may be removed from neutral solution by passage through a column of the Permutit product 'Zeo-Karb 215' (Permutit Co., London), while the anions may be removed from the resulting acidic solution by similar treatment with 'Deacidite'.

The neutral reducing sugars give rise to essentially circular spots with sharp edges under the conditions described, and although various tautomeric reactions are possible with these substances, such reactions do not appear to affect the transport of the sugar as a single component. This is not altogether the case with the amino sugars or the uronic acids. The hydrochlorides of glucosamine and chondrosamine form short trails with acidic or neutral solvents but give well formed spots under conditions of sufficient alkalinity to suppress the cation. The uronic acids show a tendency to form heart-shaped spots with basic solvents, and in the case of glucuronic acid behaviour is complicated by the presence of a slow reversible reaction between free glucuronic acid and its lactone. In acidic solvents solutions of glucuronic acid give rise to two well separated spots, one of them due to the lactone. Galacturonic acid, on the other hand, does not readily form a lactone, and for this reason it can be differentiated from glucuronic acid in spite of the fact that the R_F values of the free acids lie close together.

A number of compounds not strictly belonging to the sugar series were briefly investigated, since they may be met with in the analysis of extracts of biological origin. Three such compounds were ascorbic acid, dehydroascorbic acid and inositol; however, it was found that although all three substances travelled as fairly well defined spots, the R_F values were well outside the range occupied by the reducing sugars. Ascorbic acid showed a very characteristic reaction with the ammoniacal silver nitrate reagent, giving an immediate black coloration in the cold, while, on the other hand, inositol reduced the silver reagent very slowly at 110°, and could only be detected when present in rather high concentration. As examples of the application of the technique, a number of extracts of biological origin have been examined, and three such analyses are reported in detail in the experimental section. Glucose, fructose and sucrose were identified in an 80 % (v/v) ethanolic extract of Bramley's Seedling apples, while an examination of the soluble sugars of fresh white of egg showed the presence of glucose only, no fructose being detected. In an ethanol-chloroform extract of whole blood from the foetus of a sheep the presence of fructose and glucose was confirmed, and in addition inositol was found. In all three cases it was necessary to remove interfering electrolytes by treatment with ion-exchange reagents.

The second part of this paper (this vol. p. 251) describes two examples of the application of the method to the analysis of the hydrolysis products of polysaccharides.

SUMMARY

1. A method is described for the identification of reducing sugars in mixtures in which use is made of the filter-paper chromatogram introduced by Consden *et al.* (1944).

2. The use as a spraying reagent of a solution of silver nitrate in ammonia was found to be effective as a general method for revealing the position of the sugars on the chromatogram, and other spraying reagents useful in special circumstances are described.

3. The behaviour on the filter-paper chromatogram of twenty-two sugars and sugar derivatives has been investigated using several solvents, and the degree of separation obtained has been indicated.

4. The effect of interfering electrolytes is discussed, and a method of removing them by application of ion-exchange reagents is described.

5. As examples of the technique, the analysis of three extracts of biological origin is described; glucose only was found in an 80 % (v/v) ethanolic extract of fresh white of egg, while glucose, fructose and sucrose were shown in a fraction from Bramley's Seedling apples. The presence of glucose, fructose and inositol was shown in an extract from the foetal blood of the sheep.

The author wishes to express his thanks to Dr D. J. Bell and Dr J. S. D. Bacon for their interest in the work, and for the gift of some of the specimens of pure sugars used in this investigation.

This work forms part of the programme of the Food Investigation Board of the Department of Scientific and Industrial Research.

Correction added 12 March 1948

The R_F values for dehydroascorbic acid given in Table 2 above were noted to be anomalous and reinvestigation has revealed an error due to the interference of I′ from NaI in the solution. Although NaI gives no brown colour when sprayed with $AgNO_3$-ammonia under the conditions used, it has now been shown that on irrigation with the chromatographic solvents the ions of the salt migrate at different speeds, each forming a discrete spot on the paper. On spraying with $AgNO_3$-ammonia the reagent reacts with HI derived from I′ to give an intense purple-brown colour. Cl′ is inactive, but Br′ gives a blue-grey colour when present in high concentration.

The R_F values for dehydroascorbic acid should be corrected as follows: collidine, 0·47; phenol (additions: NH_3 (1%), HCN) 0·28; butanol-acetic acid, 0·48.

Addendum. Note on the Behaviour of Inorganic Salts on the Filter-paper Partition Chromatogram

By R. G. WESTALL, *Low Temperature Station for Research in Biochemistry and Biophysics, Cambridge*

During the course of some work on the dialyzable fraction of fresh rabbit muscle, the chromatographic method described in this paper was applied to the identification of the reducing substances. Application of the routine procedures detailed above led to the expected identification of glucose, but in addition an unknown substance was present which gave rise to a heavy spot on the chromatogram after spraying with $AgNO_3$-ammonia. The substance had an R_F value of 0·59 in phenol-1% NH_3, 0·09 in *s*-collidine and 0·20 in butanol-acetic acid mixture and was adsorbed by the cation exchange reagent 'Zeo-Karb 215'. It was therefore considered to be basic in character.

The behaviour on the filter-paper chromatogram of a number of bases known or suspected to be present in muscle juice was then investigated. The R_F values and the spraying reagents used to reveal the positions of the spots are given in Table 1. The strong bases spermine and spermidine gave brown spots when treated with $AgNO_3$-ammonia, but it was also found that strong inorganic bases, which would not be expected to reduce silver nitrate, gave similar brown colorations. In order to demonstrate this effect, various dilutions of NaOH and KOH were introduced in spots as duplicates on two strips of filter paper. One strip was heated at 105° for 1 hr. (the normal time allowed to remove the solvent from a filter-paper chromatogram), while the other was not heat treated. Both strips were sprayed with $AgNO_3$-ammonia and placed in the oven for 10 min. to develop the colour. The spots on the heat-treated paper reacted strongly to the silver reagent, whilst those on the control strip reacted only slightly. From this it appeared that the effect of the alkali was to modify the cellulose of the paper during the heat treatment required to remove the solvent from the chromatogram. This resulted in the liberation of reducing substances that were responsible for the reduction of the silver reagent.

Table 1. *R_F values of some substances present in muscle*

Substance	Phenol-NH_3	*s*-Collidine	Butanol-acetic acid	Reaction with $AgNO_3$-NH_3	Reagent used for spraying	Colour of spot
Anserine	0·92	0·10	0·18	0	Ninhydrin	Red-brown
Carnosine	0·83	0·09	0·20	0	Ninhydrin	Red-brown
Creatine	0·82	—	—	0	Picric acid reagent	Orange
Creatinine	0·94	—	—	0	Picric acid reagent	Orange
Urea	0·73	0·44	0·43	0	Ehrlich's reagent	Grey
Spermine	0·95	0·01	0·03	+	Bromothymol blue	Blue
Spermidine	0·95	0·03	0·07	+	Bromothymol blue	Blue
Glucose	0·39	0·39	0·18	+	$AgNO_3$-NH_3	Brown
NaCl-KCl	0·59	0·09	0·20	+	Bromothymol blue	Blue

Neutral inorganic salts, when introduced on to a test sheet of filter paper, gave no colour with $AgNO_3$-ammonia after similar heat treatment, but if applied to a chromatogram and irrigated with phenol-1% NH_3 they gave rise to heavy black spots after removal of the solvent and spraying with the reagent. This effect appeared to be due to hydrolysis of the salt with separation of the resulting strong acid and strong base. It was found that the area containing free base could be demonstrated by application of a number of common indicator dyes, a solution of bromothymol blue in a mixture of butanol and ethanol being particularly useful as a spraying agent. Solutions (0·01 M) of NaCl, KCl, $MgCl_2$, $CaCl_2$ and $BaCl_2$ were applied as spots to two filter-paper strips, and both strips were irrigated with phenol-1% NH_3 (18 hr.). After removal of the solvent at 105°, one strip was sprayed with bromothymol blue solution and the other with $AgNO_3$-ammonia. The paper sprayed with the indicator showed a series of dark blue spots on a green ground, while that sprayed with the silver reagent gave a corresponding series of brown spots. The spots had short backward trails, but the leading edges were sharp. There was little differentiation of R_F values, all the salts giving rise to alkaline areas at R_F 0·55–0·58. The amount of salt applied to the chromatogram was very small, being in each case not more than 2–4 μg.

Not all indicator dyes were suitable for use in showing the presence of acids and bases on the chromatograms, since the behaviour of the dyes appeared to be affected by the cellulose itself, and by the presence on it of residual traces of the solvent used in developing the chromatogram. Suitable dyes were found by trial and error. Bromothymol blue

was satisfactory for strong bases both with phenol-1% NH_3 and with collidine as chromatographic solvents; in the case of collidine chromatograms the alkaline spots showed blue on a yellow ground.

The identity of the unknown spot on the chromatograms of muscle juice and the spots given by chlorides of sodium and potassium was shown by a comparison of the chromatograms in three different solvents, phenol-1% NH_3, collidine and butanol-acetic acid mixture. When the solutions were developed side by side on the same paper strips, identical R_F values were obtained. The spot given by the inorganic salts in muscle juice was sufficiently intense to react strongly with the bromothymol blue indicator, and this reagent has since been used to confirm the basic character of other spots suspected as being due to interference by inorganic salts. However, for the purpose of identifying the reducing sugars in a mixture, it is more satisfactory to ensure the removal of all but traces of inorganic salts by preliminary treatment with suitable ion-exchange reagents.

The process of separation of the ions of a salt on the paper chromatogram may be regarded as analogous with that occurring on ion-exchange reagents, and is of particular interest since both products of hydrolysis of the salt appear as narrow moving bands.

A simple explanation of the phenomenon lies in a consideration of the equilibrium

$$NaCl + C_6H_5OH \rightleftharpoons C_6H_5ONa + HCl.$$

Here, undissociated sodium phenate would be expected to have a high R_F value in phenol, and the equilibrium would be moved to the right as a result of transport of sodium phenate out of the system. A mechanism of this sort is rendered plausible, since in phenol-NH_3 and butanol-acetic acid the sodium ion travels farther than the chloride ion, while in the basic solvent collidine the reverse is the case. Table 2 shows the separation of sodium and chloride ions obtained with a number of solvents, and it will be observed that in phenol-NH_3 the R_F values of Na^+ and Cl^- from sodium chloride are identical with the values given by Na^+ and Cl^- from sodium hydroxide and hydrochloric acid. In butanol-acetic acid and *s*-collidine, separation is not so complete, and the spots due to Na^+ and Cl^- from NaCl occupy intermediate positions between those due to the same ions from NaOH and HCl. The presence of ammonia vapour has a marked effect on the separation taking place with phenol as solvent, since in its absence separation is very poor, and with sodium chloride only light trails due to NaOH and HCl are detectable.

Table 2. *R_F values given by* Na^+ *and* Cl^- *with various solvents*

	Phenol-NH_3		*s*-Collidine		Butanol-acetic acid	
	(Na^+)	(Cl^-)	(Na^+)	(Cl^-)	(Na^+)	(Cl^-)
NaOH	0·61	—	0·07	—	0·23	—
NaCl	0·61	0·17	0·15	0·38	0·20	0·15
HCl	—	0·17	—	0·43	—	0·25

The possibility of the intervention of cellulose itself, acting as an ion-exchange reagent, should be taken into account in discussing the mechanism of the separation, and the distribution of the ions between the phases will also be affected by the potential existing at the surface of shear between the wet cellulose and the moving solvent. It is probable, therefore, that the explanation suggested above presents a considerable over simplification of the problem.

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